

Examination of Placenta and Child Saliva Samples Associated with Childhood Allergy Development

A Thesis Submitted for the Degree of Doctor of Philosophy by

Nurul Hayati Mohamad Zainal



Discipline of Obstetrics and Gynaecology
Adelaide Medical School
Robinson Research Institute
Faculty of Health and Medical Sciences
The University of Adelaide, Australia

February 2018

Table of Contents

LIST OF FIGURES	XI
LIST OF TABLES	XVII
ABSTRACT	XIX
THESIS DECLARATION	XXI
ACKNOWLEDGEMENTS	XXII
ABBREVIATIONS	XXIV
1.0 INTRODUCTION	1
1.1 Introductory background	1
1.2 The allergy epidemic	4
1.2.1 Allergy	4
1.2.1.1 Definition and prevalence	4
1.2.1.2 Mechanism of allergy	5
1.2.1.3 Treatment of allergy	9
1.2.2 Asthma	11
1.2.2.1 Definition and prevalence	11
1.2.2.2 Mechanism of asthma	11
1.2.2.3 Treatment of asthma	12
1.2.3 Atopic dermatitis	13
1.2.3.1 Definition and prevalence	13
1.2.3.2 Mechanism of atopic dermatitis	15
1.2.3.3 Treatment of atopic dermatitis	16
1.2.4 Allergic rhinitis	17
1.2.4.1 Definition and prevalence	17
1.2.4.2 Mechanism of allergic rhinitis	17
1.2.4.3 Treatment of allergic rhinitis	19
1.2.5 Food allergy	20

1.2.5.1	Definition and prevalence	20
1.2.5.2	Mechanism of food allergy	20
1.2.5.3	Treatment of food allergy	22
1.2.6	Anaphylaxis	23
1.2.6.1	Definition and prevalence	23
1.2.6.2	Mechanism of anaphylaxis	24
1.2.6.3	Treatment of anaphylaxis	26
1.2.7	Current interventions for allergy prevention	27
1.3	Developmental Origins of Allergy	29
1.3.1	DOHaD hypothesis	29
1.3.2	Mechanisms underlying developmental origins of allergy	29
1.3.2.1	Permanent structural changes	30
1.3.2.2	Epigenetic programming of gene expression	31
1.4	Development of the immune system, role of the placenta and contributions to allergic susceptibility	33
1.4.1	Role of the placenta in fetal immune system development	33
1.4.1.1	Anatomy and physiology of placenta	33
1.4.2	Normal placental function and interaction with the immune system	39
1.4.3	Basic immune system development in embryology	40
1.4.4	Fetal immune system development in relation to allergic susceptibility	44
1.4.5	The neonatal immune response at birth	45
1.4.6	Mechanisms of allergic immune development for the fetus and neonate	47
1.4.6.1	Genetics	47
1.4.6.2	Epigenetics	48
1.4.6.3	Maternal allergy	49
1.4.6.4	Maternal health	50
1.4.6.5	Environmental exposures	52
1.5	Development of the immune system at birth	57
1.5.1	Normal immune system development at birth	57
1.5.2	Immune function in children that develop allergy	59
1.5.2.1	Epigenetics	59
1.5.2.2	Environmental exposures	60

1.6	Early detection of allergy	63
1.6.1	Allergy detection in children	63
1.6.2	Salivary protein confounding factors and childhood allergy	65
1.6.3	Gaps in knowledge	67
1.6.4	The role of placenta and saliva in early life allergy detection	67
1.7	Hypotheses and aims	71
2.0	METHODOLOGY	72
2.1	Introduction	72
2.1.1	Study population	74
2.2	Materials and reagents	75
2.3	Biological marker (biomarker)	75
2.3.1	Criteria for biological marker (biomarker)	75
2.3.2	Criteria for potential salivary protein biomarkers for allergy	76
2.4	Saliva samples	77
2.4.1	Types of saliva samples	77
2.4.2	Saliva samples collection method	77
2.4.3	Saliva samples collection protocol using salivette	80
2.5	Placental tissue collection	83
2.6	Placental samples preparation for quantitative real-time polymerase chain reaction (qRT-PCR)	84
2.6.1	Placental RNA extraction	84
2.6.2	Quantitative real-time polymerase chain reaction (qRT-PCR)	84
2.7	Sample preparation for mass spectrometry (MS)	85
2.7.1	Protein precipitation with modified 2-D clean-up	85
2.7.2	Estimation of proteins concentrations with EZQ protein quantification essay	86
2.7.3	In-solution (IS) tryptic digestion of saliva samples	89
2.7.4	Filter-aided sample preparation (FASP) for placental samples	89
2.7.5	Desalting using C18 column	90

2.8	Mass spectrometry (MS)	92
2.8.1	Introduction of mass spectrometry (MS)	92
2.8.2	Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS)	94
2.8.3	Peptide separation – Nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS)	94
2.8.4	Label-free quantification (LFQ) comparative mass spectrometry (MS)	95
2.9	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)	98
2.9.1	Introduction of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)	98
2.9.2	Polymer contamination analysis using MALDI-TOF-MS	98
2.10	Mass spectrometry using LTQ Orbitrap XL	103
2.10.1	Introduction of LTQ Orbitrap XL	103
2.10.2	Peptide identification with mass spectrometry using LTQ Orbitrap XL	105
2.10.3	Standard peptides preparation for spike-in experiment to evaluate accurate quantification	106
2.10.4	Protein identification using MASCOT	112
2.10.5	Label free quantification (LFQ) using MaxLFQ	112
2.11	Validation methods	118
2.11.1	TCA/Acetone protein precipitation	118
2.11.2	Western blot	120
2.11.2.1	Introduction on Western blot	120
2.11.3	Blitz system	127
2.11.3.1	Introduction and principle of Blitz system	127
2.11.3.2	Detection methods in Blitz system	130
2.11.3.3	Applications of Blitz system	131
2.12	Data analyses	132
2.12.1	Statistical analysis for saliva and placental samples	132

3.0	WORKFLOW OPTIMISATION ON THE PLACENTAL AND SALIVA SAMPLES	133
3.1	Introduction	133
3.2	Workflow optimisation of the placental samples	134
3.2.1	Optimisation of LC-MS/MS	134
3.2.1.1	Protein resuspension buffer and types of protein digestions	134
3.2.1.2	Protein precipitation with modified 2-D clean-up	136
3.2.1.3	Desalting with C18 column	138
3.2.2	Optimisation of Western blot validation study	142
3.3	Workflow optimisation of the saliva samples	145
3.3.1	Saliva samples handling	145
3.3.2	Optimisation of LC-MS/MS	145
3.3.2.1	Guanidine hydrochloride (GdnHCl) as protein resuspension buffer	145
3.3.2.2	Salivary protein concentration for protein digestion	146
3.3.2.3	Optimisation of in-solution (IS) tryptic digestion	146
3.3.3	Optimisation of Western blot validation study	146
3.3.4	Optimisation of Blitz system validation study	151
3.3.4.1	Antibody biotinylation optimisation	151
3.3.4.2	Optimisation of biotinylated antibody immobilization to the High Precision Streptavidin (SAX) biosensor	155
3.3.4.3	Standard curve for each chosen protein	157
3.3.4.4	Optimisation of the High Precision Streptavidin (SAX) biosensor regeneration	160
3.4	Conclusion of the workflow optimisation	166
4.0	EXAMINATION OF PLACENTAL GENES AND PROTEINS TO IDENTIFY THE ASSOCIATION WITH CHILDHOOD ALLERGY	167
4.1	Introduction	167
4.2	Placental genes associated with childhood allergy	168
4.2.1	Preliminary identification of placental genes associated with allergy	168
4.2.2	Maternal characteristics during pregnancy and child characteristics in placental genes expression analysis	171

4.2.3	Placental genes associated with allergy	174
4.2.3.1	Children with allergy vs children with no allergy	174
4.2.3.2	The influence of the presence and absence of maternal asthma during pregnancy on placental gene expression and child allergy	177
4.2.3.3	Effect of sex on placental gene expression and child allergy	183
4.2.3.4	Effect of specific phenotypes on placental gene expression and child allergy	185
4.3	Placental proteins associated with childhood allergy	188
4.3.1	Maternal characteristics during pregnancy and child characteristics in LC-MS/MS	188
4.3.2	Identification of placental proteins associated with allergy	191
4.4	Validation study for placental samples with Western blot	200
4.4.1	Maternal characteristics during pregnancy and child characteristics in Western blot	200
4.4.2	Western blot validation study using placental samples from children with and without an allergy	203
4.4.2.1	Expression of chloride intracellular channel protein 3 (CLIC3) protein in the placental samples of children with and without an allergy	203
4.4.2.2	Expression of peroxiredoxin-2 (PRDX2) protein in the placental samples of children with and without an allergy	206
4.4.2.3	Expression of complement C3 (C3) protein in the placental samples of children with and without an allergy	215
4.4.2.4	Expression of haptoglobin (Hp) protein in the placental samples of children with and without an allergy	220
4.4.2.5	Expression of pan 14-3-3 protein in the placental samples of children with and without an allergy	228
4.5	Discussion	232
5.0	EXAMINATION OF SALIVARY PROTEINS TO IDENTIFY ASSOCIATION WITH CHILDHOOD ALLERGY	242
5.1	Introduction	242
5.1.1	Saliva as potential diagnostic fluid in childhood allergy	242
5.2	Proteomic analysis of saliva in LC-MS/MS	248
5.2.1	Characteristics of the cohort children and maternal asthma status in LC-MS/MS	248

5.2.2	Identification of salivary proteins associated with allergy	250
5.3	Validation study for child saliva samples with Western blot	256
5.3.1	Characteristics of the cohort children and maternal asthma status in Western blot	256
5.3.2	Western blot validation study using saliva samples from children with and without an allergy	258
5.3.2.1	Expression of pan 14-3-3 protein in the saliva of children with and without an allergy	258
5.3.2.2	Expression of cornulin (CRNN) protein in the saliva of children with and without an allergy	266
5.3.2.3	Expression of transferrin protein in the saliva of children with and without an allergy	271
5.3.2.4	Expression of involucrin protein in the saliva of children with and without an allergy	274
5.3.2.5	Expression of Haptoglobin (Hp) protein in the saliva of children with and without an allergy	279
5.3.2.6	Expression of desmoglein 1 protein in the saliva of children with and without an allergy	282
5.4	Validation study for child saliva samples with Blitz system	288
5.4.1	Characteristics of the cohort children and maternal asthma status in Blitz system	288
5.4.2	Blitz system validation study using placental samples from children with and without an allergy	290
5.4.2.1	Concentration of 14-3-3 zeta, involucrin and cornulin (CRNN) protein in the saliva of children with and without an allergy	291
5.4.2.2	Concentration of 14-3-3 zeta protein in the saliva of children with and without an allergy	294
5.4.2.3	Concentration of involucrin protein in the saliva of children with and without an allergy	297
5.4.2.4	Concentration of cornulin (CRNN) protein in the saliva of children with and without an allergy	300
5.4.3	Blitz blind test of 14-3-3 zeta and cornulin (CRNN) salivary proteins using saliva samples from children with and without an allergy	307
5.5	Discussion	308

6.0	COMPARISON OF PROTEINS ASSOCIATED WITH ALLERGY BETWEEN PLACENTA AND SALIVA SAMPLES	318
6.1	Introduction	318
6.2	Proteins identified in placenta and saliva samples	321
6.3	Proteins with differential expression associated with allergy identified in placenta and saliva samples	325
6.4	Proteins that were identified in the non-allergic population in placenta and saliva samples	328
6.5	Correlations of proteins associated with allergy between placenta and saliva samples	331
6.6	Discussion of the protein comparisons associated with allergy between placenta and saliva samples	333
6.6.1	14-3-3 proteins and allergy susceptibility	333
6.6.2	Haptoglobin (Hp) protein and allergy susceptibility	335
6.6.3	Allergy susceptibility in children with asthmatic mother	339
7.0	FINAL DISCUSSION AND CONCLUSION	342
	APPENDICES	355
	REFERENCES	395

List of Figures

Figure 1. Allergen sensitisation and IgE production ⁽²⁸⁾	8
Figure 2. Intestinal permeability and allergy: cause and/or consequence (figure adapted from ⁽⁸³⁾) ...	21
Figure 3. Summary of the pathogenesis of anaphylaxis ⁽⁹³⁾	25
Figure 4. Pathways of trophoblast differentiation and functions ⁽¹⁴⁾	34
Figure 5. Fetus and placenta inside the uterine cavity ⁽¹²²⁾	36
Figure 6. Histological image showing the junctional region between maternal decidua (left) and trophoblast shell of conceptus/embryonic placental villi (right) ⁽¹²³⁾	37
Figure 7. Cells and molecules involved in fetomaternal tolerance in mouse and human placentae ⁽¹³¹⁾	42
Figure 8. Scheme describing approximate time course of different salivary proteins and peptides ⁽²⁶⁶⁾	66
Figure 9. Samples workflows on the saliva and placental tissues.....	73
Figure 10. Commercially available saliva collection devices. Figure adapted from ⁽²⁸²⁾	78
Figure 11. Salivette's parts	81
Figure 12. Steps of salivette collection method.....	82
Figure 13. Saliva samples concentration estimation with EZQ protein quantification essay	87
Figure 14. Placental samples concentration estimation with EZQ protein quantification essay	88
Figure 15. Mass spectrometer schematic ⁽²⁸⁷⁾	93
Figure 16. Schematic representation of the two different label-free approaches ⁽²⁹⁶⁾	97
Figure 17. MALDI-TOF mass spectrometer principle ⁽²⁸⁷⁾	99
Figure 18. Polymer detection on the sample using matrix on the ultrafleXtreme MALDI-TOF/TOF MS	100
Figure 19. MS spectra in flexAnalysis	102
Figure 20. A triple quadrupole mass spectrometer ⁽²⁸⁷⁾	104
Figure 21. Spectra of 2 peptide mixture (RASG-1 and Angiotensin frag 1-7) with their retention time (RT) between sample AL355 and AL435	109
Figure 22. Spectra of 2 peptide mixture (Bradykinin and Enolase T35) with their retention time (RT) between sample AL355 and AL435	110
Figure 23. Spectra of 2 peptide mixture (Angiotensin II and Angiotensin I) with their retention time (RT) between sample AL355 and AL435	111
Figure 24. Proteome Discoverer software that was used to process MS/MS mass spectra.	113
Figure 25. MaxLFQ: Uploading the raw data and experimental groups in the "Raw files" tab	114
Figure 26. MaxLFQ: Selecting LFQ label in the "Group-specific parameters" tab.....	115

Figure 27. Uploading the “Fasta files” in “Global parameters” tab	116
Figure 28. Validation steps workflows for placental tissues and saliva samples	119
Figure 29. Novex sharp prestained markers protein standard.....	121
Figure 30. Transfer stack in electrotransfer of 1D gel with semi dry blotting using TE 77 PWR semi dry system.....	123
Figure 31. Semi-quantification analysis of Western blot band densitometry using ChemiDoc and ImageLab software.....	126
Figure 32. BLItz Pro Software interface ⁽³⁰⁵⁾	128
Figure 33. The instrument, biosensor and principle of biolayer interferometry (BLI) in Blitz system ⁽³⁰⁵⁾	129
Figure 34. Protein group, total protein and total peptides identified in placental samples with LC-MS/MS in different protein resuspension buffers (6 M GdnHCl or 2 % (w/v) SDS) with different types of protein digestions (FASP or IS).....	135
Figure 35. Protein group, membranous protein group, total protein and peptides in placental samples identified with LC-MS/MS with or without 2-D clean-up.....	137
Figure 36. Mean percentage of protein group, total protein and peptides in placental samples identified with LC-MS/MS with or without C18 column procedure	139
Figure 37. Placental sample (AL-174) visualisation using matrix on the ultrafleXtreme MALDI-TOF-MS	140
Figure 38. Workflow of Western blot using placental samples	143
Figure 39. Workflow of Western blot using saliva samples	147
Figure 40. Workflow of Blitz system using child saliva samples	152
Figure 41. Equation for calculating the required volume of 0.1 mM biotin reagent for the antibody biotinylation step	153
Figure 42. Binding curve of biotinylated antibody during immobilization to High Precision Streptavidin (SAX) biosensor	156
Figure 43. Standard curves for the three salivary proteins. A. 14-3-3 zeta protein. B. Involucrin protein. C. Cornulin (CRNN) protein.....	159
Figure 44. The optimisation results of the SAX biosensor regeneration immobilized with 14-3-3 antibody in different buffers	162
Figure 45. The optimisation results of the SAX biosensor regeneration immobilized with involucrin antibody in different buffers	163
Figure 46. The optimisation results of the SAX biosensor regeneration immobilized with cornulin (CRNN) antibody in different buffers	164
Figure 47. Preliminary workflow of placental samples.....	169

Figure 48. <i>KLHDC7A</i> gene expression level in placentae of children with (n= 29) and without (n= 7) an allergy	175
Figure 49. <i>IL1B2</i> gene expression level in placentae of children of non-asthmatic mothers (low risk of allergy) with (n= 18) and without (n= 4) an allergy	178
Figure 50. <i>IL1R2</i> gene expression level in placentae of children of non-asthmatic mothers (low risk of allergy) with (n= 18) and without (n= 4) an allergy	179
Figure 51. <i>KLHL13</i> gene expression level in placentae of children of non-asthmatic mothers (low risk of allergy) with (n= 17) and without (n= 4) an allergy	180
Figure 52. <i>IL1R2</i> gene expression level in placentae of children of non-asthmatic mothers (low risk of allergy) with (n= 10) and without (n= 3) an allergy in female sex	182
Figure 53. <i>YWHAQ</i> gene expression level in placentae of children with (n= 21) and without (n= 5) an allergy in female sex	184
Figure 54. <i>KLHDC7A</i> gene expression level in placentae with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, n= 25; eczema combination, n= 18; rhinitis combination, n= 16; and non-allergic children, n= 7	186
Figure 55. Algorithm constructing protein intensity profiles for one protein from its peptide signals ⁽³⁰¹⁾	193
Figure 56. Placental proteins in allergic children that have LFQ ratios of >2 or <0.5 fold change (fc) as compared with non-allergic group (Max-LFQ protein analysis)	198
Figure 57. Venn diagram showing placental protein distribution with significant ratios of > 2 or < 0.5 fold change according to sex of allergic children as compared with non-allergic group in placental samples	199
Figure 58. Representative Western blot of CLIC3 protein expression in the placental samples of children with (n= 40) and without (n= 28) an allergy. CLIC3 protein was detected at 30 kDa	205
Figure 59. Representative Western blot of PRDX2 protein expression in the placental samples of children with (n= 40) and without (n= 28) an allergy. PRDX2 protein was detected at 22 kDa	208
Figure 60. PRDX2 protein expression in children with (n= 40) and without (n= 28) an allergy	209
Figure 61. PRDX2 protein expression in children of asthmatic mothers (high risk of allergy) with (n= 24) and without (n= 22) an allergy	210
Figure 62. PRDX2 protein expression in children of non-asthmatic mothers (low risk of allergy) with (n= 16) and without (n= 6) an allergy	211
Figure 63. PRDX2 protein expression in children of asthmatic mother (high risk of allergy) with (n= 13) and without (n= 13) an allergy in male sex	212
Figure 64. PRDX2 protein expression in children with (n= 21) and without (n= 14) an allergy in male sex	213

Figure 65. PRDX2 protein expression in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, n= 35; eczema combination, n= 25; rhinitis combination, n= 23; and non-allergic children, n= 28	214
Figure 66. Representative Western blot of C3 protein expression in the placental samples of children with (n= 40) and without (n= 28) an allergy. C3 protein was detected at 115 kDa.....	217
Figure 67. C3 protein expression in children of non-asthmatic mothers (low risk of allergy) with (n= 16) and without (n= 6) an allergy	218
Figure 68. C3 protein expression in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, n= 35; eczema combination, n= 25; rhinitis combination, n= 23; and non-allergic children, n= 28	219
Figure 69. Representative Western blot of Hp protein expression in the placental samples of children with (n= 40) and without (n= 28) an allergy. Hp protein was detected at 52 kDa.....	222
Figure 70. Hp protein expression in children with (n= 40) and without (n= 28) an allergy.	223
Figure 71. Hp protein expression in children of asthmatic mothers (high risk of allergy) with (n= 24) and without (n= 22) an allergy	224
Figure 72. Hp protein expression in children of asthmatic mothers (high risk of allergy) with (n= 13) and without (n= 13) an allergy in male sex.....	225
Figure 73. Hp protein expression in children with (n= 21) and without (n= 14) an allergy in male sex	226
Figure 74. Hp protein expression in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, n= 35; eczema combination, n= 25; rhinitis combination, n= 23; and non-allergic children, n= 28	227
Figure 75. Representative Western blot of pan 14-3-3 protein expression in the placental samples of children with (n= 40) and without (n= 28) an allergy. Pan 14-3-3 protein was detected at 30 kDa ...	230
Figure 76. Proposed model of placental proteins mechanism underlying increased susceptibility to allergy	240
Figure 77. Salivary proteins in allergic phenotypes that have LFQ ratio of >2 or <0.5 fold change (fc) as compared with non-allergic group (Max-LFQ protein analysis).....	254
Figure 78. Venn diagram showing salivary protein distribution with significant ratios > 2 or < 0.5 fold change in relation to allergic phenotypes in child saliva samples.....	255
Figure 79. Representative Western blot of pan 14-3-3 protein expression in the saliva samples of children with (n= 38) and without (n= 24) an allergy. Pan 14-3-3 protein was detected at 30 kDa ...	260
Figure 80. Pan 14-3-3 protein expression in children with (n= 23) and without (n= 9) an allergy	261
Figure 81. Pan 14-3-3 protein expression in children of asthmatic mothers (high risk of allergy) with (n= 10) and without (n= 3) an allergy	262

Figure 82. Pan 14-3-3 protein expression in children of non-asthmatic mothers (low risk of allergy) with (n= 13) and without (n= 7) an allergy	263
Figure 83. Pan 14-3-3 protein expression in children with (n= 11) and without (n= 7) an allergy in female sex	264
Figure 84. Pan 14-3-3 protein expression in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, n= 17; eczema combination, n= 16; rhinitis combination, n= 9; and non-allergic children, n= 9.....	265
Figure 85. Representative Western blot of CRNN protein expression in the saliva samples of children with (n= 38) and without (n= 24) an allergy. CRNN protein was detected at 60 kDa.	268
Figure 86. CRNN protein expression in children of non-asthmatic mothers (low risk of allergy) with (n= 16) and without (n= 14) an allergy	269
Figure 87. CRNN protein expression in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, n= 25; eczema combination, n= 19; rhinitis combination, n= 12; and non-allergic children, n= 16.....	270
Figure 88. Representative Western blot of transferrin protein expression in the saliva samples of children with (n=38) and without (n=24) an allergy. Transferrin protein was detected at 77 kDa.....	273
Figure 89. Representative Western blot of involucrin protein expression in the saliva samples of children with (n= 38) and without (n= 24) an allergy. Involucrin protein was detected at 100 kDa....	276
Figure 90. Involucrin protein expression in children with (n= 11) and without (n= 4) an allergy in male sex	277
Figure 91. Involucrin protein expression in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, n= 20; eczema combination, n= 15; rhinitis combination, n= 9; and non-allergic children, n= 6.....	278
Figure 92. Representative Western blot of Hp protein expression in the saliva samples of children with (n= 38) and without (n= 24) an allergy. Hp protein was detected at 52 kDa	281
Figure 93. Representative Western blot of desmoglein 1 protein expression in the saliva samples of children with (n= 38) and without (n= 24) an allergy. Desmoglein 1 protein was detected at 140 kDa	284
Figure 94. Desmoglein 1 protein expression in children of non-asthmatic mothers (low risk of allergy) with (n= 11) and without (n= 7) an allergy in female sex.....	285
Figure 95. Desmoglein 1 protein expression in children with (n= 18) and without (n= 10) an allergy in female sex	286
Figure 96. 14-3-3 zeta, involucrin and CRNN proteins concentrations in the saliva of children with (n= 88) and without (n= 38) an allergy	292

Figure 97. 14-3-3 zeta protein concentration in children of asthmatic mothers (high risk of allergy) with (n= 51) and without (n= 9) an allergy.....	295
Figure 98. 14-3-3 zeta protein concentration in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, n= 68; eczema combination, n= 58; rhinitis combination, n= 38; and non-allergic children, n= 38	296
Figure 99. Involucrin protein concentration in children of asthmatic mothers (high risk of allergy) with (n= 51) and without (n= 9) an allergy.....	298
Figure 100. Involucrin protein concentration in children of non-asthmatic mothers (low risk of allergy) with (n= 18) and without (n= 17) an allergy in male sex	299
Figure 101. CRNN protein concentration in children with (n= 65) and without (n= 31) an allergy	301
Figure 102. CRNN protein concentration in children of asthmatic mothers (high risk of allergy) with (n= 37) and without (n= 7) an allergy	302
Figure 103. CRNN protein concentration in children of asthmatic mothers (high risk of allergy) with (n= 21) and without (n= 3) an allergy in male sex.....	303
Figure 104. CRNN protein concentration in children of asthmatic mothers (high risk of allergy) with (n= 16) and without (n= 4) an allergy in female sex.....	304
Figure 105. CRNN protein concentration in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, n= 52; eczema combination, n= 43; rhinitis combination, n= 27; and non-allergic children, n= 31	305
Figure 106. Proposed model of salivary proteins mechanism underlying increased susceptibility to allergy	315
Figure 107. LC-MS/MS findings on both the placental and saliva samples.....	320
Figure 108. Proposed model of 14-3-3 and Hp proteins mechanism underlying increased susceptibility to allergy in placental samples	336
Figure 109. Propose model of mechanism underlying increased allergy susceptibility in children with asthmatic mother.....	340
Figure 110. Proposed model of placental and salivary proteins mechanism underlying increased susceptibility to allergy	349
Figure 111. Summary of data analysis on potential salivary protein biomarkers for allergy	350

List of Tables

Table 1. UK diagnostic criteria for atopic dermatitis ⁽⁵⁵⁾	14
Table 2. Abbreviations and definition of common terms used in allergic rhinitis ⁽⁶⁹⁾	18
Table 3. Summary of MS-based methods used for salivary biomarker identification. Table adapted from ⁽²⁷²⁾	70
Table 4. Nanoflow LC-ESI-MS/MS multistep linear gradients.....	96
Table 5. Peptide mixture component name, retention time (RT), molecular weight (MW) and peptide sequences	107
Table 6. Peptide mixture component name detected in the saliva samples.....	108
Table 7. Primary and secondary antibodies used in this study	124
Table 8. Placental samples proteomics workflow, optimisation steps and results	141
Table 9. Placental proteins and the optimised dilution of antibodies used in the Western blot.	144
Table 10. Salivary proteins and the optimised dilution of antibodies used in Western blot.	149
Table 11. Blitz salivary proteins list and molar coupling ratio (MCR) required in antibody biotinylation step.....	154
Table 12. The optimisation results of the time duration and shaker speed for Blitz workflow	165
Table 13. Target genes selected for placental gene expression analysis	170
Table 14. Maternal characteristics during pregnancy and child characteristics in placental genes expression analysis	172
Table 15. The sixteen genes that did not change significantly in the placental samples of children with an allergy as compared to children without an allergy	176
Table 16. Significant statistical analyses on placental gene expression in relation to childhood allergy distribution and in relation to the presence (high risk of allergy) or the absence (low risk of allergy) of maternal asthma.....	187
Table 17. Maternal characteristics during pregnancy and child characteristics in LC-MS/MS	189
Table 18. Summary of placental protein identification with significant label-free quantification (LFQ) ratios in $\geq$ two groups that include placentae of allergic males, allergic females and allergic males combined with allergic females in LC-MS/MS.....	194
Table 19. Summary of placental protein identification with significant label-free quantification (LFQ) ratios in specifically placentae of allergic males and allergic females in LC-MS/MS.....	196
Table 20. Maternal characteristics during pregnancy and child characteristics for validation studies with Western blot.....	201

Table 21. Significant statistical analyses of Western blot band densitometry of child saliva samples in relation to childhood allergy distribution and in relation the presence (high risk of allergy) or the absence (low risk of allergy) of maternal asthma.....	231
Table 22. Advantages of salivary testing for diagnosis ⁽²⁶⁰⁾	243
Table 23. Saliva proteins identified according to functions. Table adapted from ⁽²⁶¹⁾	244
Table 24. Salivary proteins involved in oncological pathologies ⁽²⁵⁷⁾	246
Table 25. Salivary proteins involved in dental pathologies ⁽²⁵⁷⁾	247
Table 26. Characteristics of the cohort children and maternal asthma status in LC-MS/MS	249
Table 27. Summary of child salivary protein identification with the significant label-free quantification (LFQ) ratios in specific allergic phenotypes in LC-MS/MS	251
Table 28. Summary of child salivary protein identification with the uncalculated label-free quantification (LFQ) ratios in specific allergic phenotypes in LC-MS/MS	253
Table 29. Cohort characteristics for validation studies with Western blot.....	257
Table 30. Significant statistical analyses of Western blot band densitometry of child saliva samples in relation to childhood allergy distribution and to the presence (high risk of allergy) or the absence (low risk of allergy) of maternal asthma	287
Table 31. Characteristics of the cohort children and maternal asthma status in Blitz system	289
Table 32. 14-3-3 zeta and involucrin salivary protein that did not change significantly in the child saliva samples of children with an allergy as compared to children without an allergy.....	293
Table 33. Significant statistical analyses of estimated proteins concentrations on the Blitz system in relation to childhood allergy distribution and to the presence (high risk of allergy) or the absence (low risk of allergy) of maternal asthma	306
Table 34. LC-MS/MS comparison of proteins that were identified in both placenta and saliva samples regardless their expression in allergy	322
Table 35. LC-MS/MS comparison of proteins with differential expression associated with allergy identified in placenta and saliva samples	326
Table 36. The summary of statistical significant protein expression changes related to allergy in placental and child saliva samples analysed by qRT-PCR, LC-MS/MS, Western blot and Blitz system	327
Table 37. LC-MS/MS comparison of proteins that were identified in the non-allergic population in both placenta and saliva samples	329
Table 38. Spearman correlation coefficients for each placental protein versus each salivary protein according to Western blot data.....	332

Abstract

Allergic disease has risen to epidemic proportions during recent years. Evidence shows that prenatal events play a critical role in determining disease susceptibility via environmental influences on placental function and fetal programming. We hypothesised that childhood susceptibility to allergy is increased through significant alterations in placental gene expression involved in regulating immune system development. Furthermore, we hypothesised that products of altered placental genes are indirect measures of altered protein concentrations in the saliva of children with allergies. The aim of this PhD thesis was to identify proteins associated with childhood allergy using two sources of biological tissue: placental tissue and child saliva. The long term objectives were to identify proteins that could be measures of allergic risk in placenta and saliva, develop an assay for detection of allergic risk in newborns and target proteins for interventions that prevent allergy onset.

Placental tissue and child saliva samples were examined using a proteomic approach involving quantitative label-free comparative mass spectrometry. Data analysis were performed using Mascot database and MaxLFQ software. Placental tissue and saliva from children with no allergy were compared with children with allergic diseases (placenta, n= 16; saliva, n= 18).

Using placental samples, 1223 proteins were identified from MaxLFQ analysis. Nineteen proteins were significantly altered in allergic male placentae and 21 proteins were altered in allergic female placentae, relative to non-allergic children. Of these proteins, five candidate placental proteins associated with allergic diseases were validated with Western blot (n=68), which include chloride intracellular channel protein 3, peroxiredoxin-2, haptoglobin and complement C3. Moreover, 14-3-3 protein had very high expression in allergic children and very low expression in the non-allergic group.

Using child saliva samples, 177 proteins were identified from MaxLFQ analysis. Of these proteins, six candidate proteins with significant ratio in specific allergic phenotypes were validated with Western blot (n=62). These proteins include salivary 14-3-3, cornulin, involucrin, transferrin, haptoglobin and desmoglein 1. Based on Western blot findings, 14-3-3, cornulin and involucrin proteins were further validated using the Blitz system that uses crude saliva sample for rapid identification and quantitation as a potential non-invasive bedside allergy biomarker screening (n=126). Blitz results demonstrated that these proteins were significantly altered in allergic children with asthmatic mother (high risk of allergy), as compared to non-allergic children with asthmatic mother. Only salivary cornulin was significantly altered in allergic children as compared to non-allergic children regardless of maternal asthma status.

Findings revealed that in both placental and saliva samples, 14-3-3 proteins were significantly altered in female allergic children as compared with female non-allergic children. Interestingly, altered expression of some proteins varies in a sex-specific manner, such as observed in haptoglobin and involucrin, and in relation to presence or absence of maternal asthma. In conclusion, our findings indicate that protein expression can be altered *in-utero* in children who subsequently develop an allergy and altered expression of these proteins is detectable in saliva in early life.

Thesis Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

.....

Nurul Hayati Mohamad Zainal

Acknowledgements

This thesis would not have been possible without the inspiration and strength endowed by the God Almighty and tremendous support from a number of wonderful people; my great thanks and appreciation to all of them for being part of this remarkable journey. Foremost, I would like to express my sincere gratitude to my primary supervisor, Professor Vicki Clifton for the continuous support of my PhD study, for her great patience, continuous motivation, optimism, enthusiasm and immense knowledge. You have been a great mentor and thank you for accepting me as your student.

My work would not be completed without the help from my secondary supervisor, Professor Peter Hoffmann. Thank you for your supervision, guidance and discussion throughout my PhD. To my third supervisor, Dr Astrud Tuck, thank you for all the support you have provided over the years, for reviewing my thesis and being committed to my research project. Special thanks to our co-investigator, Dr Andrew Tai from Women's and Children's Hospital that had helped me in recruiting and collecting child saliva samples.

I am grateful to all my lab members for their help and support throughout my PhD experience in the Adelaide Proteomics Centre: Nur Aishah Bahruddin, Miao Chen, Parul Mittal, Chao Zhang and Matthew Briggs. To Mr Chris Cursaro, I have learnt so much from him and thank you for the countless times you helped me in technical assistance and with almost everything related to the lab.

I am deeply grateful to Dr Yin Ying Ho for her constant help on the technical side of this project and her kind advice on thesis writing. I would like to thank Dr Manuela Klingler-Hoffmann and Dr Florian Weiland for their willingness to share and teach their technical expertise on the protein extraction, antibodies and Western blot. Thank you to Dr Michelle Hooi and Dr Georgia Arentz for their help on the Orbitrap and Amazon. To Dr Noor Alia Lokman, I am deeply touched for your assistance in the advice on thesis writing. Your contributions was extremely valuable and much appreciated.

I would like to thank the members of Pregnancy and Development Group in Robinson Research Institute group that assist me in my PhD study, Dr Zarqa Saif for her friendship and technical assistance in the lab. To Natalie Aboustate and Isabelle-Rose Meredith, thank you for your help with the collection and organisation of the placental and saliva samples. I would also like to thank our research midwife, Ms Karen Rivers and Ms Julia Dalton; our asthma research nurse, Ms Kate Roberts-Thompson; and our asthma paediatric nurse, Ms Christine Tuckwell, for all their efforts in patient recruitment, sample collection and post-natal follow-ups.

Special thanks to Suzanne Edwards, a very approachable statistician from Adelaide Health Technology Assessment (AHTA), School of Public Health that had helped me in the statistical analysis and presentation of my data findings.

A very special gratitude goes out to Associate Professor Darryl Russell from Robinson Research Institute for lending me the Blitz machine to validate my findings.

I am really fortunate to have received scholarships from Ministry of Education Malaysia and I am also grateful to my employer, Universiti Putra Malaysia (UPM) that had granted me fully paid study leave. Thank you.

And a special acknowledgement is made to the mothers and children at Lyell McEwin Hospital and Women's and Children's Hospital for consenting to take part in this study. The significance of this work could not have been achieved without their contributions.

Thank you to my dearest Malaysian friends that are also in the same boat with me in the land Down Under, for your friendship and support: Hanan, Melati, Rose and Sarah. To my best friend, Bayah, thank you for always been there through thick and thin for the past 25 years and counting.

I am also grateful for the continuous support and encouragement as well as friendship from my UPM colleagues: Aina, Armania, Baizura, Huda, Fatin, Husna, Noraini, Raihanah, Rozaini, Salma, Shahira, Zalina and Zalinda. Thank you very much, ladies!

Finally, I am forever indebted to my beloved family who have been a constant source of love, unwavering support and pillar of strength during this challenging time in my life. To my devoted husband, Khairul Akmal, thank you for your sacrifices leaving everything behind and willing to be dragged down to another continent, the land Down Under, only for me to pursue my PhD. I love you and you mean the world to me. To my beloved children, Harith Ziqri and Adam Wafiy, I love you guys so much and my immense apologies for the time spent for this study instead for both of you. To my darling sister, Hannerita and her daughter, Syahirah, thank you for your support and love. And to my parents and family: Mama, Papa, Mak and Abah; and everyone for their love and encouragement. Thank you all for supporting me throughout my PhD.

Abbreviations

2-DE	Two dimensional electrophoresis
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
A	Asthma
AA	Amino acid
Ab	Antibody
ABCA1	ATP-binding cassette subfamily A, member 1
ACN	Acetonitrile
AD	Atopic dermatitis
ADRB2	Beta2-adrenergic receptor
AF	Allergic females
AHR	Airway hyperresponsiveness
AM	Allergic males
APC	Antigen-presenting cell
Apos	Apolipoproteins
AR	Allergic rhinitis
ASIT	Allergen specific immunotherapy
BALF	Bronchoalveolar lavage fluid
BCC	Basal cell carcinoma
BLI	Biolayer interferometry
BMI	Body mass index
bp	Base pairs
C	Control subjects, non-allergic children
C3	Complement 3
CB	Cord blood
CE	Cornified envelope
CHCA	α -cyano-4-hydroxy cinnamic acid
CID	Collision induced dissociation
CLIC3	Chloride intracellular channel protein 3
CLSP	Calmodulin-like skin protein
CMP	Cow's milk protein
CMPA	Cow's milk protein allergy
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2 gene
CRNN	Cornulin
CRP	C-reactive protein
CS-IgE	Cord serum IgE
CSR	Class-switch recombination

CTL	Cytotoxic T Lymphocytes
CYP	Cytochrome P450
Da	Dalton
DAMP	Damage-associated molecular pattern
DC	Dendritic cells
DM	Diabetes mellitus
DOHaD	Developmental origins of health and disease
<i>DSG1</i>	Desmoglein 1 gene
DTT	Dithiothreitol
E	Eczema
ECL	Enhanced chemiluminescence
EDC	Human epidermal differentiation complex
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ESI	Electrospray ionisation
ETD	Electron transfer dissociation
FA	Food allergies
Fab	Fragment antigen binding
FAP	Facilitated antigen presentation
FASP	Filter-aided sample preparation
fc	Fold change
FcεRI	Fc epsilon RI
FcRn	Neonatal Fc receptor
<i>FLG</i>	Human filaggrin gene
FOXP3	Forkhead box P3
g	G-force of relative centrifugal force
GCF	Gingival crevicular fluid
GdnHCl	Guanidine hydrochloride
GDM	Gestational diabetes mellitus
GFR	Glomerular filtration rate
H ₂ O ₂	Hydrogen peroxide
HC	High concentrate
HCl	Hydrochloric acid
HDAC	Histone deacetylase
HDL	High-density lipoprotein
HE	Hen's egg
hESCs	Human embryonic stem cells
HLA	Human leukocyte antigen
hMSCs	Human mesenchymal stem cells

Hp	Haptoglobin
HPLC	High performance liquid chromatography
HREC	Human Research Ethics Committee
IAA	Iodoacetamide
ICS	Inhaled corticosteroids
IDO	Indoleamine 2,3-dioxygenase
IEF	Isoelectric focusing
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL	Interleukin
IL-1RN	Interleukin-1 receptor antagonist protein
Imp8	Importin-8
IP	Intestinal permeability
iPLA2	Ca ²⁺ -independent phospholipase A2
iRSS-Kde	Intron recombination signal sequence–K-deleting element
IS	In-solution
ISAAC	The International Study of Asthma and Allergies in Childhood
IVA	Insect venom allergy
IVL	Involucrin gene
LBW	Low birth weight
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LCPUFA	Long chain polyunsaturated fatty acids
LDL	Low-density lipoprotein
LFQ	Label-free quantification
LFQ MS	Label-free quantification mass spectrometry
LPS	Lipopolysaccharide
m	Mass
m/z	Mass-to-charge
MALDI	Matrix assisted laser desorption/ionization
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MHC	Major histocompatibility complex
MIAMI	Marrow isolated adult multilineage inducible
min	Minutes
MMP	Matrix metalloproteinase
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry

MVCD4	Microvascular complications of diabetes 4
MW	Molecular weight
Myo10	Unconventional myosin-10
n-3	Omega-3
NA	Non-applicable/not enough sample number
NF	Non-allergic females
NH ₄ HCO ₃	Ammonium bicarbonate
NK	Natural killer
NM	Non-allergic males
NPSR1	Neuropeptide S Receptor 1
NSCLC	Non-small cell lung cancer
OFC	Oral food challenge
OMLDT	Occupational medicamentosa-like dermatitis induced by trichloroethylene
OSCC	Oral squamous cell carcinoma
OVA	Ovalbumin
PA	Phosphoric acid
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with tween-20
pDCs	Plasmacytoid dendritic cells
PG	Prostaglandin
PGS1	Biglycan (Bone/cartilage proteoglycan I)
PIC	Protease inhibitor cocktail
pI	Isoelectric point
PRDX2	Peroxiredoxin-2
PRR	Pattern recognition receptors
PVDF	Polyvinylidene fluoride
Q	Quadrupole
qRT-PCR	Quantitative real-time polymerase chain reaction
R	Rhinitis
RAS	Renin-angiotensin system
RCT	Randomised controlled trial
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RPLC	Reversed-phase liquid chromatography
RPM	Revolutions per minute
SAX	High Precision Streptavidin
SCC	Squamous cell carcinoma
SD	Standard deviation

SDS	Sodium dodecyl sulphate
SE	Standard error
SES	Socioeconomic status
sec	Seconds
SES	Socioeconomic status
SILAC	Stable isotope labeling with amino acids in cell culture
sjKRECs	Signal-joint κ receptor excision circles
SMAD3	Mothers against decapentaplegic homolog 3
SNP	Single-nucleotide polymorphism
SPT	Skin-prick test result
STAT	Signal transducer and activator of transcription
t	Time
TBST	Tris-buffered saline Tween-20
TCR	T cell receptor
TFA	Trifluoroacetic acid
TGF- β	Transforming growth factor beta
Th	T helper
TLR	Toll like receptor
TN	Tree nuts
TNF	Tumour necrosis factor
TOF	Time-of-flight
TREC	T cell receptor excision circles
Tregs	Regulatory T cells
Tris	Tris(hydroxymethyl)-aminomethane
TSDR	Treg-specific demethylated region
uNK	Uterine natural killer
UP	Unique peptides
WB	Western blot
XIC	Extracted ion chromatogram
z	Charge

1.0 INTRODUCTION

1.1 Introductory background

Allergic and autoimmune diseases have risen to epidemic proportions in the last 50 years indicating the susceptibility of immune pathways to modern environmental influences ⁽¹⁾. Expression of many of these diseases in very early childhood, particularly allergic disease, provides clear evidence that early life events have a more critical role than genomic inheritance ^(2, 3). Severity of allergic symptoms depends on the amount of immunoglobulin E (Ig)E antibodies produced, quantity of allergen, and possibly early life exposure that combines genetic and environmental factors ⁽³⁻⁶⁾. The developmental origins of allergy hypothesis suggests cycles of cascading events throughout the periconceptual period derived from interaction between maternal, placental, and fetal factors may contribute to allergic severity ⁽³⁾.

Prenatal events play a critical role in determining disease susceptibility via environmental influences on placental function and fetal programming ⁽⁷⁾. The mother provides the first environment for the developing fetus, and the range of maternal factors that may influence fetal immune and allergy development include exogenous environmental exposures and endogenous factors ⁽²⁾. Exogenous environmental exposures include maternal pre-pregnancy obesity, smoking exposure, maternal diet, *in utero* exposure to environment with high microbial burden and low socioeconomic status (SES) ^(2, 3, 8-10). More importantly, a mother with an atopic phenotype may increase the probability of her offspring developing an allergic predisposition. This is supported by several lines of evidence, including the inheritance of alleles to the offspring that promote a T helper 2 (Th)2 bias and a skewed immune system in response to inflammatory stimuli ⁽⁵⁾. During pregnancy, the maternal immune response is shifted away from the Th1-driven cell-mediated immunity towards Th2-related humoral responses, hence increasing susceptibility of allergy development in childhood. There is also evidence that perinatal differences in immune function of allergy-prone newborns include differences in neonatal regulatory T cells (Tregs) and Th17 function. Moreover, some of these pathways are epigenetically modified ^(2, 11).

Complications during pregnancy, such as maternal asthma, can alter placental cytokine expression and could predispose offspring to an allergic phenotype. Our laboratory has shown that in pregnancies complicated by maternal asthma there are reduced placental pro-inflammatory cytokine responses to an immune challenge relative to non-asthmatic controls, especially in relation to production of interleukin (IL)-1 β and tumour necrosis factor alpha (TNF)- α , regardless of fetal sex ⁽¹²⁾. Previous work has shown that baseline mRNA expression of placental cytokines, such as *TNF- α* and *IL* including *IL*-

1 β , IL-6, IL-8 and IL-5, were altered in a sex-specific manner in pregnancies complicated by asthma. Interestingly, these alterations were more prevalent in mild asthma compared to severe asthma, and these changes only occurred in mothers' pregnant with a female fetus with no changes were observed in placentae of male fetuses ⁽¹³⁾. These data suggest complications in pregnancy can alter placental gene expression in a sex-specific manner which may influence development of the fetal immune system and possibly contribute to development of childhood allergy.

The placenta has many integral functions that ensure normal development of the growing fetus. These functions include transfer of respiratory gases, transport and metabolism of nutrients, elimination of fetal waste products, hormone secretion, and most importantly, protective and immunological functions that may influence the fetal immune system resulting in allergic susceptibility in early life ⁽¹⁴⁾. For example, IgE was present in immune cells of infants with allergic mothers and placental forkhead box P3 (FOXP3) gene expression, a marker of Tregs concentration, was reduced in infants who subsequently developed an allergic disease ^(15, 16).

Fetal growth may be associated with risk of allergy in childhood ⁽⁷⁾. Alterations in fetal growth are caused by variations in nutrition and oxygen supply to the fetus in various stages of gestation forcing developmental adaptations of fetal organs with potentially detrimental long-term effects on the physiology, metabolism and, therefore, health of an individual ^(7, 17). This is known as fetal programming, which describes the process by which a stimulus or insult applied at a critical window of development results in immediate predictive adaptive responses with long term consequences on the structure or function of an organism ^(7, 17-19). Low birth weight (LBW) is an indicator of changes in fetal development and alterations in mechanisms associated with metabolism, immunity, cardiovascular function and lung function in adults ⁽⁹⁾. For example, increased risk of respiratory symptoms was reported in the first seven years of life in LBW term born children ⁽²⁰⁾. Furthermore, this developmental adaptation includes reduction in the diameter of central and small airways, resulting in impaired lung growth and could lead to an increased risk of childhood asthma or chronic obstructive pulmonary disease (COPD) throughout postnatal life ⁽²⁰⁾. On the contrary, other studies suggest LBW is protective against allergy. Villamor *et al.* reported a negative association between birth weight and asthma in twins that is unlikely to be confounded by genetic or shared environmental factors ⁽²¹⁾. These studies suggest a combination of events *in utero* may contribute to the susceptibility of childhood allergy. Such events include exposures to maternal allergy, changes in placental function that affects fetal immune development and structural alterations in organ development in association with fetal growth. Even so, the specific mechanisms associated with allergic susceptibility are yet to be identified. The contradicting studies mentioned above suggest that the role of periconceptional events on the developmental origins of allergy are not clearly understood and this knowledge gap is examined in this thesis.

This thesis examines new and novel proteins that vary in relation to childhood allergy in placental and matched saliva samples from children between six months and five years of age. The study examined gene and protein profiles of placentae from children with and without an allergy using a proteomic approach to identify novel proteins that vary in relation to allergy in placental and saliva samples. Findings were validated using several techniques in a larger population. Identification of these novel proteins could be used in future to measure childhood allergy susceptibility in early life, to develop an assay for detection of allergic risk in newborns, and furthermore to target proteins for interventions that prevent allergy onset.

1.2 The allergy epidemic

1.2.1 Allergy

1.2.1.1 Definition and prevalence

Allergy can be defined as a hypersensitivity reaction initiated by immunologic mechanisms. Atopy is a personal or familial tendency to produce IgE antibodies in heightened response to low-dose common environmental antigens (allergens), including pollens, dust and different food types, confirmed by a positive skin-prick test (SPT) result. These allergens are environmental factors that the immune system would not normally mount an immune reaction against ^(22, 23). Allergic sensitisation usually begins early in life and the number of allergens to which a patient is sensitised can increase over time ⁽²⁴⁾. Allergic diseases include asthma, atopic dermatitis (AD), allergic rhinitis (AR), food allergies (FA) and anaphylaxis.

Most concerning is the increase in allergic and autoimmune diseases in the last 50 years indicating the susceptibility of immune pathways to modern environmental influences. Allergic diseases affect more than 20 % of the population worldwide, especially in industrialised countries ⁽²⁵⁾. Moreover, allergic diseases are among the most common non communicable, chronic diseases in children and adolescents worldwide, whereby almost 50 % of school children suffer from allergic related diseases ⁽¹⁾. In Australia, 19.6 % of the population suffer from at least one allergy and this is expected to rise to 30 % of the adult population by 2050 ⁽¹⁾. Furthermore, hospital admissions for anaphylaxis with severe life threatening allergic reactions have increased four-fold in the last 20 years, food-induced anaphylaxis has doubled in the last 10 years and 10 % of infants currently have a FA ⁽¹⁾. Thus, allergy is a complex and heterogeneous disease that presents a significant burden to human health and preventative measures to reduce this burden are urgently required. Early detection of allergic susceptibility may be an approach by which prevention or interventions could be introduced to decrease allergic severity.

Asthma in children often co-exists with allergy and with other allergic diseases, including AD, AR and FA ^(26, 27). Most young children with severe AD have increased risk of developing asthma and rhinoconjunctivitis. However, most cases of AD are mild to moderate in the general population. A pattern of progression through different allergic disorders in early childhood has been termed the 'atopic march' where individuals who first present in early childhood with AD later develop AR and then atopic asthma ⁽²⁸⁾. Risk of current asthma and co-existence of allergy-related diseases at six years of age was

investigated by Saunes *et al.* ⁽²⁹⁾. Based on their findings, although most cases of AD in the general population were mild to moderate, early AD was associated with increased risk of developing childhood asthma ⁽²⁹⁾. These findings support the hypothesis of an atopic march in the general population that includes progression of atopic diseases, consisting of asthma, AD, AR and FA. Moreover, evidence shows strong epidemiological and pathophysiological association between AR and asthma in adults and children. Allergic rhinitis and impact of asthma (ARIA) classifications of symptom duration (intermittent, persistent) and severity (mild, moderate, severe) have been validated in adult and paediatric populations ⁽³⁰⁾. Moreover, clinical features and comorbidities are very important for atopic march and progression from AR to asthma is observed frequently in chronological order.

1.2.1.2 Mechanism of allergy

Hypersensitivity states and resulting reactions are generally sub classified by the Gell and Coombs classifications ⁽²³⁾. Allergic reactions vary according to each individual and may manifest as a minor skin reaction or, in an extreme case, severe anaphylactic shock that can result in death ^(31, 32). There are four types of hypersensitivity: (1) immediate (Type I) hypersensitivity response, (2) antibody-mediated (Type II) hypersensitivity reactions, (3) immune complex disease (Type III hypersensitivity) and (4) cell-mediated reactions (Type IV hypersensitivity) ⁽³³⁾. Immediate (Type I) hypersensitivity responses, such as those that occur in asthma, are characterised by production of IgE antibodies against allergens commonly found in the surrounding environment, such as pollens, mites, animal dander and house dust ⁽³³⁾. Thus, understanding the mechanisms underlying allergic disease is essential in order to have better understanding of disease pathophysiology.

The immunological pathogenesis of allergic disease is expression of T-lymphocyte, cell-mediated responses to common environmental allergens that are biased towards Th2 lymphocyte activity ⁽²⁶⁾. Th2 lymphocytes release peptide regulatory factors (cytokines), including IL-4, IL-5, IL-9 and IL-13, which coordinate IgE production by activating inflammatory cells, such as eosinophils, commonly associated with allergic inflammation ⁽²⁶⁾. The counter-regulatory pathways generated by a normal immune response to infection are coordinated by Th1 lymphocytes, which then generate cytokines, interferon (IFN)- γ and IL-2 ⁽³⁴⁾. Other pathways involved include a group of Tregs which influence both Th1 and Th2 activity either by cell–cell contact or by generation of IL-10 and transforming growth factor beta (TGF- β) ⁽³⁴⁾. Either overexpression of Th2 activity or control failure by Th1 or Tregs function causes higher probability for development of allergy and allergic inflammation. In addition, the pattern of response of T-lymphocytes is formulated by the signalling nature of antigen-presenting cells (APCs). APCs are affected by the nature of antigen exposure and generate IL-12, -15, -18 and -23 which predominantly stimulate Th1 responses, whereas IL-10 from Tregs inhibits IL-12 and therefore favours

Th2 activity ⁽²⁶⁾. New evidence shows that perinatal differences in epigenetically regulated immune function of allergy-prone newborns include differences in neonatal Tregs and Th17 function ^(2, 11). In the overall process, the maternal immune response during pregnancy is shifted away from Th1 driven cell-mediated immunity towards Th2-related humoral response, which may increase the susceptibility of childhood allergy development in at risk individuals ⁽³⁵⁾.

Allergen sensitisation and IgE production occurs via two mechanisms: initial allergen sensitisation leading to antigen-specific IgE production and amplification of antigen-specific IgE response by facilitated antigen presentation (FAP). Initial allergen sensitisation leading to antigen-specific IgE production is shown in Figure 1 (left panel) ⁽²⁸⁾. In individuals not yet exposed to a new environmental allergen, the only IgE present (blue) does not have specificity for the new antigens. Such IgE can be bound to the $\alpha\beta\gamma\gamma$ form of Fc epsilon RI (Fc ϵ RI) (high-affinity IgE receptor) on mast cells or to the $\alpha\gamma\gamma$ form of Fc ϵ RI on the surface of macrophages, monocytes or dendritic cells (DC) or to a cluster of differentiation 23 (CD23) (Fc epsilon RII, Fc ϵ RII, a low affinity receptor for IgE) on airway epithelial cells. The new antigens (orange circles in Figure 1) are either captured by DC or macrophages in the airway lumen or epithelium of the airway mucosa, or gain access to submucosal DC through disrupted epithelium or, for some antigens with intrinsic protease activity, by disrupting epithelial cell tight junctions ⁽²⁸⁾. Antigen-activated DC mature and migrate to regional lymph nodes or to sites in the local mucosa, where they present processed antigen epitopes to cognate T cells in the presence of IL-4 or IL-13, which may be derived from a variety of potential cellular sources. This induces such T cells to become differentiated and activated Th2 cells. IL-4 and IL-13, which may be derived from Th2 cells, basophils and mast cells, also activate Ig heavy chain gene class-switch recombination (CSR) for antigen-specific IgE production, designated as antigen-specific IgE (epitope A), in B cells ⁽²⁸⁾.

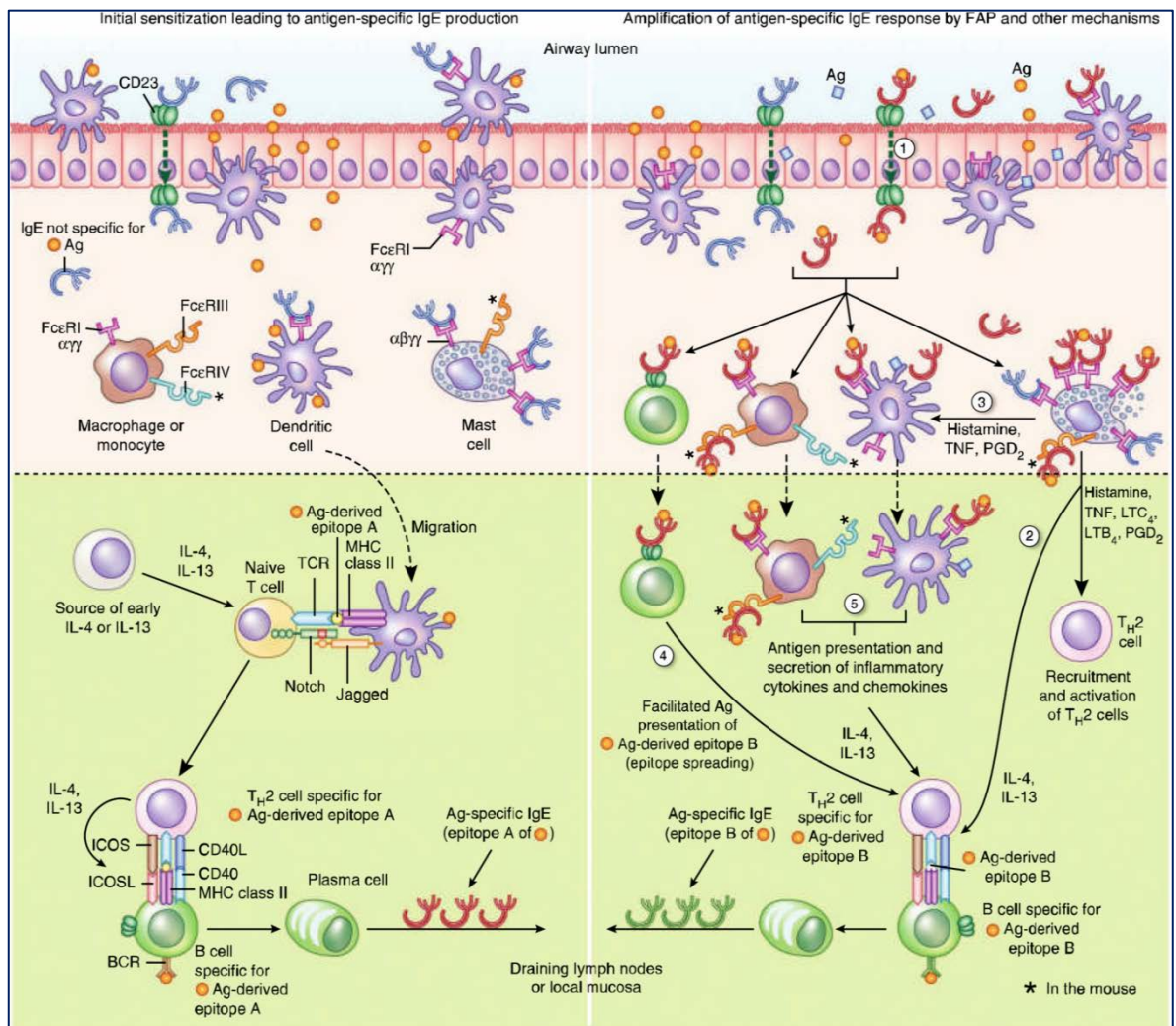


Figure 1. Allergen sensitisation and IgE production ⁽²⁸⁾. Initial allergen sensitisation results in antigen-specific IgE production (**left**). In individuals not yet exposed to a new environmental allergen (Ag), the only IgE present (**blue**) does not have specificity for the new antigen(s). Antigen-activated DC mature and migrate and they present processed antigen epitopes to cognate T cells; in the presence of IL-4 or IL-13, this induces such T cells to become differentiated and activated Th2 cells. IL-4 and IL-13, basophils, mast cells and/or other sources, also activate Ig heavy chain gene CSR for antigen-specific IgE production, designated here antigen-specific IgE (epitope A), in B cells. The antigen-specific IgE response is amplified by FAP and other mechanisms (**right**). Antigen-specific IgE can bind to multiple cell types through various IgE receptors. Antigen-induced aggregation of IgE bound to FcεRI stimulates mast cell degranulation and the release of mediators, such as histamine, PGD2 and TNF, which promote recruitment of Th2 cells, the migration, maturation and activation DC and antigen presentation. IgE and antigen-IgE complexes can cross the epithelium by transcytosis mediated by CD23 on airway epithelial cells (1), allowing them to bind to and activate FcεRI on mast cells and DC. This process contributes to the perpetuation of allergic inflammation and, potentially, through promotion of IL-4 and/or IL-13 secretion by mast cells (2) and effects of activated mast cells on DC (3), to additional local IgE CSR and IgE production in B cells, either to additional epitopes of the original antigen (shown here) or to new antigens bound by DC (square blue symbols). Antigen presentation mediated by binding of antigen-IgE complexes to CD23 on B cells, followed by antigen presentation by these B cells to cognate T cells (not shown here), is called FAP (4), a process that can result in epitope spreading, with production of IgE recognising new epitopes of the original antigen (for example, epitope B, shown here) or to epitopes of new antigens, if some IgE antibodies to that antigen already exist (not shown), and the subsequent exacerbation of allergic disorders. FcεRI αγ trimers on other APCs (for example, DC, monocytes and macrophages) permit these cells to bind and internalise IgE bound to complex antigens; epitopes derived from such antigens, including those comprising epitopes for which there is not yet a specific IgE response, are then presented to cognate T cells, which, in the presence of IL-4 and/or IL-13, can become Th2 cells that in turn promote the production of IgE against these new epitopes by B cells (5). ICOS, inducible T cell co-stimulator; ICOSL, ICOS ligand, BCR, B cell receptor.

Microbial exposure affecting childhood allergy involves the 'hygiene hypothesis' and 'early immune challenge hypothesis' ^(35, 36). The 'hygiene hypothesis' is an inverse relationship where reduced exposure to micro-organisms and parasites results in increasing prevalence of atopic disorders and autoimmune diseases, mostly in industrialised countries ^(36, 37). 'Early immune challenge hypothesis' involves a lack of appropriate immune stimulation during early childhood, resulting in disturbed alignment in the sequence of encountering antigens that influence the rise of atopy and autoimmune disease ⁽³⁷⁾. Moreover, 'early immune challenge hypothesis' emphasises the central role of training Tregs through sufficient microbial exposure that might result in abnormal immune regulatory development ⁽³⁷⁾. The 'hygiene hypothesis' may explain why children of high SES are more susceptible to allergic disorders as compared to children from lower SES. This theory suggests that a 'clean' and more microbial-free environment in early perinatal life causes Th cell phenotypes to deviate from Th1 towards Th2 dominance. Nevertheless, the 'hygiene hypothesis' does not coincide with the epidemiology of higher prevalence of childhood asthma in lower SES communities, which may be explained by cases of non-allergic asthma or other additional environmental factors ^(35, 36). A human study reported that *in utero* exposure to an environment with a high microbial burden was associated with changes in expression of innate immune genes, reduced risk of allergic disease of offspring and modification of allergen-specific IgE level in cord blood (CB) ⁽³⁾. These hypotheses explain the pathogenesis of childhood allergy development through immune activation involving microbial exposure initiated by early exposure of the fetus and neonate to the environment.

1.2.1.3 Treatment of allergy

Interventions during early life have a higher chance to affect the natural history of respiratory allergy as paediatric immune responses are more adaptable and, therefore, may respond better to treatment ⁽³⁸⁾. A study revealed that 48 % of children with persistent wheezing (the hallmark symptom of childhood asthma) and positive SPT had symptoms of AR, but none of AR symptoms in children with early-transient wheezing ⁽³⁹⁾. Another study reported that early usage of inhaled fluticasone propionate for wheezing in preschool children had no effect on the natural history of asthma or wheeze later in childhood, and did not prevent lung function decline or reduce airway reactivity ⁽⁴⁰⁾. There is compelling evidence of a link between AR and asthma and for the association of childhood AR and asthma later in life ⁽³⁸⁾. The hypothesis is that AR may itself be an asthma risk factor. Therefore, asthma burden in later life may be reduced by more focused treatment of AR in early life because the mechanism of AR usually underlies the clinical syndrome of asthma ⁽³⁸⁾.

Current therapies can control allergic symptoms, but are not a cure for allergic diseases. Current drugs, such as β_2 agonist inhalers, antihistamines and adrenaline, are used for allergy treatment and act on

Th2-immune responses to inhibit the allergic disease ⁽²⁴⁾. Recent findings indicate other immune responses are involved in allergic disease, including Th17-cells, Th1-type cytokines and innate immune system, suggesting a promising therapeutic role of new agents that can block the action of these specific cytokines to improve the management of allergy and asthma. Specific immunotherapy to desensitise patients to allergens has been used for many years. However, risks of allergic reactions, including anaphylaxis, can occur as a consequence of this therapy and result in a life threatening situation for the patient ⁽²⁴⁾. Other therapies being developed include targeting intrinsic structural defects, such as in the bronchial epithelium ⁽²⁵⁾. Nevertheless, early preventative strategies targeting allergic disease in childhood are likely to be the most effective way of reducing the overall burden of allergic disease.

Allergen specific immunotherapy (ASIT) is defined as administration of increasing amounts of specific allergens to which the patient has Type I immediate hypersensitivity. It is a disease modifying therapy used for treatment of AR, allergic asthma and hymenoptera hypersensitivity ^(41, 42). Indications for ASIT include (1) inadequate symptom control despite pharmacotherapy and avoidance measures, (2) a desire to reduce morbidity from AR and/or asthma or reduce risk of anaphylaxis from a future exposure, (3) when the patient experiences undesirable side effects from pharmacotherapy, and (4) when avoidance is not possible. Moreover, ASIT is cost effective compared with pharmacotherapy over duration of time ⁽⁴¹⁾. In AR and asthma therapy, the mode of ASIT administration is via the subcutaneous route by the physician or via sublingual route and oral route by patient ⁽⁴²⁾. Recent studies reported that immunotherapy in children with AR appears to prevent development of new allergic sensitisations and/or new-onset asthma ^(38, 41). Humoral, cellular and tissue level changes occur with ASIT, including large increases in anti-allergen IgG antibodies, a decrease in post seasonal rise of anti-allergen IgE antibodies, reduced numbers of nasal mucosal mast cells and eosinophils, induction of Tregs and suppression of Th2 more than Th1 lymphocytes. There is a corresponding increase in IL-10 and TGF- β ⁽⁴¹⁾. These recent studies suggest ASIT remains an important disease modifying therapy in patients with allergic disease.

1.2.2 Asthma

1.2.2.1 Definition and prevalence

Asthma is a complex disease comprising of atopic and non-atopic forms. There are many definitions of asthma according to components of airway diseases or a disease entity and concept ^(26, 43). Amongst all, asthma is better known as chronic inflammation of the conducting airways associated with airway hyperresponsiveness (AHR), manifested by cough, wheeze and breathlessness, particularly at night or early morning, and reversible airway obstruction, either spontaneously, with treatment, or in response to environmental triggers ^(26, 43). Airway inflammation induced by allergen exposure and most non-allergic asthma is predominantly eosinophilic. This progressive process of chronic bronchial inflammation is known as allergic asthma. Allergic asthma is characterised as a form of chronic lung inflammation by IgE-mediated hypersensitivity of bronchi to aero-allergens, with increased mucus secretion ⁽⁴⁴⁾. Childhood asthma is a heterogeneous, complex disease and pathogenesis involves alteration in airway structure and airway inflammation in early childhood, usually associated with other allergic diseases. However, not all childhood asthma is associated with allergy and non-atopic asthma may have a different natural history to allergic asthma ⁽²⁶⁾. An estimated half of all asthma cases are diagnosed by three years of age and two-thirds are diagnosed by five years ⁽⁴⁵⁾. Therefore, identification of high-risk groups for developing childhood asthma is important and identifiable by recognising the predisposing factors.

1.2.2.2 Mechanism of asthma

The mechanism of asthma is not fully understood and there are two prominent theories. One theory is that AHR leads to airway remodelling, while the other suggests that airway remodelling leads to AHR ⁽⁴⁴⁾. Both of these events may be interchangeable or even occurring continuously depending on the asthmatic event precursor. Bronchi of the lower airways in asthmatic patients are particularly hypersensitive to nonspecific stimuli, such as histamine, methacholine, cold air, perfumes, cigarette smoke and other non-specific stimuli in the air, which is referred to as AHR ⁽⁴⁶⁾. Airway hyperresponsiveness is a hallmark of asthma. It stimulates mucous hypersecretion and increases the influx of inflammatory cells which ultimately lead to airway obstruction ^(44, 47). Furthermore, thickening of bronchial wall and airway mucosa leads to narrowing of the luminal airways. Simultaneously, airway remodelling occurs. This is characterised by chronic inflammation with cellular and irreversible structural changes that include subepithelial fibrosis, increased smooth muscle mass, submucosal

gland enlargement, goblet cell hyperplasia, revascularisation and early thickening of the reticular basement membrane. All of these structural changes over time lead to thickening of smooth muscle in airway walls, which facilitates airway constriction and, consequently, airflow restriction ⁽⁴⁸⁾. Structural remodelling of bronchi accompanied by chronic inflammatory infiltrates contributes to long term narrowing of airways and underlies the major disease symptoms, such as shortness of breath, cough and sputum, in severe asthma ^(44, 48).

Airway inflammation in asthma is dominated by Th2 lymphocytes (CD4), IgE, mast cells, macrophages and cytokines, with eosinophilic infiltration being the most prominent feature. Furthermore, the disease also involves local epithelial, mesenchyma, vascular and neurologic events that influence the spread of Th2 phenotype to the lungs and via aberrant injury-repair mechanisms to remodelling of the airway wall ⁽⁴⁹⁾. In severe cases of asthma, there also can be infiltration of neutrophils into the airway walls ^(44, 47). Initially, the inflammatory process is mostly restricted to the conducting airways, but as severity of the disease increases and acute disease becomes chronic the inflammatory infiltrate spreads more extensively to the small airways and adjacent alveoli. The inflammatory response in the small airways predominantly occurs outside the airway smooth muscle, whereas inflammation of the submucosa dominates in the large airways ⁽⁴⁹⁾.

1.2.2.3 Treatment of asthma

Interventions in early life for prevention of asthma may involve allergen avoidance ⁽⁵⁰⁾. In real-life circumstances, it is not possible to reduce allergen exposure to a clinically relevant extent. Interestingly, there is contradicting evidence on whether exposure to allergens increases the risk of developing asthma. An earlier study documented that strict allergen avoidance in infancy in high risk children reduces development of allergic sensitisation to house dust mite and may prevent some cases of childhood asthma ⁽⁵⁰⁾. However, more recent meta-analysis studies indicate that early exposure to a specific allergen, including house dust mite allergen and cow milk protein (CMP), will not increase asthma susceptibility, but through induction of tolerance will decrease risk of asthma development ⁽⁵¹⁾.

Current therapies are intended for symptomatic relief by acting on Th2-type response and not on modulating immunological dysregulation in allergic diseases ⁽²⁵⁾. There are two major therapies for pharmacological treatment of asthma: preventer and reliever drugs. Preventer drugs include anti-inflammatory drugs, such as inhaled corticosteroids (ICS) and systemic steroids. Examples of commonly used ICS are beclomethasone, budesonide, fluticasone and ciclesonide. Reliever or bronchodilator drugs include beta2-agonists drugs that can be either short-acting, such as albuterol and metaproterenol, or long-acting beta2-agonist (LABA), such as formoterol. Generally,

bronchodilators drugs dilate the airway by relieving and preventing obstruction, thus are effective in reducing symptoms in asthma patients ⁽²⁵⁾.

In the past decade, two novel classes of controller medications were introduced. The first group is leukotriene receptor antagonists that include zafirlukast, montelukast and zileuton. The second group is an anti-IgE monoclonal antibody, omalizumab ⁽²⁵⁾. Omalizumab neutralises the circulating free IgE by preventing activation of IgE receptors on mast cells, basophils and DC binding the Fc portion of IgE. This leads to reduction in the quantity of cell-bound IgE, downregulation of high-affinity IgE receptors, and ultimately, prevention of mediator release from effector cells. Omalizumab is the drug of choice for patients older than 12 years with moderate to severe allergic asthma and is effective in adults with uncontrolled, persistent asthma ⁽⁵²⁾.

1.2.3 Atopic dermatitis

1.2.3.1 Definition and prevalence

Atopic dermatitis (AD) is also known as allergic dermatitis or eczema. AD can be defined as chronic inflammatory skin disease characterised by alterations of the epidermal barrier function and an IgE-mediated sensitisation to food and environmental allergens ^(22, 53, 54). AD is a clinical diagnosis and the most frequently used criteria are those of Hanifin and Rajka ^(54, 55). However, the most acceptable criterion and easiest to perform in daily clinical practice is the UK diagnostic criteria, a refinement of Hanifin and Rajka's criteria, that consists of one mandatory and five major criteria (Table 1) ⁽⁵⁵⁾.

AD is often the first atopic symptom to manifest in early in life and is commonly diagnosed in children under five years of age. These children can subsequently develop FA, asthma and AR ^(56, 57). AD affects over 20 % of children worldwide, especially in developed countries, and lifestyle is recognised as a major risk factor ⁽⁵⁸⁾. Thirty-nine percent of Caucasian children developed AD by the age of three in infants with at least one atopic parent ⁽⁵⁹⁾. Moreover in Korea, child prevalence of AD is 14.4 % and its prevalence in preschool children is significantly higher than in elementary school children ⁽⁶⁰⁾. Hypotheses proposed to explain the rise in AD prevalence include modulation of immune priming through environmental exposures, gut microbial diversity, exposure to pollution, climate change, dietary intake by mother and children, and altered expression of the filaggrin skin barrier gene ⁽⁶¹⁾. Furthermore, it is suggested that AD may be primarily caused by deterioration of the human epidermal differentiation complex (EDC) associated with altered expression of genes within ⁽⁶²⁾.

Table 1. UK diagnostic criteria for atopic dermatitis ⁽⁵⁵⁾.

UK diagnostic criteria for atopic dermatitis
Child must have itchy skin conditions in the past 12 months PLUS three or more of: History of involvement of skin creases Personal history of asthma or hayfever* History of generally dry skin in the past year Visible flexural dermatitis[#] Onset below 2 years old **
* history of atopic disease in 1st degree relative if age < 4 years. [#]as defined by photographic protocol. **not used in children < 4 years old.

Risk factors for AD may differ in different age groups of children ⁽⁶⁰⁾. In a recent study by Baek *et al.* 2013; the prevalence, burden, and risk factors of AD were surveyed among different areas and age groups in 8750 Korean children. From this study, one factor that was associated with AD in preschool children was exposure to pets in the home environment ⁽⁶⁰⁾. However, in older children; female gender, passive smoking; breastfeeding; changing the parents' house to a newly built house during the first year of life; diagnosis of asthma, and diagnosis of AR were positively associated with AD. Family history of atopic diseases, diagnosis of allergic conjunctivitis, and FA were positively associated with AD in both preschool and elementary school children. This study showed that the risk factors for AD are different in preschool and elementary school children ⁽⁶⁰⁾.

1.2.3.2 Mechanism of atopic dermatitis

Immunopathology of AD involves innate and adaptive immunity. The cutaneous innate immune system is comprised of three major components:

- 1) The physical barrier, which includes the stratum corneum and intracellular junctions
- 2) The cellular component (APC, keratinocytes, mast cells, and neutrophils)
- 3) Secretory elements (antimicrobial peptides, cytokines, and chemokines) ⁽⁶³⁾

In patients with AD, initial exposure to allergens (sensitisation phase) induces a systemic 'allergic' Th2 cell response that is amplified with each subsequent exposure (effector phase). Essential features of the Th2 immune response include local and bone marrow production of Th2 cytokines (IL-4, IL-5, and IL-13), prolonged survival and activation of eosinophils and mast cells, and production of allergen-specific IgE ^(64, 65).

Filaggrin (protein for *FLG* gene) is a major structural protein in the stratum corneum of the epidermis that facilitates terminal differentiation of the epidermis and formation of the skin barrier ^(65, 66). In patients with AD, filaggrin expression is downregulated due to increased levels of the Th2 cytokines IL-4 and IL-13, regardless of human *FLG* genotype. Furthermore, filaggrin expression is significantly downregulated because of differentiation of keratinocytes in the presence of IL-4 and IL-13. These findings support the theory that many patients with AD have a filaggrin deficiency caused by Th2 cytokine milieu ^(65, 67). Mutations of the *FLG* gene are responsible for the skin barrier defect in AD and are the most significant known genetic risk factor for AD development ⁽⁶⁵⁾. Moreover, mutations in human *FLG* increase susceptible risk for associated allergic diseases of FA, asthma and AR ⁽⁶⁵⁾.

In AD skin, the protective physical barrier, which includes stratum corneum and epidermis, is significantly compromised, resulting in increased allergen sensitisation, as well as viral and bacterial

infections ⁽⁵⁴⁾. Skin barrier dysfunction causes increased penetration of allergens and human pathogens which can augment the inflammatory response and further exacerbate the atopic disease ⁽⁵³⁾. There are specific pathways linking epidermal barrier disruption and allergen sensitisation ^(64, 65). An established hypothesis explains that epidermal disruption facilitates skin-resident APC (Langerhans and DC) in capturing environmental allergens due to either more direct exposure through a defective stratum corneum or direct effects on Langerhans cell activation. Moreover, barrier-disrupted keratinocytes release immune adjuvants that activate and cause maturation of APC and affect their ability to direct native Th polarisation, thereby influencing the character of the Th response. All these adaptive immune responses lead to further disruption of the barrier function ^(64, 65).

1.2.3.3 Treatment of atopic dermatitis

At present, treatment for AD is targeted towards symptom control, such as anti-histamines, rather than cure, and there is a strong need to identify new methods of disease prevention. Although earlier approaches focused on allergen avoidance strategies, there have been attempts to induce tolerance and enhancement of skin barrier function, as skin barrier breakdown plays an important role in AD development ⁽⁵⁸⁾. Patients with AD have higher risk of irritant and allergic contact dermatitis precipitated by topical exposures ⁽⁶⁸⁾. Therefore, careful consideration must be made in choosing skin products with a low risk of sensitisation or hypoallergenic.

According to the American Academy of Dermatology (AAD), major recommendations for paediatric and adult patients with AD of all severities include:

- 1) Application of nonpharmacological moisturisers such as aqueous cream, is an integral component in management of patients with AD, resulting in reduced disease severity and decreased use of pharmacologic interventions ⁽⁶⁸⁾
- 2) Topical corticosteroids (TCSs), such as hydrocortisone cream, are an appropriate treatment of AD not controlled by moisturisers
- 3) Topical calcineurin inhibitors (TCIs) and anti-inflammatory agents, such as acrolimus ointment and pimecrolimus cream, are an important class of topical medication allowing for steroid-sparing interventions after achieving initial disease control with TCSs. The data suggest that proactive regular, intermittent use of TCIs is additionally effective in maintaining disease remission
- 4) Bleach baths and intranasal mupirocin can decrease disease severity in those with moderate to severe AD and clinical signs of secondary bacterial infection ⁽⁶⁸⁾.

1.2.4 Allergic rhinitis

1.2.4.1 Definition and prevalence

Allergic rhinitis (AR) is an inflammatory IgE-mediated disease characterised by nasal congestion, rhinorrhea (nasal drainage), sneezing and/or nasal itching. It can be defined as inflammation of the inside lining of the nose that occurs when a person inhales something to which he/she is allergic, such as animal dander or pollen ⁽⁶⁹⁾. Symptoms of AR include sneezing, stuffy nose, runny nose, post nasal drip and itchy nose. Classification of AR may be based on: (1) temporal pattern of exposure to a triggering allergen, such as seasonal (eg, pollens), perennial/year-round (eg, dust mites), or episodic (environmental from exposures not normally encountered in the patient's environment, eg, visiting a home with pets), (2) frequency of symptoms, and (3) severity of symptoms. Classifying AR in this manner may assist in choosing the most appropriate treatment strategies for an individual patient (Table 2) ⁽⁶⁹⁾.

AR is a health problem that occurs worldwide in children, adolescents and adults and generates a significant healthcare burden ⁽³⁰⁾. Currently, AR affects up to 40 % of the global population and is one of the most common diseases affecting adults ⁽⁶⁹⁾. Recent epidemiological studies indicate that AR prevalence has increased progressively over the last three decades in industrialised, developed countries ⁽³⁰⁾. Although usually not life-threatening, AR symptoms are frequently bothersome, adversely affecting work and quality of life of affected patients, thus causing a significant burden on the individual and society. Symptoms can cause physical and mental complications, with sleep-disordered breathing in childhood and adolescence being associated with disorders in learning performance, behaviour and attention ⁽³⁰⁾.

1.2.4.2 Mechanism of allergic rhinitis

The exact mechanism of AR development and the precise way in which nasal mucosa responds to allergens is unknown. However, a few studies give some insight into how nasal mucosa plays a role in AR pathophysiology ^(70, 71). Nasal mucus is the first line of defence against pathogens and is challenged by various allergens and pathogens. A proteomics study investigated the role of nasal mucus in the pathophysiology of AR in 29 AR and non-AR subjects. Findings revealed that mucus from AR patients had an enhanced immune response and reduction in antiprotease activity ⁽⁷⁰⁾. Apolipoproteins in mucus may provide a favourable environment for cellular and humoral immune reactions, maintaining an inflammatory state of the mucosa. Proteins in healthy control subjects had protective and anti-

Table 2. Abbreviations and definition of common terms used in allergic rhinitis ⁽⁶⁹⁾.

Term	Definition
Allergic rhinitis (AR)	Disease caused by an IgE-mediated inflammatory response of the nasal mucous membranes after exposure to inhaled allergens. Symptoms include rhinorrhea (anterior or posterior nasal drainage), nasal congestion; nasal itching and sneezing.
Seasonal allergic rhinitis (SAR)	Disease caused by an IgE-mediated inflammatory response to seasonal aeroallergens. The length of seasonal exposure to these allergens is dependent on geographic location and climatic conditions.
Perennial allergic rhinitis (PAR)	Disease caused by an IgE-mediated inflammatory response to year-round environmental aeroallergens. These may include dust mites, mould, animal allergens, or certain occupational allergens.
Intermittent allergic rhinitis	Disease caused by an IgE-mediated inflammatory response and characterised by frequency of exposure or symptoms (<4 days per week or <4 weeks per year).
Persistent allergic rhinitis	Disease caused by an IgE-mediated inflammatory response and characterised by persistent symptoms (>4 days per week and >4 weeks per year).
Episodic allergic rhinitis	Disease caused by an IgE-mediated inflammatory response that can occur if an individual is in contact with an exposure that is not normally a part of the individual's environment (ie, a cat at a friend's house).

inflammatory effects promoting mucus and mucosal homeostasis. Moreover, an imbalance between cysteine proteases and antiproteases negatively influenced epithelial integrity on exposure to pollen protease activity. These findings suggest the important role of mucus as the first line defence barrier against allergens ⁽⁷⁰⁾.

A study examined recovery of Der p 1 (house dust mite) allergen following nasal administration and whether Der p 1 can be detected in nasal biopsies after natural exposure and nasal challenge to the allergen ⁽⁷¹⁾. Data shows that Der p 1 allergen was detected in nasal tissue regardless of atopic status after natural exposure. After challenge, allergen was still retained in the nose, which remains mucosally associated. There was greater Der p 1 deposition and inflammatory response in atopic than in non-atopic individuals. These findings support the hypothesis that nasal mucus and tissue act as a reservoir for the inhaled Der p 1 allergen leading to a persistent allergic inflammatory response in atopic individuals ⁽⁷¹⁾.

1.2.4.3 Treatment of allergic rhinitis

Appropriate treatment strategies are issued for adults and children that consist of patient education, allergen avoidance and pharmacological treatment based on duration and severity of individual AR disease ⁽³⁰⁾. Allergen avoidance that includes substantial reductions in house dust mite load may offer some benefit in reducing AR symptoms ^(38, 72). There are also evidence-based recommendations for diagnosis and therapy of paediatric rhinitis. Oral and intranasal antihistamines and, more effectively, nasal corticosteroids are preferred administered once daily and, given their improved safety profile, are appropriate first line AR treatment in paediatrics patients ⁽³⁸⁾. Recently, add-on therapies for AR include oral leukotriene receptor antagonists, short bursts of a nasal decongestant and saline douches, also nasal anticholinergic may be potentially useful in children ⁽⁷³⁾. Despite varying types of AR treatment, pharmacological medications remain the preferable solution to relieve severe AR symptoms.

Although current paediatric AR treatments, including non-sedating antihistamines, topical nasal glucocorticosteroids and an oral leukotriene receptor antagonist (LTRA), are effective in alleviating respiratory allergy symptoms, these treatments have not been proven to influence disease progression ^(38, 74). According to the Practical Guideline for the Management of Allergic Rhinitis in Japan, and the drugs listed for paediatric patients, other therapeutic agents for AR include additional mast cell stabiliser, chemical mediator receptor antagonists (leukotriene receptor antagonists and PGD₂) and Th2 cytokine inhibitor ⁽⁷⁵⁾.

1.2.5 Food allergy

1.2.5.1 Definition and prevalence

Food allergy (FA) is defined as an immunologically mediated hypersensitivity reaction to any food, including IgE-mediated and/or non IgE-mediated allergic reactions ⁽²²⁾. After an allergic child ingests a specific food allergen, the reaction can result in clinical symptoms ranging from mild hives to life-threatening anaphylaxis ⁽⁷⁶⁾. Sensitisation in FA may occur directly to a food allergen or indirectly through cross-reactivity with an aeroallergen ⁽⁷⁷⁾. Food allergy starts early in life with greater severity and persistence into later ages. Prevalence of FA has increased in the last 10 years, resulting in an increased incidence of anaphylaxis cases ⁽⁷⁷⁾. In Australia, challenge-proven IgE-mediated FA now affects up to 10 % of infants ⁽⁷⁸⁾. Over 90 % of FA in childhood is caused predominantly by cow milk protein allergy (CMPA) and hen egg (HE), whereas the remaining causes of FA are soy, peanuts, tree nuts (TN), wheat, fish and shellfish ^(79, 80).

Cow milk protein allergy is defined as an immunologically mediated hypersensitivity reaction to cow milk, including IgE-mediated and/or non IgE-mediated allergic reactions ⁽²²⁾. The natural history of CMPA shows heterogeneity, closely related to immunological and clinical phenotype. Children with non-IgE-mediated CMPA tend to develop tolerance at an earlier age and at a higher percentage compared to those with IgE-mediated disease. A majority of children develop tolerance against CMPA, yet individual timing of tolerance occurrence is not known. Nevertheless, some children experience severe CMPA symptoms that may persist for a lifetime ⁽⁸¹⁾.

1.2.5.2 Mechanism of food allergy

The physiology of gut barrier via the paracellular space regulates entry of molecules and microorganisms into blood circulation, and thus influences the ability to trigger a systemic immune response ⁽⁸²⁾. In normal conditions, the small intestine differentially allows absorption of small molecules while excluding larger ones. This selective permeability function depends on immunologic and non-immunologic factors including mucin, the microvilli, Ig(s) and intraepithelial lymphocytes. Alteration of intestinal permeability (IP) is hypothesised to play a key role in the pathophysiologic mechanisms of many intestinal diseases, including inflammatory bowel disease, infectious gastroenteritis, AD and FA ⁽⁸²⁾. In IgE-mediated FA, IgE sensitisation and recruitment of mast cells commonly occur in the gastrointestinal mucosa (Figure 2) ^(82, 83).

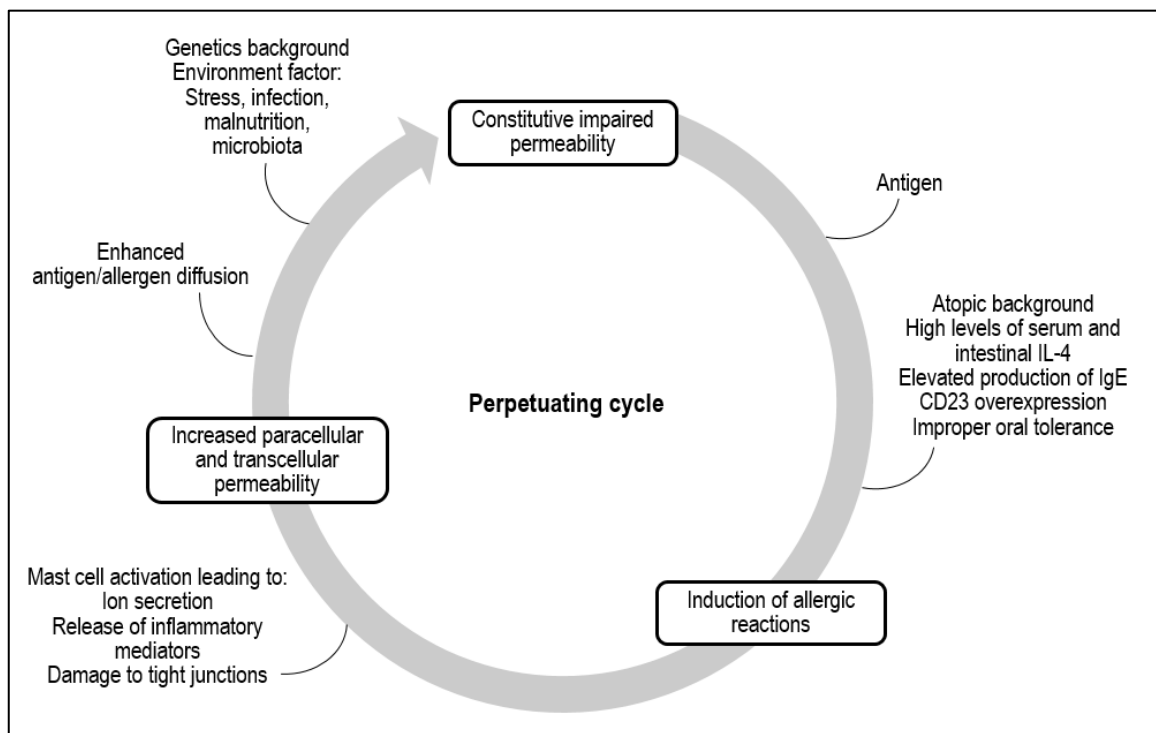


Figure 2. Intestinal permeability and allergy: cause and/or consequence (figure adapted from ⁽⁸³⁾). Poorly defined factors associated with genetic background or environment may serve as priming causes to favour antigen entry and elicit production of antigen-specific IgE in predisposed individuals. This constitutive passage in turn promotes onset and amplification of allergy-related reactions culminating in activation of mast cells and release of mast cell mediators with pro-inflammatory properties capable of negatively impacting epithelial barrier function. Further damage triggers uncontrolled passage of more antigen, now considered an allergen, leading to perpetuation of the cycle of allergen entry–IgE binding–mast cell degranulation–increased epithelial permeability.

Pre-sensitised mast cells can then be triggered by allergens in the lamina propria. During an allergic reaction, a series of inflammatory mediators, such as proteases and cytokines, are released and further affect IP (Figure 2). This leads to an increase in the passage of transepithelial allergens and perpetuates the inflammatory reaction. Furthermore, IgE-CD23-mediated allergen transport across the mucosa may play an important role in the perpetuation of sensitisation ^(82, 83). Children with FA have shown increased small IP following ingestion of the offending food as well as during elimination diets ⁽⁸²⁻⁸⁴⁾. Moreover, IP correlates negatively with age in control children and is altered in children with CMPA. When there are digestive manifestations, the IP test is most accurate in CMPA diagnosis when performed at six to 12 months of age ⁽⁸⁴⁾.

A study investigated IP in asymptomatic FA children during an elimination diet to identify clinical characteristics associated with altered IP ⁽⁸²⁾. Intestinal permeability was assessed by direct measurement of urinary clearance of two non-metabolised sugar molecules, lactulose and mannitol. Urinary recovery ratios of lactulose and mannitol (L/M) were determined 5 h following ingestion of 7.5 g of lactulose and 2 g of mannitol in 131 CMPA and HE allergic children. A total of 50 (38 %) of 131 children with FA had elevated IP while asymptomatic on strict elimination diets. Elevated IP was found in about one-third of asymptomatic food allergic children on elimination diets and was associated with shorter stature. Findings suggest that increased IP may be an intrinsic trait in a subset of food allergic children, thus IP may be an associated mechanism of FA. However, the study was not able to determine whether elevated IP is a primary defect or a consequence of long-lasting allergic insult on the intestinal mucosa in children with FA ⁽⁸²⁾.

1.2.5.3 Treatment of food allergy

Management of FA is limited to strict food allergen avoidance together with education on recognition and management of emergency allergic reactions and in some cases includes self-injectable adrenaline. Recent definitive treatment, such as oral immunotherapy, can induce desensitisation and modulate allergen-specific immune responses ⁽⁷⁷⁾. Oral immunotherapy may increase the threshold of reactivity of allergic patients, thereby improving quality of life. In single-nut-allergic patients, introduction of other nuts allows broadening of the diet, thus reducing the psychological impact of allergen avoidance ⁽⁷⁷⁾. Nevertheless, oral immunotherapy needs full supervision and participation from physicians, patients and their family to ensure FA patients benefit from this treatment.

Strategies to prevent FA include: (1) primary prevention strategies which seek to prevent onset of IgE sensitisation, (2) secondary prevention which seeks to interrupt development of FA in IgE-sensitised children, and (3) tertiary prevention which seeks to reduce expression of end-organ allergic disease in

children with established FA ⁽⁸⁵⁾. Most children are more likely to outgrow milk, egg or wheat allergy, while the majority of children will not outgrow an allergy to peanut or a TN ^(85, 86). Different approaches in management of milk and HE allergy have emerged with specific oral tolerance induction (SOTI).

Cow milk protein allergy has generally good prognosis and CMP can eventually be reintroduced in the patient diet ⁽⁸¹⁾. Current guidelines on diagnosis and management of CMPA mostly suggest re-evaluation of milk-allergic children every six to 12 months, and reintroduction of cow milk after a negative oral food challenge (OFC) ⁽⁸¹⁾. Measurements of serum IgE levels and SPT wheal sizes to crude (whole), CMP and individual milk protein components may provide useful prognostic information in the course of CMPA. Furthermore, introduction of heated milk and HE protein into the diet of allergic patients may induce tolerance development in CMPA ⁽⁸¹⁾. Therefore, a majority of infants with CMPA have many alternative interventions that may desensitise them to allergic foods.

1.2.6 Anaphylaxis

1.2.6.1 Definition and prevalence

Anaphylaxis, the most severe presentation of Type I allergy, is defined as a severe allergic reaction with rapid onset that may cause death ^(87, 88). Anaphylaxis involves an acute systemic hypersensitivity reaction affecting several organ systems, particularly the skin, respiratory; gastrointestinal and cardiovascular systems. Anaphylactic reaction severity varies and is classified into severity grades I to IV ⁽⁸⁹⁾. Prevalence of anaphylaxis is four to 50 cases per 100 000 people per year, equivalent to 0.05 % to 2 % of the global population. Moreover, anaphylaxis prevalence is increasing worldwide with a high incidence in children, especially in developed countries ⁽⁹⁰⁾. A rise in anaphylaxis prevalence in children suggests association between development of anaphylaxis and exposure to *in utero* or early life factors that increase anaphylaxis risk.

Food allergy is the main cause of anaphylaxis in children, particularly allergy to peanut and TN, then insect stings and drugs in later adult life. Presence of co-existing asthma increases the risk of severe reactions in FA children, possibly due to underlying eosinophilic inflammation of the airway ⁽⁷⁷⁾. Most food-induced anaphylactic reactions occur after ingestion of the allergic food and only a few cases are induced by inhalation of foods, such as peanut, soybean and lupine ⁽⁹¹⁾. In a large study of childhood anaphylaxis in Australia, de Silva *et al.* 2008 investigated demographic characteristics, clinical features, causative agents, settings and administered therapy involving 123 children presenting with anaphylaxis over a five year period to the Emergency Department at the Royal Children's Hospital, Melbourne ⁽⁹²⁾. Findings demonstrated that median age of presentation was 2.4 years and respiratory features were

the main presenting symptoms of anaphylaxis. Most children presenting to the Emergency Department with anaphylaxis were first-time anaphylactic reactions. Most reactions occurred in home and peanut and cashew nut were the most common causes of anaphylaxis. The authors suggested that triggers for anaphylaxis in children had not changed significantly over the last decade in Melbourne ⁽⁹²⁾. Thus, allergic food avoidance is the most important step and main priority for caregivers, especially in children with asthma.

1.2.6.2 Mechanism of anaphylaxis

Human allergic anaphylactic reaction is classified according to three main mechanisms: immunological Ig-E dependent response and immunological Ig-E independent response, and non-immunological reaction (Figure 3) ^(31, 90, 93). The main and classical pathway of anaphylaxis is commonly mediated through an exaggerated immunologic IgE-dependent mechanism that involves IgE synthesis in response to allergen exposure ^(31, 90). The IgE-dependent response consists of an allergen binding to the Fab portion of IgE, fixed to high-affinity receptors for IgE (FcεRI receptors) on surface membranes of mast cells and basophils. As a result, cross-linking of FcεRI receptors activates many proteins, including protein kinases. This activated phosphorylation cascade leads to an increase in intracellular calcium and exocytosis of granules releasing mediators. Receptor-bound IgE molecule aggregation occurs on re-exposure to the allergen and results in cell activation and mediator release. IgE also contributes to the intensity of anaphylaxis by enhancing expression of FcεRI ⁽³¹⁾.

Activation of the immunologic IgE-independent mechanisms and non-immunologic triggers also play an important role in anaphylaxis (Figure 3). The non-immunologic response induces basophil and mast cell degranulation without Ig involvement. The mechanism of non-immunologic response is still poorly understood, but could be caused by exercise, cold air or water, alcohol, or be opioid-related ⁽⁹⁰⁾. Common triggers differ according to age group ⁽⁹³⁾. Food is the most common trigger in children and adolescents, whereas medications and insect stings are more common in middle-aged and older adults. Although there are different triggers, the mechanism is similar with immune amplification via initiation of the mast cells and basophil that lead to cascade of mediators that include tryptase and histamine. Target organs in anaphylaxis pathogenesis include the skin, respiratory tract, gastrointestinal tract, cardiovascular system and central nervous system. Idiopathic anaphylaxis, currently a diagnosis of exclusion, presents opportunities for identification of previously unrecognised triggers, elucidation of pathophysiologic mechanisms and identification of patients with mastocytosis or clonal mast cell disorders (Figure 3) ⁽³¹⁾.

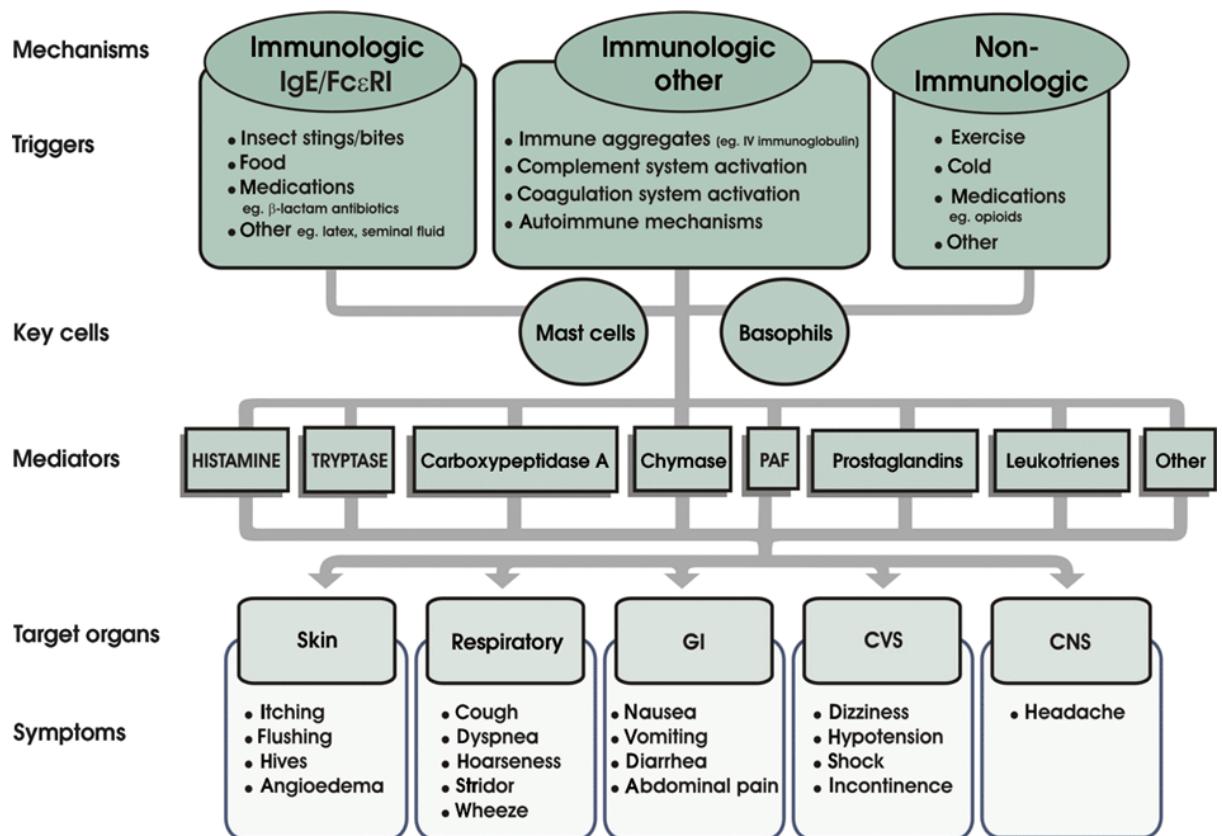


Figure 3. Summary of the pathogenesis of anaphylaxis ⁽⁹³⁾. Other potential triggers include occupational allergens, inhalants, such as horse dander or grass pollen, allergen immunotherapy, vaccines to prevent infectious diseases, hormones, colourants and enzymes. Some triggers may act through more than one mechanism. Individuals with anaphylaxis, by definition, usually have involvement of two or more body systems concurrently; the occasional exceptions are those with isolated hypotension after exposure to a known trigger.

Risk factors for severe or fatal anaphylaxis potentially include very young or old age, pregnancy, co-morbidities, such as asthma, cardiovascular disease and mastocytosis, and concurrent medications, such as β -blockers and angiotensin-converting enzyme inhibitors ⁽³¹⁾. Thus, the classical pathway of anaphylaxis is commonly mediated through an immune IgE-dependent mechanism.

Intensity of anaphylactic reaction is influenced by degree of sensitisation and other concomitant factors. This phenomenon is called augmentation or summation anaphylaxis ⁽⁸⁹⁾. Intrinsic risk factors for anaphylaxis include other associated allergic conditions, simultaneous exposure to other allergens, female gender, age, concomitant diseases, higher SES, geographic location due to vitamin D level variations, types of anaphylaxis symptoms, underlying infection and psychological stress ^(89, 94). Female gender is a risk factor for anaphylaxis reaction to latex, diagnostic agents, muscle relaxants and aspirin. In addition, type of stinging insect species and recent stings leading to mast cell or basophil priming can determine the course of anaphylaxis reactions ⁽⁹⁴⁾. Moreover, extrinsic risk factors include medications, such as beta-blockers, angiotensin converting enzyme inhibitor (ACE inhibitor), angiotensin II receptor blockers (ARBs), acetylsalicylic acid/aspirin (ASA) and non-steroidal anti-inflammatory drug (NSAIDs), as well as alcohol and exercise ^(88, 89, 94). These extrinsic factors are sometimes referred to as cofactors or augmentation factors, or cofactor-induced anaphylaxis ⁽⁸⁸⁾. Other less well-defined factors of patient-related risk factors for anaphylaxis severity and fatality include defects in mediator degradation pathways, fever, menses, emotional stress and disruption of routine ⁽³¹⁾. Thus, intrinsic and extrinsic concomitant factors influence the course and prognosis of anaphylaxis reaction.

1.2.6.3 Treatment of anaphylaxis

Anaphylaxis prevention depends primarily on optimal management of patient-related risk factors, strict avoidance of confirmed relevant allergen or other triggers and, when indicated, immunomodulation. Immediate injection of adrenaline is the current acute management for anaphylaxis reaction and it can prevent mortality ⁽⁷⁷⁾. Patients at risk for anaphylaxis recurrence in the community should be equipped with one or more epinephrine auto-injectors, a written, personalised anaphylaxis emergency action plan and up-to-date medical identification ⁽³¹⁾. Improvements in the design of epinephrine auto-injectors will help optimise ease of use and safety. Randomised controlled trials of pharmacologic agents, such as antihistamines and glucocorticoids, are needed to strengthen the evidence base for treatment of acute anaphylactic episodes ⁽³¹⁾.

1.2.7 Current interventions for allergy prevention

Dietary interventions are currently being trialled to prevent allergy in children and these approaches could be used in early life to prevent onset of inflammation and tissue remodelling observed with allergic disease. Probiotic supplementation has beneficial effects on the development of childhood allergy. Probiotics are live microorganisms which, when administered in adequate amounts as part of food, confer a health benefit on the host ⁽⁹⁵⁾. They can promote adequate balance in gut microbiota, which in turn may prevent development of allergies ⁽⁹⁶⁾. There is evidence that pre- and probiotics during pregnancy and infancy reduce atopic dermatitis (AD) risk, but they have no significant impact on AD severity in established disease ⁽⁵⁸⁾. Following perinatal supplementation of probiotic bacteria to high-risk mothers and children, a study found no allergy-preventive effect for caesarean-delivered children at five years of age ⁽⁹⁷⁾. However, a recent systematic review and meta-analysis of randomised controlled trials (RCTs) by Cuello-Garcia *et al.* reported that probiotics used by pregnant women or breast-feeding mothers and/or given to infants reduced the risk of eczema in infants; however, certainty in the evidence is low ⁽⁹⁶⁾.

A recent consensus was updated to existing guidelines on potential benefits of supporting early, rather than delayed, peanut introduction during the period of complementary food introduction in high-risk infants ⁽⁹⁸⁾. This consensus is a guideline for 12 allergy-related organisations, including the World Allergy Organization, and is based on new RCT evidence from Du Toit *et al.* in the Learning Early About Peanut Allergy (LEAP) trial ⁽⁹⁹⁾. In the trial, 640 high-risk United Kingdom infants between the ages of four and 11 months were randomised to consume peanut products at least three times per week (6 g of peanut protein, equivalent to 24 g peanuts or three teaspoons of peanut butter per week) or to completely avoid peanut products for the first five years of life. This included 542 infants with negative SPT responses to peanut and 98 infants with minimally positive SPT response ⁽⁹⁹⁾. In an intention-to-treat analysis, 17.2 % of infants in the peanut avoidance group, compared with 3.2 % in the peanut consumption group, had food challenge-proved peanut allergy by age five years, corresponding to a 14 % absolute risk reduction and relative risk reduction of 80 %. Thus, this study provides strong evidence that early introduction to peanuts significantly decreases frequency of peanut allergy development among children at high risk for this allergy and modulated immune responses to peanuts. However, the particular difficulty this study raises is how to generalise results from a trial of high-risk infants, which screened infants for the presence of peanut allergy prior to peanut introduction, to the general population ⁽¹⁰⁰⁾.

Early consumption of allergenic food is more beneficial than delayed introduction as a strategy for primary prevention of food allergy, but allergic reactions caused by early introduction are a worrying issue ⁽¹⁰¹⁾. Natsume *et al.* investigated whether or not early stepwise introduction of eggs to 147 Japanese infants with eczema at four to five months of age, combined with optimal eczema treatment, would prevent HE allergy at one year of age ⁽¹⁰¹⁾. In this RCT, 74 infants were assigned to the placebo group and 73 infants to the group that consumed orally 50 mg of heated egg powder per day from six months to nine months of age and 250 mg per day thereafter until 12 months of age. Hen egg allergy was investigated by open oral food challenge (OFC) at 12 months of age. Findings revealed significant difference between the two groups, 8 % of infants had HE allergy in the egg group, as compared to 38 % in the placebo group (risk ratio 0.222) ⁽¹⁰¹⁾. Thus, introduction of heated egg in a stepwise manner along with aggressive eczema treatment is a safe and efficacious way to prevent HE allergy in high-risk infants.

To date, there are no active interventions that prevent asthma, AD, AR, FA and anaphylaxis in the general population, especially in children. Interventions for allergy prevention are urgently required to avoid implications of severe uncontrolled childhood allergic diseases, including fatal anaphylactic shock. Therefore, the objectives of this study are to identify novel proteins that could be measures of allergic risk in placenta and saliva, to develop an assay for detection of allergic risk in newborns and target proteins for interventions that prevent allergy onset.

1.3 Developmental Origins of Allergy

1.3.1 DOHaD hypothesis

The Developmental Origins of Health and Disease (DOHaD) hypothesis, based on human epidemiologic investigations and animal studies, demonstrates that environmental exposures during critical stages of early development can alter gene expression and disease predisposition through epigenetic mechanisms ^(102, 103). The early development period includes periconceptual events and comprises of placental, fetal and maternal factors that play a critical role in determining disease susceptibility via environmental influences on placental function and fetal programming ^(7, 19, 104). Alterations occurring *in utero* during fetal programming may induce permanent changes in many physiological processes and result in chronic disease susceptibility in postnatal life such as type 2 diabetes mellitus (DM), obesity, hypertension, coronary heart disease, chronic lung disease, chronic kidney disease and metabolic and immune function disorders that include allergic diseases ^(7, 19, 103-107).

Extensive evidence from studies of human and animal models that examined the effects of early life pharmacological, surgical and nutritional stimuli, or insults on long term health, support the DOHaD hypothesis ^(17, 104). For example, both maternal undernutrition and over-nutrition may result in permanent harmful long-term effects on the physiology, metabolism and health of offspring ^(7, 17). However, there is currently limited mechanistic data on whether allergic disease is programmed *in utero*. Thus, the focus of this thesis is to determine whether there are protein differences in children who develop an allergy relative to children that do not develop an allergy, and whether these proteins are detectable in the placenta at birth, or in saliva in early life.

1.3.2 Mechanisms underlying developmental origins of allergy

The mechanisms underlying the effects of prenatal events and exposures on the long-term health of offspring are still not fully understood. Various forms of maternal constraint of fetal growth and biological processes, including immediate homeostatic responses, can contribute to the mechanistic basis of DOHaD phenomena ⁽¹⁹⁾. Potential candidate mechanisms proposed involve in allergic diseases susceptibility include permanent changes in organ structure and programmed changes in gene expression through epigenetic modifications ⁽¹⁷⁾. These mechanisms are dependent on each other and are likely to be synergistic interactions. Therefore, understanding the underlying mechanisms via the extent and nature of these processes would enable identification of individuals at risk, such as children

at risk of allergic disease, which would also provide insight into potential preventative and intervention strategies ⁽¹⁷⁾.

1.3.2.1 Permanent structural changes

Permanent changes in organ structure can consequently alter the function of critical organs. It has been suggested that an organ exposed to suboptimal nutrient or hormone levels, where that nutrient or hormone is essential for appropriate development during a critical developmental period, may permanently alter tissue structure and function ^(7, 17, 18). For example, data from small mammals and primates shows that early nutrition may have important long-term effects on blood lipids, plasma insulin, obesity and atherosclerosis, as well as behaviour and learning ⁽¹⁷⁾. Studies conducted on preterm infants revealed that diet consumed in the first month of life had a major impact on subsequent developmental attainment, growth and allergic status in early childhood ⁽¹⁸⁾. Another example is human kidney development which is greatly influenced by early fetal programming. Infants born with LBW and intrauterine growth restriction (IUGR) have a reduced number of nephrons, which could lead to increased risk of chronic hypertension and chronic kidney disease in adult life. Mechanisms involved in permanent structural changes to the kidney are not fully understood, but are suggested to be associated with impaired kidney maturation due to low nephron number, 'endocrine programming' and telomere-limited exhaustion of progenitor pools for cell turnover ⁽¹⁰⁸⁾.

Early origins of lung diseases, including asthma, may be triggered by permanent changes in fetal lung structure *in utero* ⁽¹⁰⁹⁾. Studies conducted in animal models with IUGR, preterm birth with prolonged mechanical ventilation need and maternal tobacco smoke or nicotine exposure suggest impaired alveolarisation with alveolar simplification ^(110, 111). These hypoxic conditions alter alveolar formation via mechanisms that increase elastin expression, disorder and deposit excessive lung elastin associated with decreased expression of vascular endothelial growth factor (VEGF) and its receptor in the lung (VEGFR2) ^(110, 111). Insulin-like growth factor (*IGF*)1, an important gene for alveolar formation, is increased in lungs of babies that died due to bronchopulmonary dysplasia. These babies presented with bronchopulmonary dysplasia pathological features that include increased thickness and cellularity of the mesenchyme where *IGF1* is located, caused by increased *IGF1* mRNA transcript and protein abundance in the lung ⁽¹¹²⁾. Thus, permanent changes in organ structure can consequently alter the function of critical organs, resulting in immediate and long term health consequences.

1.3.2.2 Epigenetic programming of gene expression

Epigenetic programming of gene expression controls genome function at different levels, maintains cellular memory following multiple rounds of cell division and is associated with permanent changes in gene expression ^(17, 104). Gene expression plasticity may confer adaptability to environmental change which can be advantageous, but can also adversely affect developing pathways ⁽¹¹³⁾. Most importantly, epigenetic alteration, such as DNA methylation and histone modification, can be modulated by the environment and is involved in onset of diseases including cancer ^(102, 104, 114). DNA methylation can be classified as global methylation changes and gene-specific methylation changes ⁽¹¹⁵⁾. Global methylation can be non-specifically assessed by studying overall DNA methylation of the genome, or by studying at specific CpG methylation within repeat elements. Studying specific CpG sites associated with genes known to have a functional relationship to certain diseases is the most sensitive quantification of DNA methylation in developmental origins ⁽¹¹⁵⁾.

Maternal exposures *in utero* have the capacity to activate or silence fetal genes through alterations in DNA and histone methylation, histone acetylation and chromatin structure. In response to fetal antigen during pregnancy, the maternal cellular immune system down regulates Th1 IFN- γ cell mediated immunity to Th2 state ^(35, 116). The Th2 state is achieved via multiple mechanisms and amongst them is hypermethylation or gene silencing of the *IFN- γ* gene promoter in CD4+T cells at the materno–fetal interface caused by suppression of fetal IFN- γ production ⁽⁴⁾. Another example is dietary folate, a methyl donor clearly associated with changes in gene expression and disease susceptibility through gene hypermethylation ⁽³⁾. A study conducted on individuals with periconceptional exposure to famine during the Dutch Hunger Winter in 1944 to 1945 found that, 60 years later, there was less DNA methylation of the *IGF2* gene as compared to same gender siblings unexposed to famine. This significant finding of periconceptional exposure not seen in individuals exposed to famine late in gestation identifies that there are critical time points in pregnancy at which DNA methylation can be altered and permanently influence health ⁽¹¹⁷⁾.

The role of transcription factors as potential targets of epigenetic programming through a suboptimal environment was proposed in a previous study ⁽¹⁷⁾. Modulation of transcription factors, as particularly attractive targets of developmental programming, enable modulation of other gene networks. Three key developmental transcription factors shown to be epigenetically programmed in the early environment are: *PPAR α* , *PDX-1* and *HNF4 α* . These genes are modified by DNA methylation and histone modification, with the greatest effect demonstrated by histone modifications ⁽¹⁷⁾. Moreover, increasing methylation in cytochalasin B of a functional CpG site in the *IL-2* promoter was associated with increased likelihood of severe asthma exacerbations ⁽¹¹⁸⁾. Hence, epigenetic programming of gene

expression occurs via the integration pathway of cellular memory and transcription factors *in utero* early environment.

1.4 Development of the immune system, role of the placenta and contributions to allergic susceptibility

1.4.1 Role of the placenta in fetal immune system development

The placenta is a highly specialised fetomaternal organ, acting as a communicating interphase between mother and fetus throughout pregnancy to regulate fetal growth and development. Hence, understanding placental molecular and cellular biology will provide greater insight into the role of this organ in maintenance of fetal development, specifically in relation to immunological function and mechanisms underlying allergic disease susceptibility.

1.4.1.1 Anatomy and physiology of placenta

i. Placentation

Placentation is defined as series of events following embryo implantation leading to placental development. In human, placentation is hemochorial, where maternal blood is in direct contact with the chorion ⁽¹¹⁹⁾. Thus, hemochorial placentation provides efficient transfer of oxygen and nutrients. The process of human placentation involves a sequence of intercellular interactions, starting with motile and non-motile formation of mononuclear and multinuclear trophoblast cells at various rates of proliferation, alteration of adhesive properties and further differentiation by trophoblast populations. Furthermore, mononuclear and multinuclear trophoblast cells undergo progressive local tissue remodelling in interaction with the maternal environment including decidua, smooth muscle, endothelium and blood ⁽¹²⁰⁾. Human placentation commences following proliferation, migration and invasion of extravillous trophoblast into the maternal decidua (Figure 4). Adequate transformation of the uteroplacental vasculature by the extravillous trophoblast is crucial and this occurs by the end of first trimester. Further changes occur around 10-12 weeks of gestation, where cytotrophoblasts in anchoring villi produce extravillous trophoblasts in multi-layered columns, colonising the interstitium of maternal decidua at the inner third of the myometrium ⁽¹²¹⁾.

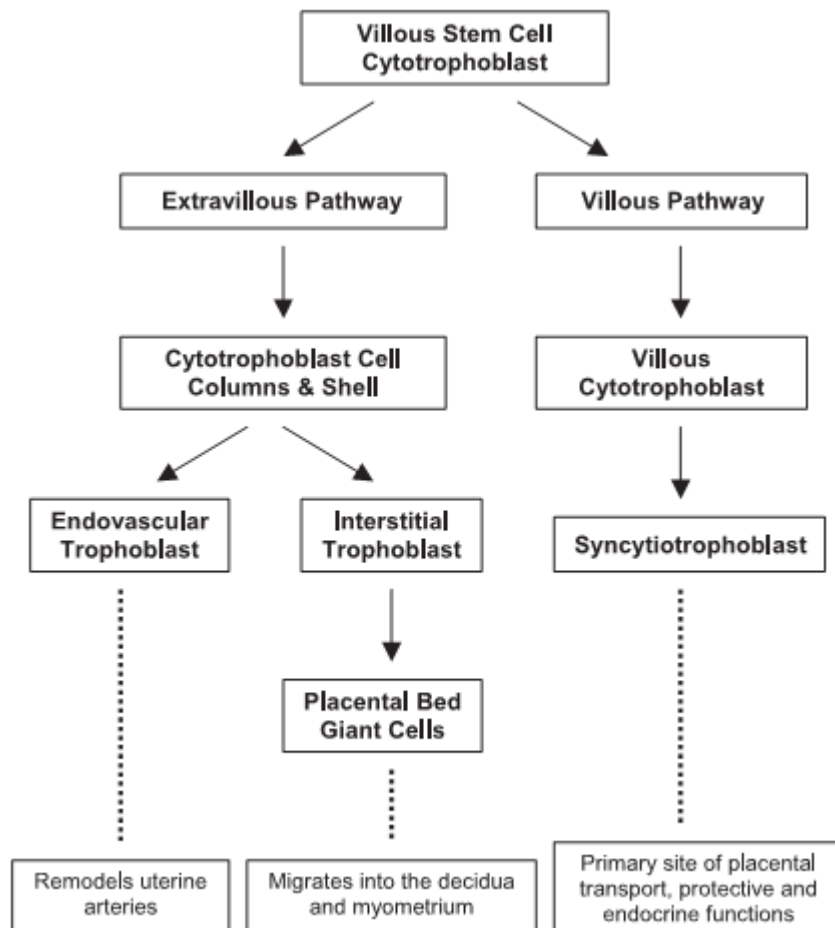


Figure 4. Pathways of trophoblast differentiation and functions ⁽¹⁴⁾. After successful implantation and initiation of placentation, trophoblast cells undergo extensive proliferation and differentiation. There are two main pathways by which trophoblast differentiation may occur, that is, villous and extravillous. By days 13-14 of pregnancy, cytotrophoblast cells penetrate the syncytiotrophoblast layer surrounding the early conceptus to form columns of extravillous cytotrophoblast cells. These contiguous cells form the cytotrophoblastic shell at the interface of fetomaternal compartments. Extravillous trophoblast cells invade the decidua and migrate, penetrating and remodelling maternal blood vessels in the uterine decidua (endovascular trophoblast). Extravillous cytotrophoblast cells also invade interstitially (interstitial trophoblast). These invasive cells promote circumferential expansion of the placental site and recruitment of maternal arterioles allowing subsequent expansion of the placental villous region below. Interstitial trophoblast cells become multinucleated and more rounded, forming placental bed giant cells as they move deeper into the decidua. These cells are the terminally differentiated end-point of the extravillous pathway. Villous (non-migratory) cytotrophoblast cells proliferate, differentiate and fuse to form the outer epithelial layer of chorionic villi, the syncytiotrophoblast. Primary villi are formed by evaginations of syncytiotrophoblasts with a cytotrophoblast core. As gestation progresses, chorionic villi grow and arborise. Exchange takes place in the most part through terminal villi that project into the intervillous space.

ii. Placental components

The two main components of the utero-placental unit are maternal and fetal tissue ⁽¹⁴⁾. Maternal tissue (decidua basalis) originates from the endometrium, while fetal tissue originates from the chorionic sac, which is derived from the blastocyst that formed the fetus (Figures 4 and 5). Fetal tissue in the mature placenta, known as the chorionic plate or chorion frondosum, is the site where fetal chorionic blood vessels are carried via branches of umbilical vessels. The basal plate comprises the maternal placenta component. Intervillous space is located between these two regions and contains intricate branches of villous structure accommodating fetal blood vessels (Figure 5). Multinucleated syncytium, known as syncytiotrophoblasts, line the intervillous space. These closely packed villous structures are the main functional units of placenta and a majority of maternal-fetal exchange occurs at the terminal region (Figure 6). This maternal-fetal exchange is comparable to exchange of oxygenated and deoxygenated blood in the alveolar region of normal adult human lungs. Deoxygenated fetal blood flows via two umbilical arteries that branch to chorionic arteries, leading to an extensive arteriocalillary venous system in the chorionic villi. Vice versa, the fetus receives oxygenated blood from maternal circulation via capillaries from various chorionic veins and a single umbilical vein ⁽¹⁴⁾.

iii. Placental functions: Transfer of respiratory gases and metabolism of nutrients

Exchange of oxygen and carbon dioxide occur via simple or passive diffusion according to concentration and electrical gradients. Transfer of oxygen to the fetus is facilitated by increased affinity of fetal haemoglobin to oxygen, while carbon dioxide transfer is facilitated by higher affinity of maternal haemoglobin to carbon dioxide compared to fetal haemoglobin ⁽¹⁴⁾.

Glucose transportation via facilitated diffusion is essential for normal fetal growth and development. As the placenta is only capable of producing glucose in late gestation, facilitated diffusion is the primary glucose source for fetal oxidative metabolism. Placental amino acid transfer and metabolism to the fetus is crucial, particularly for protein synthesis, to meet demands of the growing fetus. This process occurs via active transport, involving uptake from maternal circulation to microvillous membrane transport via trophoblast cytoplasm and then transport to umbilical circulation across the basal membrane ⁽¹²⁰⁾.

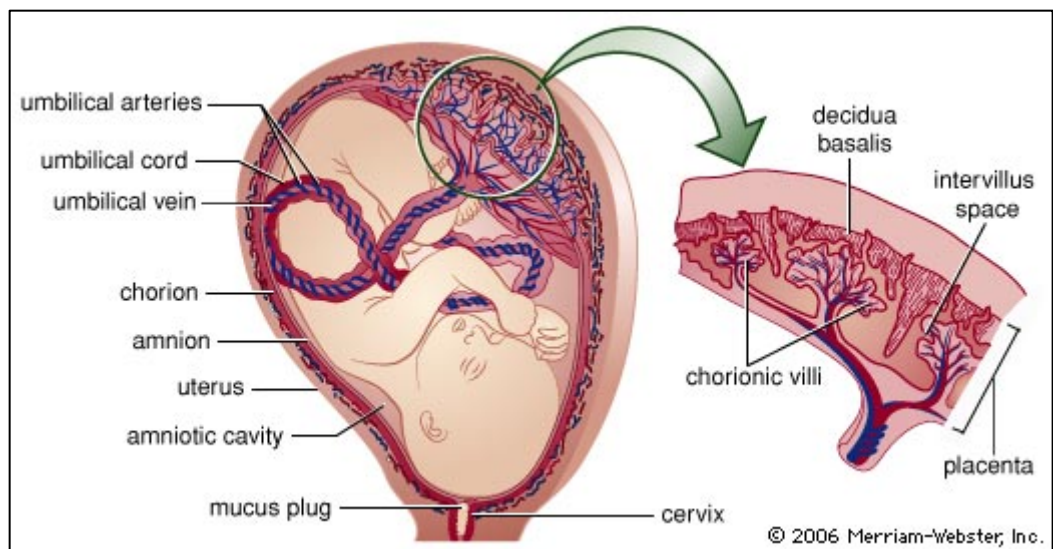


Figure 5. Fetus and placenta inside the uterine cavity ⁽¹²²⁾.

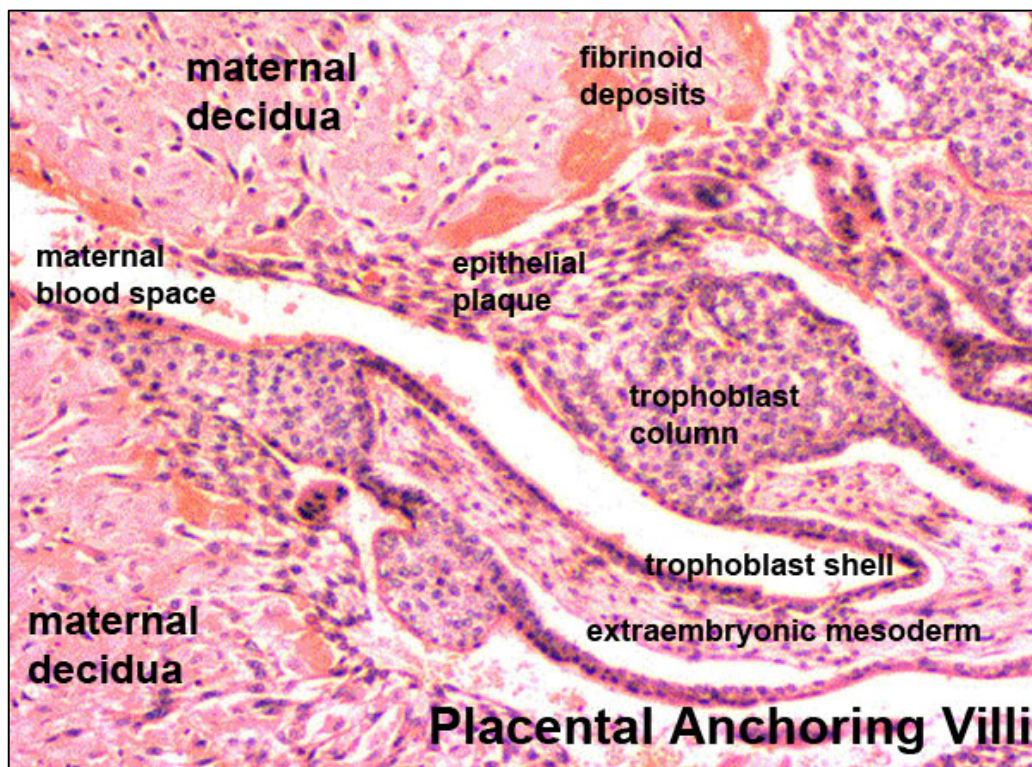


Figure 6. Histological image showing the junctional region between maternal decidua (left) and trophoblast shell of conceptus/embryonic placental villi (right) ⁽¹²³⁾.

Most lipids are bound to protein plasma. For example, free fatty acids bind to serum albumin and cholesterol to form a lipoprotein complex. Free fatty acids are released from the lipoprotein complex by lipoprotein lipase present in maternal placenta. Lipids cross the placental membrane (syncytiotrophoblast) by simple diffusion ⁽¹²⁴⁾. Water transfer is dependent on hydrostatic and osmotic pressure and occurs passively across the placenta. Inorganic ions and minerals, such as potassium, calcium and phosphate, in fetal blood are higher than in maternal blood, but levels of sodium and chloride are similar. Most inorganic ion transfer occurs actively as these ions are also involved in placental ion-transport systems, such as Na/K⁺ ATPase and Ca²⁺ ATPase ⁽¹²⁵⁾. Vitamins are transferred directly from maternal to fetal circulation. Transport and metabolism of various nutrients, such as carbohydrates, amino acids and lipids, water, inorganic ions, minerals and vitamins, occurs via three diffusion mechanisms, active, passive and facilitated diffusion, and transport and metabolism of nutrients is important to ensure optimum fetal growth and development.

iv. Placental functions: Protection against xenobiotics

The placenta acts as a barrier to protect the fetus from circulating xenobiotics, such as drugs and pollutants, in maternal blood. Most small xenobiotic molecules, such as drugs administered during pregnancy, can cross the placenta to fetal circulation by simple diffusion through transcellular or paracellular routes ⁽¹²⁶⁾. Some drugs cross the placenta by various active transporters located on fetal and maternal sides of the trophoblast layer. The placenta contains several protective features that may reduce transfer of potentially toxic substances, including export pumps in the syncytiotrophoblast, such as multidrug resistance protein 1 (MDR1) and placenta-specific ATP-binding cassette proteins (ABCP). In addition, cytochrome P450 (CYP) enzymes in the placenta can metabolise drugs and other xenobiotics ⁽¹²⁶⁾. CYP functions can be divided into oxidation/reduction of endogenous or exogenous compounds. Interestingly, the CYP enzyme superfamily, cytochrome P450 1A1 and cholesterol side-chain cleavage enzyme are linked to epigenetic modifications that occur *in utero* and are associated with childhood allergy susceptibility ^(127, 128).

Although the above mentioned protective mechanisms assist in reducing fetal exposure to certain xenobiotics, some substances freely cross the placenta and cause damaging teratogenic effects to the fetus. Amongst these harmful substances are nicotine, alcohol, certain anti-convulsants and lithium. As nicotine freely crosses the placenta, maternal active and passive smoking causes direct fetal exposure to nicotine. Recent studies suggest that maternal nicotine exposure increases susceptibility to childhood allergies, including asthma, AR and eczema ^(129, 130).

1.4.2 Normal placental function and interaction with the immune system

The human placenta plays an important role in influencing the fetal immune system and may program the fetal immune system to develop allergy in early life ⁽¹³¹⁾. Our group conducted preliminary studies to identify patterns of gene expression in the placenta that may program early immune function to increase susceptibility to allergy ⁽¹³²⁾. A set of immune genes known to be associated with asthma, allergy and inflammation were selected for analysis by qRT-PCR on placental tissue from infants who did and did not develop an allergy by two years of age. Four genes were found to be associated with the development of specific childhood allergy types. These genes were matrix metalloproteinase (*MMP*)9 for both males and females that developed eczema, toll like receptor (*TLR*)-7 for females that developed eczema, *KITL1* for males that developed rhinitis, and *ORMDL3* for females that developed asthma ⁽¹³²⁾. The data suggest that altered expression of placental genes is involved in inflammation associated with development of specific allergies in childhood which support evidence implicating the placenta in programming of the fetal immune system in early life.

Adverse conditions during pregnancy, including maternal asthma, smoking and obesity, may alter normal placental function and affect fetal immune development ⁽¹³⁾. For example, placentae of male fetuses born at less than 32 weeks were more likely to have chronic inflammation and infiltration of decidual lymphoplasmacytic cells, in contrast to placentae of female fetuses born at less than 32 weeks that appeared to be more preserved from inflammatory insult ⁽¹³³⁾. In the presence of maternal asthma, changes in placental function, including reduced placental blood flow, decreased cortisol metabolism and reduction in fetal growth, may be related to activation of pro-inflammatory pathways ⁽¹³⁾. Placental expression of *TNF*, *IL-1*, *IL-6*, *IL-8* and *IL-5* mRNA were all increased significantly in placentae of female fetuses whose mothers had mild asthma, but no changes were observed in placentae of male fetuses that have mothers with mild asthma. Pro-inflammatory cytokines, *TNF*-, *IL-1* and *IL-6*, were negatively correlated with female CB cortisol, but there were no such correlations in placentae from males. Using multivariate analysis, the strongest predictor of cytokine mRNA expression in the placenta and birth weight was fetal cortisol, but only in females. These data suggest that placental basal cytokine mRNA expression is sex-specifically regulated in pregnancies complicated by asthma and, interestingly, these changes are more prevalent in mild rather than severe asthma ⁽¹³⁾. Mechanisms that contribute to adverse outcomes for the neonate in pregnancies complicated by asthma may be mediated via changes in placental immune function ⁽¹²⁾. Maternal asthma during pregnancy induces a hyposensitive inflammatory state in the placenta which is regulated by cortisol in a sexually dimorphic manner ⁽¹²⁾. These findings suggest that male and female fetuses have different mechanisms *in utero* to cope with

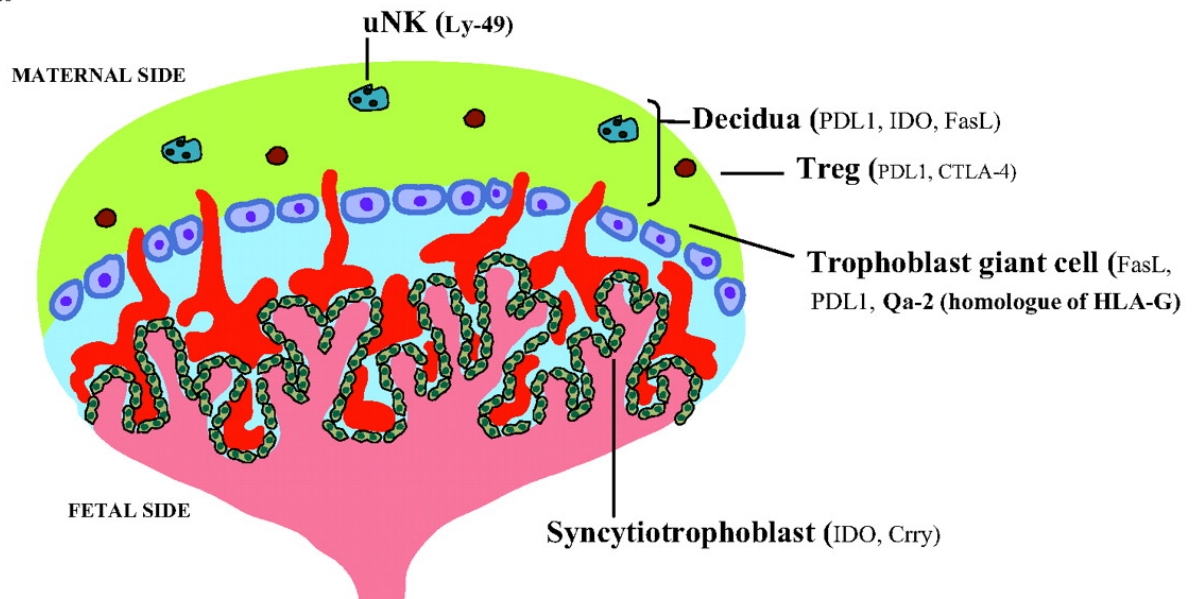
multiple adverse conditions during pregnancy causing alteration in placental function, thus influencing fetal immune system development in a sexually dimorphic manner ^(12, 13).

1.4.3 Basic immune system development in embryology

The human embryo is defined as a developing human organism from the fourth day after fertilisation to the end of the eighth week ⁽¹³⁴⁾. Exposure to paternal-fetal antigens during early pregnancy may predispose the maternal immune system to reject the fetus, thus the maternal–fetal interface develops a specialised Th2 and Tregs environment rich in cytokines, such as *IL4*, *IL10*, *IL13* and *TGF-β*, during embryology to prevent rejection ^(35, 116, 135). Placenta is the most important tissue involved in immunoregulation at the maternal-fetal interface. It is comprised of cells from maternal and fetal origin, which express molecules of human leukocyte antigen (HLA)-G by trophoblast and Fas ligand (FasL) 2 by maternal decidual cells that are crucial in fetomaternal tolerance. Mouse and human placentae are composed of layers of cells with distinct functions (Figure 7) ⁽¹³¹⁾. There are five identifiable mechanisms of maternal immune system adaptations leading to maternal tolerance: (1) nonclassical major histocompatibility complex (MHC) role by trophoblast cells; (2) T cell apoptosis; (3) immunoregulation of Tregs and indoleamine 2,3-dioxygenase (IDO); (4) B7 family of costimulatory molecules; and (5) complement and complement regulators ^(116, 131, 136, 137).

Maternal immune system adaptations leading to maternal tolerance involve components of MHC molecules on trophoblast, trophoblast HLAs and uterine natural killer (uNK) cells, trophoblast class Ib HLAs and alloantigen shedding. There are a reduced number of MHC molecules on the trophoblast. Reduced MHC expression at the maternal-fetal interface is necessary to prevent an inflammatory maternal immune response against the fetus ⁽¹³⁸⁾. During the first and second trimester, trophoblast HLAs and uNK cells are present in the decidua to increase uterine arteries blood supply to the fetoplacental unit. Direct contact of the maternal immune system with the trophoblast also plays an important role in acceptance/rejection of the fetus ⁽¹³⁹⁾

a



b

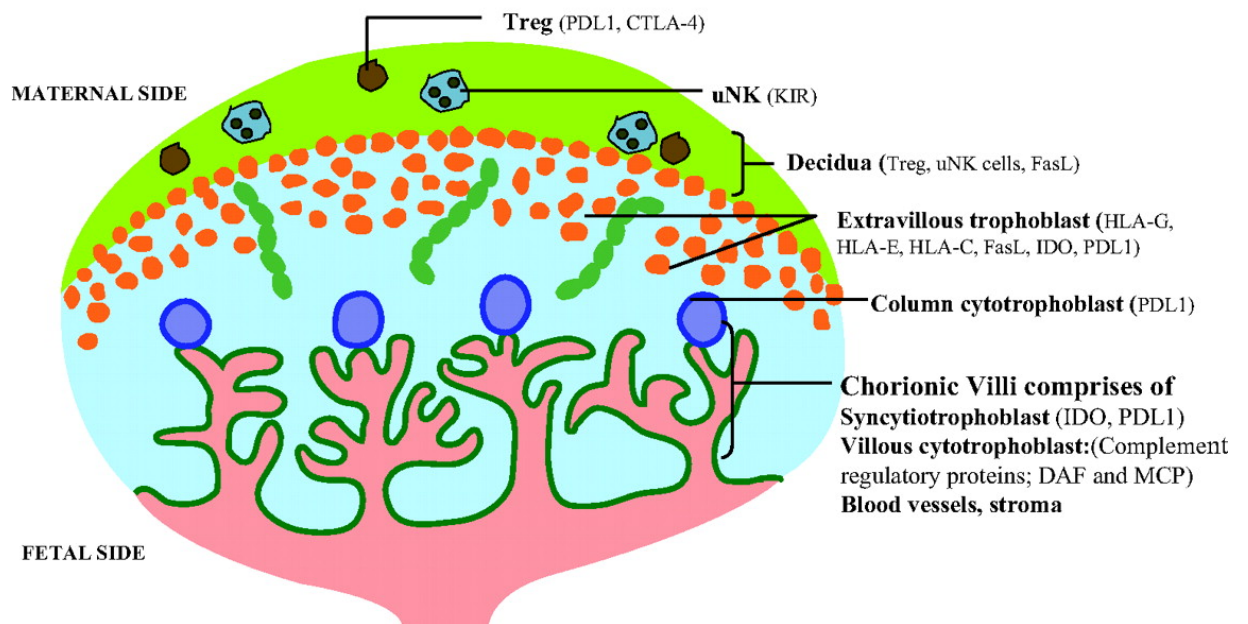


Figure 7. Cells and molecules involved in fetomaternal tolerance in mouse and human placentae
(131). Diagrammatic presentation of anatomy of mouse (a) and human (b) placentae with location of various molecules involved in fetomaternal tolerance. The outer layer of the mouse placenta, which mediates implantation and invasion into the uterus, is composed of trophoblast giant cells. The layer with analogous function in humans is composed of invasive extravillous cytotrophoblast cells. The function of the middle layer of the mouse placenta, the spongiotrophoblast, is largely unknown. However, some spongiotrophoblast cells can differentiate into trophoblast giant cells resembling the cytotrophoblast cell columns that anchor the villi of human placenta. The labyrinth layer of mouse placenta is comparable in function to chorionic villi of human placenta. In mouse and human placentas, the labyrinthine and villi, respectively, are covered by syncytiotrophoblasts that lie in direct contact with maternal blood.

T cell apoptosis in maternal immune system adaptations leads to maternal tolerance of the fetal-placental unit. Extravillous trophoblast cells express a non-classical HLA Ib gene, *HLA-G*, that inhibits cytotoxic T lymphocyte (CTL) responses and NK cell functions ^(140, 141). Allogeneic trophoblast shedding and presentation of these shed paternal Ags by maternal APCs during normal pregnancy, which is critical to establishing maternal immune tolerance to the fetal allograft ⁽¹⁴²⁾. However, the interaction of alloantigen shedding with other mechanisms of fetomaternal tolerance, including apoptosis, is still unclear ⁽¹³¹⁾. T cell apoptosis that occurs at the implantation site results in a clonal deletion of immune cells. These immune cells recognise paternal Ags present in the placenta mediated by expression of FasL on fetal trophoblast or maternal decidual cells ^(131, 143).

Tregs and indoleamine 2,3-dioxygenase (IDO) also play a role in determining progress to allergic disease in the first year of life ⁽¹⁴⁴⁾. At term, immunoregulation of the Tregs, naturally arising CD4⁺T cells expressing the IL-2R α -chain (CD25), are involved in regulation of immune responses and play important roles in maintenance of natural self-tolerance ⁽¹⁴⁵⁾. Previous studies report that decidual and/or peripheral blood CD4⁺CD25⁺high T cells increase during early pregnancy and then decrease in the postpartum period ^(146, 147). These studies support the hypothesis that pregnancy-induced Tregs play a critical role in maintaining maternal tolerance to the fetus. Smith *et al.* 2008 compared the frequency and function of CB Tregs from non-allergic children to those of children who had HE allergy in the first year of life ⁽¹⁴⁴⁾. Findings show that the magnitude of suppression was significantly lower in the allergic group. This provides preliminary evidence of differences in neonates who progress to allergic disease in the first year of life ⁽¹⁴⁴⁾. Hence, CB Tregs may be beneficial as an early biomarker to detect FA susceptibility in infants. However, the mechanisms of generation/expansion, specificity and trafficking is unclear. A potential mechanism is via indoleamine 2,3-dioxygenase (IDO), an enzyme that degrades tryptophan, is essential amino acid for T cell survival and can generate downstream tryptophan metabolites ^(131, 148). IDO is expressed on trophoblastic giant cells in mice and extravillous and villous trophoblasts in humans, where it could deprive tryptophan from T cells thereby inhibiting maternal T cell activation ^(131, 148).

The B7 family of costimulatory molecules and complement regulators in maternal immune system adaptations also lead to maternal tolerance. Optimal T cell activation requires engagement of the T cell receptor (TCR) with a cognate MHC+ peptide complex and a 'positive' T cell costimulatory signal, mediated by one of several other cell surface molecules ⁽¹⁴⁹⁾. In addition, 'negative' signal functions are provided by some T cell costimulatory molecules to regulate immune responses ⁽¹⁵⁰⁾. These are inducible molecules expressed after activation and function to eliminate maternal immune responses. A blockade of positive costimulatory, signals such as CD80 (B7-1) and CD86 (B7-2), were shown to inhibit maternal rejection of the allogeneic fetus in abortion-prone CBA/JxDBA/2 matings mice ⁽¹⁵¹⁾.

Complement regulatory proteins, such as decay-accelerating factor (CD55) and membrane cofactor protein (CD46), expressed on human trophoblast prevent damage caused by complement activation to maintain successful pregnancy ^(131, 152).

1.4.4 Fetal immune system development in relation to allergic susceptibility

To date, there is not much known about normal immune development in human fetuses. Most documented findings of fetal immune system development are based on animal studies and limited to placenta and CB immune studies in humans, because it is difficult and not feasible to access human fetus samples. In mice, the first appearance of mature α/β T cells in peripheral tissue occurs in the final stages of gestation and increase after birth ⁽¹⁵³⁾. In contrast, in human fetuses the first appearance of mature α/β T cells in peripheral tissues occurs during 10-12 weeks of gestation and increases by the end of the second trimester ⁽¹⁵⁴⁻¹⁵⁶⁾. These data indicate that human fetal T cell populations are established earlier compared to mice. Nevertheless, significance of these functional differences are not yet clear. Interestingly, a homeostatic state is achieved relatively early in development of the peripheral adaptive immune system in humans and development of fetomaternal peripheral tolerance, as well as to the response to antigens that might cross the placenta from cells bearing non inherited maternal alloantigens, infectious agents and food antigens ⁽¹⁵⁷⁾.

The ability of the adaptive immune system to specifically recognise pathogens and mount protective responses depends on expression of a largely diversified set of antigen-specific receptors, in particular T cell receptor (TCR) and Ig molecules. Production of TCR and Ig molecules requires V(D)J recombination, a process that involves rearrangement of variable (V), diversity (D) and joining (J) elements at the TCR and Ig loci ⁽¹⁵⁸⁾. Signal-joint T cell receptor excision circles (TRECs) represent by-products of the V(D)J recombination process at the TCR α/δ locus. Similarly, V(D)J rearrangement at the IGK locus in developing B cells produces signal-joint κ receptor excision circles (sjKRECs) and intron recombination signal sequence–K-deleting element (iRSS-Kde) rearrangement ⁽¹⁵⁸⁾. Rechavi *et al.* 2015 investigated levels of TRECs, sjKRECs, and iRSS-Kde rearrangement, and T and B lymphocyte repertoire clonality in human fetuses. Fetal blood samples were collected over a 12 month period from 20 fetuses at 12-26 weeks of gestational age. Blood samples were mostly from normal multifetal pregnancies requesting fetal reduction due to obstetric indications and pregnancy termination because of social indications. Using next-generation sequencing, TCR β and Ig heavy chain (IgH) repertoires in four fetuses at 12, 13, 22 and 26 weeks of gestation and in healthy full-term infants were analysed. Findings revealed a progressive increase of TREC, sjKREC and iRSS-Kde levels over time

and confirm that B cell development precedes T cell development in the human fetus ⁽¹⁵⁸⁾. Moreover, Ig somatic hypermutation and class switch recombination occur during intrauterine life. There was uneven distribution of antigen receptor specificities detected in early second trimester fetuses, with progressive attainment of a diversified and more even repertoire at 22-26 weeks of gestational age. These findings may explain why preterm neonates are more immunodeficient as compared to term infants. This is because term infants have higher leukocyte counts that are fully developed in the third trimester as compared with preterm neonates that do not complete the third trimester *in utero* ^(158, 159).

Perinatal programming is influenced by modifiable maternal exogenous environment exposures. Prescott *et al.* demonstrated that *FOXP3* expression, a marker of Tregs concentration, was reduced in infants who subsequently develop allergic disease ⁽¹⁵⁾. *FOXP3*⁺ regulatory populations play a role in early immune programming and differences in placental *FOXP3* gene expression associated with allergy reflect combined effects of maternal phenotype, fetal genotype and environmental exposures *in utero* ⁽¹⁵⁾.

Ingestion of food allergens present in maternal milk during breastfeeding has been hypothesised as a gateway to food sensitisation in early life. A recent study suggested that unborn children can also be exposed to food allergens contained in the maternal diet, giving rise to the first sensitisation to allergens occurring during pregnancy ⁽¹¹⁶⁾. The presence of food allergens *in utero* were analysed in amniotic fluid samples at two different stages of pregnancy, at 15–20 weeks and after delivery at term, using an antibody microarray that detected presence of ten allergens from milk, fruit, egg, fish, nuts and wheat ⁽¹¹⁶⁾. Presence of intact major food allergens was detected in all amniotic fluid samples. Moreover, samples from amniocentesis had a higher allergen concentration than samples after delivery at term. These findings may partly explain subsequent sensitisation of childhood allergic reactions to novel foods not consumed during pregnancy and to foods never eaten before ⁽¹¹⁶⁾.

1.4.5 The neonatal immune response at birth

The placenta acts as selective maternal-fetal barrier against transmission of microbes to the fetus. Most proteins, such as IgM, are unable to readily cross the placenta, with the exception of a few including maternal antibodies, such as IgG and IgE ⁽¹⁶⁰⁾. As IgM cannot cross the placenta, this is one reason some infections acquired during pregnancy can pose a threat to the fetus ⁽¹⁴⁾. Some examples include bacteria that cause syphilis, protozoa parasites that cause toxoplasmosis and viruses, such as human immunodeficiency virus (HIV), cytomegalovirus, varicella and coxsackie virus. Most of these infections can cause fetal demise or permanent physical and mental deformity that could affect development of the newborn in later life ⁽¹⁴⁾.

IgG is transferred using the mechanism of pinocytosis via the neonatal Fc receptor, FcRn, across the syncytiotrophoblast ⁽¹⁶¹⁾. Interestingly, IgE can be found in immune cells of the infants, transferred from their allergic mother, although maternal IgG is generally regarded as the only antibody isotype capable of crossing the placental barrier ^(16, 160). Transferred maternal antibodies provide passive immunity to the fetus as early as the second trimester ⁽¹⁶²⁾. Concentration of IgG in fetal blood increases with gestation as most of maternal antibodies are obtained in the third trimester. This passive immunity acquired by the fetus lasts several months after delivery, thus providing the newborn with a carbon copy of the mother's long-term humoral immunity before the offspring has the opportunity to establish its own immunity ^(161, 162).

Among the important sites of IgE production in allergic patients are allergy effector organs, such as nasal mucosa, lung, blood and bone marrow ⁽⁶⁾. Matson *et al.* demonstrated that percentages of CB basophils with surface-bound IgE were significantly higher in infants of allergic mothers compared with infants of non-allergic mothers ⁽¹⁶⁰⁾. Furthermore, presence of any kind of allergic disorder in the mother or her family and elevated maternal blood IgE level were associated with CB IgE of the child ⁽¹⁶³⁾. The mechanism by which maternal IgE crosses the placenta involves FcRn ⁽¹⁶⁰⁾. In mice, FcRn for IgG uptake facilitates intestinal absorption of maternal IgE in the form of IgG anti-IgE/IgE immune complexes ⁽¹⁶⁴⁾. Moreover, when IgG anti-IgE/IgE immune complexes are generated with IgG anti-IgE directed against the Cε4 domain, a region not involved in the binding of IgE to FcεRI, IgE is biologically active, retaining the capacity to bind and induce allergen-specific β-hexosaminidase release from rat basophil leukaemia cells ⁽¹⁶⁰⁾. As FcRn is expressed in human placenta, it is possible that pro-inflammatory conditions can exist during pregnancy. This favours generation and transmission of biologically active maternal IgG anti-IgE/IgE immune complexes to the fetus, with resultant binding to FcεRI-expressing fetal cells ^(160, 161, 165).

An immune response can be triggered and observed in newborns. For example, house-dust-mite allergen can be detected in amniotic fluid and umbilical-CB ⁽¹⁶⁶⁾. The only plausible explanation is that this immune capacity is developed *in utero* ^(35, 116, 135). Moreover, early desensitisation may occur *in utero*, potentially caused by transfer of maternal allergens to the fetus via fetomaternal surface ⁽³⁵⁾. Warner *et al.* showed early evidence that peripheral blood mononuclear cell (PBMC) sensitivity to allergens can be detected just after birth and Jones *et al.* showed that specific allergen-induced responses have been detected as early as 22 weeks of gestation ^(167, 168). Another study examined pregnant mothers exposed to common inhalant allergen (Der p 1) throughout pregnancy. ELISA was performed on maternal blood and matched amniotic fluid samples at 16-17 weeks of gestation, and in matched maternal and umbilical-CB at term (37 ≥ weeks of gestation) ⁽¹⁶⁶⁾. Der p 1 was detectable in amniotic fluid samples, maternal blood and cord-plasma samples and mononuclear cells in umbilical-

CB displayed allergen-specific reactivity. Detection of Der p 1 in amniotic fluid and fetal circulation provides direct evidence of trans-amniotic and trans-placental allergen exposure ⁽¹⁶⁶⁾. Thus, these findings indicate that early desensitisation to allergens may occur *in utero*.

1.4.6 Mechanisms of allergic immune development for the fetus and neonate

Genetics, epigenetics, maternal well-being and environmental factors may influence susceptibility to childhood allergic disease ^(36, 109, 169). Factors affecting the *in utero* environment, such as maternal atopy and infections, and bacterial exposure in pregnancy or early life may act as immunomodulators enhancing or inhibiting development of asthma and atopy in childhood ⁽¹⁰⁹⁾. These factors can be altered through the course of respiratory or immune system development *in utero* and at birth ^(5, 170).

1.4.6.1 Genetics

Genetic inheritance is a significant factor in development of allergic disease ⁽³⁶⁾. Genetic factors, including allelic variants of gene encoding cytokines, cytokine receptors and pattern recognition receptors (PRR), determine susceptibility in potential genetic predisposition of developing childhood asthma ^(5, 171). In animal models, these specific genotypes can result in Th2-biased adaptive immune responses and hyperinflammatory responses in tissues, factors that influence asthma susceptibility ^(35, 171). Most genes involved are related to immune and inflammatory signalling or functional responses. These alleles include *Nrf2*, *TNF- α* , *IL-13*, *TLR-2* and *TLR-4* genes, whereas genetic marker polymorphisms are *GPR154*, *ADAM33* and *IL-1 Receptor-Like 1* polymorphisms ⁽¹⁷²⁻¹⁷⁶⁾. Children with specific *TLR* alleles are more susceptible to traffic-related air pollutants and more likely to have doctor-diagnosed asthma up to eight years of age ⁽¹⁷³⁾. Polymorphisms and haplotypes in the 70-kb haplotype block in the G protein-coupled receptor on chromosome 7p are associated with asthma, rhinoconjunctivitis and sensitisation with elevated total serum IgE levels in European children ⁽¹⁷⁷⁾. Thus, childhood allergies are influenced by genetic factors that include allelic variants of gene encoding cytokines, cytokine receptors and PRR.

Neuropeptide S Receptor 1 (*NPSR1*) gene was associated with multiple allergic phenotypes in several patient populations ⁽¹⁷⁸⁾. The *NPSR1* gene single nucleotide polymorphism (SNP), rs546333, was associated with high IgE-associated AD in 796 children at age two years born to family at high risk for allergic diseases. Moreover, allergic disease in both parents doubled the risk for IgE-mediated allergic disease in offspring ⁽¹⁷⁸⁾. Another study evaluated risk factors of childhood AD development in 762

infants with at least one atopic parent showing that children carrying the *CD14-159C/T* CC genotype and exposed to a pet dog were less likely to have AD ⁽⁵⁹⁾. Carriers of the *CD14-159C/T* and *IL4R alpha* /75V SNPs had an increased risk of AD at ages two and three, especially among children who were SPT positive. These results support a protective role for early exposure to dogs in high risk children, especially among those carrying the *CD14-159C/T* SNP ⁽⁵⁹⁾. These studies indicate genetics augmented with environmental exposures work together to influence AD susceptibility.

1.4.6.2 Epigenetics

Environmental changes at critical times in prenatal human development influence developmental pathways and induce permanent changes in metabolism and chronic disease susceptibility ⁽¹⁰²⁾. Allergic disease susceptibility is epigenetically determined by maternal exposures, with the capacity to activate or silence fetal genes via alterations in DNA and histone methylation, histone acetylation, and chromatin structure ^(113, 179). In pregnancy, the underlying mechanisms of *in utero* exposures, such as maternal diet, microbial exposure, smoking and air pollution exposure, have epigenetic effects on fetal gene expression, thereby altering subsequent development of allergic diseases ⁽¹⁷⁹⁻¹⁸¹⁾. Furthermore, this altered genetic state may be long-lasting depending on exposure to certain environmental factors during early childhood, or it may be reversed by mechanisms not well understood ⁽¹¹¹⁾. This heritable altered genetic state may be modified by environmental stimuli that include maternal diet (dietary methyl donors and cofactors), such as folic acid and vitamins B12, B6, B2 and zinc that are required for DNA methylation ⁽¹¹¹⁾. In animal studies, evidence indicates that allergy protective effects of microbial exposure in pregnancy may be mediated by changes in methylation of *Th1* genes of offspring. Moreover, this epigenetic regulation is involved in perinatal differences of immune function pathways in allergy-prone newborns which include differences in effector T cell (Th1/Th2) and in neonatal Tregs and Th17 function ^(113, 179).

Exposure to diesel exhaust particulate (DEP) has been demonstrated to exacerbate asthma and COPD in the human bronchial epithelial cell line, partly via epigenetic modifications ⁽¹⁸²⁾. Increased DEP was associated with increased acetylation of histone H4 in the promoter region of the cyclooxygenase-2 (*COX-2*) gene, an inflammatory mediator. In addition, chromatin modification and degradation of histone deacetylase 1 (*HDAC1*) gene were observed ⁽¹⁸²⁾. In another study, differential methylation status of 30 DNA sequences were identified dependent on maternal polycyclic aromatic hydrocarbon exposure levels using umbilical cord white blood cell DNA and fetal placental tissues ⁽¹⁸³⁾. These DNA sequences include methylation of a CpG island in acyl-CoA synthetase long-chain family member 3 that is also associated with childhood asthma. Prenatal exposure to the pollutant phthalate, an endocrine disruptor, increased susceptibility of childhood asthma mediated via alterations in DNA

methylation ⁽¹⁸⁴⁾. Differential methylation in three genes, androgen receptor (*AR*), *TNFα* and *IL-4*, were identified in urine and blood samples from 256 children. Higher exposure of phthalate was associated with lower methylation of *TNFα* and higher risk of childhood asthma ⁽¹⁸⁴⁾.

Although epigenetic mechanisms likely contribute to asthma pathogenesis, their role in asthma inception, whether the trajectory to asthma begins at birth and whether epigenetic mechanisms, specifically DNA methylation, contribute to asthma inception is not well understood ⁽¹⁸⁵⁾. In three methylation studies using neonatal immune cells, of 589 differentially methylated regions, SMAD3 protein is the most connected node within the network of asthma-associated and differentially methylated regions ⁽¹⁸⁶⁾. SMAD3 was selectively increased in asthmatic children of asthmatic mothers, associated with childhood asthma risk and was strong positively associated with neonatal production of IL-1β, an innate inflammatory mediator. These findings support the hypothesis that trajectory to childhood asthma begins at birth and involves epigenetic modifications in immunoregulatory and pro-inflammatory pathways associated with presence of maternal asthma ⁽¹⁸⁵⁾.

Epigenetic changes play a role in pathogenesis of allergic diseases, including childhood AD and FA, via multifactorial gene-environment interaction ^(111, 169, 187). DNA methylation is one of the most common epigenetic modifications associated with FA. 'Signatures' of aberrant methylation have been identified as a promising specific marker of disease-related processes, including immune cell phenotypes, biological aging and environmental exposures, each modifiable by underlying host genotype. Recent evidence suggests that methylation signatures may be developed with clinical utility for discriminating types of allergic phenotypes, such as FA ⁽¹⁶⁹⁾. Using CB of 303 children, Curtin *et al.* reported significant interaction between age at asthma exacerbation or hospital admission and methylation of *IL-2* ⁽¹¹⁸⁾. Moreover, increased methylation of a functional CpG site in the *IL-2* promoter was associated with increasing age and likelihood of severe asthma exacerbations and hospital admissions for asthma or wheeze between ages of two and eight years ⁽¹¹⁸⁾. Thus, epigenetic modifications, especially DNA methylation, is associated with pathogenesis of allergic diseases via multifactorial gene-environment interaction.

1.4.6.3 Maternal allergy

Maternal allergy is associated with regulation of neonatal immune function, where immaturity of Th1 function has been suggested to influence susceptibility of childhood allergy ^(160, 166, 179, 188). Acute atopic symptoms during pregnancy were associated with increased risk of early AD and AR in offspring ^(5, 109, 189). Maternal acute atopic symptoms during pregnancy were associated with increased risk of early AD and AR at five years of age in an urban population ⁽¹⁰⁹⁾. Moreover, presence of endotoxin in a child's

mattress was inversely associated with atopic sensitisation and asthma. A prospective birth cohort study investigated potential maternal atopy and maturation of offspring immune system and resulting disease risks ⁽¹⁸⁹⁾. Concentrations of Th1/Th2/Th17 and inflammatory cytokines/chemokines, as well as IgE, were measured in phytohemagglutinin and lipopolysaccharide (LPS) stimulated maternal blood in the 34th week of gestation and in corresponding children's blood at birth and one of age in 353 mother-child pairs. Findings revealed that maternal inflammatory cytokines (MCP-1, IL-10, TNF- α) during pregnancy correlated with children's corresponding cytokines at the age of one, but were not related to IgE or AD. Moreover, while maternal IgE predicts children's IgE, AD in children is only associated with maternal AD ⁽¹⁸⁹⁾.

Cell types and mechanisms associated with maternal atopic disease that influence allergic susceptibility in offspring are poorly understood ^(160, 179). A study investigated the relationship between maternal allergy and frequencies of CB basophils and plasmacytoid DC (pDCs) ⁽¹⁶⁰⁾. Findings revealed that percentages of CB basophils with surface-bound IgE were significantly higher in infants of allergic mothers compared with infants of non-allergic mothers. The high percentages of CB basophils in infants of allergic mothers correlated with CB IgE levels, but not with maternal IgE levels. The finding of increased IgE on CB basophils from infants of allergic mothers suggests that cell-bound IgE may be a more sensitive indicator (than free IgE) of total IgE in the fetal compartment ⁽¹⁶⁰⁾.

1.4.6.4 Maternal health

Asthma is one of the most common chronic diseases among pregnant women ^(3, 9, 26, 27, 170). Cells of the innate immune system, such as monocytes and neutrophils, increase during pregnancy, which also exacerbate pre-existing maternal asthma ⁽¹⁹⁰⁾. Maternal asthma triggers perinatal programming of asthma in offspring and the childhood asthma phenotype could be programmed long before birth ^(3, 9, 12, 26, 27, 170, 191, 192). During pregnancy, the maternal immune response shifts away from the Th1-driven cell-mediated immunity towards Th2-related humoral response providing an environment essential for fetal survival, but simultaneously increasing susceptibility of childhood allergy development ^(2, 11). Moreover, placenta may adapt to the presence of maternal asthma to ensure continued fetal growth, but may compromise fetal organ system development, including the immune system ^(132, 181, 192, 193). Genetic and environmental factors *in utero* affect maturation of the fetal immune system during pregnancy and early childhood and may increase the risk of offspring developing an allergy ^(3, 5, 170). Thus, these initial inflammatory processes aggravated by maternal asthma and altered reactivity to stimuli are characteristic of initiation of chronic asthma in offspring ⁽⁴⁵⁾.

Mechanistic changes in placental immune function are suggested to contribute to adverse outcomes for the neonate in pregnancies complicated by asthma ⁽¹²⁾. Previously, we conducted a study to determine whether maternal asthma during pregnancy influenced placental pro-inflammatory immune response ⁽¹²⁾. Placental explants were exposed to an immune challenge (LPS) in the presence and absence of cortisol *in vitro*. The study revealed that placentae from pregnancies complicated by maternal asthma had a reduced pro-inflammatory cytokine response to an immune challenge relative to controls, especially in relation to production of *IL-1 β* and *TNF- α* , regardless of fetal sex ⁽¹²⁾. Moreover, cortisol inhibition of placental cytokine production was dependent on timing of exposure, fetal sex and presence and absence of maternal asthma. These findings suggest that maternal asthma during pregnancy induces a hyposensitive inflammatory state in the placenta which was regulated by cortisol in a sexually dimorphic manner ⁽¹²⁾. Based on this study, adverse outcomes for the neonate in pregnancies complicated by asthma may be influenced by changes in placental immune function, but the exact mechanism involved is unclear.

The presence of maternal asthma during pregnancy can alter placental and CB cytokine expression ^(9, 179, 181). Placental adaptation triggers compensatory mechanisms involved in alteration of placental protein production associated with childhood allergy. Moreover, maternal atopy interacts with the *TLR1*, *TLR2* and *TLR10* genotype of offspring to influence Tregs marker gene expression and Th2 cytokine levels in CB ^(181, 194). However, a recent human study demonstrated that maternal allergy has no significant effect on frequency of Tregs in CB, but is associated with impaired function of Tregs on inhibition of Th2 cytokine (IL-4) response to allergen stimulation ⁽¹⁹⁵⁾. This augments predominant Th2 deviation in CB of children with maternal allergy which increases subsequent childhood AD and allergy development. These data further support evidence that maternal asthma is a significant risk factor that increases likelihood of offspring to develop childhood allergy, thus these children are categorised as high risk of allergy ^(193, 195).

Gestational diabetes (GDM) is associated with development of childhood allergy. GDM significantly increased the risk of AD and early childhood allergen sensitisation in term birth infants up to 3.2 years of age ⁽¹⁹⁶⁾. Recently, a Canadian study involving children aged seven to eight years showed that maternal diabetes may contribute to perinatal programming of child asthma by amplifying detrimental effects of environmental tobacco smoke (ETS) and maternal asthma ⁽¹⁹¹⁾. Children with maternal asthma and maternal history of diabetes have higher likelihood of childhood asthma in those exposed to ETS as compared to children with maternal asthma but without maternal history of diabetes ⁽¹⁹¹⁾. GDM mothers are usually obese. A probable mechanism involved in early childhood atopy is that insulin resistance in GDM may have mediated the association of obesity on allergen sensitisation ^(196, 197). Moreover, altered levels of adipokines associated with GDM may have an effect on immunologic

development in infancy ⁽¹⁹⁷⁾. Interestingly, there are no documented associations of GDM with AD and allergen sensitisation in preterm infants ⁽¹⁹⁶⁾. This is because maternal hyperglycemia usually occurs in the last trimester of pregnancy, not achieved in preterm infants. Hence, maternal history of diabetes aggravated by maternal asthma may increase susceptibility of childhood allergy development, especially in AD and asthma.

Maternal pre-pregnancy overweight or obesity influences the risk of developing childhood allergy ^(196, 198-200). High body mass index (BMI) in pregnancy has a significant relationship with early childhood wheeze which may develop to childhood asthma. This relationship increased with increasing maternal BMI, irrespective of child BMI ^(199, 200). Another cohort study observed that of 20.7 % mothers with pre-pregnancy obesity, 5 % of children developed recurrent wheeze up to three years of age. Moreover, maternal pre-pregnancy obesity was associated with recurrent wheeze in early childhood irrespective of the effects of fetal growth and early life atopy ⁽⁸⁾. The mechanism involved is that maternal obesity affects pulmonary and immunological development of the fetus *in utero* via increased levels of inflammatory substances in obese mothers during pregnancy, or via impaired placental function ^(199, 200). Furthermore, maternal obesity in pregnancy increased the risk of pregnancy complications, caesarean sections and adverse birth outcomes, including respiratory illness in children ⁽²⁰⁰⁾. These studies suggest that maternal pre-pregnancy overweight or obesity increase risk of developing childhood allergy.

1.4.6.5 Environmental exposures

Numerous exogenous environmental exposures *in utero* and early postnatal life influence fetal immune development and can have persistent effects on lung development, thus influencing lung function and respiratory health throughout later life ⁽¹⁷⁰⁾. These exogenous environmental exposures include maternal tobacco smoking and drug exposures, maternal SES, maternal diet, urban environment, maternal microbial exposure, early exposure to farm animals, maternal psychological stress and maternal exposure to heavy metals ^(5, 26, 201). Such exogenous environmental exposures usually occur in combination and produce synergistic elevated risk factors for childhood allergic disease. In addition, changing the *in utero* environment can increase risk of childhood allergy susceptibility, such as observed in assisted reproduction technologies (ART). ART is associated with increased susceptibility of childhood asthma as demonstrated in a large prospective study of 18 818 children ⁽²⁰²⁾. Findings suggested that *in utero* events contributed to alterations of fetal organ development and can influence susceptibility to an asthmatic phenotype.

i. Maternal smoking and environmental tobacco smoke (ETS)

Maternal smoking in pregnancy increases risk of wheezing and asthma in childhood, IUGR and LBW, and is associated with reduced childhood lung function ^(9, 203). A significant limitation in past studies was inability to differentiate between the effects of *in utero* and postnatal exposure, and only limited studies have focused on the effects of maternal smoking ^(129, 130, 201, 203, 204). However, using ultrasound to assess fetal growth, a study revealed that maternal smoking was associated with reduced fetal measurements in the second and third trimesters, but not in the first trimester ⁽²⁰³⁾. Moreover, mothers who did not quit smoking during the first trimester delivered smaller infants with adverse respiratory outcomes in childhood ⁽²⁰³⁾. Changes observed are based on fetal measurements, thus it is possible that functional changes may still occur in the first trimester but not manifest anatomically, for example, epigenetic modifications. FA has been reported to be influenced by maternal tobacco smoke via epigenetic modifications ^(111, 205, 206). During pregnancy, exposure to tobacco smoke is associated with aberrant global methylation in the placenta and CB, as well as in children. Furthermore, tobacco smoke exposure is linked to reduced HDAC expression and activity, which may cause the augment expression of inflammatory mediators (IL-8 and TNF- α) ^(111, 186).

Maternal nicotine exposure during pregnancy alters histone H3 and H4 modifications in fetal lung and gonad in the next two generations of rat offspring ^(181, 207). It is suggested that the detrimental effect of *in utero* nicotine exposure on lung growth and development are mostly caused by specific signalling pathway alterations involved in lung development, instead of being due to irreversible disruption by teratogenic or toxicological effects ⁽²⁰⁷⁾. Methylation of H3/H4 results in downregulation of peroxisome proliferator-activated receptor gamma (*PPAR*) gene expression linked to the epigenetic effect of nicotine with decreased lipofibroblast expression and asthma susceptibility ⁽²⁰⁷⁾. In rats, nicotine exposure during pregnancy is not restricted only to the offspring of the exposed pregnancy, but are also transmitted to the subsequent generation, possibly through germline epigenetic alterations ⁽²⁰⁷⁾.

Active or passive maternal smoking in pregnancy is a significant risk factor for increased susceptibility to childhood allergy ^(129, 130). Maternal smoking during pregnancy was associated with increased risk of wheeze and asthma via alterations in lung development among children who were not exposed to maternal smoking after birth ⁽¹³⁰⁾. Moreover, the likelihood of childhood wheeze and asthma increased linearly in relation to maternal daily cigarette consumption during the first trimester of pregnancy ⁽¹³⁰⁾. Interestingly, fetal exposure to maternal passive smoking was significantly associated with wheeze, AR and AD, while exposure to maternal active smoking was associated with asthma and wheeze, but not with AR or AD ⁽¹²⁹⁾. Furthermore, tobacco smoke has been demonstrated to affect beta2-adrenergic receptor (*ADRB2*) gene expression ⁽²⁰⁸⁾. *ADRB2* gene encodes beta-2-adrenergic receptor which is a

member of the G protein-coupled receptor superfamily. In the airway, catecholamines, or β 2-agonists, activate the β 2-adrenergic receptors, which results in bronchodilation and relief of wheezing symptoms. A SNP in the *ADRB2* gene reduced bronchodilation and relief of wheezing symptoms and thus is associated with increased susceptibility to asthma ⁽²⁰⁸⁾. Both *in utero* and in early postnatal life exposure to tobacco smoke was associated with increased risk of wheeze in children, and the risks were greater for children with the *Arg16Arg* genotype or two copies of the *Arg16-Gln27* diplotype of *ADRB2* gene ⁽²⁰⁸⁾. Therefore, maternal smoking in pregnancy, either active or passive, can increase susceptibility of childhood asthma due to effects on lung development and interaction with genotype.

ii. Maternal dietary intake

Fish and fish oil are major sources of n-3 (or omega 3) long chain polyunsaturated fatty acids (LCPUFA). Maternal fish consumption during pregnancy influences the fetal immune system through anti-inflammatory effects of n-3 LCPUFA ⁽²⁰⁹⁾. Dietary consumption of n-3 LCPUFA during pregnancy may play an important role in susceptibility of developing allergic disease in childhood, particularly for those with a genetic predisposition to production of the IgE antibody ^(36, 210, 211). Gunaratne, Makrides *et al.* conducted a Cochrane Review in RCTs to evaluate the effect of n-3 LCPUFA supplementation of pregnant and/or lactating women (compared with placebo or no treatment) on allergy outcomes of infants or children ⁽²¹²⁾. In the review, eight trials involving 3366 women which randomly allocated women to either a n-3 LCPUFA supplement or a control group, and their 3175 children were included. In these trials, women were supplemented with n-3 LCPUFA during pregnancy (five trials), lactation (two trials) or both pregnancy and lactation (one trial). Overall, results showed little effect of maternal n-3 LCPUFA supplementation during pregnancy and/or breastfeeding for reduction of allergic disease in children ⁽²¹²⁾. There were reductions in some outcomes, such as FA in children at 12 months and IgE-mediated eczema in children between 12 and 36 months of age. The review concluded that there is limited evidence to support maternal n-3 LCPUFA supplementation during pregnancy and/or lactation for reducing allergic disease in children ⁽²¹²⁾. The possible mechanisms underlying the effect of maternal n-3 LC PUFA status on childhood allergy are unclear. A recent study showed CB DNA methylation levels of genes encoding Th1 and Th2 cytokines were increased in infants of supplemented mothers, suggesting that n-3 LCPUFA may modulate immune function of progeny through altered methylation ^(36, 213).

The relationship between peanut, TN and sesame seed intake during pregnancy and allergic disease development in children has been controversial. Maslova *et al* showed that maternal intake of peanuts and TN were inversely associated with asthma or medication-related asthma diagnosis and self-reported AR in children at 18 months of age ⁽⁸⁰⁾. They suggest consumption of peanuts and TN during

pregnancy may decrease risk of allergic disease development in children ⁽⁸⁰⁾. In contrast, Kemp *et al.* measured serum-specific IgE at age 16 and reported that maternal antenatal peanut consumption was strongly correlated with increased peanut and rye sensitisation in those with a family history of asthma ⁽²¹⁴⁾. These sensitisations were likely related to IgE antibodies cross-reacting with carbohydrate epitopes common to rye and peanut allergens in later life ⁽²¹⁴⁾. Moreover, Hsu *et al.* suggested that only maternal ingestion of peanut, TN and sesame seed during pregnancy were associated with increased risk of similar food allergen sensitisation in children, unlike non-ingested maternal exposure to these allergens through progesterone treatment during ART ⁽²¹⁵⁾. The possible mechanism for prevention of allergic diseases by maternal food restriction is related to avoidance of prenatal allergen exposure and prevention of prenatal sensitisation ⁽²¹⁶⁾.

Recent studies provide evidence that maternal vitamin D intake during pregnancy is associated with asthma and allergic outcomes in offspring ⁽²¹⁷⁻²¹⁹⁾. Vitamin D regulates multiplication of bronchial smooth muscle cells, which may be important in asthma and COPD ⁽²¹⁷⁾. Recent studies implicated the importance of vitamin D in innate immune defence and pathogenesis of allergic diseases ^(218, 219). Using CB samples collected at birth and analysed for 25-hydroxyvitamin D (25[OH]D) levels in 239 newborns, findings revealed that cord serum 25(OH)D levels were inversely associated with risk of transient early wheezing and AD by five years of age ⁽²¹⁸⁾. Another recent study identified a similar relationship in 498 AD Chinese children ⁽²¹⁹⁾. Childhood AD was associated with childhood vitamin D deficiency with presence of high total serum IgE. Moreover, serum 25(OH)D levels were correlated inversely with severity of AD in short and long term duration ⁽²¹⁹⁾. Thus, vitamin D intake during pregnancy and vitamin D intake by children can influence susceptibility of developing AD in childhood.

iii. Maternal microbial and allergen exposure

Maternal microbial exposure plays a key role in allergy development and allergic disease ⁽²²⁰⁾. According to Prescott *et al.*, maternal allergy is associated with significantly higher neonatal IL-12 and IFN- γ responses to TLR-2, TLR-3 and TLR-4 activation in CB mononuclear cells, whereas TNF- α and IL-6 responses to TLR-2, TLR-4 and TLR-5 activation are significantly higher in newborns who subsequently have allergic disease. Consistent with previous reports, atopic newborns have lower Th IFN- γ response to phytohaemagglutinin (PHA) mitogens. This relative hyperresponsiveness could be the product of intrauterine influences, including maternal atopy, functional genetic polymorphisms, or both ⁽²²⁰⁾. Thus, maternal microbial exposure is associated with childhood allergy development via a mechanism that involves increased perinatal to TLR responses.

Maternal allergen exposure during pregnancy or in early postnatal life may contribute to risk of childhood AD. A study examined the relationship of the perinatal home environment and risk of suspected AD among 865 Japanese parent-child pairs where the child was under the age of one ⁽⁵⁷⁾. Findings demonstrated that high house dust mite allergen levels and mould in the kitchen during pregnancy increased risk of infantile AD, whereas frequent vacuuming practices during pregnancy and giving the infant a bath or shower at least once a day may protect against infantile AD ⁽⁵⁷⁾.

In utero and early postnatal farm exposures increase expression of innate immune system receptors and have a protective effect against childhood allergic diseases ^(221, 222). Underlying immunologic mechanisms are still unclear, but are most likely modulated by immune-regulatory cells early in life, such as Tregs ⁽²²²⁾. Roduit *et al.* examined the association between *in utero* farm-related exposures, expression of innate immune genes and AD at birth in 1063 European children ⁽²²¹⁾. Data showed that elevated expression of *TLR-5* and *TLR-9* genes in CB was associated with decreased AD. Moreover, maternal contact with farm animals and cats during pregnancy had a protective effect on development of AD in early life, associated with lower expression of innate immune receptors, such as *TLR-2* polymorphism at birth ⁽²²¹⁾. A recent study by Lluís *et al.* revealed that Tregs were significantly increased and inversely associated with asthma and perennial IgE in farm-exposed children after immune and LPS stimulation. This study suggested that farm milk exposure in children was associated with increased Treg numbers and accompanied induction of a regulatory phenotype early in life ⁽²²²⁾. Based on these studies, childhood asthma and AD development are influenced by *in utero* and postnatal environmental farm exposures that may contribute to a protective effect for childhood allergic diseases development.

1.5 Development of the immune system at birth

1.5.1 Normal immune system development at birth

Fetal and neonatal immune systems are related to physiological demands which are three-fold: (1) protection against infection, including viral and bacterial pathogens at the maternal–fetal interface ⁽²²³⁾; (2) avoidance of potentially harmful pro-inflammatory/Th1-cell-polarising responses that can induce alloimmune reactions between mother and fetus; and (3) mediation of the transition between the normally sterile *in utero* environment to the foreign antigen-rich environment of the outside world, including primary colonisation of the skin and intestinal tract by microorganisms ⁽²²³⁾.

The newborn immune system has not fully matured relative to older children and adults at the time of term birth ⁽²²⁴⁾. The neonatal innate immune system is biased against production of pro-inflammatory cytokines that impairs responses to many vaccines ⁽²²³⁾. This immature immune system can increase susceptibility of the newborn to infections by a wide range of pathogens that in turn may influence susceptibility to allergic disease. Data suggests that depressed Th1-dependent anti-microbial immunity in the neonatal period is the consequence of fetomaternal tolerance or intrauterine survival ^(131, 137). The naive adaptive immune system must develop specificity and memory, which can only be completed in the early childhood years ⁽²²⁵⁾. Therefore, normal term neonates depend almost completely on their innate immune response during the first weeks after birth ⁽²²⁴⁾.

Adaptive immunity maturation occurs mostly after term birth, thus all newborn infants have deficiencies in T cell activation and cytokine production, B cell Ig production and interactions between T and B cells, as compared with adults ⁽²²⁴⁾. At birth, lymphocyte subpopulations are lower than in adults and require clonal expansion in the first few weeks of postnatal life ⁽²²⁶⁾. Term neonates have deficient T cell function as a result of a greater proportion of naïve T cells in the circulation and a low subpopulation of memory T cells ⁽²²⁶⁾. The proportion of naïve T cells is increased, a result of inefficient DC antigen uptake and presentation caused by a reduction in MHC II expression on APCs ⁽²²⁴⁾.

APC in neonates show weaker responses to innate stimuli than do those in adults, except for TLR-mediated responses to TLR8 agonists ⁽²²⁷⁾. APC reactivity at birth in children born under modern environmental conditions is increased as compared with children born under traditional environmental conditions ⁽²²⁸⁾. A study identified differences in the function of neonatal APCs and T cell phenotype and function in CB mononuclear cells (CBMC) in children born in more traditional settings versus those of modern societies. CBMC samples were collected from newborns from Papua New Guinea (PNG;

traditional) and Australia (modern) ⁽²²⁸⁾. In Australian newborns, cord naive T cells (CD4(+)CD25(-)CD127(+) cells) showed an enhanced and more rapid proliferative response in an autologous, APC-dependent culture system, a result of differences in neonatal APCs rather than T cell function. Moreover, there was an increased capacity to process antigen and to upregulate activation markers post stimulation. In contrast, resting PNG APCs exhibited higher baseline levels of activation and inhibitory markers and were less responsive to *in vitro* stimulation. The study suggests that the functionally more dormant nature of APCs in PNG newborns is possibly an immune regulation mechanism to safeguard against development of harmful inflammatory responses in early life and to prepare newborns for the immune challenges in their postnatal environment ⁽²²⁸⁾.

There is a universal immunological memory to aeroallergens by adulthood, whereby the presence of allergic disease is related to cell cytokine responses from Th2 cytokines ⁽¹⁷¹⁾. According to Prescott *et al.*, low-level Th2 skewed allergen-specific responses at birth with minimum IFN- γ present in atopic and non-atopic infants' CBMC ⁽²²⁹⁾. Non-atopic newborns had higher Th2 responses to house dust mite which was downregulated by the first year of life, whereas atopic infants had distinct neonatal patterns of Th2 polarised allergen specific immunity and displayed declined capacity for production of Th1 cytokine IFN- γ . Neonates who subsequently developed atopy initially had reduced capacity to mount Th2 responses. In contrast to non-atopics who selectively downregulate their fetal Th2 polarised allergen-specific responses, atopic children displayed age-associated upregulation of Th2 immunity ⁽²²⁹⁾.

Seroreactivity to aeroallergens early in infancy is associated with increased risk of childhood asthma susceptibility ⁽²³⁰⁾. Maturation of Th responses to aeroallergens occurs postnatally in atopic and non-atopic infants ⁽²³¹⁾. Prescott *et al.* measured mononuclear-cell proliferative from CBMC and fresh PBMC, and cytokine responses to aeroallergens, such as *Feld 1* (cat allergen) and tetanus toxoid in atopic and non-atopic infants ⁽²³¹⁾. In both groups there was a low-level Th2-skewed allergen-specific response at birth, with little concomitant specific IFN- γ production. Neonatal Th2 cytokine responses, including IL-4, IL-6, IL-10 and IL-13 responses, were lower in the atopic group relative to the non-atopic group. Overall, there was a persistent Th2 response in atopic children associated with defective neonatal IFN- γ production which may drive development of an allergic phenotype ⁽²³¹⁾.

Evidence suggests that fetal exposures to maternal infection may exert long-term effects on skin function of the skin and immune system, especially in pathogenesis of AD and CMPA ^(232, 233). Prevalence of AD was significantly higher in children born to mothers who had experienced febrile or gynaecological infections compared with those without mothers suffering from that specific pregnancy complication ⁽²³²⁾. Infections during pregnancy may interfere with fetal and perinatal programming of skin immune development with different mechanisms, such as via exogenous toxins or inflammation,

or from inflammatory mediators derived from the placenta ⁽²³²⁾. A study demonstrated that elevated maternal C-reactive protein (CRP) levels in pregnancy was associated with a higher risk of AD in 4984 Dutch preschool children ⁽²³³⁾. During early pregnancy, skin of the fetus is highly permeable to components of amniotic fluid, thus cytokines from the surrounding micro-environment could be absorbed in immunoreactive forms ⁽²³⁴⁾. Furthermore, fetal exposure to paracetamol has been associated with a greater risk to develop allergic diseases in childhood and usage of antibiotics during pregnancy increases risk of eczema ^(232, 234, 235). Prevalence of CMPA is also associated with history of maternal infection and antimicrobial use during pregnancy ⁽²³⁶⁾. These studies suggest that infections and maternal inflammation can increase susceptibility of AD and CMPA *in utero* in offspring.

1.5.2 Immune function in children that develop allergy

Early-life environmental conditions can alter the course of immune and respiratory system development by: (1) sustaining Th2 bias during gestation; (2) arresting innate immune cell maturation; and (3) creating inflammatory dysfunction in the infant ⁽⁵⁾. Alteration of immune function development influences childhood allergy susceptibility predominantly via a mechanism involving epigenetic modifications and postnatal environmental exposures.

1.5.2.1 Epigenetics

Epigenetic mechanisms may facilitate gene susceptibility to develop asthma and atopic diseases via gene-environment interactions ^(169, 237). Using a rural birth cohort study, Michel *et al.* examined whether epigenetic patterns in asthma candidate genes are influenced by farm exposure in general or change over the first years of life, and whether these changes may contribute to asthma development, using DNA extracted from CB and whole blood ⁽²³⁸⁾. In CB, regions in *ORMDL1* and *STAT6* were hypomethylated while regions in *RAD50* and *IL-13* were hypermethylated in DNA from farmers' as compared to non-farmers' children. Changes in methylation over time occurred in 15 gene regions. These differences clustered in the genes were highly associated with asthma (*ORMDL* family) and IgE regulation (*RAD50*, *IL-13*, and *IL-4*), but not in Tregs genes (*FOXP3*, *RUNX3*). Therefore, findings suggest that DNA methylation patterns change significantly in early childhood and early environmental exposures play a key role in determining which gene-associated allergy is altered ⁽²³⁸⁾.

In mice, epigenetic changes can enhance development of allergic airway disease ⁽²³⁹⁾. Using DNA and RNA from PBMCs, Yang *et al.* reported 81 regions that were differentially methylated in inner-city children with persistent atopic asthma, as compared with healthy control subjects ⁽²³⁷⁾. Several immune

genes were hypomethylated in asthma, including *IL-13*, *RUNX3* and specific genes relevant to T lymphocytes (*TIGIT*). Amongst asthmatic patients, 11 differentially methylated regions were associated with higher serum IgE concentrations and 16 were associated with percent predicted FEV1. Data suggested that DNA methylation marks in specific gene loci are associated with asthma, thus epigenetic changes might be involved in establishing the asthma associated immune phenotype ⁽²³⁷⁾.

1.5.2.2 Environmental exposures

Early postnatal environmental exposures include breastfeeding, dietary intake and intestinal flora exposure during childhood, and environmental allergens, substances and climate changes.

i. Breastfeeding

Evidence suggests that exclusive breastfeeding offers some protection against AD and asthma, as compared with CMP formulas ⁽⁸⁵⁾. Breastfeeding for at least the first four months of life offers some protection against allergic disease, especially eczema and asthma, but the protective effect of exclusive breastfeeding beyond four months of age is uncertain ⁽⁸⁶⁾. The American Academy of Paediatrics recommends exclusive breastfeeding for at least four months in high risk infants and delaying complementary foods until four to six months of age to prevent or delay AD, CMPA and wheezing in the first two years of life ^(240, 241). There is also substantial evidence of the protective effect of breastfeeding on development of asthma and wheezing in early infancy and childhood, but evidence for its protective effect on other allergic disease is not as conclusive ^(240, 242, 243). Thus, exclusive breastfeeding is an established dietary intervention to reduce development of infant and childhood allergy.

Breastfeeding plays an important role in lung development and prevention of childhood asthma ^(242, 244, 245). A short period of exclusive breastfeeding and early introduction of bottle feeding or solid food may cause lung and airway growth impairment and increased risk of childhood asthma ⁽²⁰⁾. Breast milk consists of IgA, antimicrobial proteins and peptides, including lactoferrin and α -defensin, cytokines, glycans, lysozyme and long-chain fatty acids that stimulate the infant innate immune system, thus short breastfeeding duration may remove the protective effect against atopy and asthma ⁽²⁴⁶⁾. Nevertheless, the protective effect of breastfeeding remains controversial as some studies suggest that breastfeeding increases later risk of atopic disease and asthma ^(242, 244, 245). However, age of the child and parental allergy may be the confounding factors in conflicting results observed in these cross-sectional studies. A study suggested that breastfeeding has a protective effect on wheeze in the first three years of life, but has no effect on wheeze, atopy or bronchial hyperresponsiveness at ages seven to eight years ⁽²⁴²⁾.

Most importantly, the study indicates that breastfeeding does not increase risk of asthma or atopy in children, even when their mothers are asthmatic ⁽²⁴²⁾. Based on these studies, environmental exposures in postnatal life that include exclusive breastfeeding can reduce the risk of developing childhood asthma and atopic diseases, regardless of maternal asthma presence.

There is no evidence to support feeding with a hydrolysed formula for prevention of allergy compared to exclusive breastfeeding ^(240, 243). However, in high risk infants unable to be completely breastfed there limited evidence suggests that prolonged feeding with a hydrolysed infant formula (soy formula and other mammalian milks) compared to a cow milk formula reduces development of infant and childhood allergy ^(240, 243). This protective effect may better reflect tertiary prevention that includes reduction of disease expression in infants with pre-existing CMPA, rather than in infants without known CMPA.

ii. Dietary intake and intestinal flora exposure

Different factors have been hypothesised as postnatal risk factors for FA, including exposure to food allergens in early postnatal life, microbial factors in the gut and dietary factors ⁽⁷⁷⁾. Conventional intervention knowledge is that early exposure to allergenic food proteins during pregnancy, lactation, or infancy leads to FA in childhood. Thus, previous prevention strategies aimed to eliminate allergenic food proteins during these periods of immunologic vulnerability, especially in children with high risk of allergy ^(85, 247). However, Kattan recently found that early food avoidance leads to higher rates of FA ⁽⁸⁶⁾. Further, Du Toit *et al.* studied 640 infants aged four to 11 months with severe eczema, HE allergy, or both. The infants were randomly assigned to regularly consume or avoid peanuts until 60 months of age ⁽⁹⁹⁾. In both infant groups with negative and positive SPT results, prevalence of peanut allergy at 60 months of age was significantly lower in the consumption group (1.9 % and 10.6 %, respectively) than in the avoidance group (13.7 % and 35.5 %, respectively). The study demonstrated that early introduction of peanut significantly decreased frequency of peanut allergy among children at high risk for peanut allergy and modulated immune responses to peanuts ^(86, 99). In addition, there is evidence to support the use of dietary restriction in atopic pregnant and/or lactating women, but benefits are mostly restricted to the outcome of mild atopic eczema ^(86, 247). There is also a risk that food avoidance interventions may compromise maternal and fetal nutrition.

Less microbial exposure in early childhood is associated with more allergic disease later in life ^(97, 248). Allergic children have different fecal microflora, with less lactobacilli and bifidobacteria, as compared to non-allergic children. Food intake diversity during infancy may affect bacterial diversity of intestinal flora and is linked to development of allergic diseases ⁽²⁴⁸⁾. Nwaru *et al.* investigated associations between food diversity during the first year of life and development of asthma and allergies in 3142

Finnish children. Findings suggested that less food diversity during the first year of life may increase risk of asthma and allergies in childhood ⁽²⁴⁸⁾. The mechanisms for this association were unclear, however increased dietary antigen exposure may be a contributing factor of allergic disease development ⁽²⁴⁸⁾. Hence, reduced diversity of food intake during infancy reduces bacterial diversity of intestinal flora and increases asthma and allergy progression in childhood.

iii. Environmental allergens, substances and climate changes

Among the most extensively studied environmental factors influencing allergy are airborne allergens, such as dust mites, pollens, fungi and animal dander ⁽²⁴⁹⁾. Foods may elicit true IgE-mediated allergy and various non-immunological reactions associated with direct release of mediators or toxic activity. Over 85 000 chemicals are recognised in the human environment and they may act as contact allergens or irritants, causing allergic or non-allergic contact dermatitis including metals, fragrances, preservatives, botanicals and paraphenylenediamine ⁽²⁴⁹⁾. Moreover, infections can occur and may contribute to an exacerbation of allergic disease, especially in contact dermatitis ⁽²⁴⁹⁾.

Environmental allergens that influence development of AR in children can vary between urban and rural areas in different regions worldwide ⁽²⁵⁰⁾. Wang *et al.* studied inhaled allergen distribution and relevant factors of AR in 626 Guangzhou children diagnosed with AR, showing that the main inhaled allergens tested by SPT were dust mite, dog hair and cat hair. Furthermore, other correlated variables related with pathogenesis and development of AR in childhood were age distribution, living environment, family history and, most importantly, coexisting diseases of AD and asthma ⁽²⁵⁰⁾. Therefore, variations of postnatal factors exist between urban and rural areas that influence development of AR in children according to common airborne allergens within that area.

Domestic water hardness, swimming in chlorinated pools and climate changes may increase prevalence of childhood AD ^(251, 252). Exposure to hard water increased childhood AD in 358 Belgian children between the age of five to six years via mechanisms of epidermal barrier breaching by detergents, or salts in hard water, and by chlorine-based oxidants in swimming pool water ⁽²⁵¹⁾. AD prevalence was significantly lower with the highest-quartile mean annual relative humidity and increased UV exposure in a study involving 91 642 American children aged up to 17 years. However, AD susceptibility was increased with third-quartile mean annual indoor heating degree days ⁽²⁵²⁾. Hence, environmental exposures that influence skin integrity may increase prevalence of childhood AD. Therefore, early postnatal environmental exposures that include breastfeeding, dietary intake and intestinal flora exposure, environmental allergens, substances and climate changes may cause alteration of immune function development which influences childhood allergy susceptibility.

1.6 Early detection of allergy

1.6.1 Allergy detection in children

As allergic diseases usually occur in early life, early biomarkers of atopic susceptibility are required to target allergy and introduce preventive measures to high-risk infants. However, to date there is no reliable general or specific allergy phenotype detection method that is easy and non-invasive for children. Most reported allergic phenotype detection methods are invasive in nature, such as SPT, OFC and blood test, and many involve not readily accessible biological samples, such as CB, maternal blood or newborn vernix ^(56, 77, 163, 253-255).

Early markers of atopic predisposition, such as cord serum IgE (CS-IgE) levels and maternal blood concentrations of IgE, have been used to target allergy preventive measures in high risk infants ⁽²⁵⁴⁾. Elevated CS-IgE level is considered a risk factor for subsequent allergy in childhood and is useful in predicting subsequent atopic manifestations up to 20 years of age. The combination of elevated CS-IgE and positive family history of allergy is strongly associated with subsequent atopic manifestations in Finnish populations ⁽²⁵⁴⁾. Nabavi *et al.* studied 181 Iranian neonates and their mothers showing that IgE maternal blood concentration was correlated with IgE concentrations in CB ⁽¹⁶³⁾. Further results showed that presence of any kind of allergic disorder in the mother and elevated maternal blood IgE level was associated with CB IgE in the child ⁽¹⁶³⁾.

In AD, SPT is the gold standard method for allergy diagnosis. SPT is invasive in nature, but is the main tool in allergy diagnostics. However, there is mixed opinion regarding clinical usefulness of SPT ⁽²⁵⁵⁾. SPT allows identification of individuals at risk for FA and of the particular allergen inducing eczematous flare. Nevertheless, when performed by a non-specific specialist, positive SPTs to foods may wrongly lead to prolonged elimination diets which may induce nutritional deficiencies, tolerance loss to avoided foods and increased health costs ⁽²⁵³⁾. Therefore, there is a great need to find early, preferentially non-invasive, biomarkers to identify individuals susceptible to AD. Protein abundances in newborn vernix, such as Polyubiquitin-C and Calmodulin-like protein 5, show strong negative correlation to the AD group ⁽⁵⁶⁾. Polyubiquitin-C and Calmodulin-like protein 5 are promising candidates as biomarkers for identification of newborns predisposed to develop AD and have the potential to replace SPT as a non-invasive allergy diagnostic in children.

Diagnostic decision levels and component-specific IgE are useful in diagnosis of FA. OFC remains the gold standard diagnostic for FA, but it is time consuming, expensive and risky in terms of the child developing a severe allergic reaction during test ⁽²⁵⁵⁾. Nevertheless, OFC may also be an alternative

way to reduce parental anxiety and improve education ⁽⁷⁷⁾. An ideal *in vitro* test, such as clinical performance of microarray for specific IgE detection in children with challenge-proven/excluded CMPA or HE allergy, may be safer substitute for OFC ⁽²⁵⁶⁾. D'Urbano *et al.* showed that the microarray has a good ability to predict OFC results in children with suspected CMPA or HE allergy ⁽²⁵⁶⁾. Furthermore, this approach decreases the number of OFCs performed and decreases risk of a severe reaction, however it is not cost effective ⁽²⁵⁶⁾. Therefore, owing to severe reaction risk that may be caused by OFC, other cost effective and non-invasive pre-tests are needed.

Anaphylaxis diagnosis is based primarily on clinical criteria and is not dependent on an abnormal finding of laboratory tests that include serum total tryptase levels. Anaphylaxis diagnosis is not fully excluded regardless of normal results in laboratory tests ⁽³¹⁾. Positive SPT or increased serum specific IgE levels that test for potential triggering allergens confirm sensitisation, but do not diagnose anaphylaxis because asymptomatic sensitisation is common in the general population ⁽³¹⁾. Therefore, identifying a biological test that is non-invasive, safe and cost effective is urgently required. This thesis considers whether the placenta or saliva could be suitable biological samples for early detection of allergic risk.

Current evidence suggests there may be some genes altered in the placenta that are associated with allergic risk ^(15, 172-176). The placenta is an organ that is easily obtainable after delivery and could be used in the future as an important organ for identifying future disease risk. Saliva is a biological sample that is easy to collect via a painless procedure. Furthermore, saliva is the best approach for biomarker measurement as it is easily accessible, non-invasive and an easy bed side screening for real time measurement of the ongoing human physiological system ⁽²⁵⁷⁻²⁵⁹⁾. All of these advantages emphasise saliva as a very promising diagnostic candidate for detection and monitoring of disease biomarkers, especially in infants, toddlers, children and anxious or uncooperative patients ⁽²⁶⁰⁾. However, to date saliva is not considered a medically acceptable biomarker. Interestingly, recent studies of saliva suggest it can be used for detection of head and neck carcinoma, breast and gastric cancers, salivary gland disease, Sjögren syndrome, systemic sclerosis, dental and gingival pathology, preeclampsia and psychiatric and neurological diseases ^(257, 261-265). Furthermore, protein biomarkers have the advantages as modifiable influencing factors rather than genetic and epigenetic factors that are mostly non-modifiable factors for allergic diseases susceptibility in childhood.

1.6.2 Salivary protein confounding factors and childhood allergy

In the process of identifying potential salivary biomarkers for allergy, meticulous attention should be given to some salivary proteins that are dependent on age, sex or state of disease, especially caries lesions or periodontal disease ^(266, 267). Messana *et al.* investigated the human salivary proteome in a large group of subjects over a wide range of ages, from preterm newborns of 180 days of postconceptional age up to 17 year old subjects. The study defined the appearance and level changes in proteins typically found in adult saliva from the last months of fetal development to adulthood ⁽²⁶⁶⁾. Evidence suggested that individual variability of the salivary proteome is very high in the paediatric age, especially in preterm newborns, such as S100 proteins (Figure 8) ⁽²⁶⁶⁾. The study was based on the principle of important contribution of proteome variability due to physiological modifications occurring according to time dimension. Exploration of proteomic temporal changes is termed 'chrono-proteomics' ⁽²⁶⁶⁾. In relation to sex-specificity, a study evaluated inter-individual biochemical variation in a population of 268 systemically healthy young students ⁽²⁶⁷⁾. Findings revealed that salivary pH, buffering capacity, protein content, MUC5B, secretory IgA and chitinase activity were lower in female subjects compared to male subjects, whereas MUC7 and lysozyme activity were higher in female subjects ⁽²⁶⁷⁾. Findings suggest there are distinctive distributions and inter-relations of important salivary biochemical variables in a systemically healthy young adult population, highlighting significant gender differences in salivary biochemistry ⁽²⁶⁷⁾.

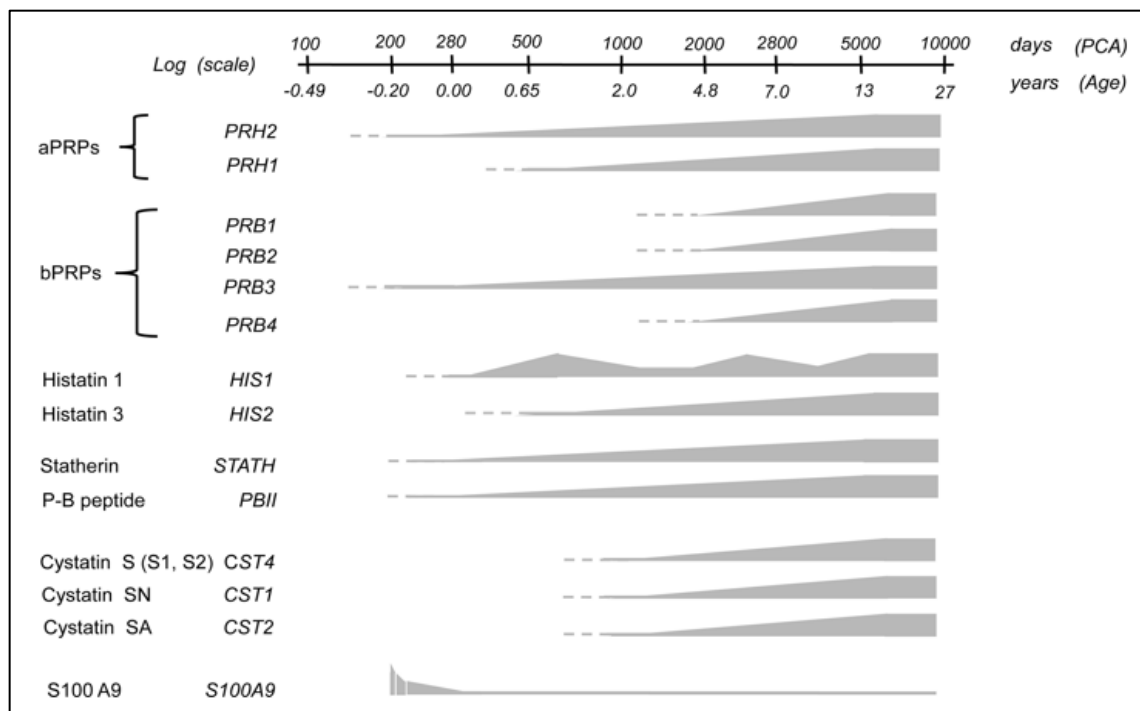


Figure 8. Scheme describing approximate time course of different salivary proteins and peptides ⁽²⁶⁶⁾. The sum of extracted ion chromatogram (XIC) peak areas of all members belonging to the same family of proteins/peptides, grouped on the basis of locus, is reported as a function of age. A logarithmic scale was used in the abscissa axis to better highlight major changes occurring in preterm newborns during the first months of life, which corresponds to the last months of fetal development, and those occurring immediately after the normal term of delivery and in the first years of life. Dashed lines represent the age range in which the proteins were detected in at least one baby. The upper limit of this range corresponds to the age in which saliva of all babies displayed the protein in the chromatographic profile, even if at small amount.

1.6.3 Gaps in knowledge

To date, there are very few studies that show *in utero* changes, especially protein changes in placental tissues associated with increased susceptibility to childhood allergy. In order to obtain more detailed evidence on how protein alterations correlate with development of childhood allergy, this research focuses on identification of proteins altered in placental tissue from children who subsequently developed an allergy and are compared to placentae from non-allergic children. Furthermore, the study identifies protein biomarkers in children's saliva that may predict risk of allergic susceptibility at birth or early childhood. This work provides evidence for development of clinical tools that measure protein biomarkers in biological samples for detection of allergic susceptibility. Such tools may be used as justification for targeted interventions in early childhood for disease prevention and assist in reducing morbidity and mortality caused by childhood allergy.

1.6.4 The role of placenta and saliva in early life allergy detection

Allergic disease has risen to epidemic proportions since the last decade ^(25, 30, 228, 237). Recent evidence shows that prenatal events play a critical role in determining disease susceptibility via environmental influences on placental function and fetal programming. Furthermore, evidence suggests that allergic disease is associated with immune system deviations that occur *in utero* ^(36, 157). Th1-dependent anti-microbial immunity suppression in the neonatal period is a consequence of fetomaternal tolerance to prevent fetus rejection, leading to Th2 dominance and, in combination with other events and exposures, could predispose children to an allergic disease ^(131, 137). This thesis supports the hypotheses that childhood susceptibility to allergy is increased through significant alterations in placental gene expression involved in regulating immune system development. Furthermore, this thesis proposes that altered protein concentrations in saliva samples collected from children with allergies in early life provide indirect measures of altered placental genes.

Saliva is a human body fluid with complicated constituents and multiple biological functions ^(258, 261, 268). Saliva contains analytes in concentrations that are 1000-fold less than those in human serum ⁽²⁶⁸⁾. Similar to serum, saliva contains hormones, amino acids, electrolyte, Ig, antibodies, growth factors, enzymes, microbes and their products. Most of these constituents enter saliva through blood barriers of capillary walls via passive diffusion, active transport or extracellular ultra-filtration ⁽²⁶⁸⁾. Moreover, expectorated saliva also contains a large proportion of the total gingival crevicular fluid (GCF) from the periodontal pockets in the whole mouth ⁽²⁶⁹⁾. GCF, an inflammatory serum exudate, caused by inflammation of periodontal tissues, originates from the blood vessels in the gingival connective tissue

(270). It carries biological molecular markers gathered from the systemic and surrounding circulation (265). The composition of the GCF is a complex combination of molecules coming from the blood, host tissues and subgingival biofilm, including; leucocytes, proteins, enzymes, tissue breakdown products, inflammatory mediators and cytokines produced locally in response to bacterial biofilm (270). GCF is as a source of biochemical disease indicators as it offers great potential reflecting the ongoing response generated by cells and tissues in the periodontium. Hence, saliva, which also contains GCF is an important body fluid that reflects the physiological and pathological function of the human body (258).

The main function of saliva in the oral cavity include lubrication and binding, followed by solubilisation of dry food, oral hygiene, initiation of starch digestion and immunity (263). Lubrication, through the provision of a lubricious film that contains mucins, proline-rich proteins, and water; aids in lubricating the hard and soft oral surfaces and is essential for speech, mastication and swallowing (268). Saliva helps bolus formation by moistening food which protects the oral mucosa against mechanical damage and help in the preliminary digestion of food through the presence of salivary alpha amylase which breaks up carbohydrates into sugars while salivary lipase initiates fat digestion (268). It also facilitates taste perception and digestion of carbohydrates through salivary alpha amylase. This process enables soluble food-derived molecules to reach the gustative papillae and buffer the acid components of food with the bicarbonates (271).

Saliva also contains lysozyme, an enzyme that lyses bacteria and prevents overgrowth of oral microbial populations (268). The maintenance of tooth and mucosal integrity by saliva and its antibacterial and antiviral activities are mainly attributable to salivary mucins, which bind to bacteria and prevent bacterial adhesion to tooth enamel. Saliva proteins are identified according to their functions. Interestingly, the main known function of salivary proteins is immunity, with 21 % of salivary proteins being involved in immune function (261). Therefore, saliva is a promising biological sample that may contain biomarkers of allergic diseases risk, which is the focus of this thesis.

Proteomic technologies with advanced instruments and refined analytical techniques are widely used as powerful approaches for biomarker-related clinical applications. Mass spectrometry (MS) based methods used for salivary biomarker identification have been explored in the past decade and have become one of the core technologies for proteomics due to high sensitivity and accuracy for mass measurement (272). These include variety of MS techniques, such as two-dimensional gel electrophoresis–mass spectrometry (2-DE/MS), liquid chromatography tandem mass spectrometry (LC–MS/MS), matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) and surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF/MS), which have been extensively applied to identification biomarkers in saliva, but not in relation to allergic diseases (Table 3) (272).

Although MS has been used extensively, most studies have moderate sample sizes (less than 45) as MS is an expensive method. Therefore, validation methods used, such as ELISA and Western blot, with larger sample sizes have been used to confirm MS findings (Table 3) and this is the approach taken in this research.

Current evidence shows that prenatal events play a critical role in determining childhood allergic disease susceptibility via environmental influences on placental function and fetal programming. Therefore, the hypothesis for this thesis is that childhood susceptibility to allergy is increased through significant alterations in placental gene and protein expression involved in regulating immune system development due to placental adaptation to the maternal environment. Further, products of altered placental genes and proteins are associated with altered protein concentrations in the saliva of children with allergies.

Saliva is a non-invasive and stress-free alternative to blood sampling, thus eliminating any discomfort and pain associated with blood venepuncture. It is a readily accessible secretion and is widely accepted as a potential medium for clinical diagnostics ⁽²⁷²⁾. Moreover, as compared with blood, saliva contains a smaller quantity of proteins, therefore decreasing any potential risk of non-specific interference and hydrostatic interactions ⁽²⁷³⁾. Within blood, the protein concentration can vary over several orders of magnitude, with protein half-lives ranging from a few seconds to several months or longer. Interestingly, the composition of saliva is not as complex or varying as serum, and should more accurately reflect the current condition of the body at any given time ⁽²⁷³⁾. Furthermore, as compared to blood, other advantages include easy and multiple sampling opportunities, reduced need for sample pre-processing and thus is cost-effective, minimal risk of contracting infectious organisms, and it is also an ideal biofluid for collecting specimens from patients in developing countries ^(272, 274). Thus, saliva has the promising potential to replace blood as the gold standard in diagnosing allergic diseases.

This research aimed to identify proteins associated with childhood allergy using two sources of biological tissue: placental tissue and saliva, collected from two populations of women whose children have different risks of allergic disease susceptibility. The long term objectives of the study were to:

1. Identify proteins that could be measures of allergic risk in placenta and saliva,
2. Develop an assay for detection of allergic risk in newborns,
3. Target proteins for interventions that prevent allergy onset.

Table 3. Summary of MS-based methods used for salivary biomarker identification. Table adapted from ⁽²⁷²⁾.

Disease	Saliva	Stimulation	Proteomics approach	Biomarkers	Verification methods	References	Sample size
Breast cancer	Whole	Stimulated	SELDI-TOF/MS	• c-erbB-2	• ELISA • Western blot	Streckfus, Bigler, & Zwick, 2006	Control – 3 Disease – 3
Caries	Whole	Unstimulated	2-DE/MS	• Statherin 5 • Cystatin	• Western blot	Rudney, Staikov, & Johnson, 2009	Control – 18 Disease – 23
Gastric cancer	Whole	Unstimulated	MALDI-TOF/MS	• 1472.78 Da • 2936.49 Da • 6556.81 Da • 7081.17 Da	–	Wu, 2009	Control – 18 Disease – 23
Graft versus host disease	SM/SL	Stimulated	SELDI-TOF/MS MALDI-TOF/MS	• Lactoferrin • SLPI • IgA • b2-microglobulin	• ELISA	Imanguli <i>et al.</i> , 2007	Control – 0 Disease – 41
HNSC	Whole	Stimulated	LC–MS/MS	• Complement factor B	• Western blot	Ohshiro <i>et al.</i> , 2007	Control – 5 Disease – 3
Oral lichen planus	Whole	Unstimulated	2-DE MALDI-TOF/MS	• Urinary prokallikrein • PLUNC	–	Yang, Liu, Liu, Cheng, & Li, 2006	Control – 6 Disease – 6
OSCC	Whole	Unstimulated	2-DE/MS LC–MS/MS	• M2BP • Catalase • Profiling1 • CD59 • MRP14	• ELISA • Western blot	Hu <i>et al.</i> , 2008	Control – 64 Disease – 64
Pulmonary disease	Whole	Unstimulated	2-DE/MS	• Lipocalin • Apolipoprotein A1	–	Nicholas <i>et al.</i> , 2010	Control – 20 Disease – 25
Type-1 Diabetes	Whole	Stimulated	2-DE MALDI-TOF/MS	• α-amylase • Cystatin • PIP	–	Hirtz <i>et al.</i> , 2006	Control – 8 Disease – 8
Type-2 diabetes	Whole	Unstimulated	LC–MS/MS	• A1AT • α-2-macroglobulin • Cystatin C • Transthyretin	• ELISA	Rao <i>et al.</i> , 2009	Control – 10 Disease – 30

° Abbreviations: SM represents submandibular, SL represents sublingual, HNSC represents head and neck squamous carcinoma, OSCC represents oral squamous cell carcinoma and SS represents Sjögren's syndrome.

1.7 Hypotheses and aims

HYPOTHESIS 1: Childhood susceptibility to allergy may be programmed *in utero* due to differences in placental adaptation to the maternal environment. Susceptibility to allergy may be identifiable through alterations in placental gene and proteomic profiles.

AIM 1: To identify differentially expressed genes in placenta collected from children with and without allergy.

AIM 2: To optimise a proteomic method for identification of placental proteins associated with allergic disease in childhood.

AIM 3: To identify different proteomic profiles in children who subsequently develop allergy.

AIM 4: To determine whether presence of maternal asthma alters the proteomic profile of children who subsequently develop allergy.

AIM 5: To determine whether proteomic profiles vary in a sex-specific manner in children who subsequently develop allergy.

HYPOTHESIS 2: Childhood susceptibility to allergy can be detected in saliva.

AIM 1: To optimise a proteomics method for identification of salivary proteins associated with allergic disease in childhood.

AIM 2: To determine if saliva is a biological fluid that can be used to detect allergy in susceptible children.

AIM 3: To determine whether the proteomic profile varies with different allergic phenotypes.

AIM 4: To identify whether there is a common protein present for all allergic phenotypes.

HYPOTHESIS 3: Changes in placenta proteomic profiles are reflected in a similar change in saliva in early childhood.

AIM 1: To determine proteins with similar expression profile in placenta and saliva samples of children with allergic diseases.

AIM 2: To determine whether differential expression of proteins that are altered in placenta can also be detected in saliva of children with allergic diseases.

2.0 METHODOLOGY

2.1 Introduction

Two biological samples were used in this study: placenta and saliva. Preliminary workflow of placental sample analysis was conducted by comparing data between previous preliminary assessment of microarray on placental samples of children with allergy, and LC-MS/MS on saliva sample of asthmatic mothers. Samples workflows performed on both of the saliva and placental tissues are as described in Figure 9. The methodology for placental samples from children with and without an allergy include quantitative real-time polymerase chain reaction (qRT-PCR) and Proteomics (LC-MS/MS); and validation study of Western blot. For saliva samples, optimisation methods include Proteomics (LC-MS/MS); and two validation studies which were Western blot and Blitz system.

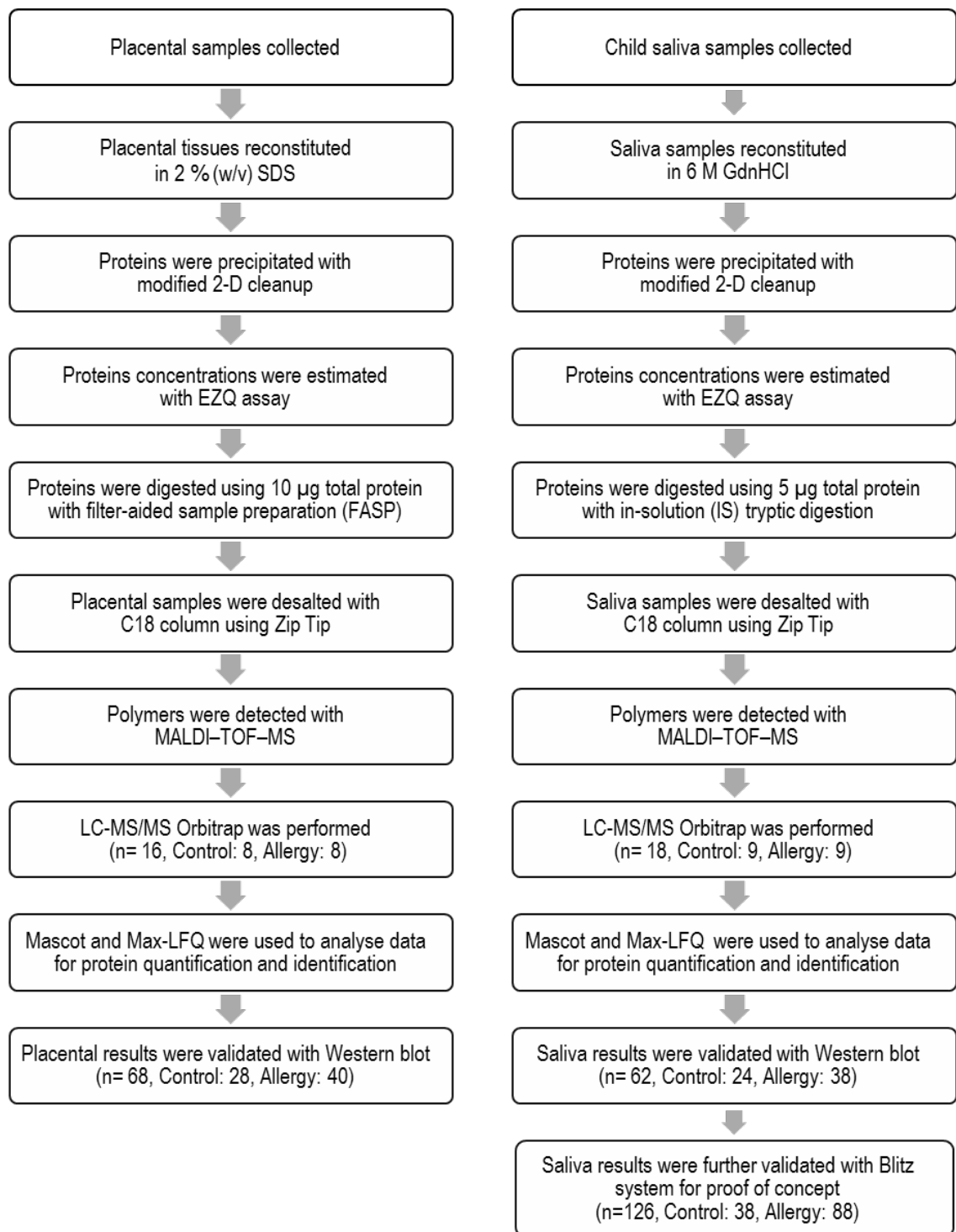


Figure 9. Samples workflows on the saliva and placental tissues. (GdnHCl represents guanidine hydrochloride, SDS represents sodium dodecyl sulfate, MALDI-TOF-MS represents matrix assisted laser desorption ionization-time of flight mass spectrometry and LC-MS/MS represents liquid chromatography tandem mass spectrometry).

2.1.1 Study population

Women with asthma or no asthma were recruited at their first antenatal visit at Lyell McEwin Hospital in a prospective cohort from the Asthma and Pregnancy Cohort study (n= 287). Consent was obtained prior to entry into the study and ethics approval was provided by Lyell McEwin Hospital Human Research Ethics Committee (HREC) with HREC reference number: 2009045, as attached in **Appendix 1**. During antenatal visits at 12, 20, 28, and 36 weeks of pregnancy, women were assessed by a midwife, with additional respiratory training; information related to atopy, asthma, diet and fetal growth were assessed. The midwife utilised a standardised data collection tool to collect demographic data including maternal weight, height, previous obstetric history, medical history. Maternal smoking was assessed by maternal self-report, with women classified according to smoking status during the first antenatal visit. Socioeconomic status for each woman was determined using her residential postcode at the time of delivery ⁽²⁷⁵⁻²⁷⁷⁾. Placental tissue was collected at delivery. Children were followed-up every year from 6 months of age and assessment on atopy was conducted using a modified International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire, as attached in **Appendix 2**. ISAAC questionnaires are self-reported by parents to evaluate asthma, allergic rhinitis and eczema in childhood. It contains 17 items and core questionnaires are to assess the prevalence and severity of asthma, rhinitis and eczema in defined populations. Saliva samples were collected from the infants between 6 months to 5 years of age. Allergy determined by doctor diagnosis was documented.

In addition to this Asthma and Pregnancy Cohort study, other samples of child saliva were collected from patients between 6 months to 5 years old that were receiving treatment in the Women's and Children's Hospital. Suitability of the potential participants for the study were screened through modified ISAAC questionnaires while these children were attending a Paediatric Clinic for assessment of allergic status. Consent was obtained prior to saliva collection and ethics approval was provided by Women's and Children's Hospital HREC, as attached in **Appendix 3**. Parent information sheet and consent form are also attached in **Appendix 4 and 5**.

As this study was using human biological samples, the sample size were based on the availability of the placental and saliva samples collected in the Asthma and Pregnancy Cohort study and Women's and Children's Hospital. The sample size differed for both placental and saliva samples. For placental samples, 54 in qRT-PCR, 16 in LC-MS/MS and 68 in Western blot whereas the sample size for saliva samples were 18 in LC-MS/MS, 62 in Western blot and 126 in Blitz method.

2.2 Materials and reagents

All the chemicals, solutions, enzyme and peptide used in this project are listed in the Appendix 6.

2.3 Biological marker (biomarker)

2.3.1 Criteria for biological marker (biomarker)

According to the National Institutes of Health, a biological marker (biomarker) is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmaceutical responses to a therapeutic intervention ^(273, 278). A biomarker can be defined as any biomolecule or a specific characteristic, feature, and indicator of a change in any biological structure and function that can objectively measure the state of a living organism ⁽²⁷⁹⁾.

Criteria for biomarker include:

- 1) A major product of oxidative modification that may be implicated directly in the development of a disease.
- 2) A stable product, not susceptible to artefactual induction, not easy to lose, or not changeable during storage.
- 3) Representative of the balance between oxidative damage generation and clearance.
- 4) Determined by an analytical assay that is specific, sensitive, reproducible and robust.
- 5) Free of confounding and interference factors from dietary intake.
- 6) Accessible in a target tissue or a valid surrogate tissue such as a leukocyte.
- 7) Detectable and measurable within the limits of detection of a reliable analytical procedure ^(273, 280).

Based on this criteria, suitable placental and salivary protein biomarkers for allergic diseases that can be identified in early life will be the focus of the research.

2.3.2 Criteria for potential salivary protein biomarkers for allergy

Therefore, to choose salivary protein targets for this current study, specific criteria for selection were defined. The criteria include:

- 1) Exclude proteins that are:
 - a. Abundantly expressed in saliva.
 - b. Associated with other pathologies.
 - c. Altered in an inflammatory response in a nonspecific manner.
 - d. That change with age and sex.
- 2) Include proteins that are:
 - a. Known to be associated with the disease of interest.
 - b. Are not commonly observed in saliva.

In addition, confounding factor that should be considered includes proteins that are produced by the salivary glands versus proteins that enter saliva by diffusion from the circulation.

2.4 Saliva samples

2.4.1 Types of saliva samples

There are 2 types of salivation, unstimulated and stimulated salivation. Unstimulated salivation is watery saliva produced by salivary gland at rest, reflects basal salivary flow rate and stimulated by parasympathetic innervation. Unstimulated salivation occurs for about 14 hours a day and 90 % of this saliva is produced by major salivary glands ⁽²⁸¹⁾. On the contrary, stimulated salivation represents the thicker secretion during food intake and stimulated by sympathetic innervation. This saliva is present in our mouths for up to 2 hours and higher in the afternoon than in the morning salivary protein; its concentration follows this diurnal pattern ⁽²⁸¹⁾.

In this particular study, stimulated salivation was chosen; in which the children chewed on the swab to stimulate the saliva production.

2.4.2 Saliva samples collection method

There are 4 commercially available saliva collection devices that are described in Figure 10 and include:

- 1) Unstimulated saliva: drool, SalivaBio Oral Swab (SOS) (Salimetrics, Carlsbad, California, United States).
- 2) Mechanically stimulated saliva: Salivette with cotton swab (Sarstedt, Thermo Fisher Scientific, California, United States).
- 3) Mechanically stimulated saliva: Salivette with synthetic swab (Sarstedt, Thermo Fisher Scientific, California, United States).
- 4) Acid stimulation saliva: GBO Saliva Collection System (Greiner Bio-One, Kremsmünster, Austria).

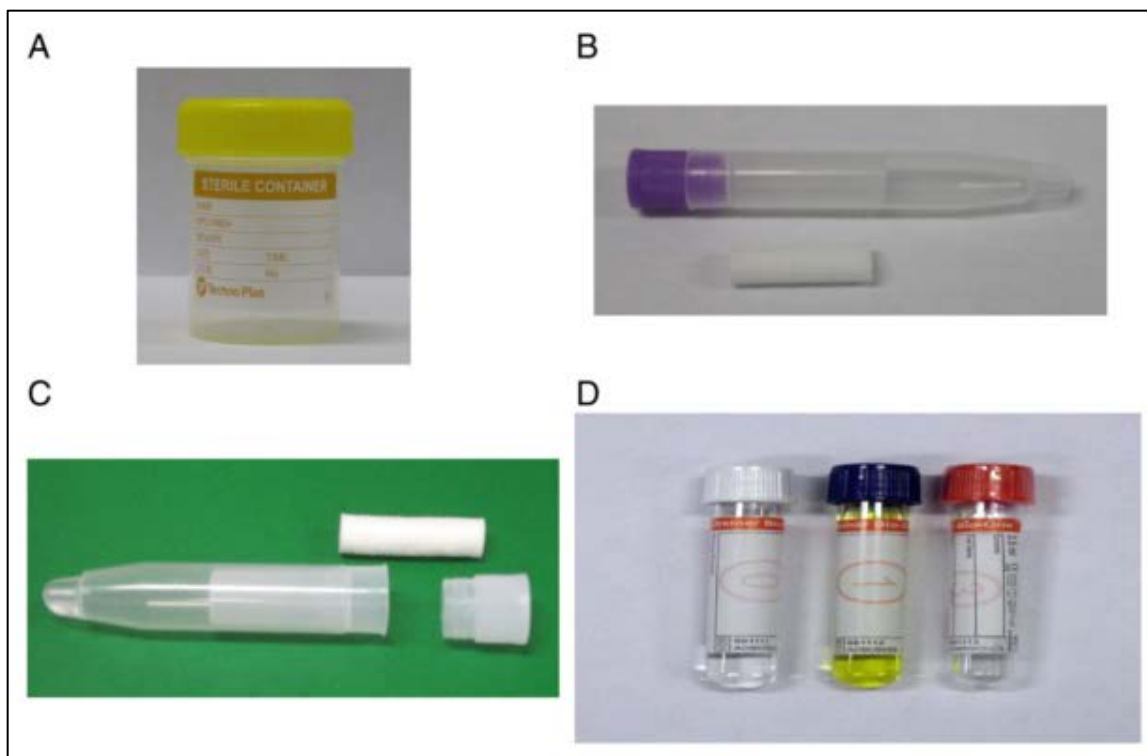


Figure 10. Commercially available saliva collection devices. Figure adapted from ⁽²⁸²⁾. (A) Drool collected in a sterile specimen container. (B) SalivaBio Oral Swab (SOS) (Salimetrics, Carlsbad, California, United States). (C) Salivette: Cotton and Synthetic swab (Sarstedt, Thermo Fisher Scientific, California, United States). (D) GBO Saliva Collection System (Greiner Bio-One, Kremsmünster, Austria).

There are variation of salivary protein composition and salivary flow rate depending on the methods used in saliva samples collection ⁽²⁸²⁾. Standard drool method had significantly higher salivary protein concentration as compared with GBO Saliva Collection System method. Furthermore, salivary flow rates were significantly lower in unstimulated saliva which includes drool and SalivaBio Oral Swab (SOS), when compared with mechanically stimulated methods. These results demonstrated significantly relevant differences in analyte levels and in the salivary flow rates depending on the saliva collection method ⁽²⁸²⁾. However, based on the current study population that were children between 6 months to 5 years old, mechanically stimulated saliva using salivette with cotton swab (Sarstedt, Thermo Fisher Scientific, California, United States) was the most suitable saliva collection device to be used in this study. This method was chosen as it was an easy, non-invasive and painless procedure, which favours its use in this population of study ⁽²⁵⁹⁾. Unstimulated saliva using drool or SOS and acid stimulation saliva using GBO Saliva Collection System are not feasible and can be extremely difficult to perform on these children.

2.4.3 Saliva samples collection protocol using salivette

I have used 2 methods to collect the saliva, depending on the child's age, using salivettes with cotton swab (Sarstedt, Thermo Fisher Scientific, California, United States) (Appendix 7) and standard drool method. These 2 methods were chosen and conducted according to the flexibility and cooperation of the child during saliva samples collection procedure. The saliva samples collection procedure was conducted during a fixed time between 8.30 in the morning to 12.00 in the afternoon to enable the researcher to minimise the baseline variations, thus to reduce the diurnal variations of salivary proteins.

If the child is less than 3 years old, the drooling saliva was collected from the child's mouth using the tube or swab. First, the salivette was held at the rim of the suspended insert (Figure 11c) and the stopper is parted (Figure 11a) by slightly pushing it to the side. Then, the swab (Figure 11b) is removed from the salivette and the tube is placed at the tip of the child's mouth to collect the drooling saliva in younger children (Figures 11 and 12). Older children were asked to spit inside the tube, or saliva was collected from the child's mouth by inserting the cotton swab and allowing them to hold or chew it in their mouth for 1 minute. Approximately 1 mL saliva volume was collected with both methods.

After completion of the saliva collection, the salivette was centrifuged at 3500 rpm for 30 minutes at 4 °C. The centrifugation was performed within 4-6 hour after the saliva collection to avoid further protein degradation. A 10 µl aliquot of protease inhibitor cocktail (PIC) (v/v 1:100), Sigma Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, Missouri, United States) was added to the salivette prior to saliva collection to inhibit protein degradation. The salivette and saliva were kept on ice (0 to -4 °C) during the whole collection procedure as PIC will degrade if keep in temperature higher than 4 °C. After centrifugation, the solution of saliva and PIC was mixed and aliquoted to several cryotubes containing at least 200 µL of saliva and stored at -80 °C. At least 200 µL of saliva was required for the proteomic analysis, therefore only 1 tube was thawed at one time and this minimized the protein degradation effect on thawing and re-freezing of the saliva samples.

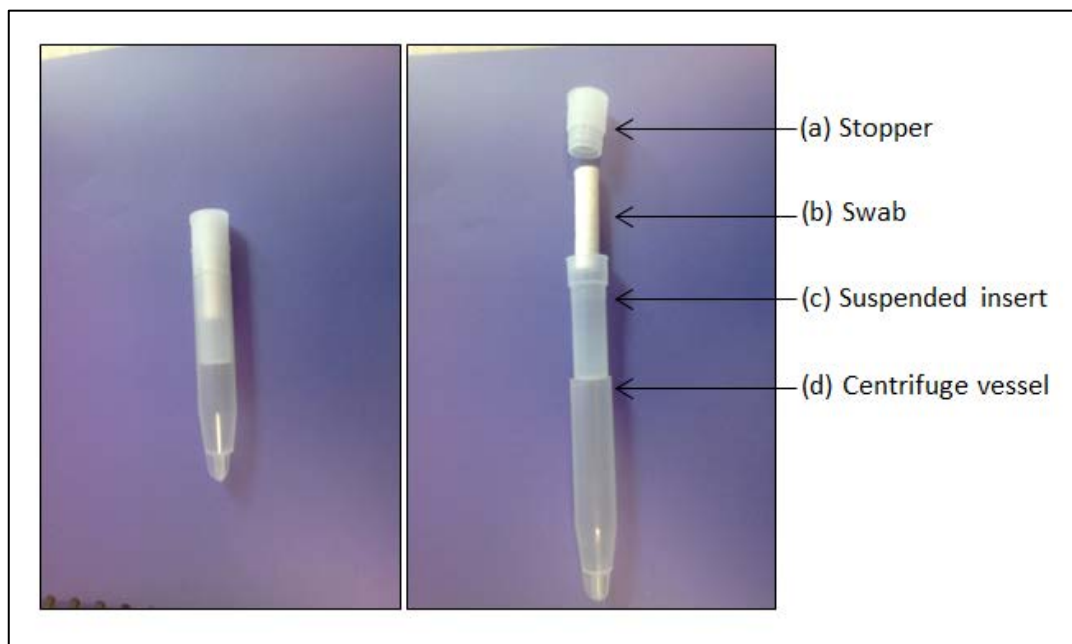


Figure 11. Salivette's parts. (a) Stopper. (b) Swab. (c) Suspended insert. (d) Centrifuge vessel.

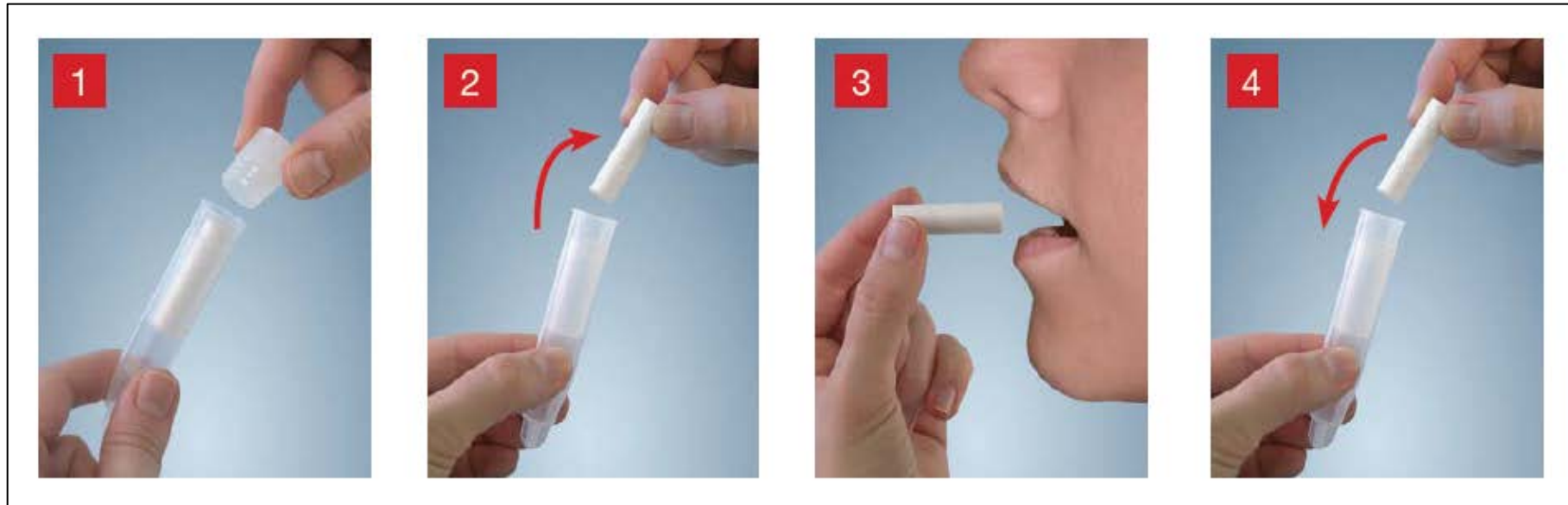


Figure 12. Steps of salivette collection method. (1) The salivette is held at the rim of the suspended insert and the stopper is parted by slightly pushing it to the side. (2) The swab is removed from the salivette. (3) Let the child chew the swab in his or her mouth for 1 minute or, the tube is placed at the tip of the child's mouth to collect the drooling saliva. (4) The swab is put back to the suspended insert and the salivette is closed firmly with the stopper.

2.5 Placental tissue collection

Placentae were collected within 45 minutes of delivery on ice to stop protein degradation and multiple samples were pooled together from the central and peripheral regions of the maternal side of the placenta. Tissues were washed in saline, mixed together and cut into small pieces before being placed in 5 ml tubes and snap frozen in liquid nitrogen. Samples were stored at -80 °C until required.

In preparation for protein extraction and protein precipitation for mass spectrometry (MS), placental tissues were grounded using pestle and mortar using liquid nitrogen to ensure that the tissues were not abruptly thawed causing the tissue protein to be degraded. Approximately 50 mg of placental tissue was suspended in a 1.5 mL tube containing 900 μ L 2 % (w/v) SDS and 100 μ L PIC (1:10 dilution). The samples were aliquoted into 300 μ L aliquots and placed in the Bioruptor on high speed for 15 minutes. The samples were then centrifuged at 20 000 g for 30 minutes and supernatant collected and stored at -80 °C until further use.

2.6 Placental samples preparation for quantitative real-time polymerase chain reaction (qRT-PCR)

2.6.1 Placental RNA extraction

Total RNA was extracted from 0.2 g of crushed frozen placenta using the TRIzol method (Invitrogen, Life Technologies, Carlsbad, California, United States). Briefly, 1 mL trizol/0.1 g tissue was added to the crushed frozen placenta prior to homogenizing (Polytron homogenizer, Kinematica, Luzern, Switzerland) ⁽²⁸³⁾. The homogenate was centrifuged (7800 g, 10 minutes) and 0.2 mL chloroform/1 mL trizol was added to the supernatant. Then, the mixture was shaken vigorously by hand for 15 seconds, then centrifuged again (7800 g, 15 minutes). Next, the supernatant was removed, and 0.5 mL isopropanol/1 mL trizol was added. After the solution was incubated at room temperature for 10 minutes, the solution was centrifuged (7800 g, 10 minutes), the supernatant was removed, and 1 mL 75 % (v/v) ethanol/1 mL trizol was added. RNA concentration was measured using spectrophotometer (Implen, Munich, Germany) and RNA quality and integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, United States). As a mandatory requirement, only RNA samples demonstrating intact 28S and 28S rRNA peaks and a high RNA integrity number (RIN) (>6) were included in the study. Reverse transcription was performed on 1 µg of total RNA using the iScript cDNA Synthesis kit (Biorad, Hercules, California, United States) according to the manufacturer's instructions.

2.6.2 Quantitative real-time polymerase chain reaction (qRT-PCR)

Taqman Gene Expression Assays (Life Technologies, Carlsbad, California, United States) were used to measure expression of putative allergy associated genes. qRT-PCR was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Mulgrave, VIC, Australia) according to the manufacturer's instructions, and two technical replicates were included for each sample per assay. PCR data was analysed using the 2- $\Delta\Delta C_t$ method: ΔC_t sample - ΔC_t calibrator, with expression of each gene normalized to housekeeping gene, beta-actin for gene expression analyses of placental tissue.

2.7 Sample preparation for mass spectrometry (MS)

There are 4 steps involved in sample preparation for MS that include modified 2-D clean-up, EZQ protein quantification assay, protein digestion and desalting using C18 column (refer Figure 9, Methodology section 2.1).

2.7.1 Protein precipitation with modified 2-D clean-up

Protein extraction was performed using the ReadyPrep™ 2-D Cleanup Kit (Bio-Rad, California, United States). This kit facilitates the preparation of low conductivity samples through methods of concentrating protein from samples that are too dilute, allowing for higher protein loads that can improve spot detection. This procedure works to quantitatively precipitate and concentrate protein in the sample while washing away substances like ionic detergents, salts; nucleic acids; lipids and plant-derived phenolic compounds. Additionally, the precipitation is not affected by the presence of detergents, or other common buffers, salts and reagents used to extract or treat proteins. The entire process can be completed in approximately 2 hours, depending on how many samples being processed.

The protocol of ReadyPrep™ 2-D Cleanup Kit was modified from the original manual supplied by the manufacturer to optimise the protein precipitation. Both of samples, placental tissue and saliva were thawed and kept on ice (0 to -4 °C) during the whole process. Firstly, 200 µL of sample was added with 600 µL precipitating agent 1 with a 3:1 ratio and incubated for 30 minutes on ice. Next, precipitating agent 2 was added in the same volume of precipitating agent 1, centrifuged at 20 000 g for 20 minutes at -9 °C and then the supernatants were discarded. This step is followed by adding 40 µL of wash reagent 1, incubated on ice for 5 minutes, centrifuged at 20 000 g for 10 minutes at -9 °C and then the wash reagents were removed. Then, protein pellets were washed with 1 mL of wash reagent 2 (acetone) and 5 µL of wash additive 2 and incubated on ice for 30 minutes. The samples were vortexed every 10 minutes during the incubation for at least 30 seconds. After that, the samples were centrifuged down at 20 000 g, 30 minutes at -9 °C and the supernatants were discarded. Finally, the protein pellets were air-dried for up to 5 minutes and resuspended in either 20 µL 6 M GdnHCl for the saliva sample or in 50 µL 2 % (w/v) SDS for the placental tissue. The reconstituted samples were then vortexed, incubated at room temperature for 5 minutes and vortexed again for about 1 minute to ensure optimum suspension. Alternatively, sonication for 5 minutes was also used if the pellets were too large or the pellets had been over dried. Aliquots of the protein extracts were stored at -80 °C until further analysis.

2.7.2 Estimation of proteins concentrations with EZQ protein quantification essay

Fluorescence-based EZQ® protein assay (Invitrogen, Thermo Fisher Scientific, California, United States) was used to determine the sample protein concentration prior to tryptic digestion. Ovalbumin, a lyophilised standard protein was used as the protein standard reference. Two-fold serial dilutions of 5 µg/µL ovalbumin in 1 % (w/v) SDS stock solution was prepared using either GdnHCl for saliva samples and 2 % (w/v) SDS buffer for placental tissue, down to 0.078125 µg/µL of protein concentration. Sample concentrations needed to be less than 5 µg/µL and spotted as neat and serial dilutions of 1/10 and 1/20. One microliter of protein standards, samples and buffer as the no-protein control were pipetted in triplicate on the assay paper that was already clipped on the EZQ 96-well micro plate cassette. The protein spotted assay paper was dried on a 56°C heating block, then fixed and washed in 40 mL methanol for 5 minutes with gentle shaking on the orbital shaker. This step removes contaminating substances including urea, SDS; reducing agents; salts and dyes.

Next, the assay paper was dried on a 56 °C heating block and then stained with 40 mL of EZQ protein quantitation reagent (Invitrogen, Thermo Fisher Scientific, California, United States) for 30 minutes on the orbital shaker with minimum light exposure. After staining, the assay paper was rinsed 3 times for 1.5 minutes in rinse buffer (10 % (v/v) methanol/7 % (v/v) acetic acid), lastly with reverse osmosis (RO) water and kept wet by soaking it in RO water. The results were then analysed using the Typhoon fluorescence scanner, Typhoon Imager (GE Healthcare, Little Chalfont, United Kingdom) or ChemiDoc (Life Science, Bio-Rad Laboratories, California, United States) using the wet assay paper (Figures 13 and 14). The scanning parameters for Typhoon fluorescence scanner were set to: fluorescence acquisition mode, 300 V photo multiplier tube (PMT), emission filter of 610 BP, excitation wave length of laser blue 488 and 200 µm pixel resolution. Whereas the scanning parameters for ChemiDoc (Life Science, Bio-Rad Laboratories) using Image Lab software, Version 5.2.1 (Life Science, Bio-Rad Laboratories, California, United States) were set to: single channel protocol, gel imaging acquisition setting, blots application of SYPRO Ruby, Bio-Rad Mini Ready Agarose gel imaging area, optimise image exposure time for intense band and highlight saturated pixels display option.

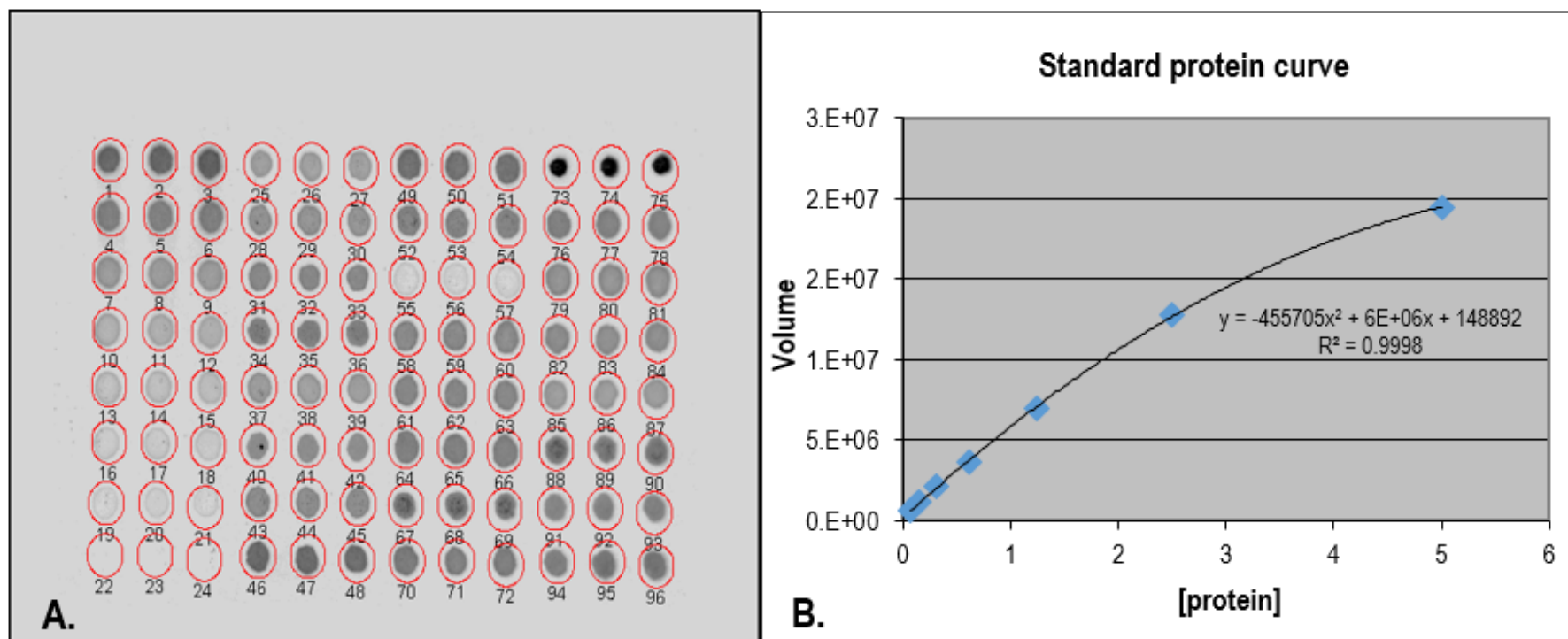


Figure 13. Saliva samples concentration estimation with EZQ protein quantification assay. A. Saliva samples spotting viewed on ChemiDoc. B. Saliva samples standard protein curve.

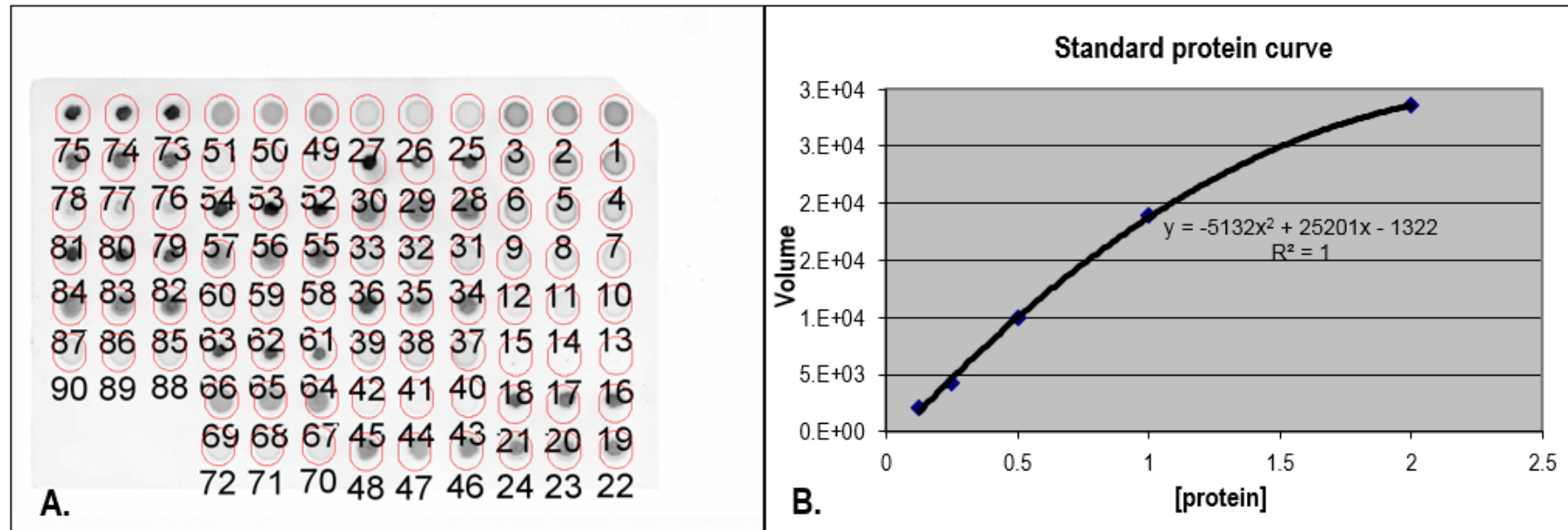


Figure 14. Placental samples concentration estimation with EZQ protein quantification assay. A. Placental samples spotting viewed on Typhoon Imager. B. Placental samples standard protein curve.

2.7.3 In-solution (IS) tryptic digestion of saliva samples

Protein in saliva samples were digested using method of in-solution (IS) tryptic digestion. For saliva, 5 µg total proteins were used for each sample. The protein sample was reduced in final DTT concentration of 20 mM by incubating for 45 minutes at 56 °C. Samples were cooled to room temperature and then alkylated in 55 mM IAA in 100 mM ammonium bicarbonate (NH₄HCO₃), 15 minutes at room temperature in dark. Protein digestion depends on digestion buffer and its pH (7.5 to 8.5), enzyme to substrate ratio, as well as temperature and time.

The saliva samples in 6 M GdnHCl were diluted to at least 0.5 M with 100 mM NH₄HCO₃. The sample protein was digested with sequencing grade trypsin (Promega, Wisconsin, United States) in a ratio of 1:50 (enzyme to protein) by incubating at 37 °C overnight. Protein digestion was stopped by adding 100 % (v/v) TFA to 0.5 % (v/v) final concentration. The peptide-containing extracts were dried-up in the 1.5 mL centrifuge tube using a vacuum centrifuge, the SpeedVac (Thermo Fisher Scientific, Massachusetts, United States), until approximately 1 µL in volume. The extracted peptides were then reconstituted to 25 µL with TA2 (2 % (v/v) ACN/0.1 % (v/v) TFA) and stored at -20 °C until the desalting step.

2.7.4 Filter-aided sample preparation (FASP) for placental samples

FASP has both the advantage of in-gel and IS protein digestion for mass-spectrometry (MS) based proteomics⁽²⁸⁴⁾. SDS was used to solubilize the proteome and then exchanged with urea on a standard filtration device. Advantages of FASP are: 1) provide protein digest that are free from nucleic acids and other cell components, 2) can be applied to samples containing high concentrations of detergents; 3) no precipitation thus the concentration of sample is kept constant; 4) up to 0.2-200 µg of total protein can be processed in a single filter device, and 5) the yield and purity of peptides can be monitored by UV-spectrometry allowing quality control of the digest⁽²⁸⁴⁾.

For placental tissue, 10 µg total proteins and 200 µL freshly made 7M urea were loaded to spin filter, Vivacon 500 (Sartorius, Göttingen, Germany) and then centrifuged at 14000 g for 15 minutes at room temperature. Proteins were reduced by adding 100 µL of 20 mM DTT in 7 M urea in the spin column and then incubated for 60 minutes at room temperature. Spin column was spun at 14 000 g for 15 minutes. Alkylation was performed by adding 100 µL of 55 mM IAA in 7 M urea in the spin column and

then incubated for 20 minutes at room temperature in the dark. The spin column was centrifuged at 14000 g for 10 minutes. Spin column was washed with 100 μ L of 7 M urea and centrifuged at 14000 g for 15 minutes. This washing step was repeated three times. The same wash step was performed using 100 μ L of 50 mM NH_4HCO_3 and repeated three times. Proteins were digested with sequencing grade trypsin (Promega, Wisconsin, United States) at a ratio of 1:50 (enzyme to protein) diluted in 100 μ L of 10mM NH_4HCO_3 and then incubated at 37 °C overnight with mixing. Digested peptides were collected in a fresh collection tube by adding 50 μ L of 10 mM NH_4HCO_3 and then centrifuged at 14000 g for 10 minutes). This step was repeated with 50 μ L 1 % (v/v) FA. Samples were dried-down up to ~1 μ L using a vacuum centrifuge. Finally, the samples were reconstituted in 10 μ L TA2 (2 % (v/v) ACN/0.1 % (v/v) TFA) and stored at -20 °C until ready to use. Desalting was required for the end samples with the addition of matrix-assisted laser desorption/ionization (MALDI) to ensure the samples were polymer-free.

2.7.5 Desalting using C18 column

After the saliva and placental samples underwent IS tryptic digestion and FASP, desalting using C18 column was performed to remove contaminants that may interfere with mass spectrometric analysis of peptides. C18 column, ZipTip® Pipette Tips (Merck Milipore, Massachusetts, United States) is used for sample concentration between 1.0 to 10.0 μ g (typically 5 μ g). ZipTip® Pipette Tips was used for both saliva and samples as the sample concentration was 5 and 10 μ g. The ZipTip® Pipette Tip C18 is a 10 μ L tip with a 0.6 μ L bed of chromatography media (resin) fixed at its end for sample elution in 1 to 4 μ L. It provides a reproducible, high-recovery method for concentrating and purifying peptide, protein or oligonucleotide samples for improved analytical data prior to the sensitive analyses such as MALDI-TOF-MS.

The whole desalting process was performed using only one ZipTip® Pipette Tip for each one of saliva and placental sample with extra care taken to ensure that the tip was always wet. ZipTip® Pipette Tip was placed on 1-10 μ L pipette that had been depressed to a dead stop using the maximum volume setting of 10 μ L. Then, the wetting solution (100 % (v/v) ACN) was aspirated and dispensed twice. The same step was repeated with equilibration solution (0.1 % (v/v) TFA). After that, the sample binding was performed by steps of aspirating and dispensing samples without introducing air into the sample. This binding step was repeated for at least 7-10 cycles to ensure maximum binding of complex mixtures. Then, the wash solution (0.1 % (v/v) TFA) was aspirated into the tip and dispensed to waste. The sample washing was repeated 3 times. Following that, the peptides were eluted in elution solution (0.1 % (v/v) TFA/50 % (v/v) ACN) in the HPLC vials. This elution step was repeated at least 8 times.

Finally, the samples were dried-up up to $\sim 1 \mu\text{L}$ using SpeedVac (Thermo Fisher Scientific, Massachusetts, United States). The samples were reconstituted in $10 \mu\text{L}$ TA2 to a final concentration of $0.5 \mu\text{g}/\mu\text{L}$.

2.8 Mass spectrometry (MS)

2.8.1 Introduction of mass spectrometry (MS)

Mass spectrometry (MS) is an analytical technique that can provide both qualitative (structure) and quantitative (molecular mass or concentration) information on analyte molecules based on their movement in an electric or magnetic field ^(285, 286). Conversion of sample molecules into ions in the gas phase occurs and separation of these sample molecules are based on their mass-to-charge (m/z) ratio, therefore positively and negatively charged ions can be formed. This technique is performed by mass spectrometer. A mass spectrometer is basically consists of three main components: an ion source, a mass analyzer, and a detector; and these components determine the level of performance and the specific type of analysis a mass spectrometer can be perform ^(286, 287) (Figure 15). Initially, the sample is ionized and the ions produced by MALDI or electrospray ionization (ESI) source are separated in the mass analyzer based on their mass-to-charge (m/z) ratio. The mass analyzer mainly determines the MS performance and functionality. Then, the ions are then detected by the detector. The end product is a mass spectrum, which is a plot of ion abundance versus m/z (Figure 15). Resolution, and mass accuracy are standard MS instrument specifications and tandem MS is an essential functionality. All of these are very crucial for successful protein identification (ID) and characterization ^(286, 287).

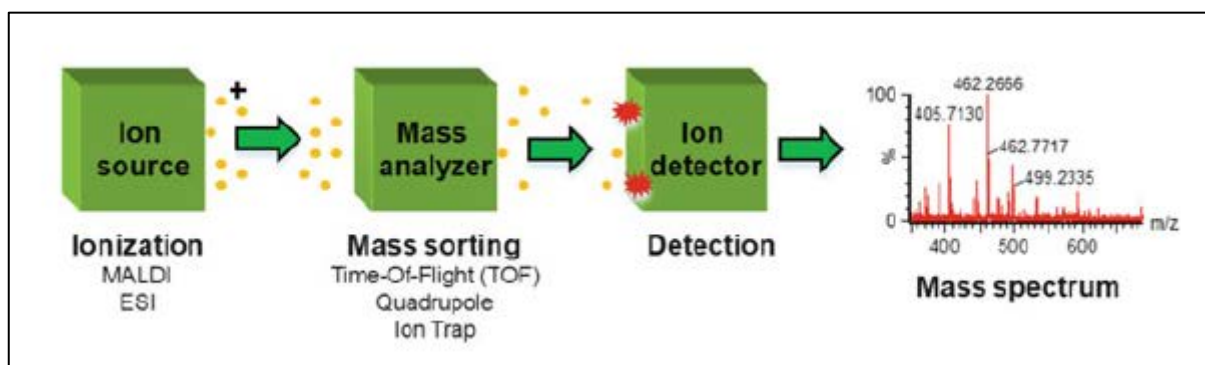


Figure 15. Mass spectrometer schematic ⁽²⁸⁷⁾.

2.8.2 Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS)

Liquid chromatography (LC) is a complementary approach whereby proteins are separated using chromatography techniques according to differences in their specific properties in the protein pre-fractionation stage. Electrospray ionization (ESI) is a technique used in MS that assist the transfer of ions using an electrospray in which a high voltage is applied to a liquid to create an aerosol ⁽²⁸⁵⁾. ESI is the ion source of choice to couple LC with MS, thus the method's name: LC-ESI-MS/MS or usually known as LC-MS/MS. Separation principles in protein chromatography include charge, size, hydrophobicity and biorecognition (ligand specificity). Amongst all, reversed-phase liquid chromatography (RPLC) technique that use hydrophobicity principle has many advantages as compared to other protein chromatography technique. These advantages include high enrichment of sample, high peak spectra capacity and sample desalting capacity ⁽²⁸⁸⁾. Separation mechanism in RPLC depends on the hydrophobic binding interaction between salute molecule in the sample (mobile phase) and immobilized matrix hydrophobic ligand (stationary phase) by using gradient elution ^(288, 289).

2.8.3 Peptide separation – Nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS)

Peptide separation via the Nanoflow LC-ESI-MS/MS method was performed based on the established method optimised in previous study conducted in the same lab ⁽²⁹⁰⁾. Peptide concentration of 500 ng was separated by online RPLC using a Dionex Ultimate 3000 NanoLC system equipped with a separation column (Acclaim PepMap RSLC, C18, pore size 100 Å, particle size 2 µm, 75 µm inner diameter (ID) × 50 cm length) and a trapping column (Acclaim PepMap100, C18, pore size 100 Å, particle size 3 µm, 75 µm ID × 2 cm length). Both columns were purchased from Thermo Scientific. The chromatography system was coupled to a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany), equipped with a nano-ESI source (Thermo Scientific) and a nano-ESI needle O.D 150 µm, I.D 30 µm (Thermo Scientific).

The buffer system used was as follows:

- A) 2 % (v/v) ACN, 0.1% (v/v) FA in water.
- B) 80 % (v/v) ACN, 0.1% (v/v) FA in water.

Flow rate was set to 300 nL/min and column temperature was set to 45 °C. Each sample was analysed in triplicate to assess the reproducibility of the analytical platform. Intermittent blank runs were performed to prevent carry-over between samples. The following multistep linear gradients is detailed in Table 4. Based on the previously established method, the optimal conditions for LC-MS/MS salivary peptide analysis were using 4-60% of Buffer B for 125 minutes gradient ⁽²⁹⁰⁾.

2.8.4 Label-free quantification (LFQ) comparative mass spectrometry (MS)

Label-free quantification (LFQ) aims at quantifying peptides and proteins without the use of stable isotope containing compound to chemically bind to and thus label the protein ⁽²⁹¹⁾. The basis of LFQ is to make the assumption that under well controlled conditions with sufficient data redundancy, identical peptides across multiple LC-MS/MS experiments can be compared directly ⁽²⁹²⁾.

Label-free LC-MS for quantitative proteomics has more advantages than label-based experiments, however it must be followed by suitable software for data analysis. This is because the data analysis for label-free LC-MS is challenging especially when quantification approach relies on detection and identification of known, unique peptides for each protein of interest ⁽²⁹³⁾. The major advantages of the label-free approaches are that they typically do not require extra experimental steps and that comparative quantification can be performed across many samples simultaneously. Moreover, by incorporating information regarding the detectability of a peptide, absolute quantitation is also possible by label-free methods ⁽²⁹²⁾. Most label-based quantification approaches such as stable isotope labeling with amino acids in cell culture (SILAC) have potential limitations: complex sample preparation, the requirement for increased sample concentration, and incomplete labelling ⁽²⁹⁴⁾. There are two different approaches in LFQ: 1) measurement of chromatographic peak areas (also termed mass spectrometric signal intensities) of peptide precursor ions, or 2) those based on spectral counting and implies a counting, and a comparison of the number of fragment-ion spectra (MS/MS) acquired for peptides of a given protein (Figure 16) ^(295, 296). In this particular study, quantitative label-free comparative MS with ion-intensity-based approach was chosen as the most suitable method.

Table 4. Nanoflow LC-ESI-MS/MS multistep linear gradients.

Linear gradient	Time interval (min)	% of buffer B	% of buffer A
60 min LC-gradient	0-10	4	96
	10.0-70.0	4-60	96-40
	70.5-80.0	90	10
	80.5-95.0	4	96
125 min LC-gradient	0-10.0	4	96
	10.0-125.0	4-40	96-60
	125.0-135.0	40-60	60-40
	135.5-145.0	90	10
Wash gradient	0-10.0	4	96
	10.0-20.0	14-40	86-60
	20.0-30.0	40-90	60-10
	30.0-35.0	90	10

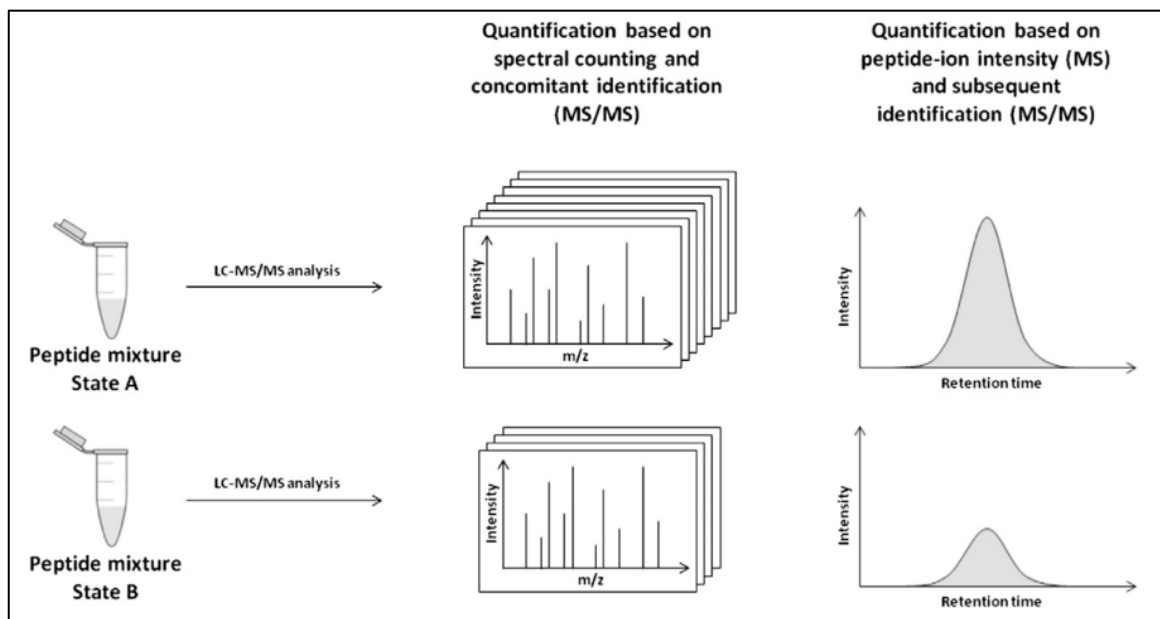


Figure 16. Schematic representation of the two different label-free approaches ⁽²⁹⁶⁾. In the spectral-counting approach (left), peptide and protein abundances can be estimated based on the number of acquired peptide spectrum matches. In the ion-intensity-based approach (right), the changes of peptide abundances are determined by measuring and comparing the chromatographic peak areas of the corresponding peptides. In this example, both results would indicate a higher peptide abundance in State A.

2.9 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

2.9.1 Introduction of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) can be described as a “soft” ionization technique that under suitable conditions succeed to transfer essentially non-volatile, fragile molecules into the gas phase that allows the molecular ions of analytes to be identified even in complex mixtures of biopolymers with no or only limited fragmentation ^(297, 298). The ionization–desorption principle of MALDI-MS is based on the of analytes that had been co-crystallized with an organic, light-absorbing matrix such as α -cyano-4-hydroxy cinnamic acid (CHCA) or sinapinic acid which ionizes the analyte when activated by a laser as it enters the gas phase. The ions once formed are accelerated in an electric field and accordingly separated to their mass-to-charge ratio (m/z) in the mass spectrometer analyser (Figure 17) ⁽²⁸⁷⁾. Usually, MALDI is coupled with time-of-flight (TOF) analysers that indicate the mass of intact biopolymers ⁽²⁹⁸⁾.

2.9.2 Polymer contamination analysis using MALDI-TOF-MS

MALDI-TOF-MS was performed on both saliva and placental samples to detect polymer contamination. 5-10 mg/mL CHCA in TA70 (70 % (v/v) ACN/ 30 % (v/v) H₂O/0.1 % (v/v) TFA) was weighted and prepared as the matrix solution. Next, the matrix solution was diluted 1:20 in TA90 (90 % (v/v) ACN/10 % (v/v) H₂O /0.1 % (v/v) TFA). Similarly, peptide standard, PepCall II (Bruker Daltonik, Bremen, Germany) was also diluted 1:20 in TA70. The samples were then diluted to 1/10 and 1/100 with TA90. After that, 0.8 μ L of neat and diluted samples were spotted on the Anchor Chip according to samples sequence. Approximately 0.8 μ L of PepCal II was spotted at the middle of each four samples (Figure 18). The samples and PepCal II were allowed to dry up and then each dried samples were overlaid with 1 μ L of matrix 1/20 CHCA.

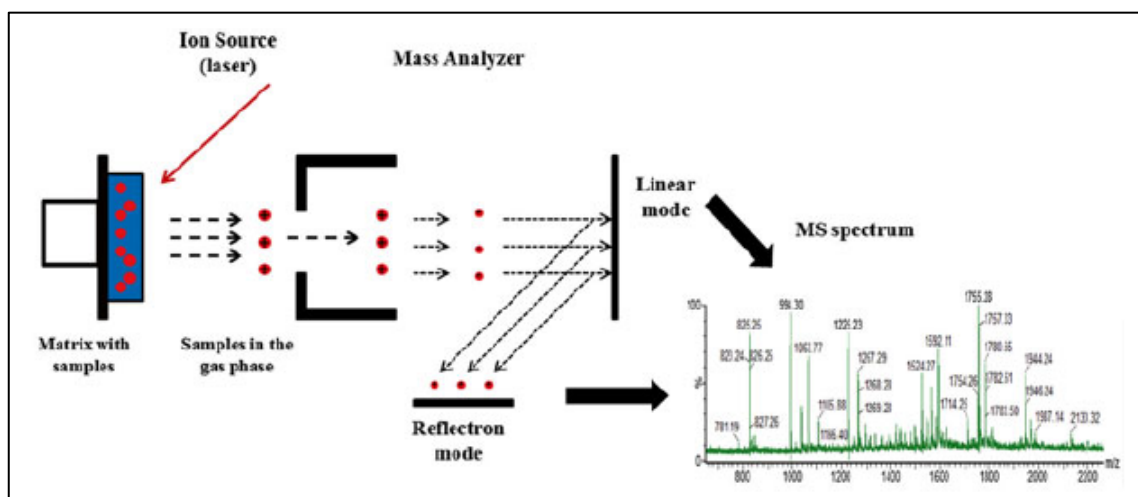


Figure 17. MALDI-TOF mass spectrometer principle ⁽²⁸⁷⁾. An ion source, a mass analyser, and detector are present on the instrument. At the detector the mass spectrum is detected/recorded. The mass analyser is a time-of-flight (TOF) and can be used in linear mode or reflective mode.

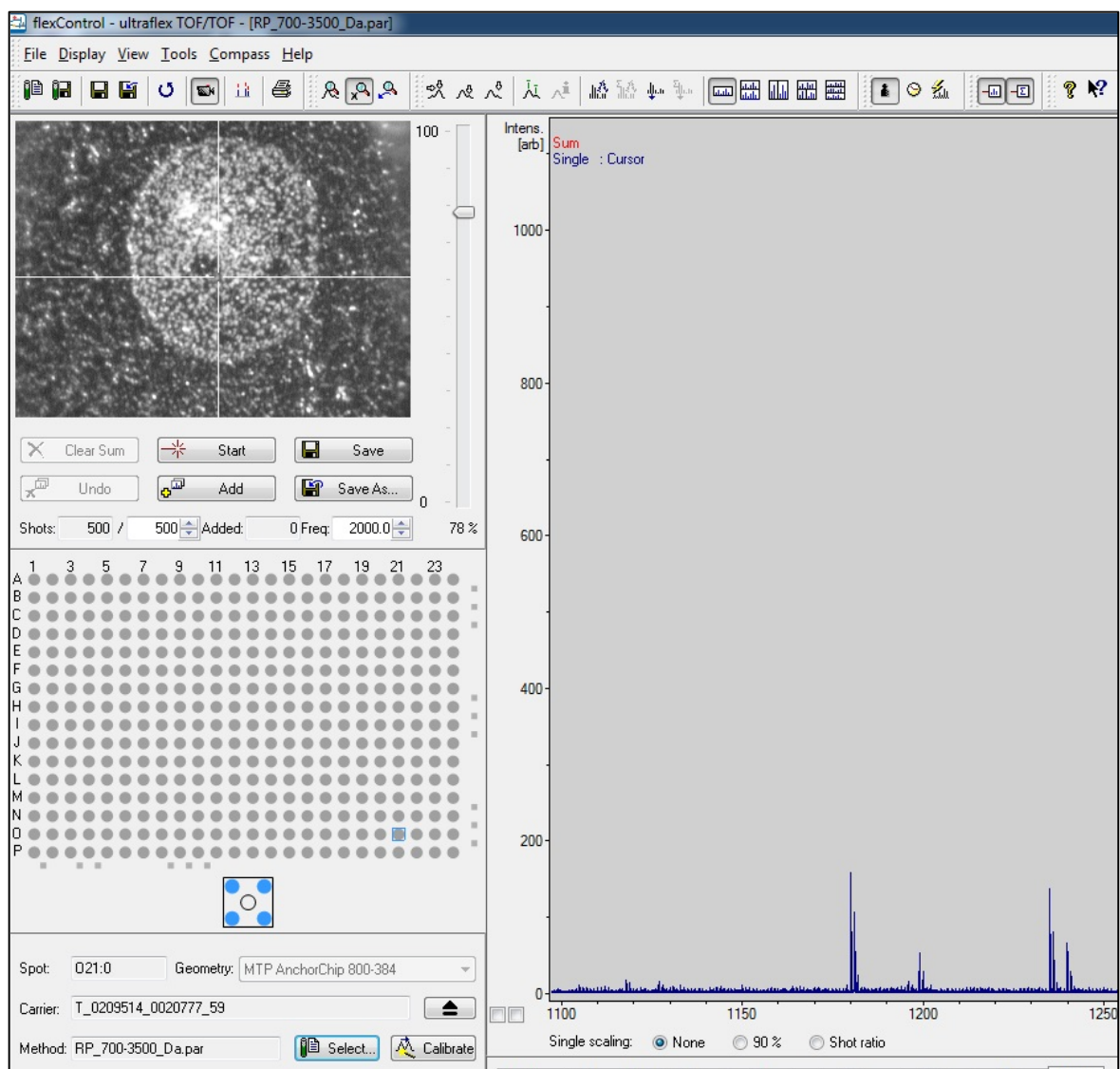


Figure 18. Polymer detection on the sample using matrix on the ultrafleXtreme MALDI-TOF/TOF MS. MALDI-TOF MS was performed on the Anchor Chip once the matrix dried up.

MALDI-TOF MS was performed on the Anchor Chip once the matrix dried up. MS data was acquired using an ultrafleXtreme MALDI-TOF-MS, (Bruker Daltonics, Bremen, Germany) controlled by flexControl (v3.4, Bruker Daltonics). MS spectra were processed in flexAnalysis (Figure 19).

Instrument-specific settings were as follows: positive reflector (RP) detection range of 700–3500 m/z, 500 Da matrix suppression; 2.371 kV reflector detector voltage; 2 kHz laser repetition rate; 57 % laser power and with 500 shots taken at each spot and summed.

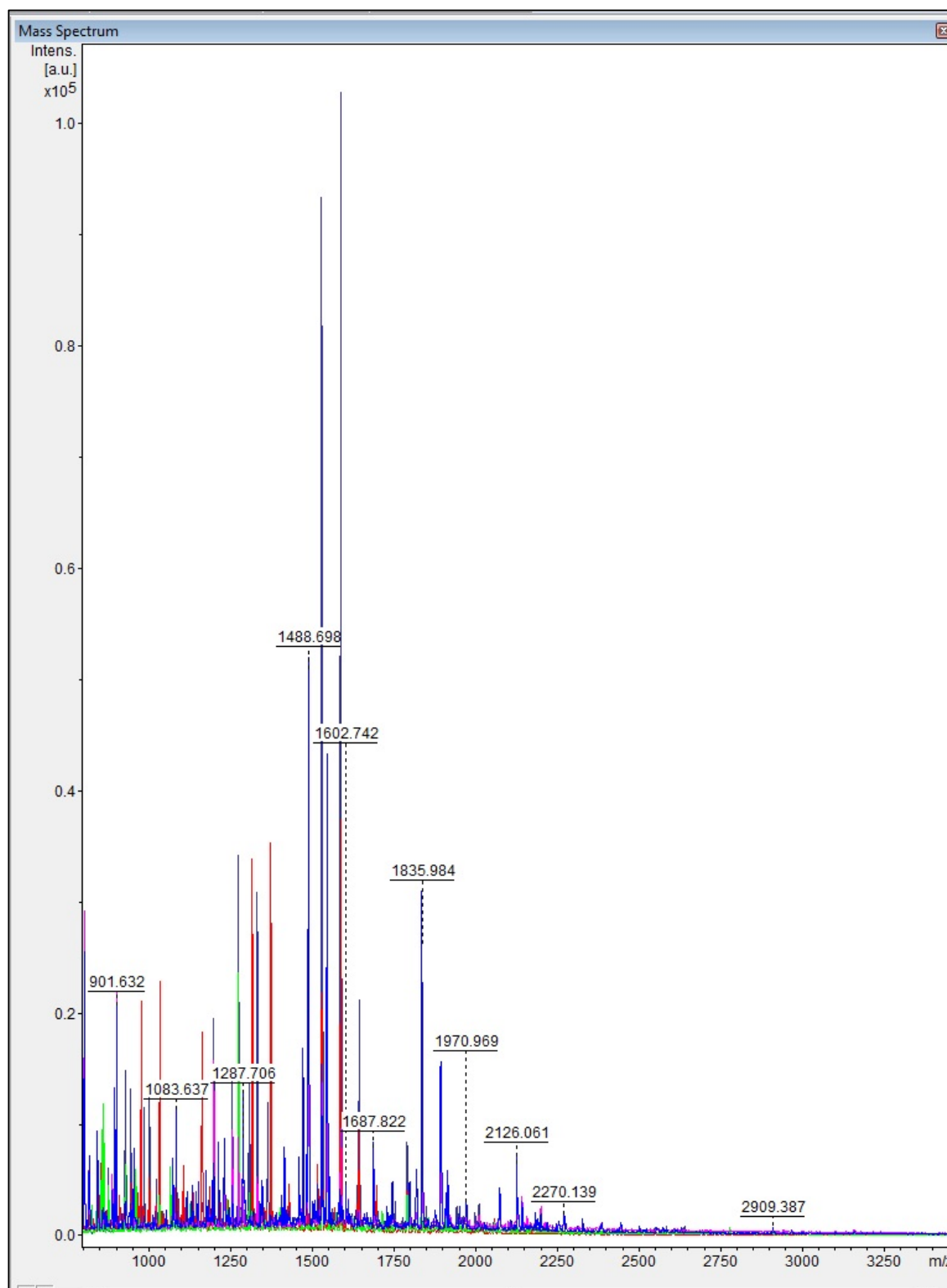


Figure 19. MS spectra in flexAnalysis. Instrument-specific settings were as follows: positive reflector (RP) detection range of 700–3500 m/z, 500 Da matrix suppression; 2.371 kV reflector detector voltage; 2 kHz laser repetition rate; 57 % laser power and with 500 shots taken at each spot and summed.

2.10 Mass spectrometry using LTQ Orbitrap XL

2.10.1 Introduction of LTQ Orbitrap XL

The quadrupole analyser consists of a set of four parallel metal rods (Figure 20) ⁽²⁹⁹⁾. A combination of constant and varying (radio frequency) voltages allows the transmission of a narrow band of m/z values along the axis of the rods. By varying the voltages with time, it is possible to scan across a range of m/z values, resulting in a mass spectrum. Most quadrupole analysers operate at $<4000\ m/z$ and scan speeds up to $1000\ m/z$ per sec or more. They usually operate at unit mass resolution, which means that the mass accuracy is seldom better than $0.1\ m/z$ ⁽²⁹⁹⁾. Ions can be induced to undergo fragmentation by collisions with an inert gas such as nitrogen or argon, a process called collision induced dissociation (CID). One type of collision cell is a quadrupole that has been designed to maintain the low pressure of the collision gas required for dissociation and transmit most of the fragment ions that are produced ⁽²⁹⁹⁾. A particularly useful MS configuration is obtained by placing a collision cell between two quadrupole mass analysers. This combination is called a triple quadrupole MS and is an example of tandem MS in which two or more stages of mass analysis are independently applied (Figure 20) ⁽²⁹⁹⁾.

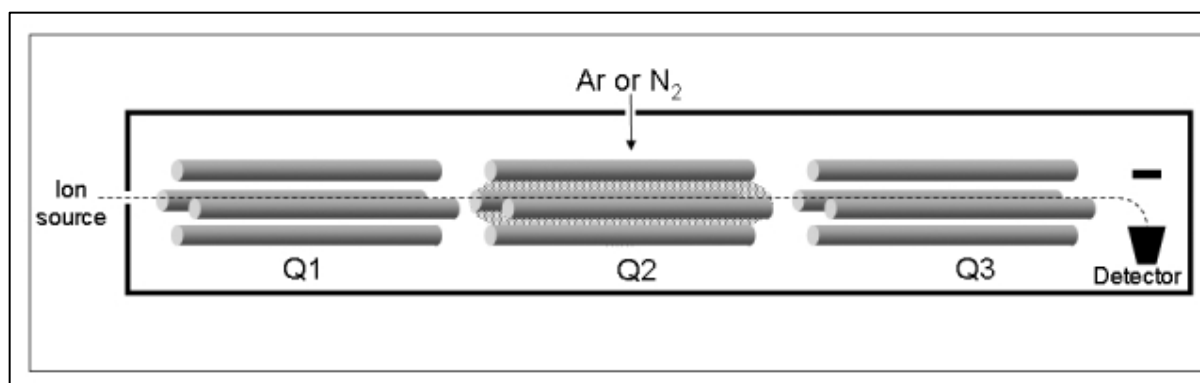


Figure 20. A triple quadrupole mass spectrometer ⁽²⁸⁷⁾. Q1 and Q3 act as mass filters and can be independently fixed, scanned or stepped. Q2 is a collision cell that contains a low pressure inert gas. N₂ represents nitrogen and Ar represents argon.

2.10.2 Peptide identification with mass spectrometry using LTQ Orbitrap XL

In this particular study, Linear Trap Quadrupole (LTQ) Orbitrap XL instrument was managed in data-dependent mode to automatically switch between full scan MS and acquiring MS/MS. Thermo Tune Plus and Xcalibur software (Thermo Scientific, Massachusetts, United States) were used to operate the instrument. Orbitrap analyzer was used to acquire full scan mass spectra (from m/z 300–2000) and Orbitrap system resolution was set to $r= 60,000$ at m/z 400. All experiments were conducted on the standard mass spectrometric conditions that include: spray voltage, 1.25 kV; no sheath and auxiliary gas flow; heated capillary temperature, 200 °C; predictive automatic gain control (AGC) enabled and an S-lens RF level of 50–60 %. All unassigned charge states and charge state of one were rejected for MS/MS. The 6 most intense peptide ions with minimum signal intensity of 1000 were sequentially isolated and fragmented in the linear ion trap by CID.

Based on the previous established MS method in our lab, the optimal conditions for LC-MS/MS salivary peptide analysis were a concentration of 500 ng of sample per analysis, duration of 120 min; separation column of 50 cm length and fragmentation technique of CID ⁽²⁹⁰⁾.

2.10.3 Standard peptides preparation for spike-in experiment to evaluate accurate quantification

The spike-in experiment was performed to evaluate reproducibility of LC-MS data generated by label-free LC-MS method including retention time (RT), peak intensity and peak width ⁽²⁹⁰⁾. Two saliva samples, AL355 and AL435 were spiked with peptide mixture consisting nine standard peptides, MassPREP Peptide Mixture (Waters, Milford, Massachusetts, United States). The details of peptide mixture component name, RT, molecular weight (MW) and peptide sequences are shown in Table 5. The peptide mixture contains approximately 1.5 µg (~1 nmole) of each peptide. When reconstituted in 100 µL of solvent (0.1 % (v/v) TFA or 0.1 % (v/v) formic acid (FA)), the final sample concentration is ~15 µg/ml per peptide. All standard peptides were diluted with TA2 to the desired concentration. Out of nine peptides, only six detected: RASG-1, Angiotensin frag1-7, Bradykinin, Angiotensin II, Angiotensin I and Enolase T35 (Table 6). The spectra of these six standard peptides are listed in Figures 21, 22 and 23.

Table 5. Peptide mixture component name, retention time (RT), molecular weight (MW) and peptide sequences.

Peak #	Component Name	Retention Time *(minutes)	Molecular Weight **(g/mol)	pKa	Peptide Sequence
1	Allantoin (VO marker)	0.76	158.0440	-	-
2	RASG-I	4.78	1000.4938	9.34	RGDSPASSKP
3	Angiotensin frag.1-7	9.59	898.4661	7.35	DRVYIHP
4	Bradykinin	12.04	1059.5613	12.00	RPPGFSPFR
5	Angiotensin II.	13.06	1045.5345	7.35	DRVYIHPF
6	Angiotensin I.	14.92	1295.6775	7.51	DRVYIHPFHL
7	Renin substrate	16.82	1757.9253	7.61	DRVYIHPFHLVYS
8	Enolase T35	19.18	1871.9604	7.34	WLTGSQLADLYHSLMK
9	Enolase T37	21.55	2827.2806	3.97	YPIVSIEDPPFAEDDWEAWSHFFK
10	Melittin	24.93	2845.7381	12.06	GIGAVLKVLTTGLPALISWIKRKRQQ

Table 6. Peptide mixture component name detected in the saliva samples. The bold font are the charges detected in the LC-MS/MS either doubly or triply.

Component name	Molecular weight (g/mol)	Doubly charge	Triply charge
RASG-1	1000.49	501.25	334.50
Angiotensin frag1-7	898.47	450.23	300.49
Bradykinin	1059.56	530.78	354.19
Angiotensin II	1045.54	523.77	349.51
Angiotensin 1	1295.68	648.84	432.89
Enolase T35	1871.96	936.98	624.99

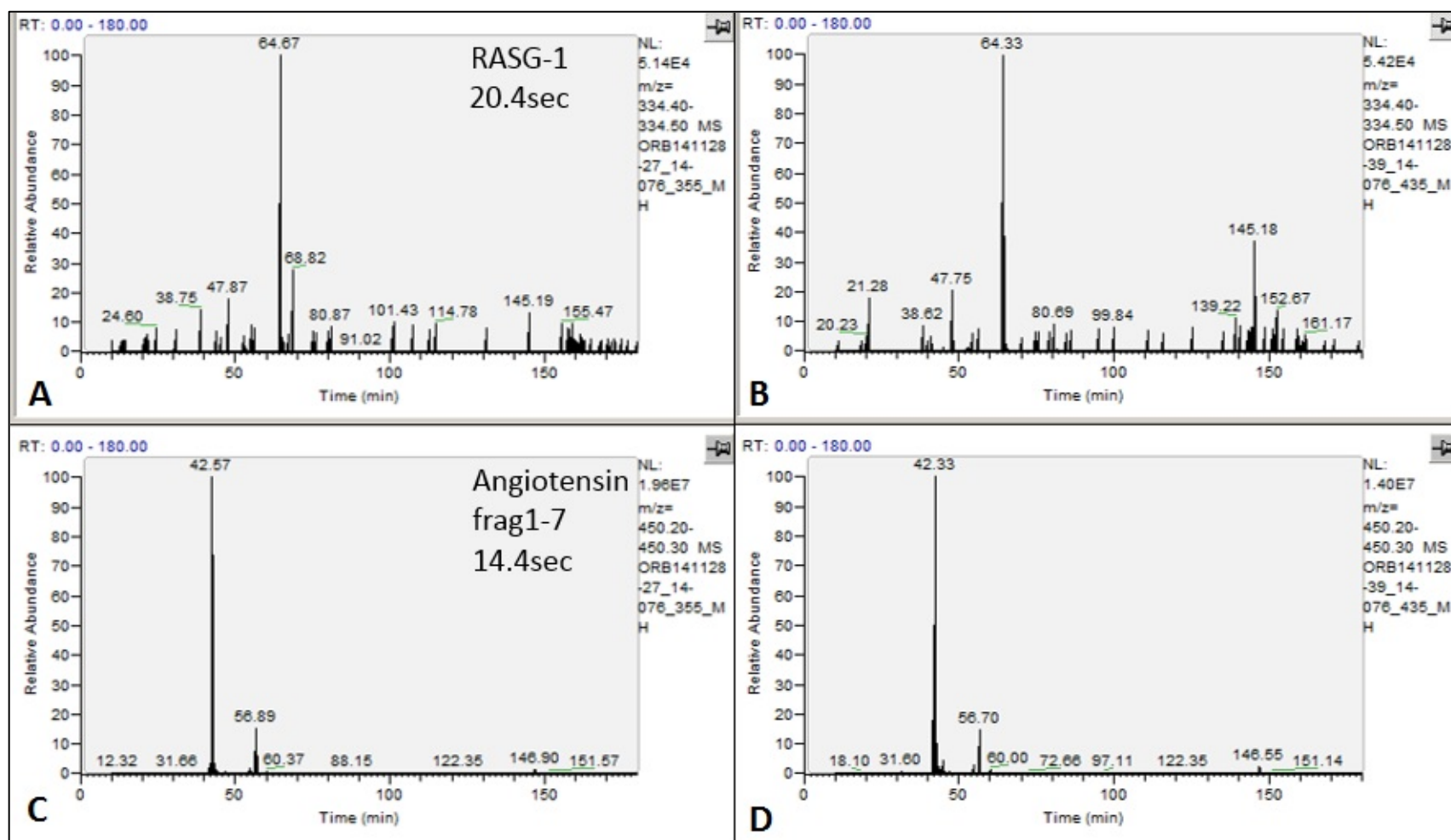


Figure 21. Spectra of 2 peptide mixture (RASG-1 and Angiotensin frag 1-7) with their retention time (RT) between sample AL355 and AL435. A and B: RASG-1, RT 20.4 seconds. C and D: Angiotensin frag 1-7, RT 14.4 seconds.

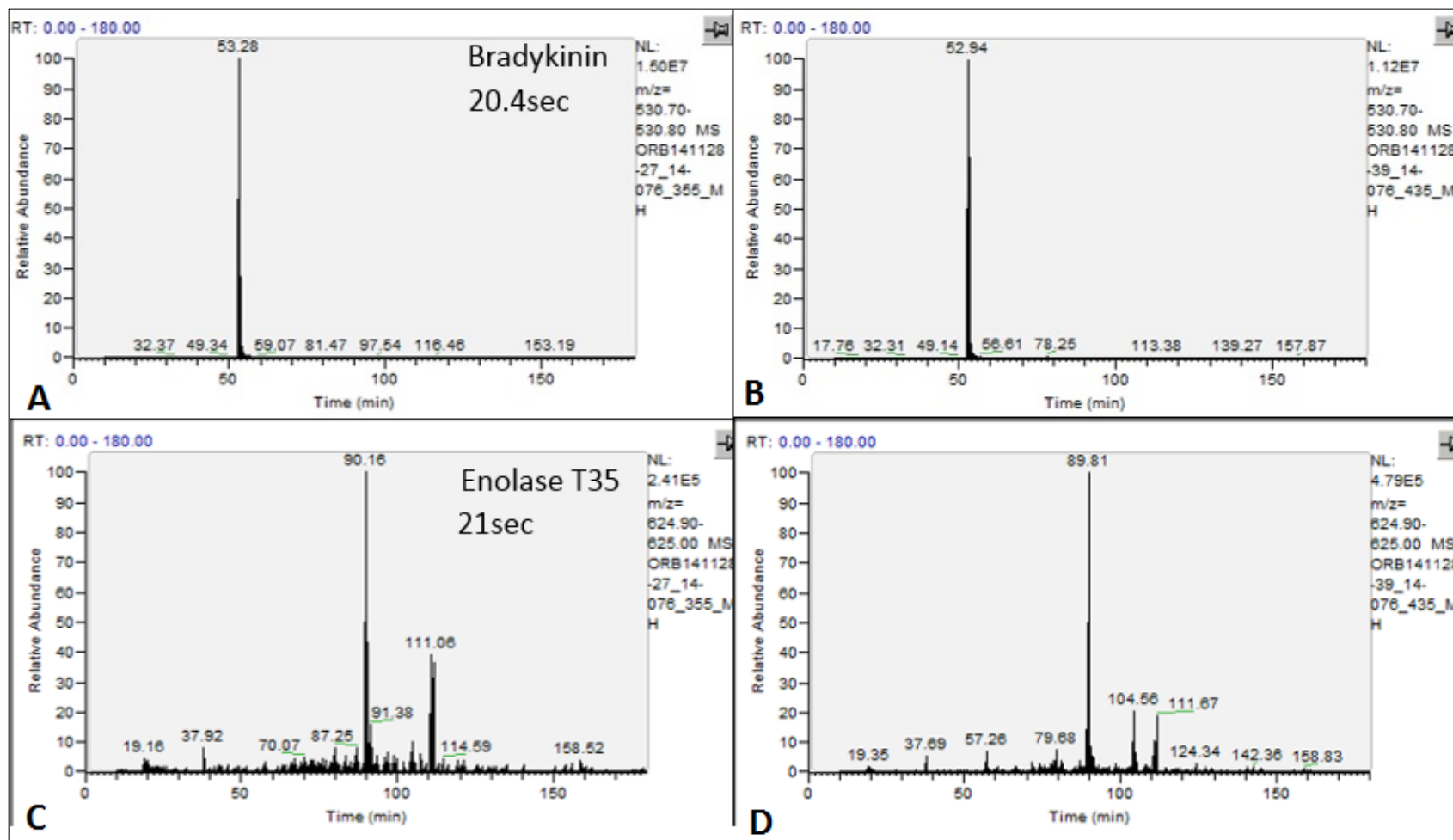


Figure 22. Spectra of 2 peptide mixture (Bradykinin and Enolase T35) with their retention time (RT) between sample AL355 and AL435. A and B: Bradykinin, RT 20.4 seconds. C and D: Enolase T35, RT 21 seconds.

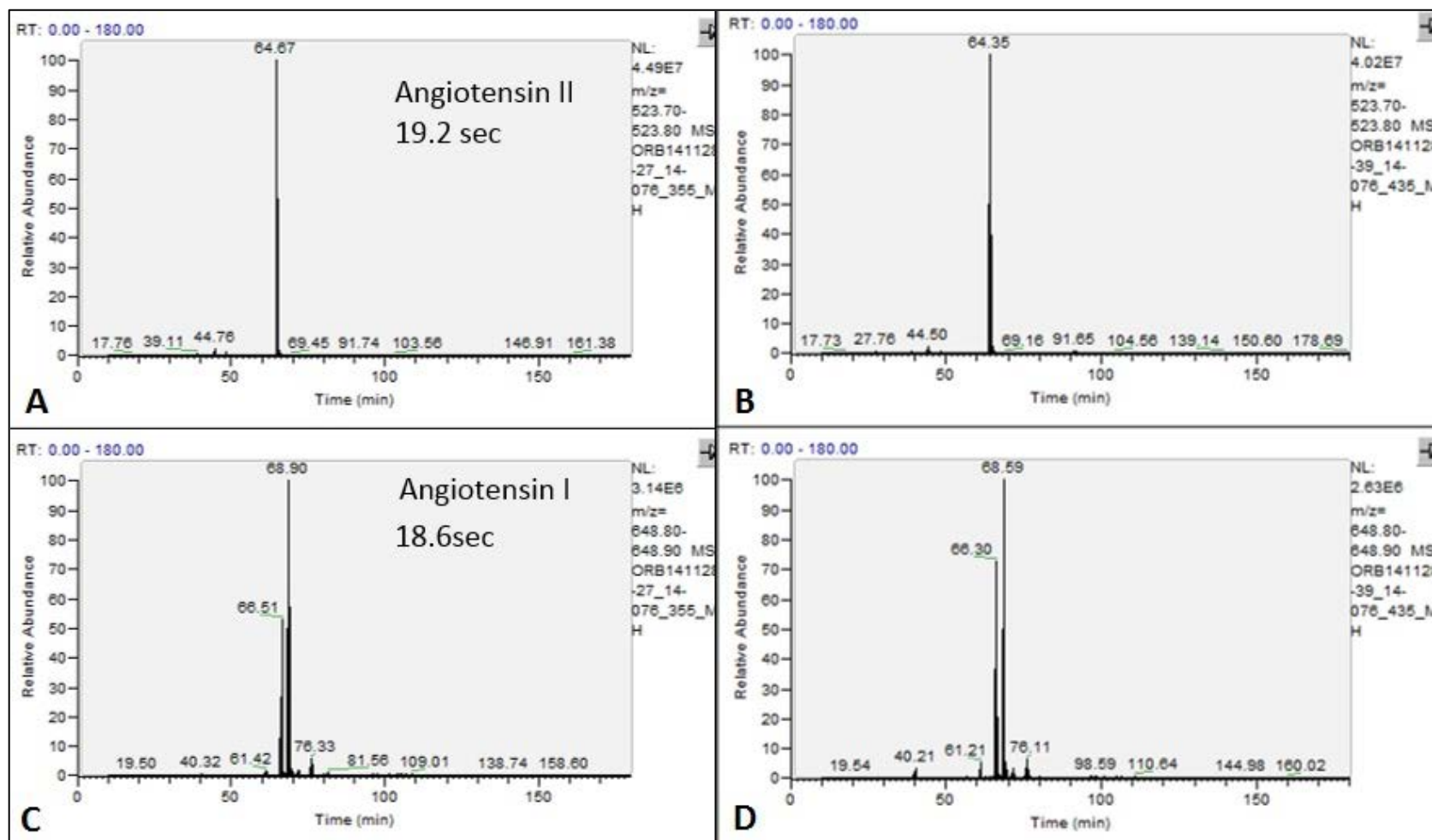


Figure 23. Spectra of 2 peptide mixture (Angiotensin II and Angiotensin I) with their retention time (RT) between sample AL355 and AL435. A and B: Angiotensin II, RT 19.2 seconds. C and D: Angiotensin I, RT 18.6 seconds.

2.10.4 Protein identification using MASCOT

Proteome Discoverer v1.4 software (Thermo Scientific, Massachusetts, United States), a database searching platform, was used to search MS/MS spectra against the Swiss-Prot 2013_04 database, Homo sapiens taxonomy (Figure 24). Spectra were searched using the Mascot algorithm, Mascot server v2.2 search engine (Matrix Science, Boston, United States) by collecting gene ontology data from databases and existing literature. Searches were performed using the following parameters: trypsin digestion with maximum two missed cleavages sites allowed, ESI Trap instrument, 10 ppm precursor mass tolerance, 0.8 Da fragment-ion tolerance, fixed carbamidomethylation of cysteine and variable oxidation of methionine. Identified proteins were considered deregulated based upon the following criteria: minimum of two peptides per protein, count only rank 1 peptides and count peptide only in top scored protein. To increase confidence in peptide identification, MASCOT search results on the MS/MS spectra were subjected to false discovery rate (FDR) of $\leq 1\%$ employing a decoy (randomised sequence) search strategy utilising a reversed protein sequences database.

2.10.5 Label free quantification (LFQ) using MaxLFQ

MaxQuant is a free, quantitative proteomics software that works on an integrated suite of algorithms specifically developed for high-resolution quantitative mass spectrometry data. MaxQuant detects peaks, isotope clusters and SILAC peptide pairs as three-dimensional objects in m/z , elution time and signal intensity space by using correlation analysis and graph theory ⁽³⁰⁰⁾. Recently, a subgroup of MaxQuant, an accurate proteome-wide LFQ by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ had been developed ⁽³⁰¹⁾.

Important parameters to adjust prior to analyze data using MaxLFQ include:

- 1) Uploading the raw data and experimental groups (control vs allergic phenotypes) in the "Raw files" tab (Figure 25).
- 2) Selecting LFQ label in the "Group-specific parameters" tab (Figure 26).
- 3) Uploading the "Fasta files" in "Global parameters" tab (Figure 27).
- 4) Setting the numbers of thread for the program to run.

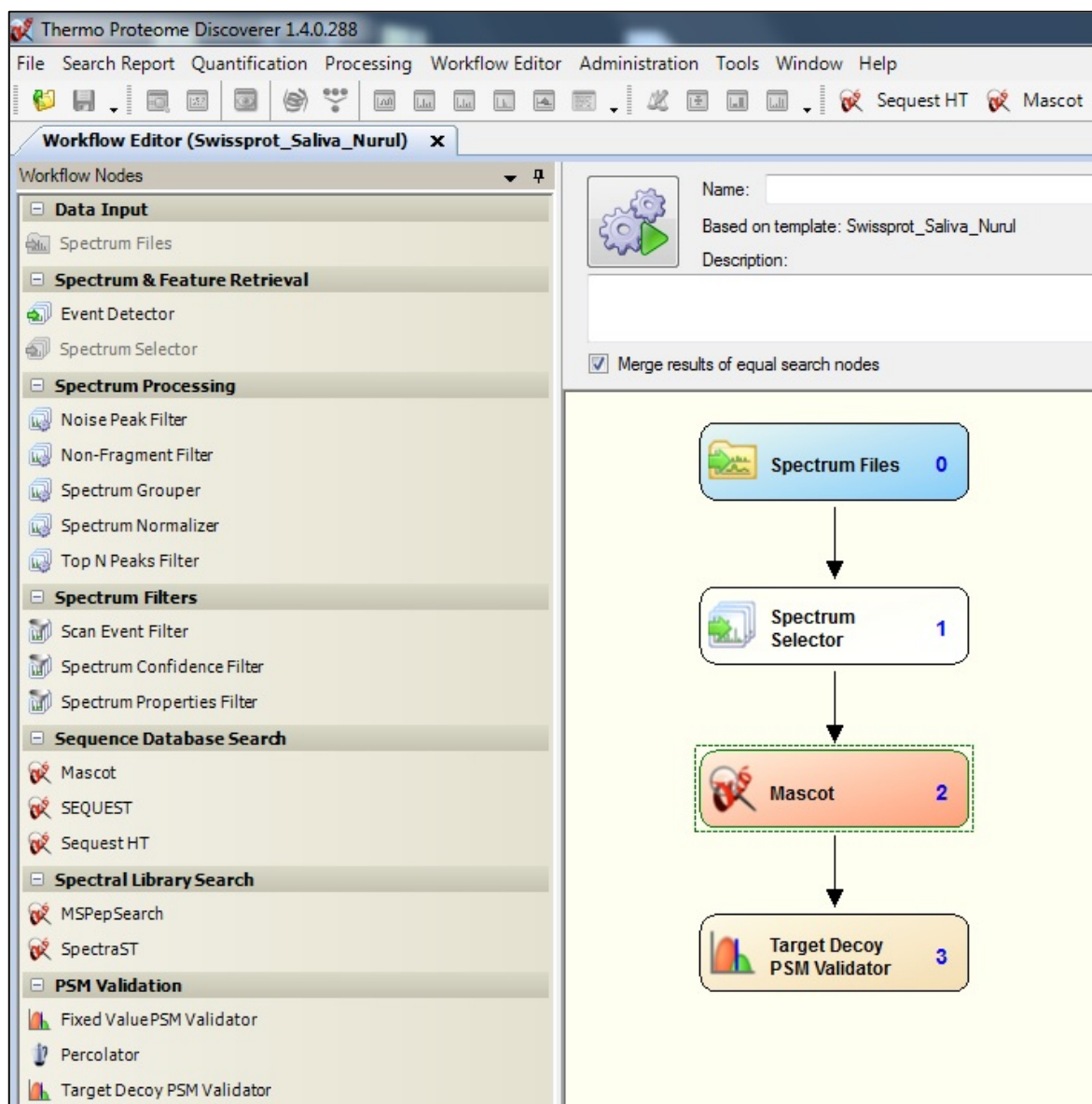


Figure 24. Proteome Discoverer software that was used to process MS/MS mass spectra. Spectra were searched using MASCOT search engine.

Session1 - MaxQuant

Raw files Group-specific parameters Global parameters Performance Viewer Andromeda configuration

Load Remove Write template Set experiment Set parameter group
 Load folder Change folder Read from file Set fractions No fractions

Input data Exp. design file Edit exp. design

	File	Size	Exists	Parameter group	Experiment	Fraction
1	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-09_14-076_134B_MH.raw	606.5 MB	True	Group 0	C2	32767
2	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-12_14-076_211B_MH.raw	589.5 MB	True	Group 0	E1	32767
3	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-15_14-076_279_MH.raw	601.6 MB	True	Group 0	A1	32767
4	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-18_14-076_282_MH.raw	602.6 MB	True	Group 0	R1	32767
5	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-21_14-076_295_MH.raw	578.5 MB	True	Group 0	E3	32767
6	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-24_14-076_320_MH.raw	528.7 MB	True	Group 0	A2	32767
7	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-27_14-076_355_MH.raw	538 MB	True	Group 0	R2	32767
8	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-30_14-076_356_MH.raw	623.7 MB	True	Group 0	C3	32767
9	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-33_14-076_404_MH.raw	589.3 MB	True	Group 0	A3	32767
10	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-36_14-076_421_MH.raw	494.7 MB	True	Group 0	R3	32767
11	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-39_14-076_435_MH.raw	550.2 MB	True	Group 0	E2	32767
12	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-42_14-076_503_MH.raw	534.4 MB	True	Group 0	C1	32767
13	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-45_14-076_320_MH.raw	524.7 MB	True	Group 0	A2	32767
14	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-48_14-076_355_MH.raw	531.5 MB	True	Group 0	R2	32767
15	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-51_14-076_356_MH.raw	641.2 MB	True	Group 0	C3	32767
16	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-54_14-076_211B_MH.raw	593.2 MB	True	Group 0	E1	32767
17	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-57_14-076_279_MH.raw	602.9 MB	True	Group 0	A1	32767
18	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-60_14-076_282_MH.raw	595.5 MB	True	Group 0	R1	32767
19	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-63_14-076_295_MH.raw	569.8 MB	True	Group 0	E3	32767
20	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-66_14-076_404_MH.raw	586.9 MB	True	Group 0	A3	32767
21	D:\NHMZ Storage\MaxQuant\MaxQuant 14-076 and 15-021\ORB141128-69_14-076_421_MH.raw	498.7 MB	True	Group 0	R3	32767

items 15 selected

Number of threads: 2 Start Stop Partial processing Send email when done Details

Figure 25. MaxLFQ: Uploading the raw data and experimental groups in the “Raw files” tab.

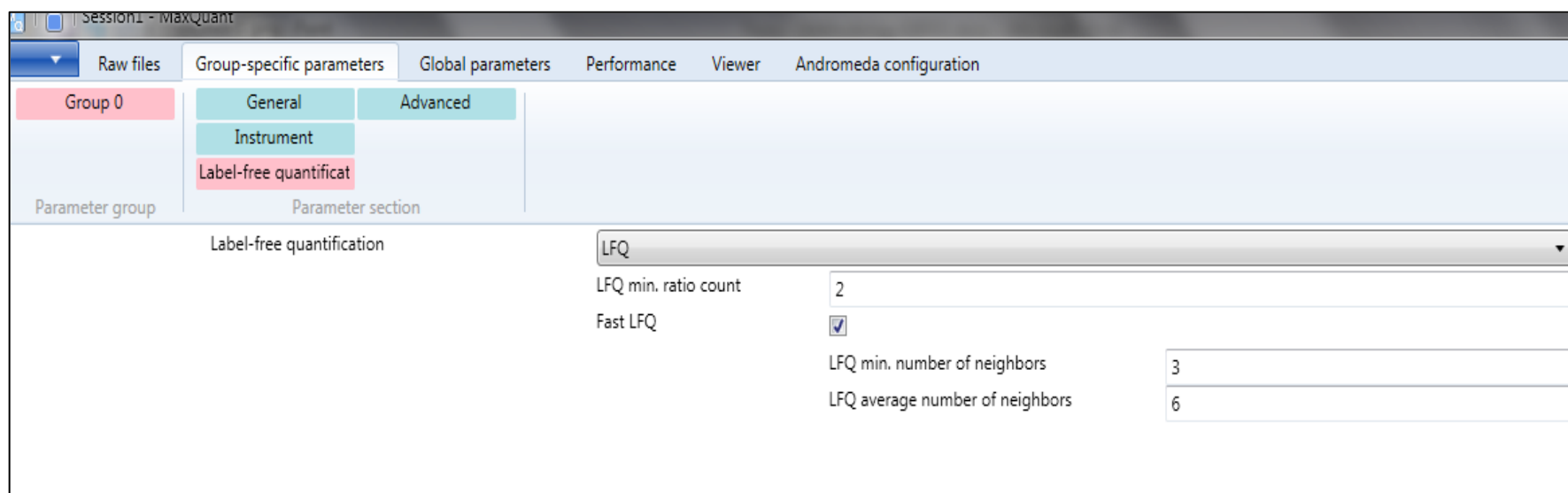


Figure 26. MaxLFQ: Selecting LFQ label in the “Group-specific parameters” tab. LFQ represents label-free quantification.

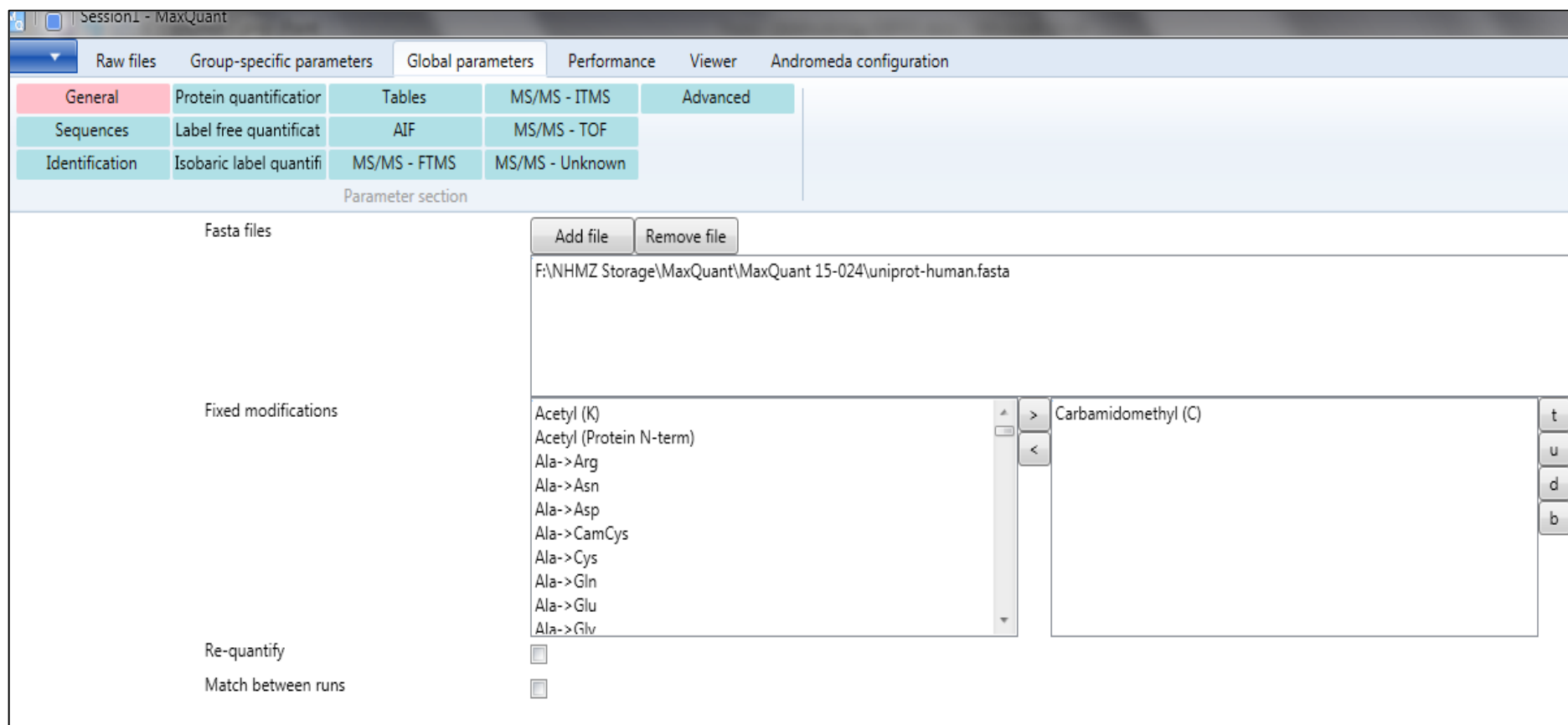


Figure 27. Uploading the “Fasta files” in “Global parameters” tab.

There are many text files generated after MaxLFQ had been executed that include “allPeptides.txt”, “evidence.txt” and “parameters.txt” but most importantly is “proteinGroups.txt” whereby LFQ intensities variables are created. LFQ intensities are the output of the MaxLFQ that are based on the (raw) intensities and normalized on multiple levels to ensure that profiles of LFQ intensities across samples accurately reflect the relative amounts of the proteins ^(300, 301). These LFQ intensities were used to generate the LFQ ratio between the control and the allergic samples that will be further explained in the result chapters.

2.11 Validation methods

Using LC-MS/MS with LTQ Orbitrap XL, placentae of children with no allergy were compared to placentae of children with allergic diseases (male $n=8$ and female $n=8$). For saliva samples, 9 child saliva with no allergy were compared to 9 child saliva with specific allergic phenotypes including asthma, eczema and rhinitis (total $n=18$). After LC-MS/MS data analysis, the potential protein candidates for allergy biomarkers identified in the placental and saliva samples were chosen according to the inclusion and exclusion criteria (refer Methodology section 2.3.1 and 2.3.2) and were validated using Western blot in much larger sample size. Three out of six salivary proteins used in the Western blot were selected and these three salivary proteins were further validated with Blitz system using 126 child saliva samples. Samples workflows performed on the placental tissues and saliva samples in the validation steps are as described in Figure 28.

2.11.1 TCA/Acetone protein precipitation

TCA/Acetone protein precipitation is similar to the modified 2-D clean-up protein precipitation, which was performed on the placental and saliva samples in the initial workflow of LC-MS/MS. The main difference is that modified 2-D clean-up is a facilitated kit, therefore it is easier and more time effective as compared with TCA/Acetone protein precipitation. However, because of the larger sample size for the validation steps ($n=68$ for placenta and $n=62$ for saliva samples), TCA/Acetone protein precipitation that is more cost effective is the preferred method.

Average placental tissues of ~ 50 mg or 200 μ L saliva sample was suspended in a precooled (-20°C) centrifuge tube containing 1.6 mL 20 % (w/v) TCA and 0.2 % (v/v) DTT in ice-cold (-20°C) acetone. The samples were kept at -20°C overnight to ensure complete protein precipitation. Next day, the sample was centrifuged at 20 000 g, 60 minutes at -10°C . The supernatant were discarded and the pellet was resuspended in 1.6 mL of 80 % (v/v) ice-cold acetone containing 0.2 g DTT. Then, the sample was vortexed and centrifuged again at 20 000 g, 60 minutes at -10°C . Afterwards, the supernatant was discarded and the pellet was air dried for 5 minutes. Finally, the sample was reconstituted in 25 μ L of 1 % (v/v) SDS and solubilized by repeated vortex, then sonicated for 15 minutes and then incubated on ice (-20°C) for 30 minutes. However, if the pellet was still not fully dissolved, the sample was placed in the Bioruptor to until dissolved.

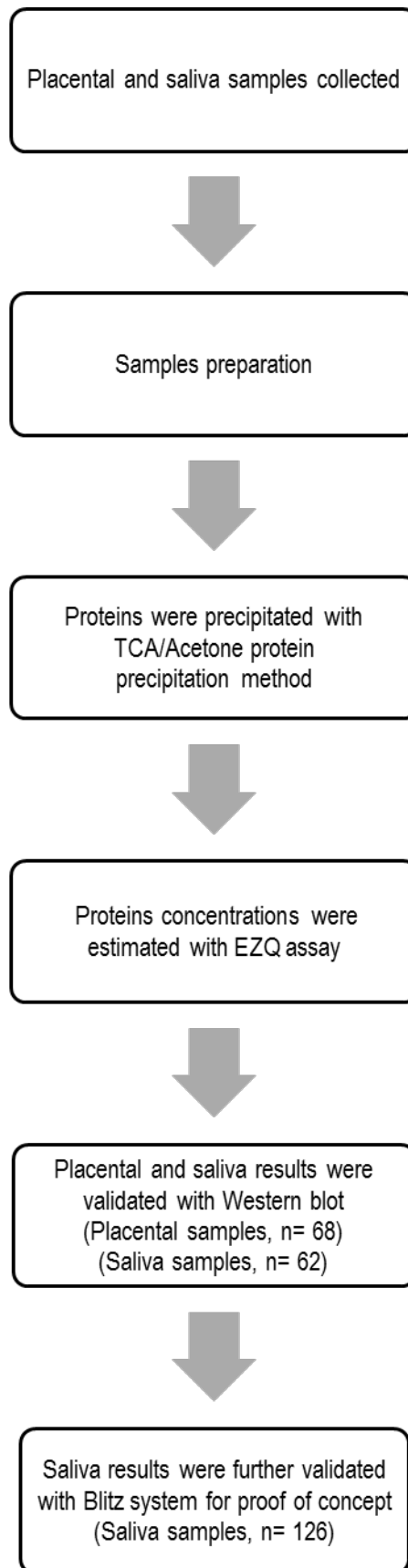


Figure 28. Validation steps workflows for placental tissues and saliva samples.

2.11.2 Western blot

2.11.2.1 Introduction on Western blot

Western blot is a multistep method that determines the presence or absence, size and modification; degradation states of target proteins; as well enables the quantitation of proteins from complex protein mixtures or homogenates ⁽³⁰²⁾. In this technique, a mixture of proteins is separated based on MW, and therefore by type of proteins, through 1D gel electrophoresis. These separated proteins are then transferred or blotted to a membrane, either nitrocellulose or polyvinylidene fluoride (PVDF), producing a band for each protein. The blotting membrane is then incubated with labels antibodies specific to the protein of interest. Western blot uses three elements to accomplish this task: (1) separation by size, (2) transfer to a solid support, and (3) marking target protein using a proper primary and secondary antibody to visualize ⁽³⁰³⁾.

i. 1D gel electrophoresis

1D gel electrophoresis was performed using 4-12 % SDS gel (Invitrogen, Thermo Fisher Scientific, California, United States), and MOPS running buffer 1x. Eight micrograms of protein, 5 μ L of 4x (Lithium dodecyl sulfate) (LDS) buffer and 1 μ L 1 M DTT were loaded into the SDS gel well after protein denaturation for 5 minutes at 95 °C or 10 minutes at 70 °C using dry block heater. 5 μ l of Novex sharp prestained markers (Thermo Fisher Scientific, Massachusetts, United States) were also loaded as the MW marker (Figure 29). The electrophoresis was set to 200 V for 50 minutes. Upon 1D gel electrophoresis completion, the SDS gel was removed from the casing carefully and placed inside a plastic container filled with miliQ water until the next step.

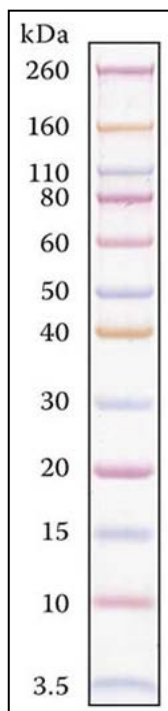


Figure 29. Novex sharp prestained markers protein standard.

ii. Western blotting

The next step was the Western blotting transfer of 1D gel using semi dry blotting method on TE 77 PWR semi dry system, Amersham ECL Semi-Dry Blotters (GE Healthcare, Little Chalfont, United Kingdom). PVDF membrane (Immobilon-FL Membrane, Merck Millipore, Billerica, Massachusetts, United States) was wet in 100 % (v/v) methanol, water and Transfer buffer 1x (192 mM glycine, 25 mM Tris-base, 0.1 % (w/v) SDS, 10 % (v/v) methanol) according to the sequence. Simultaneously, Whatman filter paper (GE Healthcare, Little Chalfont, United Kingdom) was incubated in Transfer buffer 1x.

The sandwich sequence from the lower electrode (anode) was 3 sheets of Whatman filter paper, PVDF membrane, SDS gel and another 3 sheets of Whatman filter paper (Figure 30). Western blotting transfer was performed with the standard settings of 0.8 mA/cm² membrane for 90 minutes. When the transfer finished, the cassette was disassembled and the band and MW marker on the blotting membrane were then marked as required with pencil.

Non-specific binding on the membrane was blocked using diluted skim milk blocking solution (5 % (w/v) skim milk in TBST (50 mM Tris-base, 138 mM sodium chloride, 2.7 mM potassium chloride, 0.05 % (v/v) Tween-20, pH 8.0). The blotting membrane was then rocked on the orbital shaker for 1 hour in the milk and then rinsed in TBST (2 times, 10 minutes each). Then, the blotting membrane was placed inside a plastic bag filled with 2-3 mL of primary antibody solution placed on orbital shaker with a marble overnight at 4 °C (12-18 hours). Next day, the blotting membrane was removed from the plastic bag and washed in TBST (4 times, 10 minutes). Secondary antibody was also diluted accordingly in skim milk blocking solution (5 % skim milk in TBST). Afterwards, the blotting membrane was placed inside a plastic bag filled with 2-3 mL of secondary antibody solution and then sealed. The blotting membrane was placed on orbital shaker with marble to react for 1 hour at room temperature (22-28 °C). After that, the blotting membrane was removed from the plastic bag and washed in TBST (4 times, 10 minutes). Primary and secondary antibodies used in this study are listed in Table 7.

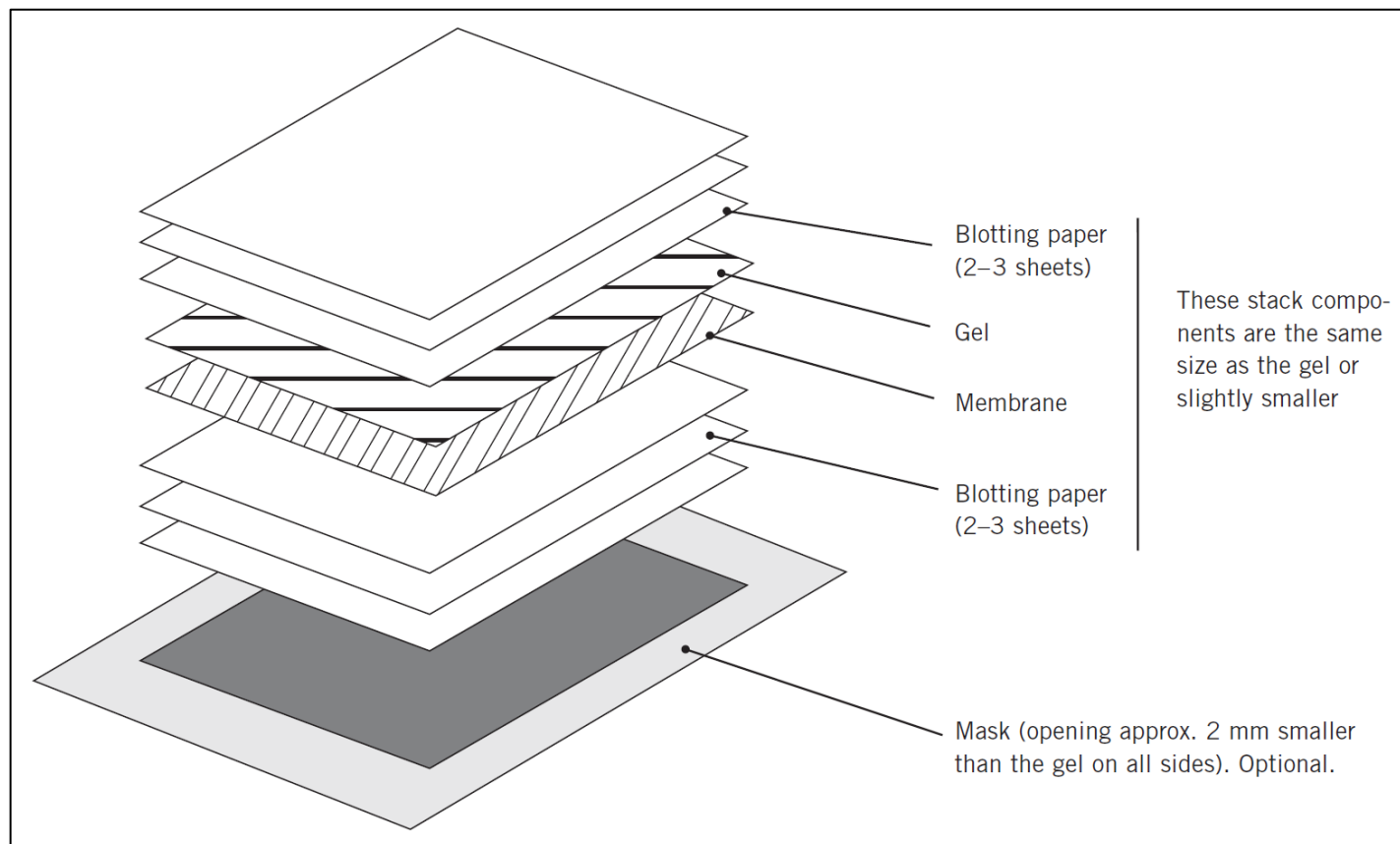


Figure 30. Transfer stack in electrotransfer of 1D gel with semi dry blotting using TE 77 PWR semi dry system. The sandwich sequence from the lower electrode (Anode) is 3 sheets of Whatman filter paper, PVDF membrane, SDS gel and another 3 sheets of Whatman filter paper.

Table 7. Primary and secondary antibodies used in this study.

Antibody	In which sample used	Catalogue numbers	Final concentration used (µg/mL)	Antibody company
Primary antibody				
Anti-CLIC3 antibody	Placenta	Ab56364	5.000	Abcam, Cambridge, United Kingdom
Anti-Peroxiredoxin 2 antibody	Placenta	Ab109367	0.879	Abcam, Cambridge, United Kingdom
Anti-C3 antibody	Placenta	Ab200999	0.396	Abcam, Cambridge, United Kingdom
Anti-Biglycan antibody	Placenta	Ab58562	??	Abcam, Cambridge, United Kingdom
Monoclonal Anti- β -Actin-Peroxidase antibody	Placenta	A3854	2.000	Sigma-Aldrich, St. Louis, Missouri, United States
Hpt/Haptoglobin Polyclonal antibody	Placenta and saliva	Bs-1808R	2.000	Bioss Antibodies, Massachusetts, United States
Pan 14-3-3 (K-19)	Placenta and saliva	SC-629	0.100	Santa Cruz, Dallas, Texas, United States
CRNN Polyclonal antibody	Saliva	11799-1-AP	0.213	Proteintech, Rosemont, IL, United States
Anti-Transferrin antibody [11D3] (HRP)	Saliva	Ab10208	1.600	Abcam, Cambridge, United Kingdom
Anti-Involucrin antibody [SY5]	Saliva	Ab80530	2.000	Abcam, Cambridge, United Kingdom
Anti-Desmoglein 1 antibody	Saliva	Ab124798	0.377	Abcam, Cambridge, United Kingdom
CALML3 monoclonal antibody (M04), clone 2A11	Saliva	H00000810-M04	??	Abnova, Taipei, Taiwan
IPO8 monoclonal antibody (M01), clone 3D5	Saliva	H00010526-M01	??	Abnova, Taipei, Taiwan
Anti-Salivary Amylase antibody [2D4]	Saliva	Ab128195	0.500	Abcam, Cambridge, United Kingdom
Secondary antibody				
Mouse IgG (H&L) Antibody Peroxidase Conjugated	Placenta and saliva	610-703-124	0.500	Rockland, Limerick, PA, United States
Donkey Anti-Rabbit IgG H&L (HRP)	Placenta and saliva	Ab6802	0.040	Abcam, Cambridge, United Kingdom
Rabbit Anti-Goat IgG H&L (HRP)	Placenta	Ab6741	0.400	Abcam, Cambridge, United Kingdom

For visualisation of target proteins using enhanced chemiluminescence (ECL) detection steps, Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, Massachusetts, United States) was used. The two components of ECL reagents, Solution A, luminol reagent; and Solution B, peroxide solution were mixed in equal volumes 30 minutes prior to use. The solution was allowed to warm to room temperature. Next, the blotting membrane was placed in the plastic bag filled with ECL reagents and then open end was sealed, followed by incubation for 15 minutes at room temperature. Finally, the visualization and further semi-quantification densitometric analysis of the band intensity on the blotting membrane was carried out using ChemiDoc and Image Lab software Version 5.2.1 (Life Science, Bio-Rad Laboratories, California, United States) (Figure 31). The ECL reaction is stable for another 30 minutes.

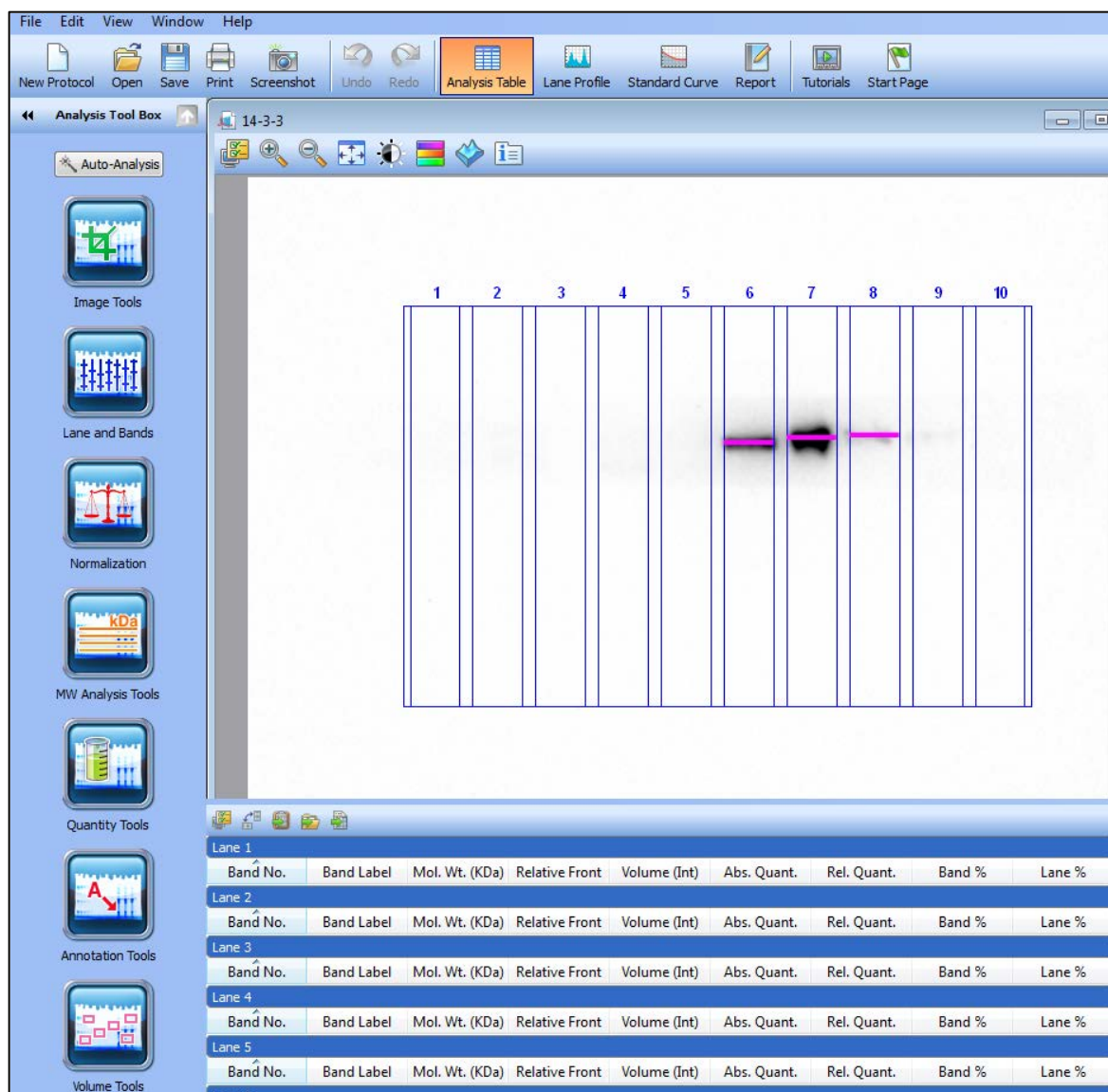


Figure 31. Semi-quantification analysis of Western blot band densitometry using ChemiDoc and ImageLab software.

v. Antibody stripping

PVDF membrane can be used few times using different antibodies with the same host such as mouse or rabbit after the antibody stripping method performed. For each 100 mL of stripping reagents used in this study, 1.5 g Glycine was added with 1 mL HCl (pH is 2.2), 1 mL 10 % (w/v) SDS, 1 mL Tween-20 and remaining is miliQ water. Firstly, the stripping reagent was heated in the microwave up to boiling. Then the stripping reagent was poured on the PVDF membrane on the orbital shaker (45 rpm, 30 mins). The PVDF membrane is then ready to be reused and the primary antibody binding steps can be performed.

2.11.3 Blitz system

2.11.3.1 Introduction and principle of Blitz system

Blitz system (Pall FortéBio Corp., Menlo Park, California, United States) uses a reliable principle of biolayer interferometry (BLI) for analyzing protein-protein and other biomolecular interactions ^(304, 305) (Figure 32). Like other label-free techniques, BLI has the advantage of probing unmodified or native proteins, thus eliminating potential perturbation by labels of the interactions being studied. Blitz system with BLI principle allows rapid identification, quantitation and characterization of expressed protein from as little as 4 microliters of sample ⁽³⁰⁴⁾ (Figure 33). Streptavidin (SA) biosensor provide a simple and straightforward method of molecular immobilization used in the Blitz system. Utilized with BLI, SA biosensors enable quick and easy modification and customization of the biosensor with any biotin-tagged molecule for quantitative and kinetic measurements. Amongst the SA biosensors, the High Precision Streptavidin (SAX) biosensor (Pall FortéBio Corp., Menlo Park, California, United States) that was selected in this study has the most stringent assay precision requirements with precision-controlled coefficient of variation (CV) specification of <4 % ⁽³⁰⁶⁾.

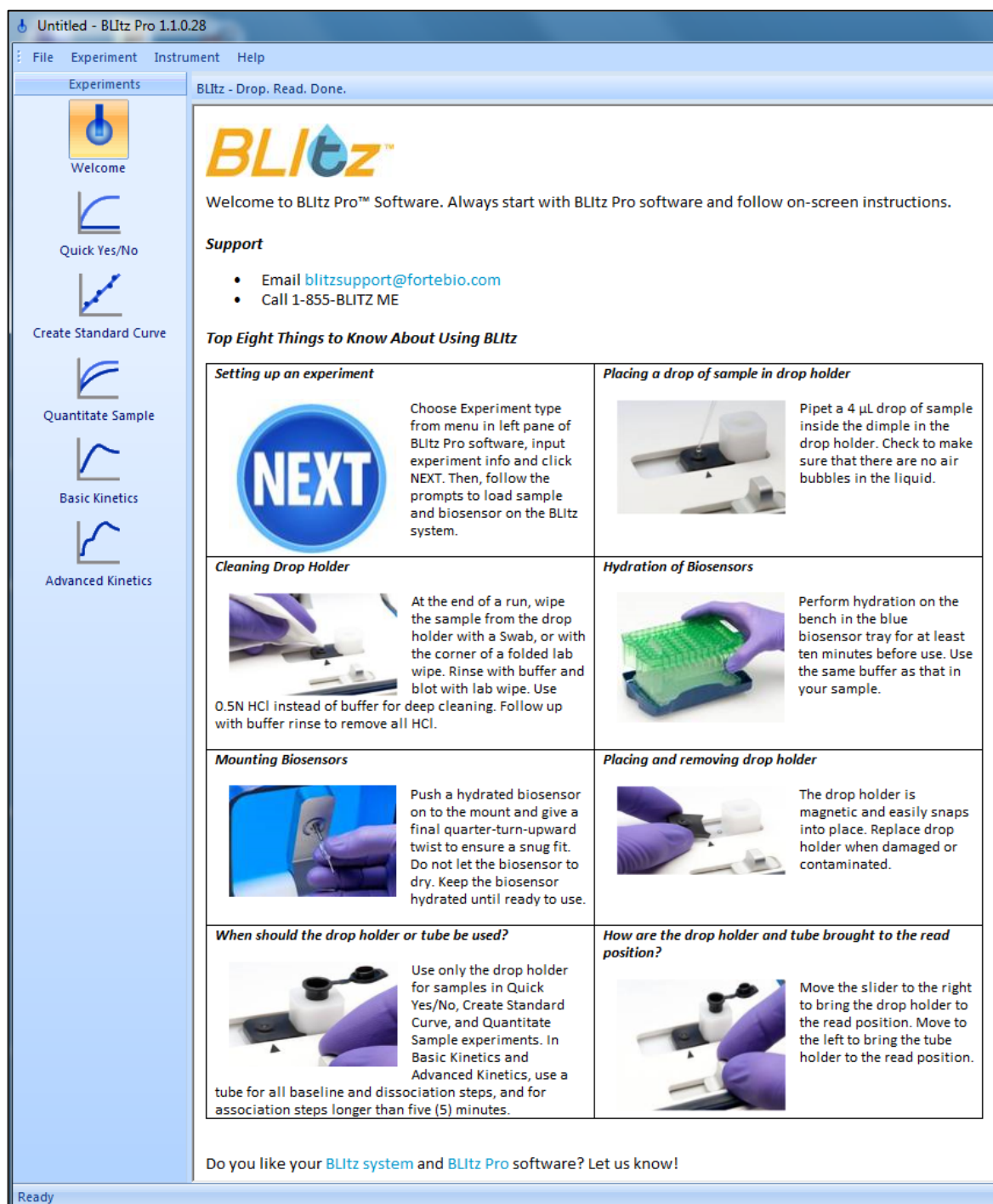


Figure 32. BLItz Pro Software interface ⁽³⁰⁵⁾. Data processing and interferometry data (curve fitting) were globally fitted to a 1:1 binding ratio for calculating kinetic parameters.

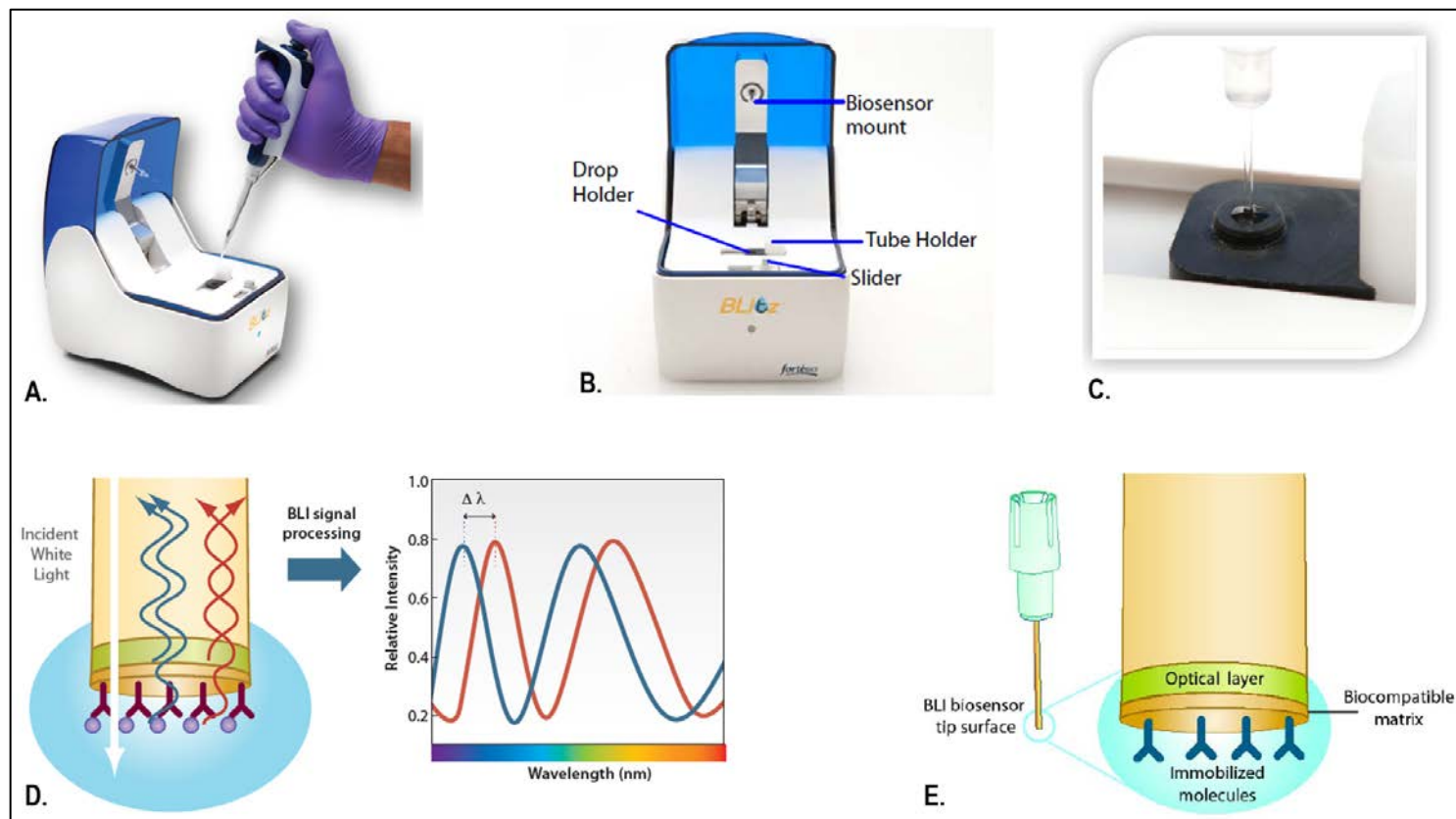


Figure 33. The instrument, biosensor and principle of biolayer interferometry (BLI) in Blitz system ⁽³⁰⁵⁾. A. The Blitz instrument is portable and small with size of approximately 15 X 22 cm. B. BLItz system components (front). C. Biosensor testing 4 microliters sample. D. Principle of biolayer interferometry (BLI) technology by displaying constructive and destructive interference. D. Dip and read assays in Blitz system.

2.11.3.2 Detection methods in Blitz system

There are 3 detection methods that can be used in Blitz system:

- 1) Direct detection: antibody is on the biosensor (for target protein concentration between 0.5-2000 $\mu\text{g/mL}$).
- 2) Sandwich assay: polyclonal antibody is added after the biomarker is bound to the antibody coated sensor (for target protein concentration between 1-10 ng/mL).
- 3) Precipitation assay: polyclonal antibody has a precipitation reagent on it that will help amplify the signal (for target protein concentration below 1 ng/mL).

In this study, direct detection method was selected because the salivary proteins concentrations of the three target proteins (pan 14-3-3, involucrin and cornulin (CRNN)) were estimated between 0.5-2000 $\mu\text{g/mL}$, hence the range of level that can be detected in this detection method.

2.11.3.3 Applications of Blitz system

Application of Blitz system involve several few steps:

- 1) Antibody biotinylation: to determine suitable antibody concentration, molar coupling ratio (MCR) of 1:1, 1:3 or 1:5.
- 2) Immobilization of the antibody to the SA biosensor: to optimise binding time (300-600 seconds) and shaker speed (0-3000 rpm).
- 3) Creating a standard curve for each chosen protein: 8 to 12 fold dilution of known concentration for each protein.
- 4) Quantitate sample experiment: to optimise binding time (300-600 seconds) and shaker speed (0-3000 rpm).
- 5) Regeneration of the SA biosensor: to optimise suitable buffer binding time and shaker speed (0-3000 rpm).

Binding interactions were analysed in a Blitz machine (Pall FortéBio Corp., Menlo Park, California, United States) and were measured as a wavelength shift in nanometers. A buffer reference or blank sample, or no-protein control was subtracted from all binding curves before curve fitting. Data processing and interferometry data (curve fitting) were globally fitted to a 1:1 binding ratio for calculating kinetic parameters using the BlitzPro Software, version 1.1 (ForteBio Inc.) (Figure 32) ⁽³⁰⁷⁾.

2.12 Data analyses

2.12.1 Statistical analysis for saliva and placental samples

The data from the study were statistically analysed using Excel 2013 (Microsoft Corp., Redmond, Washington, United States), GraphPad Prism 7.00 for Windows, Version 7.02, (GraphPad Software, La Jolla California, United States, www.graphpad.com) and IBM SPSS Statistics version 24 (IBM, Armonk, New York, United States). Statistical non-parametric analyses for the data acquired in the qRT-PCR, Western blot and Blitz system methods were conducted using a Mann-Whitney U test, with a two-tailed test, Kruskal-Wallis ANOVA with post-hoc Dunn's multiple comparisons test and Spearman correlation coefficients. For the comparison of 2 groups, the Mann-Whitney U test was used. When multiple testing was required, the Kruskal-Wallis ANOVA with the post-hoc Dunn's multiple comparisons test was performed. In addition, ROC curve was performed for the Blitz blind test data. P-values smaller than 0.05 was regarded as statistically significant.

In this study, both placental and the saliva samples were analysed in relation to childhood allergy distribution based on these following groups:

- 1) Control vs allergy.
- 2) The influence of the presence and absence of maternal asthma during pregnancy on protein expression and child allergy
 - a. Children of asthmatic mothers (high risk of allergy) with and without an allergy.
 - b. Children of non-asthmatic mothers (low risk of allergy) with and without an allergy.
 - c. The effect of sex and the presence and absence of maternal asthma on salivary protein expression.
- 3) Effect of sex on protein expression and child allergy
 - a. Children with allergy vs children with no allergy.
- 4) Effect of specific allergic phenotypes on protein expression and child allergy
 - a. Control vs specific allergy.

3.0 WORKFLOW OPTIMISATION ON THE PLACENTAL AND SALIVA SAMPLES

3.1 Introduction

The optimisation methods for placental samples from children with and without an allergy include quantitative real-time polymerase chain reaction (qRT-PCR) and Proteomics (LC-MS/MS); and validation study of Western blot. For saliva samples, optimisation methods include Proteomics (LC-MS/MS); and two validation studies which were Western blot and Blitz system. Samples workflows performed on both of the saliva and placental tissues are as described in Methodology section 2.1.

MS-based methods such as LC-MS/MS is a reliable discovery tool due to its high sensitivity and accuracy for mass measurement and is an established technique used for salivary biomarker identification ⁽²⁷²⁾. qRT-PCR was conducted in preliminary workflow to measure expression of allergy-associated genes in the placental samples whereas Western blot validated the presence of specific proteins from placental and salivary samples ⁽³⁰²⁾. Blitz system used crude saliva samples as little as 4 microliters and has the advantages of not requiring expertise skill, easy to use, feasible and applicable to be used in clinical setting ⁽³⁰⁴⁾. Therefore, it may serve as a practical screening method of allergic risk detection in infants.

In this study, optimisations were essential to ensure the data was reliable and reproducible. In both placental and saliva samples, optimisations of protein resuspension buffer and types of protein digestion were conducted and distinguished. The necessity of performing precipitating protein with 2-D clean-up and desalting protein with C18 clean-up (Zip Tip) prior to LC-MS/MS were compared for the coverage of protein group, membranous protein group, total protein and peptides. Moreover, in the Western blot, the optimised dilution of primary and secondary antibodies was established. For the Blitz system validation method, optimisation involved several steps including antibody biotinylation, immobilization of the antibody to the SAX biosensor, creating a standard curve for each chosen protein, the optimum running and regeneration buffers and regeneration of the High Precision Streptavidin (SAX) biosensor.

3.2 Workflow optimisation of the placental samples

3.2.1 Optimisation of LC-MS/MS

The steps of LC-MS/MS that needed optimisation include choices of protein resuspension buffer, types of protein digestions and the necessity to perform precipitation (modified 2-D clean-up) and desalting steps (C18 clean-up). Tandem mass spectra of the placental samples were processed with Proteome Discoverer v1.4 software (Thermo Scientific, Massachusetts, United States), a database searching platform. Spectra were searched using the MASCOT v2.2 search engine (Matrix Science, Boston, United States) (refer Methodology section 2.10.4).

3.2.1.1 Protein resuspension buffer and types of protein digestions

Placental samples were reconstituted with two types of buffer: 6 M GdnHCl and 2 % (w/v) SDS. Then, two types of protein digestions, in-solution (IS) tryptic digestion and filter-aided sample preparation (FASP) were performed on each of these placental samples prior to the LC-MS/MS (n=16). Tandem mass spectra of the placental samples in different protein resuspension buffers with two different types of protein digestions were annotated and compared. Data variables that include protein group, total protein and total peptides were analysed and presented in means percentage values.

Overall, FASP had better protein coverage than the IS method in both 6 M GdnHCl and 2 % (w/v) SDS protein resuspension buffer (Figure 34). Furthermore, findings revealed that placental samples with FASP protein digestion has slightly better protein group coverage when reconstituted in 2 % (w/v) SDS (95.50 %) as compared with placental samples reconstituted in 6 M GdnHCl (95.16 %).

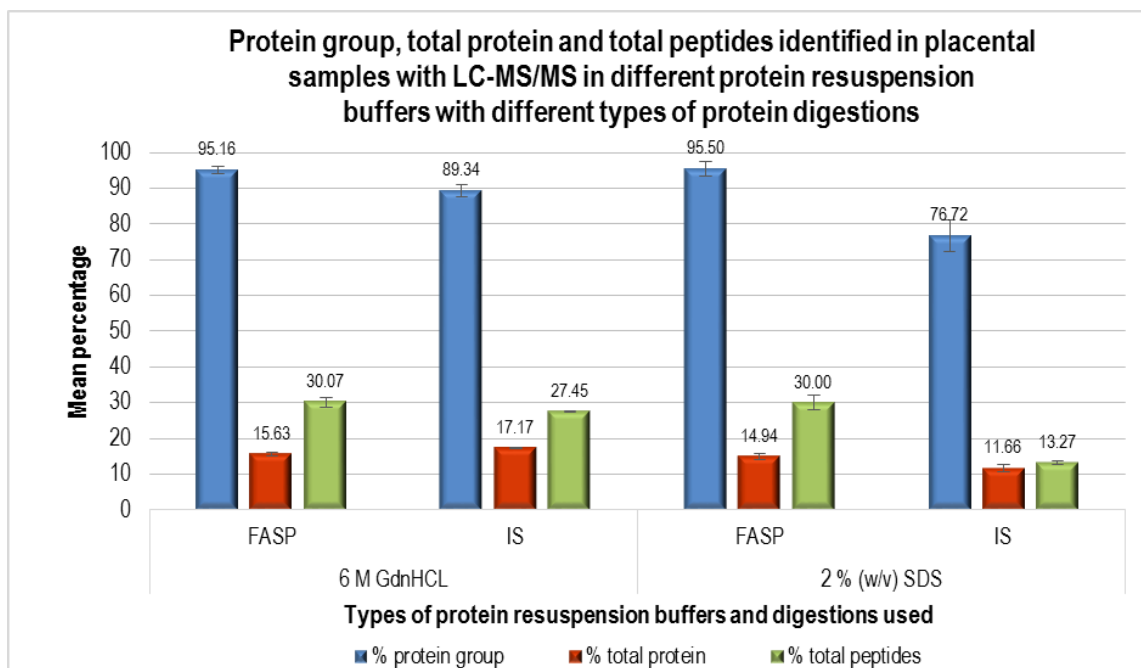


Figure 34. Protein group, total protein and total peptides identified in placental samples with LC-MS/MS in different protein resuspension buffers (6 M GdnHCL or 2 % (w/v) SDS) with different types of protein digestions (FASP or IS). (GdnHCL represents guanidine hydrochloride, SDS represents sodium dodecyl sulfate, FASP represents filter-aided sample preparation and IS represents in-solution tryptic digestion).

3.2.1.2 Protein precipitation with modified 2-D clean-up

The purpose of the 2-D clean-up is for concentrating protein from samples that are too dilute by quantitatively precipitating and concentrating protein in the sample, while washing away substances such as ionic detergents, salts, nucleic acids, lipids and plant-derived phenolic compounds (refer Methodology section 2.7.1). However, there is concern that while doing this step, protein loss may occur. To determine whether performing 2-D clean-up outweigh the risk of protein loss, placental samples were reconstituted in 2 % (w/v) SDS with or without 2-D clean-up procedure prior to protein digestion and desalting step. After LC-MS/MS, membranous protein group was annotated manually, according to UniProtKB/Swiss-Prot protein database from the list in the Mascot results file. Findings revealed that placental samples with 2-D clean-up had a higher protein group (98.32 %) and membranous protein group coverage (55.20 %) as compared with placental samples without 2-D clean-up (98.30 % and 44.33 %), respectively (Figure 35).

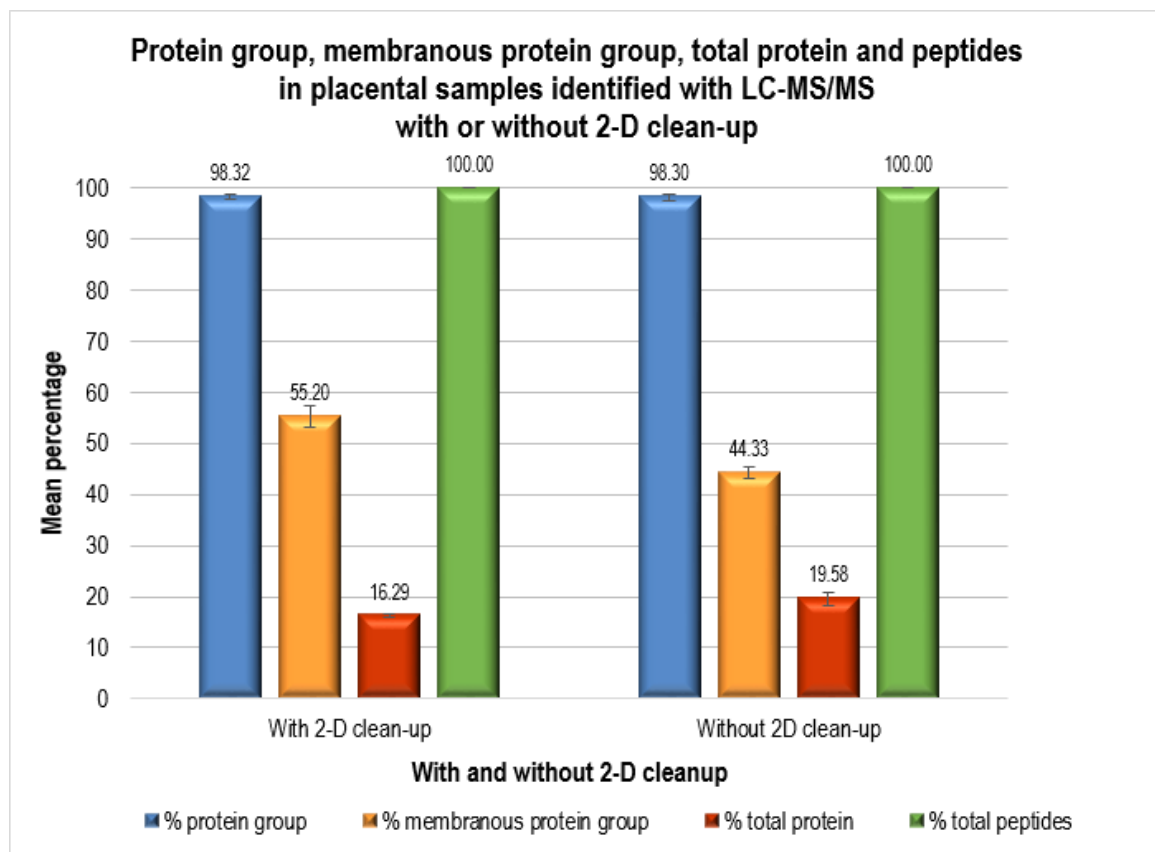


Figure 35. Protein group, membranous protein group, total protein and peptides in placental samples identified with LC-MS/MS with or without 2-D clean-up.

3.2.1.3 Desalting with C18 column

C18 column using Zip Tip is used for desalting purposes to remove contaminants that interfere with mass spectrometric analysis (refer Methodology section 2.7.5). To determine whether performing desalting step is an essential requirement, placental samples were reconstituted in 2 % (w/v) SDS with or without C18 column procedure. Data analysis demonstrated that placental samples that underwent C18 column procedure had higher protein group coverage (97.85 %) and a higher number of total peptides (100 %) as compared with placental samples without C18 column procedure (93.34 % and 31.71 %) (Figure 36).

In addition, MALDI–TOF–MS was also performed on these placental samples with and without C18 column procedure (refer Methodology section 2.7.5). Findings revealed that placental samples without C18 column procedure were very dense and crystallized as compared with C18 column procedure, most probably because of the high salt content (Figure 37A). Moreover, the image of those placental samples with C18 column procedure performed had more visibility on the ultrafleXtreme indicating possibly less content of salt (Figure 37B).

Summary of placental samples proteomics workflow, optimisation steps and results are as detailed in Table 8.

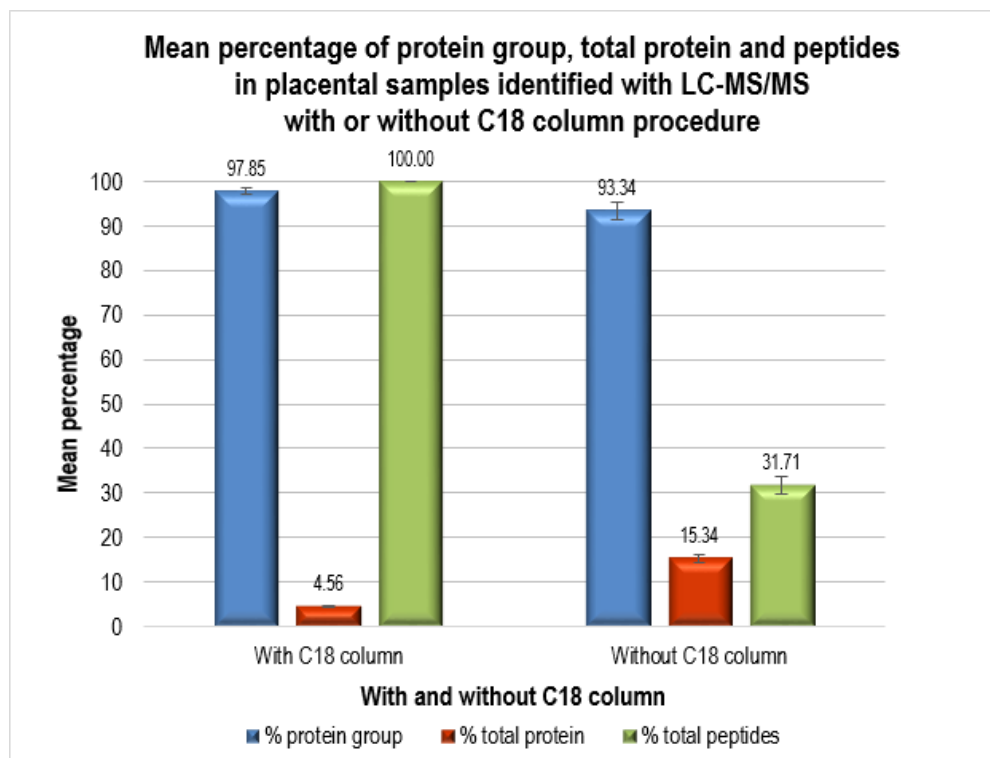


Figure 36. Mean percentage of protein group, total protein and peptides in placental samples identified with LC-MS/MS with or without C18 column procedure.

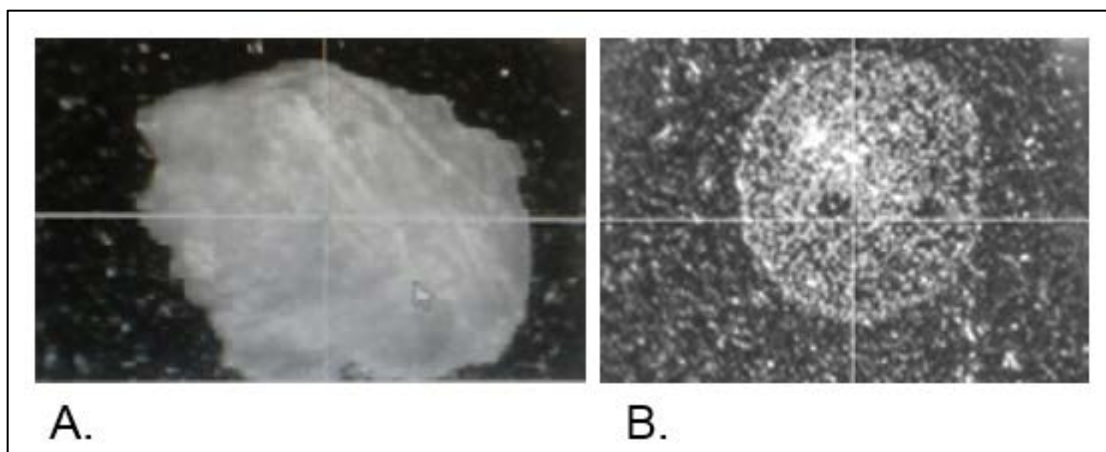


Figure 37. Placental sample (AL-174) visualisation using matrix on the ultrafleXtreme MALDI-TOF-MS. A. Without C18 column step. B. With C18 column step.

Table 8. Placental samples proteomics workflow, optimisation steps and results.

Placenta samples proteomics workflow	Optimisation steps	Optimisation result
Placental samples were collected	➤ Olfactory protein was used as target protein ➤ Protein resuspension buffers was compared between 6 M GdnHCl and 2 % (w/v) SDS.	➤ 2 % (w/v) SDS had better protein coverage than 6 M GdnHCl (as depicted in Figure 35).
Proteins were precipitated with modified 2-D clean-up	➤ Protein recovery was compared with or without 2-D cleanup.	➤ With 2-D clean-up had better protein coverage than without 2-D clean-up (as depicted in Figure 36).
Proteins concentrations were estimated with EZQ assay	NA	NA
Proteins were digested using 10 µg total protein	➤ Protein recovery after digestion was compared between IS and FASP.	➤ FASP had better protein coverage than IS (as depicted in Figure 35).
Placental samples were desalted with C18 column using Zip Tip	➤ Protein recovery was compared with or without C18 column procedure.	➤ With C18 column had better protein coverage than without C18 column (as depicted in Figure 37).
Polymers were detected with MALDI–TOF–MS	NA	NA
LC-MS/MS Orbitrap was performed	NA	NA
Mascot and Max-LFQ were used to analysed data for protein quantification and identification	NA	NA

° Abbreviations: GdnHCl represents guanidine hydrochloride, SDS represents sodium dodecyl sulfate, IS represents in-solution tryptic digestion, FASP represents filter-aided sample preparation, MALDI-TOF MS represents matrix assisted laser desorption ionization-time of flight mass spectrometry and NA represents non-applicable.

3.2.2 Optimisation of Western blot validation study

Initially, six placental proteins candidates were chosen according to inclusion and exclusion criteria for the potential proteins biomarker for allergy (refer Methodology section 2.3.1 and 2.3.2) but only five placental antibodies were able to be optimised and can be visualized in the Western blot (n= 68). Biglycan (Bone/cartilage proteoglycan I) (PGS1) was not able to be optimised in the Western blot, hence was not used in the validation study. Workflow of Western blot using placental samples is depicted in Figure 38. Placental proteins and the optimised dilution of antibodies used in Western blot are as listed in Table 9. These five placental proteins include chloride intracellular channel protein 3 (CLIC3) (ratio of >2 relative to non-allergic samples); peroxiredoxin-2 (PRDX2); haptoglobin (Hp) and complement 3 (C3) (ratio <0.5 fold change relative to non-allergic samples). Moreover, there is also 14-3-3 protein eta that had very high expression in allergic children and very low expression in the non-allergic group. Each of these proteins were normalised with the housekeeping protein, beta-actin, which has been well-established in the literature as an appropriate housekeeping gene for human placenta (132, 308). Although there were some discrepancy between the MW and the observed band size of some of the proteins used in Western blot such as C3 (Table 9) and Involucrin (Table 10), the similar findings were observed in the manufactures' datasheet. Moreover, positive and negative controls were used in the optimisation to validate the antibodies. Therefore, the specificity of the antibodies are reliable.

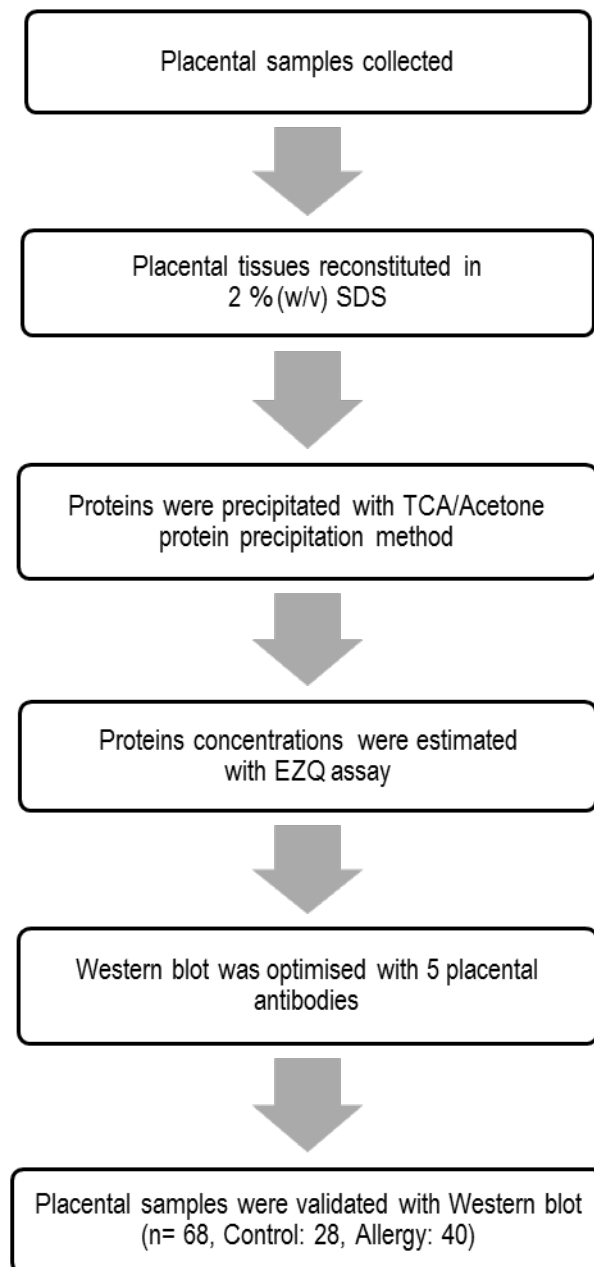


Figure 38. Workflow of Western blot using placental samples. (SDS represents sodium dodecyl sulfate).

Table 9. Placental proteins and the optimised dilution of antibodies used in the Western blot.

Placental proteins	Primary antibody company	Swiss-Prot entry	MW (kDa)	WB band observe (kDa)	Primary antibody dilution	Secondary antibody (host)	Secondary antibody company	Secondary antibody dilution	Ratio LFQ MS	Allergic disease related	Notes
Chloride intracellular channel protein 3 (CLIC3)	Abcam, Cambridge, United Kingdom	O95833	26.65	30	1/200	Mouse	Rockland, Limerick, PA, United States	1/2000	AM/NM= 2.80 AM+AF/NM+NF= 2.52	Asthma	
Peroxiredoxin 2 (PRDX2)	Abcam, Cambridge, United Kingdom	P32119	22	22	1/1000	Rabbit	Abcam, Cambridge, United Kingdom	1/50 000	AM/NM= 0.48	Asthma	
Complement C3 (C3)	Abcam, Cambridge, United Kingdom	P01024	187.15	115	1/2000	Rabbit	Abcam, Cambridge, United Kingdom	1/50 000	AF/NF= 0.46	Asthma	
Haptoglobin (Hp)	Bioss Antibodies, Massachusetts, United States	P00738	45.21	52	1/500	Rabbit	Abcam, Cambridge, United Kingdom	1/50 000	AF/NF= 0.46	Asthma, rhinitis	Present in both samples, saliva ratio LFQ E/C 0.07
Pan 14-3-3	Santa Cruz, Dallas, Texas, United States	Varies	30	30	1/1000	Rabbit	Abcam, Cambridge, United Kingdom	1/50 000	Uncalculated ratio of 14-3-3 eta in AM and AF	Contact dermatitis, asthma	14-3-3 eta present in both samples, saliva ratio LFQ E/C 2.50
Biglycan (Bone/cartilage proteoglycan I) (PGS1)	Abcam, Cambridge, United Kingdom	P21810	41.65	42	??	Goat	Abcam, Cambridge, United Kingdom	1/5000	AF/NF= 2.39 AM/NM= 2.39 AM+AF/NM+NF= 2.39	Asthma, rhinitis	Unable to be optimised
Actin	Sigma-Aldrich, St. Louis, Missouri, United States	P60709	41.74	42	1/1000	Mouse	Rockland, Limerick, PA, United States	1/2000	Housekeeping protein	NA	

° Abbreviations: MW represent molecular weight, WB represents Western blot, LFQ MS represents label-free quantification mass spectrometry, Mono represents monoclonal, Poly represents polyclonal, NA represents non- applicable/not enough sample number, AM represents allergic males, AF represents allergic females, AM+AF represents allergic males combined with allergic females, NM represents non-allergic males, NF represents non-allergic females and NA represents non-applicable/not enough sample number.

3.3 Workflow optimisation of the saliva samples

3.3.1 Saliva samples handling

Sample handling imposes a real challenge to saliva analysis. Saliva samples need to be collected and stored under conditions of minimal proteolysis, deglycosylation and dephosphorylation to minimise protein degradation ⁽²⁹⁰⁾. Protein degradation may be caused by several factors: bacterial proteases that may present in saliva, high temperature; pH condition and freezing and thawing cycle ⁽³⁰⁹⁾. The recommended protocol to prevent any degradation is that sample processing should be performed on ice (0 to 4 °C), PIC is added immediately, centrifuged to remove insoluble material and stored in -20 or -80 °C ⁽³¹⁰⁾. However, without PIC, short-term storage (less than 24 hours) of freshly collected samples on ice is effective to prevent protein degradation without interfering with the chemistry of the proteome ⁽³⁰⁹⁾. Furthermore, minimising the time elapsing between sample collection and analysis will potentially reduce the risk of protein degradation. All of these influencing factors were carefully considered in this current study to prevent protein degradation as minimal as possible.

3.3.2 Optimisation of LC-MS/MS

3.3.2.1 Guanidine hydrochloride (GdnHCl) as protein resuspension buffer

Saliva samples were reconstituted in a protein resuspension buffer consisting of 6 M GdnHCl; according to previous protocols, conducted on saliva samples in the same laboratory ⁽²⁹⁰⁾. GdnHCl is the hydrochloride salt of guanidine, a strong chaotropic agent and one of the strongest denaturants used in physiochemical studies of protein folding. GdnHCl denaturing completely unfolds the misfolded protein in the inclusion bodies ⁽³¹¹⁾. At high concentrations of GdnHCl such as 6 M, proteins lose their ordered tertiary structure, and tend to become randomly coiled as they do not contain any residual structure ⁽³¹²⁾. Therefore, because we are targeting membrane proteins, the ionic nature of GdnHCl plays a role in destabilizing salt bridges of the protein making it easier to be digested later. The denaturant, 6 M GdnHCl, was then reduced and removed from the solubilized protein solution by dilution in a huge volume of refolding buffer to recover soluble and active proteins ⁽³¹¹⁾. In this step, protein folding/unfolding intermediates are usually formed; these intermediates often aggregate due to contiguous hydrophobic groups ⁽³¹³⁾. This process of protein aggregation often leads to a significant amount of protein precipitation, which is beneficial for protein digestion ⁽³¹¹⁾.

3.3.2.2 Salivary protein concentration for protein digestion

Child saliva concentrations for the salivary protein cellular pellet resuspended in 6 M GdnHCl varied between children who were 6 months relative to 5 years, between 0.60–7.07 $\mu\text{g}/\mu\text{L}$. This is slightly less than adult saliva concentrations that vary between 3.38–7.00 $\mu\text{g}/\mu\text{L}$. Hence, with regards to precious sample and limitation in starting material, 5 μg of samples were used and sufficient for IS digestion tryptic digestion.

3.3.2.3 Optimisation of in-solution (IS) tryptic digestion

Prior to in-solution tryptic digestion, protein samples were reduced and alkylated (refer Methodology section 2.7.3). The volume of 6 M GdnHCl was increased as the saliva protein concentration increased. Following the denaturation of the saliva proteins, the concentration of GdnHCl was lowered from 6 M to at least 1 M for optimal trypsin digestion. High molarity of 6 M GdnHCl can cause incomplete trypsin digestion. Thus, a pH neutral of ~ 7.8 was created for trypsin protein digestion. To do this, each of the saliva samples were adjusted individually, added to acetonitrile (ACN) in 10 % of final volume solution and remaining total volume made up with 100 mM NH_4HCO_3 . This was to ensure that the molarity of GdnHCl was reduced to 0.5 M prior adding trypsin. The IS protein digestion calculation used for the 18 saliva samples in LC-MS/MS is as shown in Appendix 8, Table 1.

3.3.3 Optimisation of Western blot validation study

With regards to salivary confounding factors, eight salivary protein candidates were chosen with careful consideration given to ensure that the selected proteins were not dependent on age, sex or other state of diseases (refer Introduction section 1.6.2). The selection is made based on the inclusion and exclusion criteria (refer Methodology section 2.3.2). However, only six salivary antibodies were able to be optimised and visualized by Western blot. Calmodulin-like protein 3 and importin-8 (Imp8) were not able to be optimised in the Western blot, thus were not used in the validation study. Workflow of Western blot using childhood saliva samples is depicted in Figure 39.

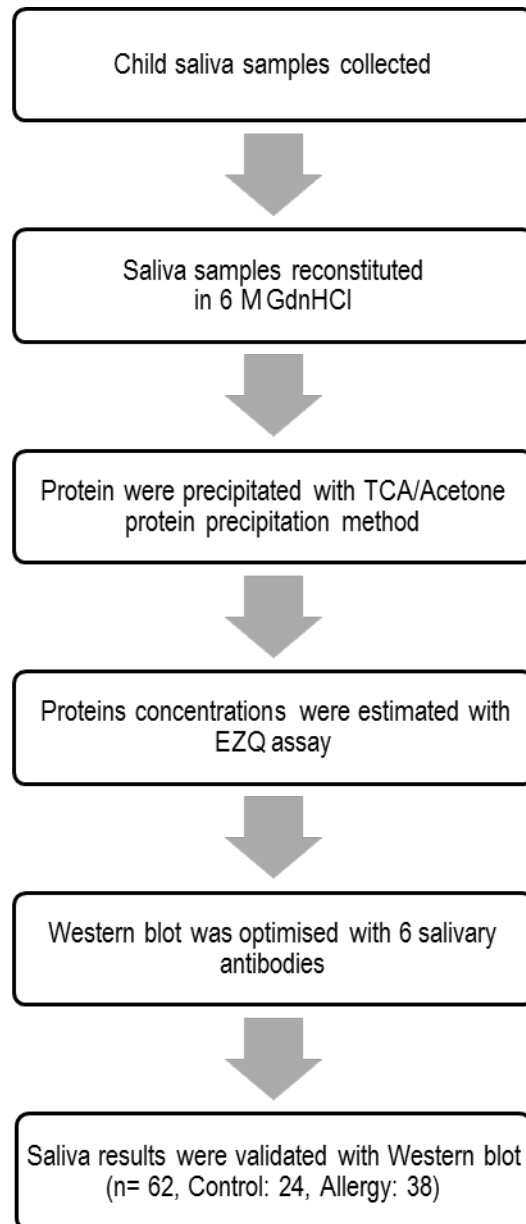


Figure 39. Workflow of Western blot using saliva samples. (GdnHCl represents guanidine hydrochloride).

Salivary proteins and the optimised dilution of antibodies used in Western blot are as listed in Table 10. These salivary proteins include cornulin (CRNN) and 14-3-3 protein that were found to be in significant ratio >2 and transferrin; involucrin and Hp with the ratio <0.5 fold change relative to non-allergic samples; and also desmoglein 1 that had very low expression with no calculated ratio when compared with samples of non-allergic group. Each of these proteins were normalised with the housekeeping protein, alpha amylase, which has been well-established in the literature as an appropriate housekeeping gene for human salivary samples ^(264, 272, 273).

Table 10. Salivary proteins and the optimised dilution of antibodies used in Western blot.

Salivary proteins	Primary antibody company	Swiss-Prot entry	MW (kDa)	WB band observe (kDa)	Primary antibody dilution	Secondary antibody (host)	Secondary antibody company	Secondary antibody dilution	Ratio LFQ MS	Allergic disease related	Notes
Pan 14-3-3	Santa Cruz, Dallas, Texas, United States	Varies	30	30	1/1000	Rabbit	Abcam, Cambridge, United Kingdom	1/50 000	E/C= 2.50	Contact dermatitis, Asthma	14-3-3 eta present in both samples, uncalculated ratio of 14-3-3 eta in placental AM and AF
Cornulin (CRNN)	Proteintech, Rosemont, IL, United States	Q9UBG3	53.56	60	1/1000	Rabbit	Abcam, Cambridge, United Kingdom	1/50 000	A/C= 2.08	Eczema	
Transferrin	Abcam, Cambridge, United Kingdom	P02787	77.08	77	1/500	Mouse	Rockland, Limerick, PA, United States	1/2000	A/C= 0.04 E/C= 0.14 R/C= 0.10	Chronic asthma	
Involucrin	Abcam, Cambridge, United Kingdom	P07476	68.48	100	1/500	Mouse	Rockland, Limerick, PA United States	1/2000	R/C= 0.41	Atopic dermatitis	
Haptoglobin (Hp)	Bioss Antibodies, Massachusetts, United States	P00738	45.21	52	1/500	Rabbit	Abcam, Cambridge, United Kingdom	1/50 000	E/C= 0.07	Asthma, rhinitis	

Desmoglein 1	Abcam, Cambridge, United Kingdom	Q02413	113.75	140	1/1000	Rabbit	Abcam, Cambridge, United Kingdom	1/50 000	Uncalculated ratio in A, E	Atopic dermatitis, complex autoimmune disease (pemphigus vulgaris and foliaceus), chronic rhinosinusitis	
Calmodulin-like protein 3	Abnova, Taipei, Taiwan	P27482	16.89	??	??	Mouse	Rockland, Limerick, PA, United States	1/2000	A/C= 2.16	Atopic dermatitis, psoriatic epidermis	Unable to be optimised
Importin-8 (Imp8)	Abnova, Taipei, Taiwan	O15397	119.94	??	??	Mouse	Rockland, Limerick, PA, United States	1/2000	Uncalculated ratio in A	Asthma	Unable to be optimised
Salivary alpha amylase	Abcam, Cambridge, United Kingdom	P04745	57.75	58	1/1000	Mouse	Rockland, Limerick, PA, United States	1/2000	Housekeeping protein	NA	

° Abbreviations: MW represent molecular weight, represents Western blot, LFQ MS represents label-free quantification mass spectrometry, Mono represents monoclonal, Poly represents polyclonal, C represents control subjects who are children with no allergy, A represents children with asthma, E represents children with eczema, R represents children with rhinitis, NA represents non-applicable/not enough sample number, AM represents allergic males, AF represents allergic females and NA represents non-applicable.

3.3.4 Optimisation of Blitz system validation study

Optimisation of Blitz system involved several steps including antibody biotinylation, immobilization of the antibody to the SAX biosensor, creating a standard curve for each chosen protein, and regeneration of the High Precision Streptavidin (SAX) biosensor as depicted in Figure 40 (refer Methodology section 2.11.3.3).

3.3.4.1 Antibody biotinylation optimisation

The antibody or protein immobilisation onto the Streptavidin biosensors must be biotinylated prior to assaying on the Blitz system ⁽³¹⁴⁾. The high-affinity protein-ligand interactions between Streptavidin and biotin is extensively used as a reliable system for the rapid, stable and irreversible non-covalent binding of biological molecules ⁽³¹⁵⁾. The volume of 0.1 mM biotin reagent required for the biotinylation step is dependent on the molar coupling ratio (MCR) of the biotin reagent to protein as shown in Figure 41 ⁽³¹⁴⁾. As a general protocol, it is recommended to use a 1:1 ratio (1 biotin for every protein molecule). If the protein concentration is less than 500 µg/mL or if the extent of biotinylation at an MCR of 1:1 is found to be insufficient, a 3:1 or 5:1 ratio is recommended ⁽³¹⁴⁾.

Salivary alpha amylase was used as the first antibody to be biotinylated because this housekeeping protein is expressed in high levels and is easily detected in the saliva samples. Then, the antibodies were biotinylated according to Blitz system protocol. The reaction was stopped by removing the excess biotin reagent using the Micro Desalt Spin Column 7K MWCO (Thermo Fisher Scientific, Massachusetts, United States), as suggested by the manufacturer's instructions. The summary of Blitz salivary proteins list and the optimised MCR required in antibody biotinylation step are as detailed in Table 11.

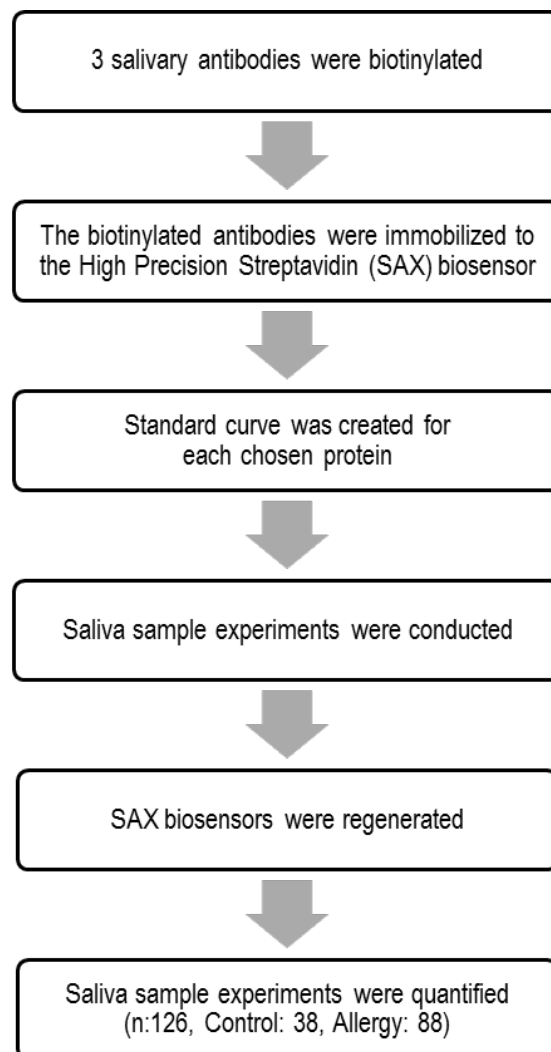


Figure 40. Workflow of Blitz system using child saliva samples.

$$\mu\text{L } 0.1 \text{ mM Biotin reagent} = \frac{\text{Protein Concentration (mg/mL)} \times \text{MCR} \times \text{Volume protein } (\mu\text{L}) \times 10}{\text{MW Protein (kDa)}}$$

Figure 41. Equation for calculating the required volume of 0.1 mM biotin reagent for the antibody biotinylation step. (MCR represents molar coupling ratio, MW represents molecular weight).

Table 11. Blitz salivary proteins list and molar coupling ratio (MCR) required in antibody biotinylation step.

Salivary proteins	Antibody company	Allergic disease related	Ratio LFQ MS	Significant Western blot statistical analysis using saliva samples	Swiss-Prot entry	MW (kDa)	Antibody concentration (µg/mL)	Antibody volume used in Blitz (µL)	MCR ratio	Volume of 0.1 mM Biotin (µL)
Salivary alpha amylase	Abcam, Cambridge, United Kingdom	NA	NA	NA	P04745	58	500	10	5:1	4.31
Pan 14-3-3	Santa Cruz, Dallas, Texas, United States	Contact dermatitis, asthma	E/C= 2.50	Control vs allergy AM control vs AM allergy NAM control vs NAM allergy Control female vs allergy female Control vs specific allergy (asthma combination, eczema combination and rhinitis combination)	Varies	30	100	10	5:1	1.67
Involucrin	Abcam, Cambridge, United Kingdom	Atopic dermatitis	R/C= 0.41	Control male vs allergy male Control vs specific allergy (eczema combination)	P07476	68.48	1000	8	3:1	3.50
Cornulin (CRNN)	Proteintech, Rosemont, IL, United States	Eczema	A/C= 2.08	NAM control vs NAM allergy Control vs specific allergy (rhinitis combination)	Q9UBG3	53.56	213	10	5:1	1.99

° Abbreviation: LFQ MS represents label-free quantification mass spectrometry, MW represent molecular weight, MCR represents molar coupling ratio, NA represents non-applicable, C represents control subjects who are children with no allergy, A represents children with asthma, E represents children with eczema, R represents children with rhinitis, AM represents children with asthmatic mothers (high risk of allergy) and NAM represents children with non-asthmatic mothers (low risk of allergy).

3.3.4.2 Optimisation of biotinylated antibody immobilization to the High Precision Streptavidin (SAX) biosensor

After serial attempts of biotinylated antibody immobilized to the SAX biosensor, it was found that the optimal binding time was 300 seconds with the shaker speed of 2200 rpm. When performing the step of loading/binding the antibody to the SAX biosensor, slow loading for a longer period of time is preferable to rapid loading in a shorter time or rapid ligand immobilization⁽³¹⁶⁾. Ideally, the binding curve in the loading step will show a gradual increase in signal and should not be allowed to reach saturation, but instead plateau (Figure 42). However, in the Blitz machine, the drop holder only enabled a maximum volume of 4 μL biotinylated antibody, so, the maximum time limit for application was 300 seconds. For longer times of more than 300 seconds, tube holders instead must be used with the minimum biotinylated antibody volume of 250 μL . Therefore, due to the limited volume of antibody available for this study, the drop holder with biotinylated antibody volume of 4 μL was the most optimal.

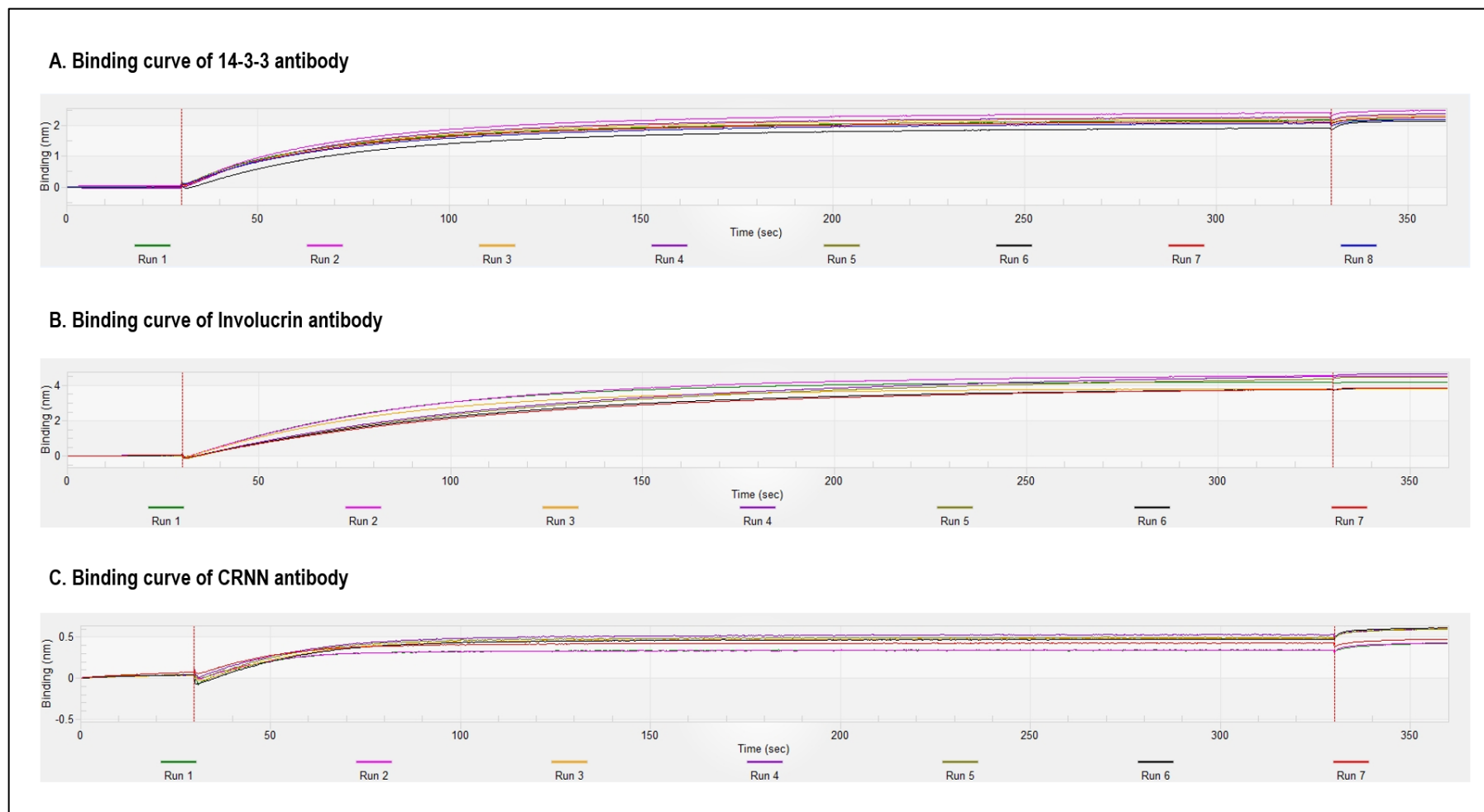


Figure 42. Binding curve of biotinylated antibody during immobilization to High Precision Streptavidin (SAX) biosensor. A. 14-3-3 antibody. B. Involucrin antibody. C. Cornulin (CRNN) antibody.

3.3.4.3 Standard curve for each chosen protein

Unknown saliva sample concentrations of the specified protein are interpolated from the standard curve, generated from serial known concentration of the specific protein recombinant. Protein concentrations can be calculated from the initial binding rate of the interaction based on the initial slope and binding rate at equilibrium ⁽³¹⁷⁾. The protein concentration of saliva samples being analysed should fall within the concentration range of the standard curve for accurate quantification. Therefore, saliva samples being analysed may be run neat or diluted, as appropriate, to bring the concentration within the linear range of the standard curve for the highest accuracy and precision.

A standard curve must be generated for:

- 1) Each protein or antibody molecule, the same protein as the samples to be quantified.
- 2) Known amounts of the protein or antibody in 2 or 3 replicates.
- 3) Dilution in a buffer matrix that exactly matches the percentage of the standard and unknown samples.
- 4) At least 3 different concentrations of spiked samples that fall into the high, middle and low end of the usable standard range should be evaluated.
- 5) A series of 8 or 12 dilutions of a known concentration of purified analyte is diluted in a matrix that matches the samples.
- 6) A reference sample that matches the matrix of the samples to be analysed but does not contain the protein of interest ⁽³¹⁷⁾.

Standard curves can be saved and utilized for subsequent experiments, as long as the standard protein exactly matches the analyte in isotype and species, and the biosensors are from the same manufacturing lot as those used to run the samples ⁽³⁰⁵⁾.

In this study, standard curves for these three proteins were generated with 120 seconds binding time and shaker speed of 2200 rpm, similarly as in the quantification step. Protein recombinants that were used as the standard protein for involucrin and CRNN were manufactured by Abcam, Cambridge, United Kingdom, and pan 14-3-3 protein recombinant was synthesized in the laboratory, a gift from Joanna Woodcock from Centre for Cancer Biology SA Pathology, Adelaide.

Standard curves for 14-3-3 zeta, involucrin and CRNN proteins are as depicted in Figure 43. The Blitz protocol suggested that coefficient of determination (R^2) in the standard curve equation should be more than 0.8, as shown in Figure 43 (R^2 for 14-3-3 zeta: 0.9998, involucrin: 0.9418 and CRNN: 0.8862). Protein recombinants were provided at concentrations of 2700 $\mu\text{g/mL}$ for 14-3-3 zeta, 100 $\mu\text{g/mL}$ for

involucrin and 500 µg/mL for CRNN. Therefore, any readings beyond these concentrations are considered inaccurately measured. The assays were performed over a broad range of concentrations with five to seven dilution series were prepared for these protein recombinants. Each diluted standard were measured three times on the Blitz system and the mean values were plotted after subtracting background values. TBST (50 mM Tris-base, 138 mM sodium chloride, 2.7 mM potassium chloride, 0.05% Tween-20, pH 8.0) was used as the running buffer to spike the recombinant proteins.

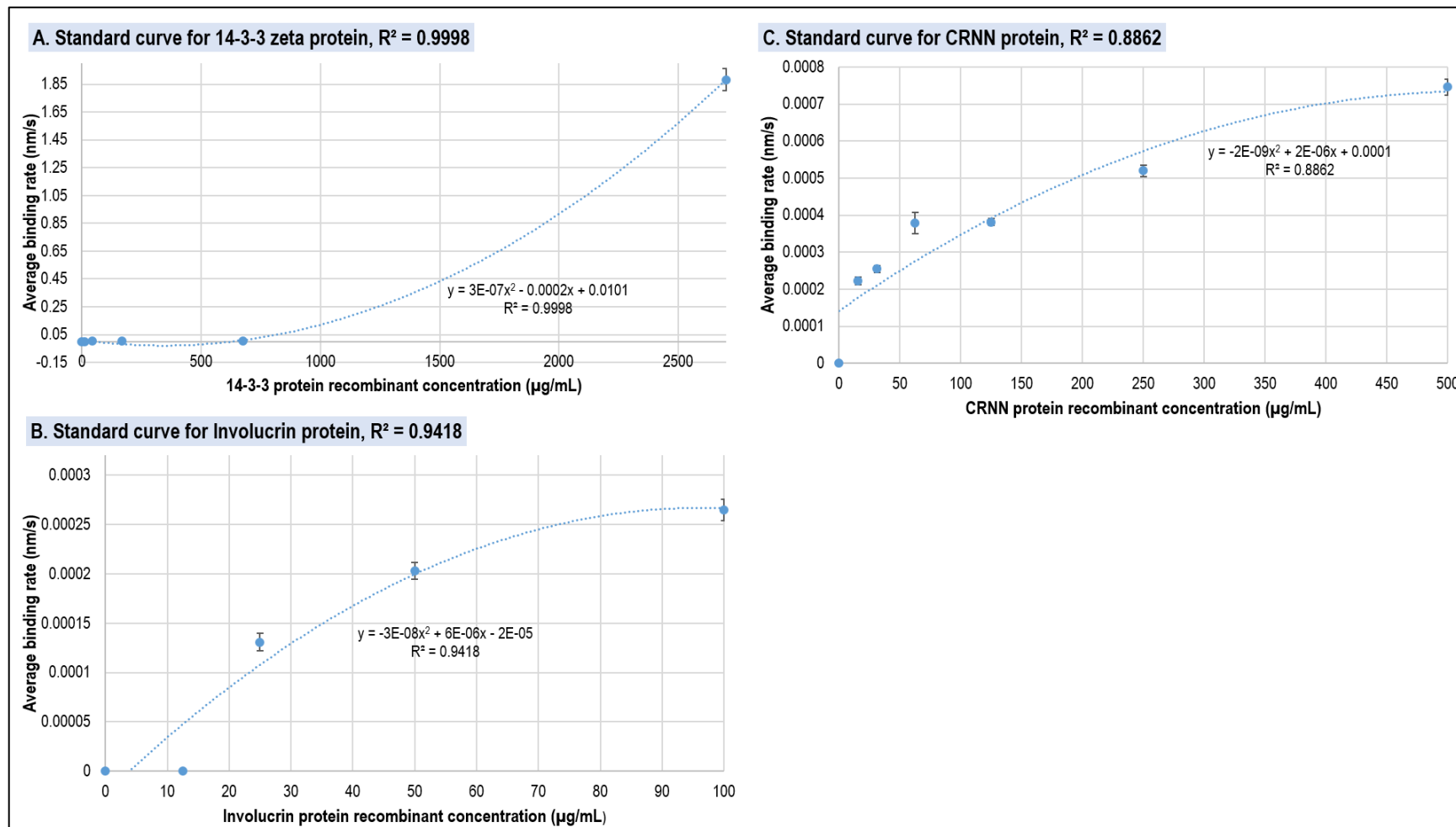


Figure 43. Standard curves for the three salivary proteins. A. 14-3-3 zeta protein. B. Involucrin protein. C. Cornulin (CRNN) protein. (R^2 for 14-3-3 zeta: 0.9998, involucrin: 0.9418 and CRNN: 0.8862).

3.3.4.4 Optimisation of the High Precision Streptavidin (SAX) biosensor regeneration

SAX biosensor can be regenerated and reused up to eleven times provided that the valid regeneration protocol was established ⁽³¹⁸⁾. This is evidence by the binding efficiency studies conducted to reevaluate the capture antibody surface coverage. The underlying surface chemistry of the Streptavidin biosensor is robust and stable over a broad range of pH and ionic strengths ⁽³¹⁸⁾. Any “loss” occurring after the first regeneration can be minimized by having one mock binding cycle as the first assay as shown in blue line in Figure 46A. Then the rest of the assays should be the same or within acceptable variations ⁽³¹⁷⁾.

There were ten types of regeneration solutions recommended by the Blitz protocol based on protein-dependent suitability that may need low pH, high salt, presence of detergent, or even high pH ⁽³¹⁷⁾. However, with regards to the suitability of the biosensor selected, which is SAX, and types of antibodies, three buffers were selected: 10 mM Glycine (pH 1), 500 mM Phosphoric Acid (~pH 1) and 0.01% (w/v) SDS (~ pH 5). Each SAX biosensor was subjected to ten times regeneration using these three types of buffers. The shaker speed used was 1000 rpm and the regenerating buffer was alternated with the running buffer, TBST every 30 seconds, so that the shaking and the acidic buffer will not destroy the biosensor surface.

Criteria needed to successfully regenerate the ligand-coated Streptavidin biosensor surface include:

- 1) Biosensor surface chemistry must be stable under the regeneration conditions.
- 2) Immobilized ligand must be stable under the regeneration conditions and retain activity over multiple regeneration cycles (ligand-dependent).
- 3) Ligand-target protein interaction must dissociate during regeneration (protein pair dependent) ⁽³¹⁸⁾.

The optimisation results of the SAX biosensor regeneration immobilized with 14-3-3, involucrin and CRNN antibodies are as depicted in Figures 44, 45, and 46. Based on these results:

- 1) A regeneration condition of 3 X 30 seconds cycles of 10 mM Glycine pH 1 with shaker speed of 1000 rpm, alternating with 3 X 30 seconds cycles of TBST running buffer was identified as an excellent method for regenerating SAX biosensors that had been immobilized with 14-3-3, involucrin and CRNN.

- 2) SAX biosensors regeneration may be repeated up to ten times for optimum result, which mean binding rate remained similar where they are seemingly on top of each other after every biosensor regeneration process without compromising the antibody-protein binding.

In this study, the optimal binding time of 120 instead of 200 seconds in standard curves and quantitate samples steps was chosen because at 120 seconds, the antibody-protein binding had reached maximum capacity. The summary of the optimisation results of the time duration and shaker speed for Blitz workflow are as detailed in Table 12.

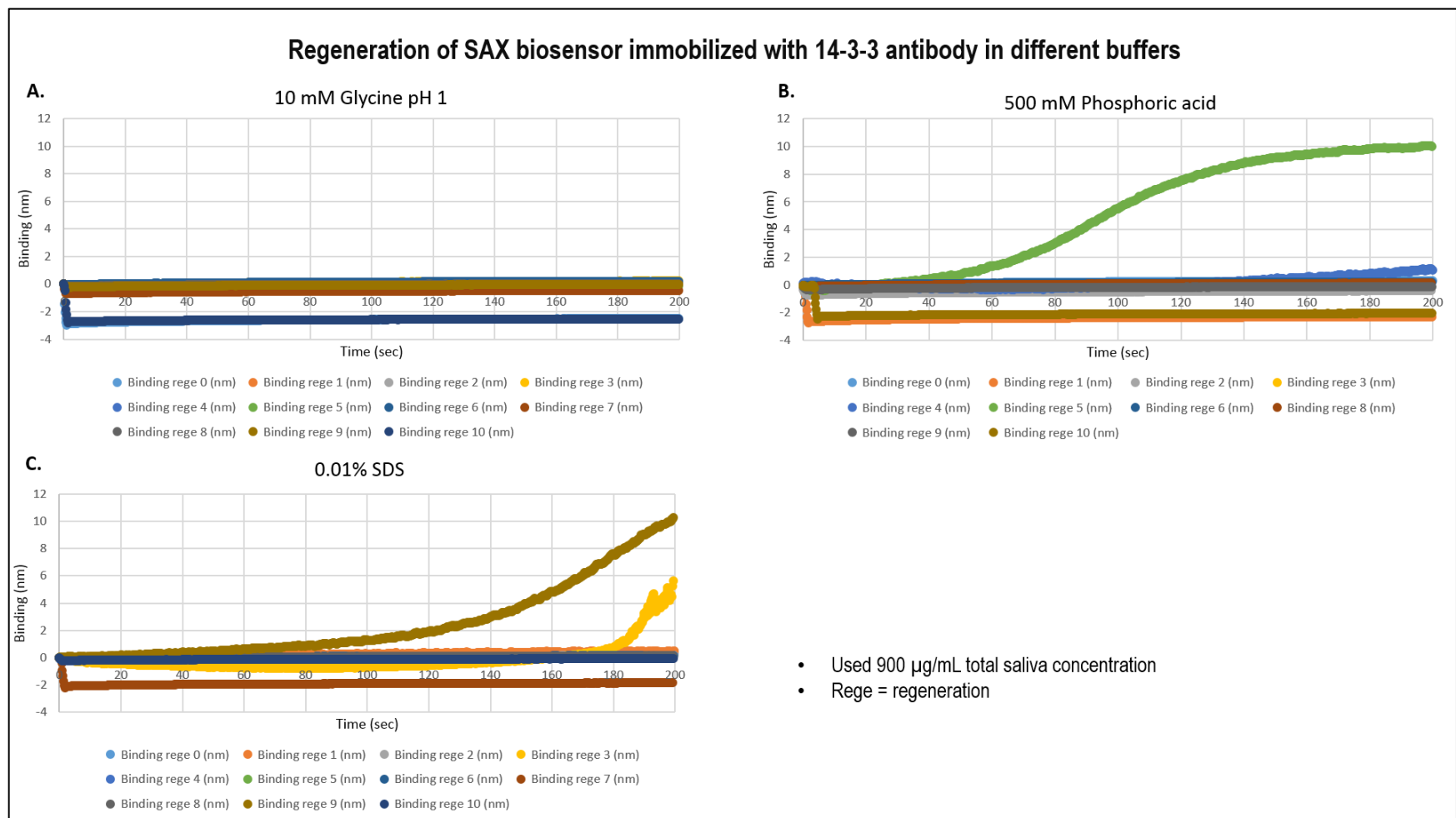


Figure 44. The optimisation results of the SAX biosensor regeneration immobilized with 14-3-3 antibody in different buffers. A. 10 mM Glycine (pH 1). B. 500 mM Phosphoric Acid ($\sim$ pH 1). C. 0.01% (w/v) SDS ($\sim$ pH 5).

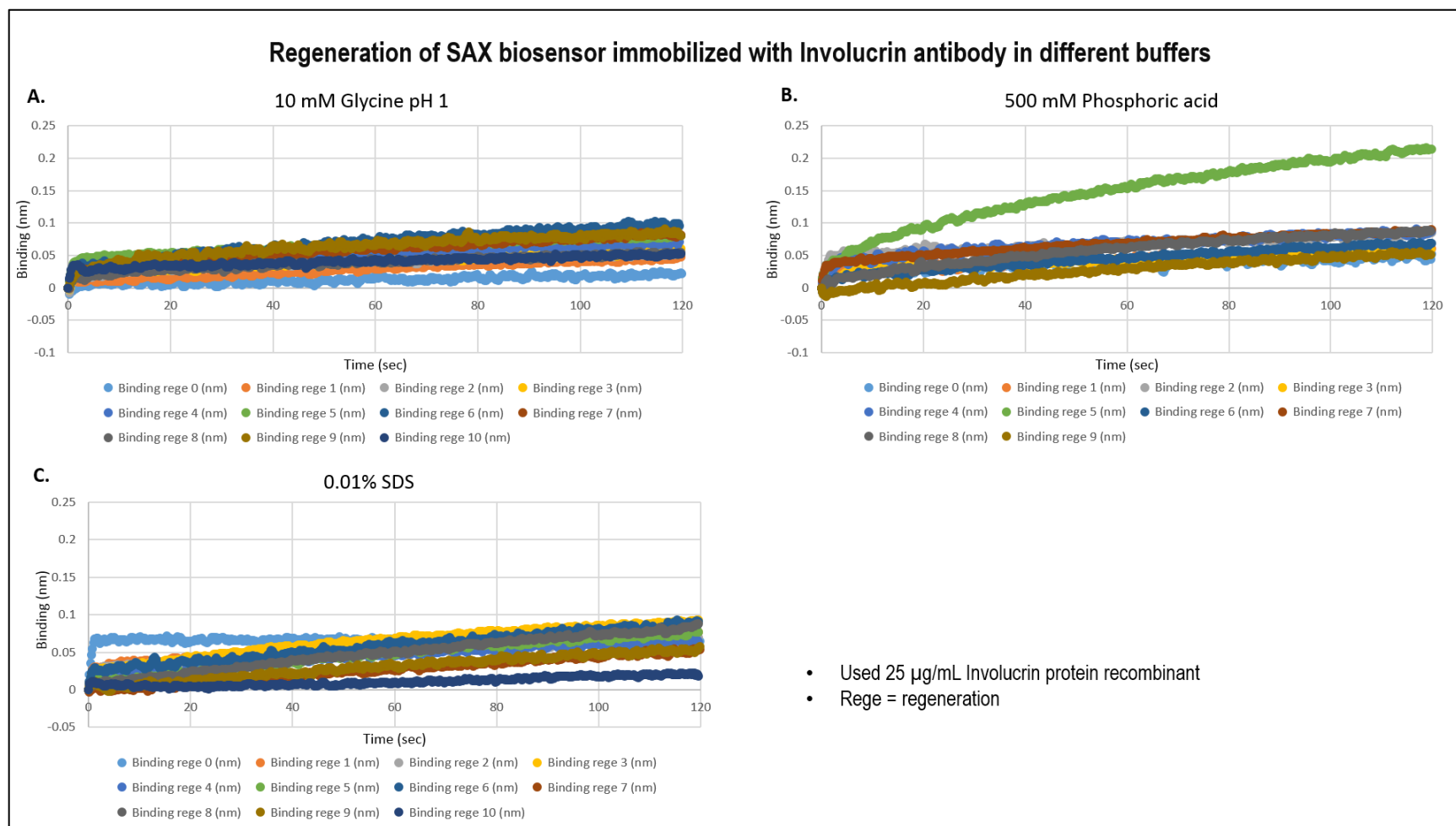


Figure 45. The optimisation results of the SAX biosensor regeneration immobilized with involucrin antibody in different buffers. A. 10 mM Glycine (pH 1). B. 500 mM Phosphoric Acid ($\sim$ pH 1). C. 0.01% (w/v) SDS ($\sim$ pH 5).

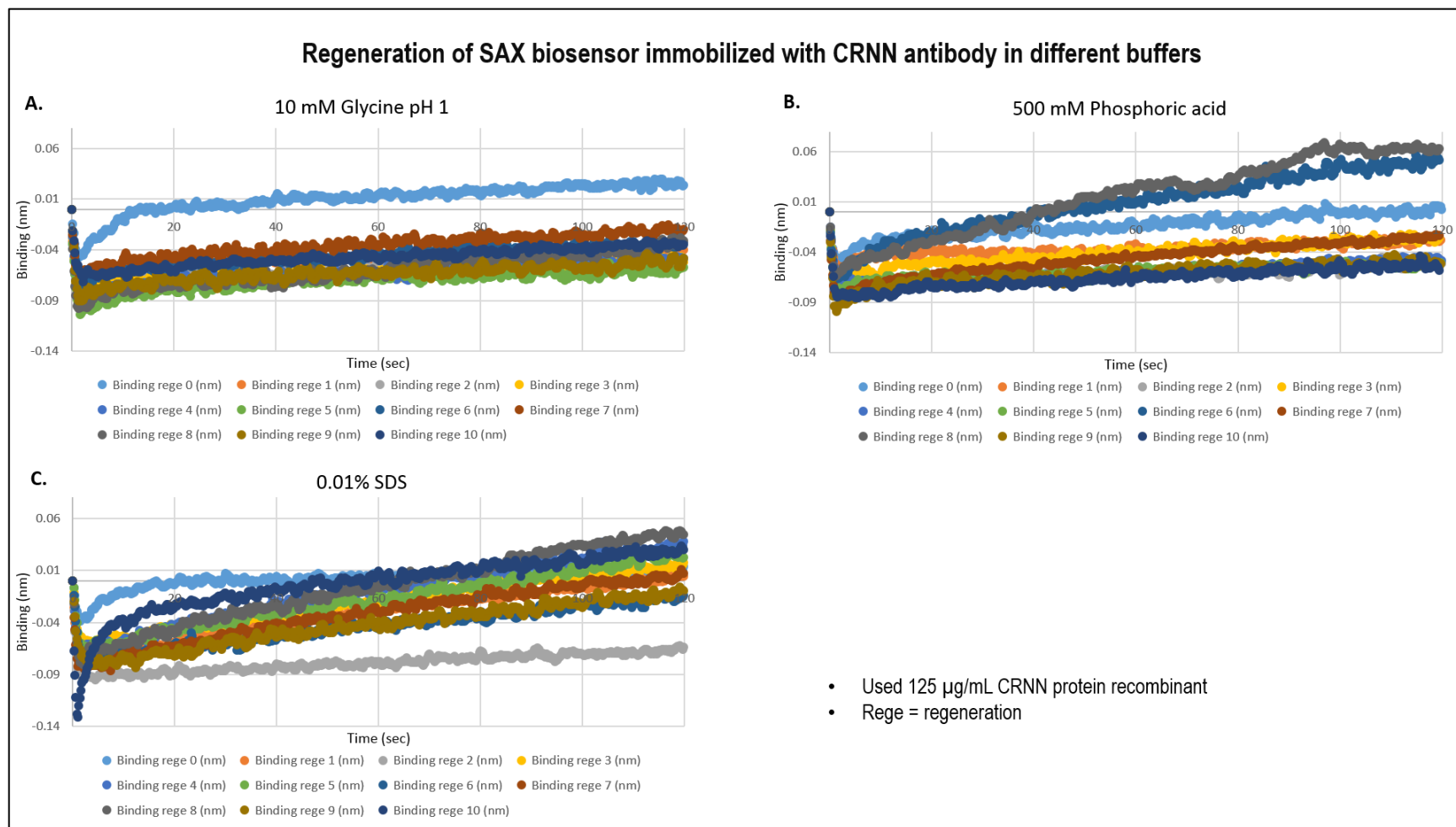


Figure 46. The optimisation results of the SAX biosensor regeneration immobilized with cornulin (CRNN) antibody in different buffers. A. 10 mM Glycine (pH 1). B. 500 mM Phosphoric Acid ($\sim$ pH 1). C. 0.01% (w/v) SDS ($\sim$ pH 5).

Table 12. The optimisation results of the time duration and shaker speed for Blitz workflow.

Blitz workflow	Optimised time duration in seconds			Shaker speed (rpm)
	Initial baseline	Binding time	Baseline	
Immobilization of biotinylated antibody to SAX biosensor	30	300	30	2200
Creating standard curves	-	120	-	2200
Quantitate saliva samples	-	120	-	2200
Regeneration with 10 mM Glycine, pH 1	30	30X3	30X3	1000

3.4 Conclusion of the workflow optimisation

Based on the optimisation results in LC-MS/MS, placental samples had better protein group coverage when using protein resuspension buffer of 2 % (w/v) SDS; digesting 10 µg total protein with FASP; precipitating protein with 2-D clean-up and desalting protein with C18 clean-up (Zip Tip). The 2-D clean-up method that enables detection of a higher number of membranous protein group was preferable because these proteins were easier to be detected, especially for this particular protein biomarker study. Moreover, protein precipitation using the 2-D clean-up method and desalting procedure using C18 column did not cause protein loss but on the contrary provided more protein coverage as compared to not performing these 2 steps. Out of six placental proteins candidates chosen, only five placental antibodies were able to be optimised and can be visualized in the Western blot.

Using saliva samples, the optimisation results for LC-MS/MS demonstrated that the best protein group coverage was with protein resuspension buffer of 6 M GdnHCl and digesting 5 µg total protein with IS tryptic digestion. As I am targeting membrane proteins for this particular protein biomarker study, the ionic nature of GdnHCl plays a role in destabilizing salt bridges of the protein making it easier to be digested to peptides later. Similarly as in placental samples, saliva samples also had better protein group coverage when precipitating protein with 2-D clean-up and desalting procedure with C18 clean-up were performed. In the Western blot optimisation, only six out of eight salivary antibodies were able to be optimised and visualized. For the Blitz system optimisation step, 10 mM Glycine pH 1 with TBST were identified as the best regeneration and running buffer for SAX biosensors regeneration process. These solutions were chosen because when they were used in the biosensor regeneration process, the mean protein binding rate on the biosensor remained similar after every cycle without compromising the antibody-protein binding. The SAX biosensors regeneration may be repeated up to ten times for optimum result without compromising the antibody-protein binding, therefore Blitz method is relatively cost effective.

In the next chapter, qRT-PCR, LC-MS/MS and Western blot method will be used to examine placental samples whereas LC-MS/MS, Western blot and Blitz method will be used to examine saliva samples. This study aim to examine similar proteins expression profile instead of similar genes expression profile in both placenta and saliva samples of children with allergic diseases. Therefore, qRT-PCR was not performed with saliva samples as it is not applicable for future proteomic clinical screening method.

4.0 EXAMINATION OF PLACENTAL GENES AND PROTEINS TO IDENTIFY THE ASSOCIATION WITH CHILDHOOD ALLERGY

4.1 Introduction

Childhood allergic disease susceptibility can be detected as early as 16-17 weeks of gestation. During this stage of gestation, fetal biological samples, including amniotic fluid and umbilical-CB, have been identified to contain house dust mite allergen ⁽¹⁶⁶⁾. These *in utero* exposures may result in alteration in placental gene expression and contribute to subsequent development of allergic diseases. For example, placental *FOXP3* expression, a marker of T regulatory cell concentration that plays a role in early immune programming, was reduced in infants who subsequently developed an allergic disease ⁽¹⁵⁾. Based on this information, the placenta may be used as an indicator of allergic susceptibility. Thus, placental tissue may be used for early diagnosis of allergic diseases for the purpose of introducing a targeted intervention and reducing or preventing the allergy.

The hypothesis for this analysis was that childhood susceptibility to allergy may be programmed *in utero* due to differences in placental adaptation to the maternal environment. Susceptibility to allergy may be identifiable through alterations in placental gene and proteomic profiles. Therefore, the purpose of the following studies in placentae was to examine gene and protein profiles of placentae from children with and without an allergy using a proteomic approach, identify novel proteins that vary in relation to allergy and validate findings with several techniques in a larger population.

The aims for this work were:

- 1) To identify different gene profiles of placentae from children with and without an allergy.
- 2) To optimise a proteomic method for identification of placental proteins associated with allergic diseases in childhood.
- 3) To identify different proteomic profiles in children who subsequently develop an allergy.
- 4) To determine whether presence of maternal asthma alters the proteomic profile of children who subsequently develop an allergy.
- 5) To determine whether proteomic profiles vary in a sex-specific manner in children who subsequently develop an allergy.

4.2 Placental genes associated with childhood allergy

4.2.1 Preliminary identification of placental genes associated with allergy

Preliminary workflow of placental sample analysis is described in Figure 47. Data analysis was conducted comparing data between previous preliminary assessment of microarray on placental samples of children with allergy, and LC-MS/MS on saliva sample of asthmatic mothers ⁽²⁹⁰⁾. Both of these studies were conducted by our group and collaborators ⁽²⁹⁰⁾. Seventeen genes were present in the microarray data that correlate with the proteins in the LC-MS/MS data and these genes were selected to be further validated in the qRT-PCR in placenta samples of children with and without an allergy. The target genes selected were as listed in Table 13. These genes had been normalized with the housekeeping gene, beta-actin, as used previously for gene expression analyses of placental tissue which has been well-established in the literature as an appropriate housekeeping gene for human placenta ^(132, 308).

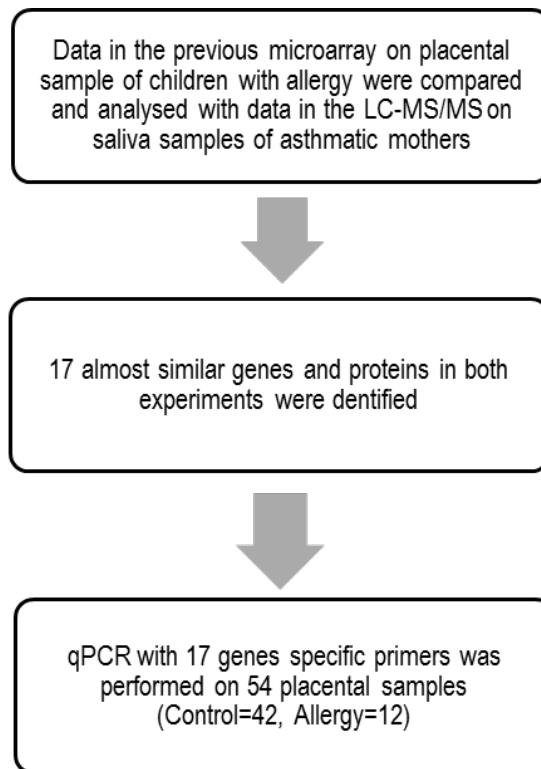


Figure 47. Preliminary workflow of placental samples.

Table 13. Target genes selected for placental gene expression analysis.

Gene name	Description
<i>ACTL8</i>	Actin like 8
<i>ACTRT2</i>	Actin related protein T2
<i>CCDC27</i>	Coiled-coil domain containing 27
<i>ENO2</i>	Enolase 2
<i>IL1B</i>	Interleukin 1 beta
<i>IL1R2</i>	Interleukin 1 receptor type 2
<i>KLHDC7A</i>	Kelch domain containing 7A
<i>KLHL13</i>	Kelch like family member 13
<i>KLHL17</i>	Kelch like family member 17
<i>MAP3K5</i>	Mitogen-activated protein kinase kinase kinase 5
<i>MAPK8IP1</i>	Mitogen-activated protein kinase 8 interacting protein 1
<i>OR4F16</i>	Olfactory receptor family 4 subfamily F member 16
<i>OR4F17</i>	Olfactory receptor family 4 subfamily F member 17
<i>PER3</i>	Period circadian clock 3
<i>PRDM2</i>	PR/SET domain 2
<i>PTCHD2</i>	Patched domain containing 2
<i>YWHAQ</i> (14-3-3 protein theta)	Tyrosine 3-monooxygenase/Tryptophan 5-monooxygenase activation protein theta

4.2.2 Maternal characteristics during pregnancy and child characteristics in placental genes expression analysis

Samples of placenta for quantitative real-time polymerase chain reaction (qRT-PCR) were part of an existing prospective cohort from the Asthma and Pregnancy Cohort study that was designed to examine the effects of asthma during pregnancy on the mother, placenta and baby (refer Methodology section 2.1.1). The cohort consisted of women with and without asthma that were recruited at the first antenatal visit from the Lyell McEwin Hospital antenatal clinics between May 2009 and May 2012. They consented to take part in a non-interventional prospective asthma study evaluating the impact of maternal asthma on perinatal outcomes ⁽²⁷⁵⁻²⁷⁷⁾. Children were followed up from 6 months of age and allergic status identified using a modified ISAAC questionnaire.

Maternal and child characteristics are detailed in Table 14. The mean age, body mass index (BMI), gravida and parity of the mothers was 27.02 years, 27.70, 2.07 and 0.65 respectively, while the mean gestational days and birthweight was 277.22 days and 3564.63 grams, respectively. There were no significant differences between male and female offspring in relation to maternal age, maternal BMI, gravida, parity, gestational age and birthweight. There were 42 placental samples from allergic children (males: n= 21, females: n= 21) and 12 placental samples from non-allergic children. The majority of specific allergic phenotypes was the AER group, which combined the allergies of asthma, eczema and rhinitis. This group consisted of 15 samples and the least was the eczema and rhinitis group that contributed to only 1 sample. Allergy combination is the presence of a specific allergy, for example asthma, in one specific phenotype or in combination with other allergies. Therefore asthma combination consists of a group of children with asthma (A); asthma and eczema (AE); asthma and rhinitis (AR); and asthma, eczema and rhinitis (AER).

Table 14. Maternal characteristics during pregnancy and child characteristics in placental genes expression analysis. (Significant result was P<0.05).

Maternal and child characteristics	Males	Females	Both sexes	* Significance differences between males and females
Maternal age in years (mean $\pm$ SE)	26.25 $\pm$ 1.09	27.85 $\pm$ 1.21	27.02 $\pm$ 0.82	p= 0.3675
Maternal BMI (mean $\pm$ SE)	26.74 $\pm$ 0.84	28.74 $\pm$ 1.21	27.70 $\pm$ 0.74	p= 0.3057
Gravida (mean $\pm$ SE)	1.96 $\pm$ 0.23	2.19 $\pm$ 0.24	2.07 $\pm$ 0.17	p= 0.4716
Parity (mean $\pm$ SE)	0.61 $\pm$ 0.21	0.69 $\pm$ 0.15	0.65 $\pm$ 0.13	p= 0.1572
Gestational age in days (mean $\pm$ SE)	277.79 $\pm$ 1.62	276.62 $\pm$ 2.07	277.22 $\pm$ 1.31	p= 0.8467
Birthweight in gram (mean $\pm$ SE)	3597.86 $\pm$ 76.06	3528.85 $\pm$ 97.40	3564.63 $\pm$ 61.45	p= 0.4115
Birthweight centile (mean $\pm$ SE)	52.25 $\pm$ 5.36	57.35 $\pm$ 5.97	54.71 $\pm$ 4.01	NA
Number of asthma (n)	3	5	8	NA
Number of eczema (n)	0	2	2	NA
Number of rhinitis (n)	0	2	2	NA
Number of asthma and eczema (n)	5	2	7	NA
Number of asthma and rhinitis (n)	4	3	7	NA
Number of eczema and rhinitis (n)	0	1	1	NA
Number of asthma, eczema and rhinitis (n)	9	6	15	NA
Number of asthma combination (n)	21	16	37	NA
Number of eczema combination (n)	14	11	25	NA
Number of rhinitis combination (n)	13	12	25	NA
Number of allergic children (n)	21	21	42	NA
Number of non-allergic children (n)	7	5	12	NA
% of asthmatic mothers (high risk of allergy) in allergic children	61.90	52.38	57.14	NA
% of asthmatic mothers (high risk of allergy) in non-allergic children	85.71	40.00	66.67	NA

% of non-asthmatic mothers (low risk of allergy) in allergic children	38.10	47.62	42.86	NA
% of non-asthmatic mothers (low risk of allergy) in non-allergic children	14.29	60.00	33.33	NA

* Data were analysed by Mann-Whitney test with two-tailed p-value.

° Abbreviations: NA represents non-applicable.

4.2.3 Placental genes associated with allergy

Each of these genes were normalised with the housekeeping gene, beta-actin, and the gene expression analyses were performed in relation to general allergy, the presence or absence of maternal asthma during pregnancy, child sex and child allergy phenotypes (refer Methodology section 2.12.1 and Optimisation section 3.2.1).

4.2.3.1 Children with allergy vs children with no allergy

From data analysis between previous preliminary assessment of microarray on placental samples of children with allergy, and LC-MS/MS on saliva sample of asthmatic mothers, seventeen placental genes were present in the microarray data that correlate with the proteins in the LC-MS/MS data ⁽²⁹⁰⁾. These genes were further validated in the qRT-PCR. Out of the seventeen placental genes, only *KLHDC7A* (Mann Whitney U test, $P=0.022^*$) gene was significantly decreased in the placental samples of children with an allergy as compared to children without an allergy (Figure 48). The genes selected for placental gene analysis that did not change significantly in relation to allergy are listed in Table 15.

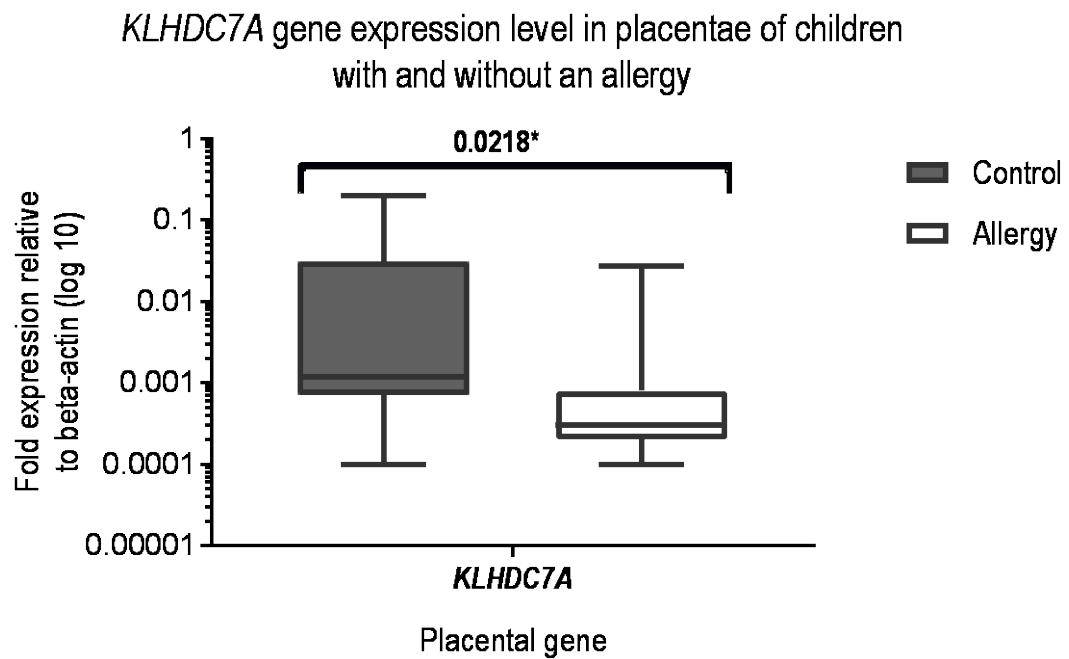


Figure 48. *KLHDC7A* gene expression level in placentae of children with (n= 29) and without (n= 7) an allergy. Genes are expressed relative to beta-actin. Data of *KLHDC7A* gene fold expression was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

Table 15. The sixteen genes that did not change significantly in the placental samples of children with an allergy as compared to children without an allergy.

Placental genes	Relative abundance to beta-actin in children with no allergy		Relative abundance to beta-actin in children with allergy	
	Medians	Interquartile range	Medians	Interquartile range
<i>YWHAQ</i>	0.7235	0.7738	0.2986	0.8066
<i>ENO2</i>	0.0043	0.0044	0.0028	0.0039
<i>IL1B</i>	0.0344	0.0410	0.0182	0.0588
<i>IL1R2</i>	0.0228	0.0351	0.0149	0.0223
<i>KLHL13</i>	0.0026	0.0032	0.0013	0.0030
<i>KLHL17</i>	0.0028	0.0026	0.0024	0.0022
<i>MAP3K5</i>	0.0620	0.1150	0.0382	0.0939
<i>OR4F16</i>	0.0148	0.0299	0.0084	0.0138
<i>PER3</i>	0.0088	0.0136	0.0062	0.0066
<i>PRDM2</i>	0.0778	0.1334	0.0430	0.0904
<i>ACTRT2</i>	0.0007	0.0210	0.0005	0.0009
<i>OR4F17</i>	0.0029	0.0194	0.0010	0.0030
<i>ACTL8</i>	0.0007	0.0000	0.0001	0.0003
<i>MAPK8IP1</i>	NA	NA	0.0001	0.0004
<i>PTCHD2</i>	0.0009	0.0012	0.0001	0.0004
<i>CCDC27</i>	0.0003	0.0000	0.0001	0.0000

° Abbreviations: NA represents non-applicable, no value.

4.2.3.2 The influence of the presence and absence of maternal asthma during pregnancy on placental gene expression and child allergy

i. Children of non-asthmatic mothers (low risk of allergy) with and without an allergy

When comparing the difference in gene expression in the allergic placentae of non-asthmatic mothers (low risk of allergy) with and without an allergy, there were five placental proteins that were significantly decreased in the allergic placentae of non-asthmatic mothers regardless of sex. These placental proteins were *IL1B* (Mann Whitney U test, $P= 0.019^*$) (Figure 49), *IL1R2* (Mann Whitney U test, $P= 0.0003^{***}$) (Figure 50) and *KLHL13* (Mann Whitney U test, $P= 0.030^*$) (Figure 51).

There were no placental genes altered significantly in the presence of maternal asthma (high risk) between children of asthmatic mothers that developed an allergy relative to children of asthmatic mothers who did not have an allergy.

IL1B2 gene expression level in placentae of children of non-asthmatic mothers (NAM) with and without an allergy

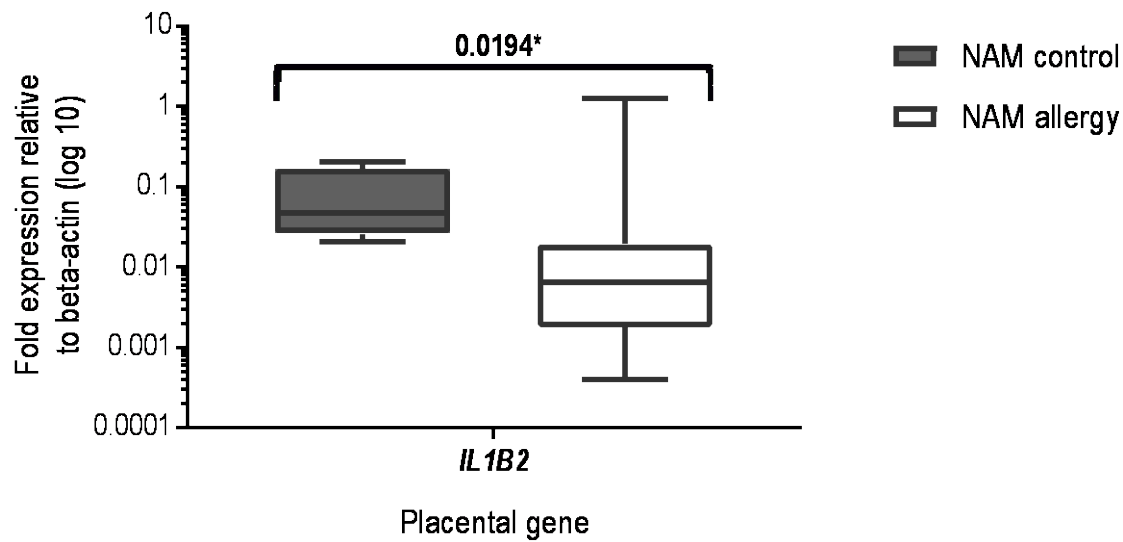


Figure 49. *IL1B2* gene expression level in placentae of children of non-asthmatic mothers (low risk of allergy) with (n= 18) and without (n= 4) an allergy. Genes are expressed relative to beta-actin. Data of *IL1B2* gene fold expression was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

IL1R2 gene expression level in placentae of children of non-asthmatic mothers (NAM) with and without an allergy

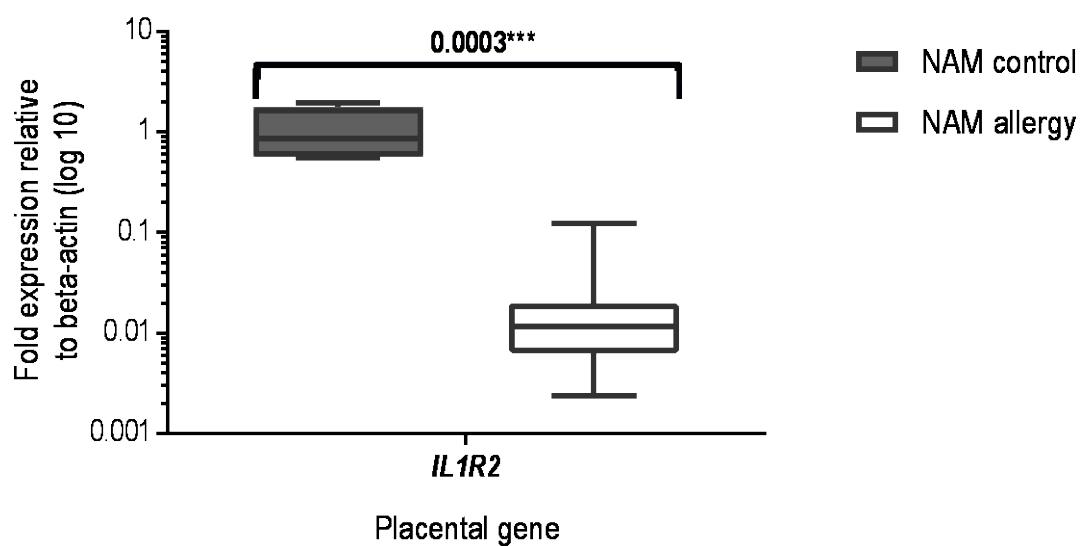


Figure 50. *IL1R2* gene expression level in placentae of children of non-asthmatic mothers (low risk of allergy) with (n= 18) and without (n= 4) an allergy. Genes are expressed relative to beta-actin. Data of *IL1R2* gene fold expression was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

KLHL13 gene expression level in placentae of children of non-asthmatic mothers (NAM) with and without an allergy

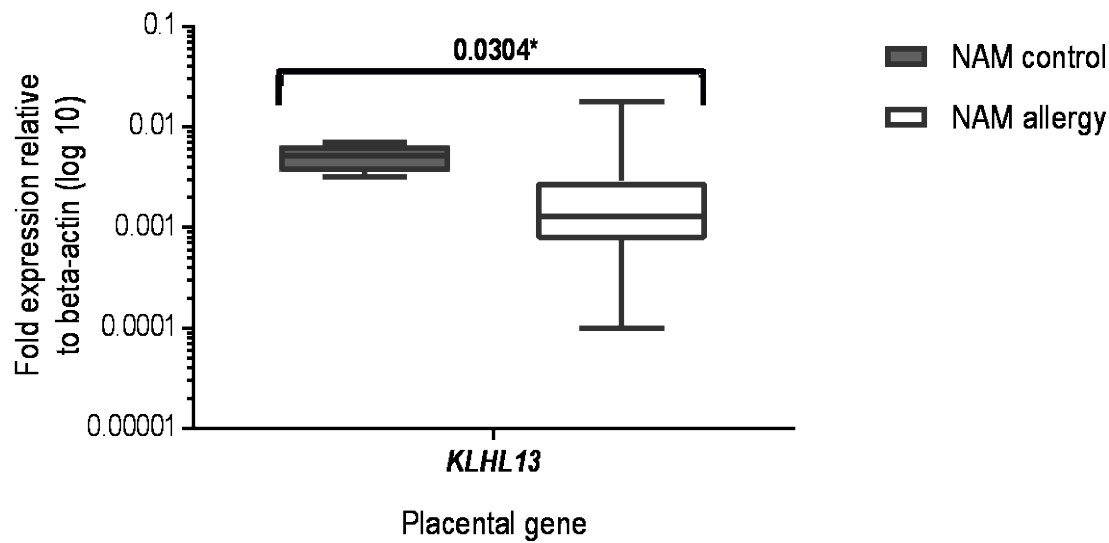


Figure 51. *KLHL13* gene expression level in placentae of children of non-asthmatic mothers (low risk of allergy) with (n= 17) and without (n= 4) an allergy. Genes are expressed relative to beta-actin. Data of *KLHL13* gene fold expression was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

ii. The effect of sex and the presence and absence of maternal asthma on placental gene expression

When comparing the difference in gene expression in the allergic placentae of non-asthmatic mothers (low risk of allergy) with and without an allergy in relation to female sex, only *IL1R2* (Mann Whitney U test, $P=0.049^*$) (Figure 52) placental genes that was significantly decreased in the allergic placentae of non-asthmatic mothers in female sex.

There were no significant differences in gene expression in male placentae in the presence and absence of maternal asthma and in the presence and absence of child allergy.

IL1R2 gene expression level in placentae of children of non-asthmatic mothers (NAM) with and without an allergy in female sex

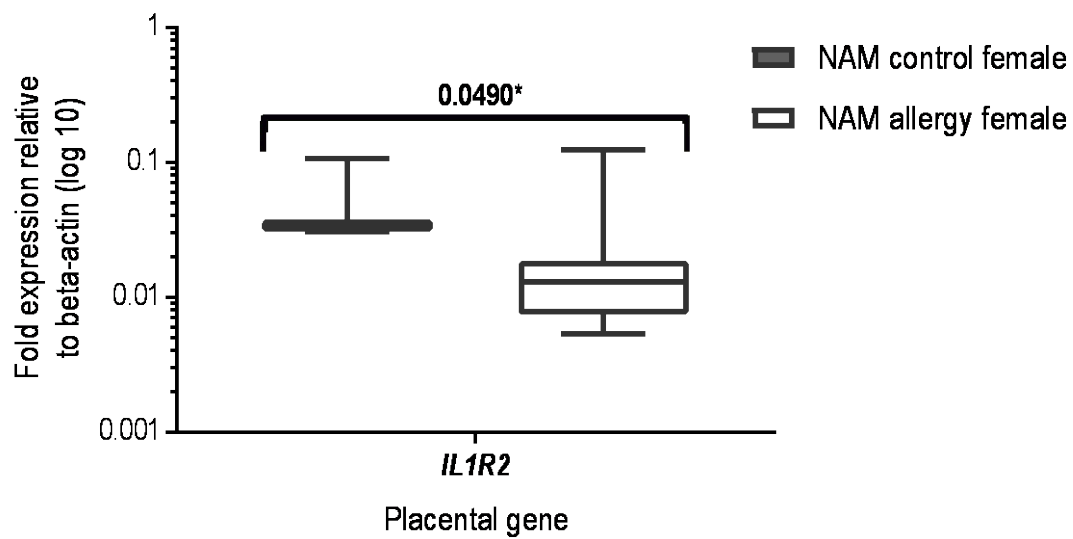


Figure 52. *IL1R2* gene expression level in placentae of children of non-asthmatic mothers (low risk of allergy) with (n= 10) and without (n= 3) an allergy in female sex. Genes are expressed relative to beta-actin. Data of *IL1R2* gene fold expression was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

4.2.3.3 Effect of sex on placental gene expression and child allergy

i. Children with allergy vs children with no allergy

YWHAQ (Mann Whitney U test, $P= 0.023^*$) (Figure 53) gene expression was significantly decreased in the female allergic placentae as compared to female non-allergic placentae. There was no significant difference of placental *YWHAQ* gene expression in other groups.

There were no differences in placental gene expression in relation to the presence and absence of allergy in males.

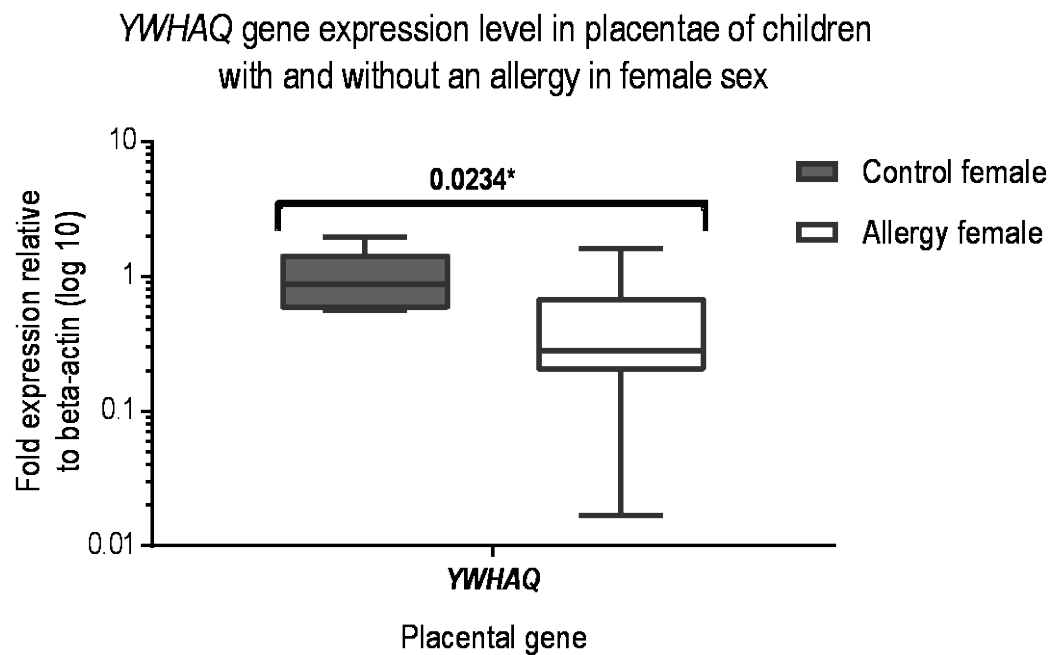


Figure 53. *YWHAQ* gene expression level in placentae of children with (n= 21) and without (n= 5) an allergy in female sex. Genes are expressed relative to beta-actin. Data of *YWHAQ* gene fold expression was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

4.2.3.4 Effect of specific phenotypes on placental gene expression and child allergy

When comparing the difference in placental gene expression between children without an allergy to children with specific allergic phenotypes, only *KLHDCA* (Mann Whitney U test, $P = 0.020^{**}$, $P = 0.048^{*}$ and $P = 0.018^{*}$) was significantly increased in the allergic placentae with any asthma, eczema and rhinitis combination allergic phenotypes regardless of sex (Figure 54).

The summary of significant statistical analyses on placental gene expression in relation to childhood allergy distribution and in relation to the presence (high risk of allergy) or the absence (low risk of allergy) of maternal asthma is detailed in Table 16.

KLHDC7A gene expression level in placentae of children with and without any asthma, eczema or rhinitis allergy combination

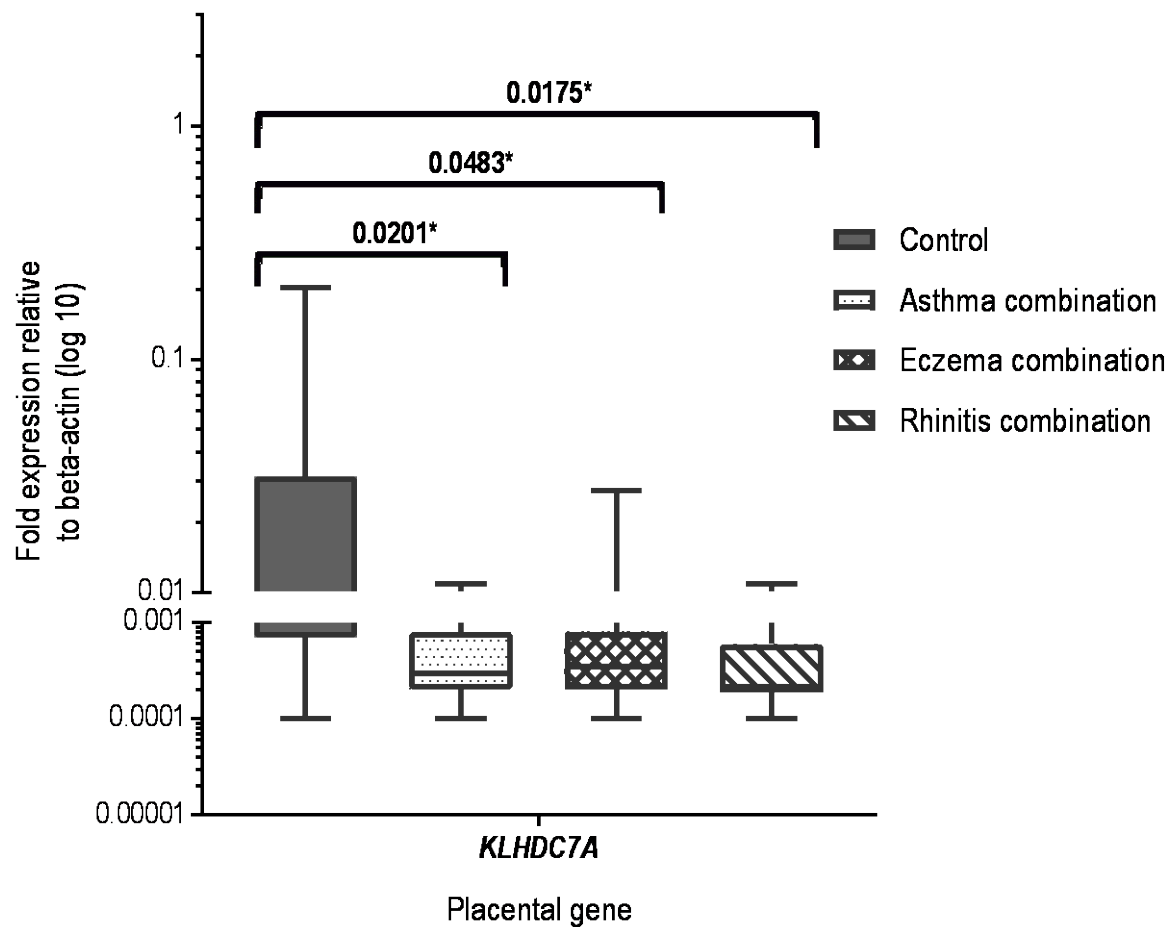


Figure 54. *KLHDC7A* gene expression level in placentae with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, n= 25; eczema combination, n= 18; rhinitis combination, n= 16; and non-allergic children, n= 7. Genes are expressed relative to beta-actin. Data of *KLHDC7A* gene fold expression was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

Table 16. Significant statistical analyses on placental gene expression in relation to childhood allergy distribution and in relation to the presence (high risk of allergy) or the absence (low risk of allergy) of maternal asthma. (Significant result was $P < 0.05$).

Placental genes	Significant statistical analysis	Statistical analysis used	P values
<i>YWHAQ</i>	Control female vs allergy female	MWU	0.0234*
<i>KLHDC7A</i>	Control vs allergy	MWU	0.0218*
	Control vs specific allergy (asthma combination)	MWU	0.0201*
	Control vs specific allergy (eczema combination)	MWU	0.0483*
	Control vs specific allergy (rhinitis combination)	MWU	0.0175*
<i>IL1B</i>	NAM control vs NAM allergy	MWU	0.0194**
<i>IL1R2</i>	NAM control vs NAM allergy	MWU	0.0003***
	NAM control female vs NAM allergy female	MWU	0.0490*
<i>KLHL13</i>	NAM control vs NAM allergy	MWU	0.0304*

° Abbreviations: NAM represents children with non-asthmatic mothers (low risk of allergy) and MWU represents Mann Whitney U test with two-tailed p-value.

4.3 Placental proteins associated with childhood allergy

4.3.1 Maternal characteristics during pregnancy and child characteristics in LC-MS/MS

Placental samples for LC-MS/MS were part of an existing prospective cohort from the Asthma and Pregnancy Cohort study and retrospectively collected (refer Methodology section 2.1.1).

LC-MS/MS was performed using 16 placental samples. The mean age, BMI, gravida and parity of the mothers was 27.94 years, 27.30, 2.38 and 0.75 respectively, while the mean gestational days and birthweight was 274.94 days and 3409.63 gram, respectively. There were no significant differences between male and female offspring in relation to maternal age, maternal BMI, gravida, parity, gestational age and birthweight. There were equal numbers of 8 placental samples for both allergic children and non-allergic children. The majority of specific allergic phenotypes was the AER group, which combined the allergies of asthma, eczema and rhinitis. This group consisted of 3 samples, while the smallest group was rhinitis that contributed to only 1 sample. Rhinitis often occurs as a co-morbidity with other allergies and is rarely found to be present alone. There were similar numbers of children with (high risk of allergy) and without asthmatic mothers (low risk of allergy) with allergies. Maternal characteristics during pregnancy and child characteristics in LC-MS/MS are summarised in Table 17.

Table 17. Maternal characteristics during pregnancy and child characteristics in LC-MS/MS. (Significant result was P<0.05).

Maternal and child characteristics	Males	Females	Both sexes	* Significance differences between males and females
Maternal age in years (mean $\pm$ SE)	29.13 $\pm$ 2.12	26.75 $\pm$ 1.85	27.94 $\pm$ 1.31	p= 0.4559
Maternal BMI (mean $\pm$ SE)	29.65 $\pm$ 1.22	24.94 $\pm$ 1.31	27.30 $\pm$ 1.07	p= 0.0650
Gravida (mean $\pm$ SE)	2.50 $\pm$ 0.40	2.25 $\pm$ 0.42	2.38 $\pm$ 0.29	p= 0.7568
Parity (mean $\pm$ SE)	0.88 $\pm$ 0.37	0.63 $\pm$ 0.25	0.75 $\pm$ 0.23	p= 0.8825
Gestational age in days (mean $\pm$ SE)	279.13 $\pm$ 1.61	270.75 $\pm$ 4.47	274.94 $\pm$ 2.60	p= 0.2906
Birthweight in gram (mean $\pm$ SE)	3615.00 $\pm$ 108.97	3204.25 $\pm$ 108.65	3409.63 $\pm$ 92.50	p= 0.0477*
Birthweight centile (mean $\pm$ SE)	47.43 $\pm$ 9.23	47.70 $\pm$ 10.27	47.56 $\pm$ 6.90	NA
Number of asthma (n)	0	0	0	NA
Number of eczema (n)	0	0	0	NA
Number of rhinitis (n)	0	1	1	NA
Number of asthma and eczema (n)	1	1	2	NA
Number of asthma and rhinitis (n)	1	1	2	NA
Number of eczema and rhinitis (n)	0	0	0	NA
Number of asthma, eczema and rhinitis (n)	2	1	3	NA
Number of asthma combination (n)	4	3	7	NA
Number of eczema combination (n)	3	2	5	NA
Number of rhinitis combination (n)	3	3	6	NA
Number of allergic children (n)	4	4	8	NA
Number of non-allergic children (n)	4	4	8	NA
% of asthmatic mothers (high risk of allergy) in allergic children	50	50	50	NA
% of asthmatic mothers (high risk of allergy) in non-allergic children	100	50	75	NA

% of non-asthmatic mothers (low risk of allergy) in allergic children	50	50	50	NA
% of non-asthmatic mothers (low risk of allergy) in non-allergic children	0	50	25	NA

* Data were analysed by Mann-Whitney test with two-tailed p-value.

° Abbreviations: NA represents non-applicable.

4.3.2 Identification of placental proteins associated with allergy

From MaxLFQ and Mascot software analysis, 1223 proteins were identified to be present in placental samples of children with allergic diseases (Appendix 9, Table 2). Out of these 1223 proteins, 926 protein had more than 2 unique peptides (UP). A UP are defined as peptides, irrespective of its length, that exist only in one protein of a proteome of interest ⁽³¹⁹⁾. Despite the fact that these peptides may appear more than once in the same protein, they are not shared with another protein group ⁽³¹⁹⁾. Therefore, proteins that have more than 2 UP have a higher probability to be a genuine protein, and not a false positive result. In addition, unique sequence coverage is the percentage of the sequence that is covered by the identified UP of the best protein sequence contained in the protein group.

Using the LFQ volume intensity in the MaxLFQ software, fold change (fc) ratio was calculated between LFQ intensity of allergic sample to LFQ intensity of non-allergic sample. However, LFQ intensity may be zero (0) when there is no calculated ratio existing from peptides in sample A as described in Figure 55 ⁽³⁰¹⁾. Peptides present in sample A may not fulfil all the requirements to distinguish peptide species between two samples (A and B), hence generating the zero protein LFQ intensity (Figure 55). Peptide species are a distinct combination of peptide sequence, modification state and charge; therefore, inability to fulfil one of these characteristics might prohibit identification of the peptide species. This may indicate that the uncalculated ratio represents important information, as one of the sample group may have none of the protein present, while the other sample group has an abundant volume of the same protein. The summary of LC-MS/MS result with significant LFQ ratios in placentae of allergic children in 2 or all 3 groups allergic males, allergic females and allergic males combined with allergic females are shown in Table 18 and the summary of LC-MS/MS result with significant LFQ ratios specifically in placentae of allergic males and allergic females are shown in Table 19.

From the fold change LFQ intensity ratios in MaxLFQ analysis, there were 219 proteins identified with a significant ratio (ratio > 2 or < 0.5 fold change) when compared to the non-allergic group in placental samples. Allergic male combined with allergic female groups had 126 proteins with a significant ratio, the highest number of proteins as compared with other allergy groups (Figure 56). Eleven placental proteins were identified to be present in all three groups of allergic children (Figure 57 and Table 18). There was one protein identified in both allergic males and allergic females that was four and a half LIM domains protein 1 (FHL-1) with <0.5 fold change as compared with the non-allergic group. There were nineteen proteins significantly altered in the placentae of allergic males and 21 proteins altered in placentae of allergic females relative to non-allergic children (Figure 57 and Table 19). Out of these 219 placental proteins, six candidate placental proteins associated with allergic diseases were chosen

and further validated with Western blot (n= 68) according to inclusion and exclusion criteria (refer Methodology section 2.3.1 and 2.3.2).

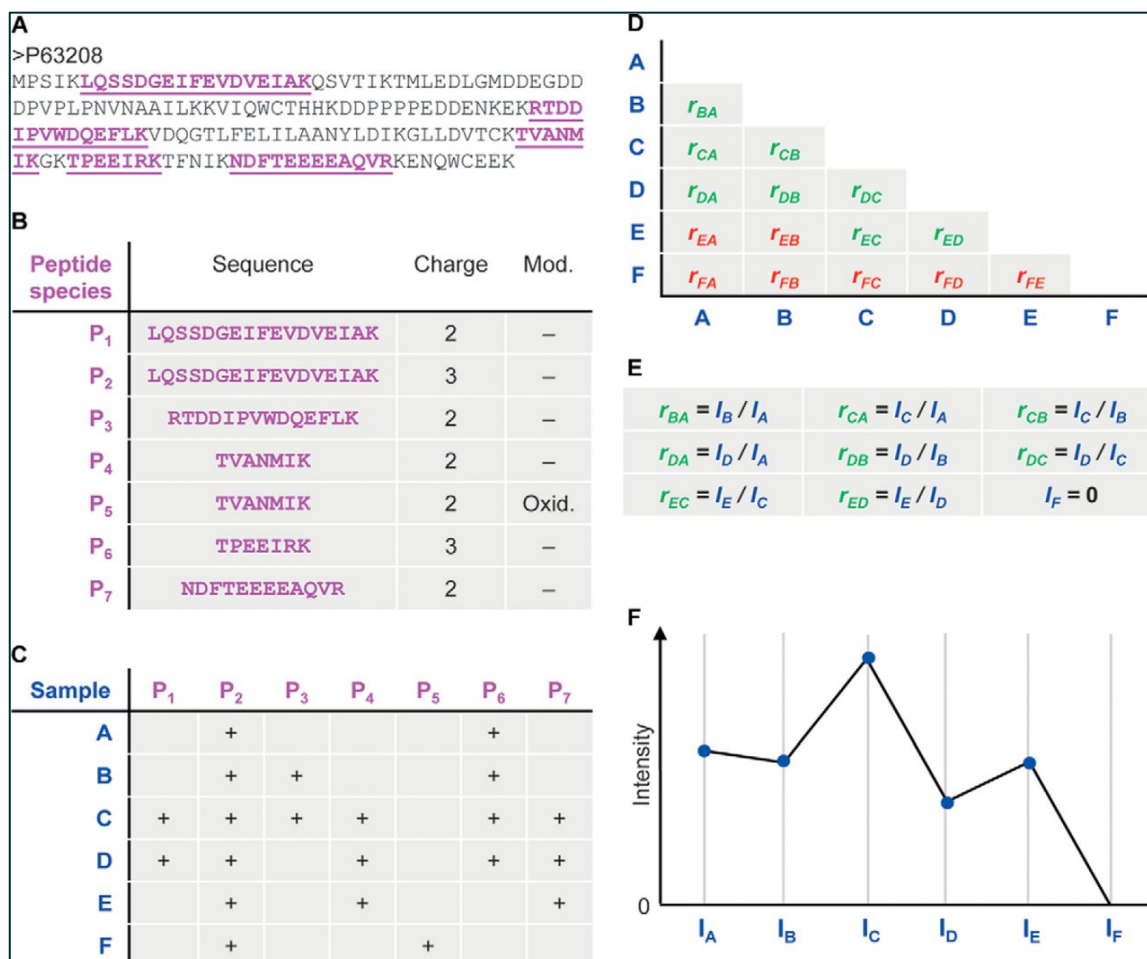


Figure 55. Algorithm constructing protein intensity profiles for one protein from its peptide signals⁽³⁰¹⁾. A, an exemplary protein sequence. Peptides with an XIC-based quantification are indicated in magenta. B, the five peptide sequences give rise to seven peptide species. For this purpose, a peptide species is a distinct combination of peptide sequence, modification state, and charge, each of which has its own occurrence pattern over the different samples. C, occurrence matrix of peptide species in the six samples. D, matrix of pair-wise sample protein ratios calculated from the peptide XIC ratios. Valid/invalid ratios are coloured in green/red based on a configurable minimum ratio count cut-off (2). If a sample has no valid ratio with any other sample, like sample F, the intensity will be set to zero (0). E, system of equations that needs to be solved for the protein abundance profile. F, the resulting protein abundance profile for one protein. The absolute scale was adapted to match the summed-up raw peptide intensities.

Table 18. Summary of placental protein identification with significant label-free quantification (LFQ) ratios in $\geq$ two groups that include placentae of allergic males, allergic females and allergic males combined with allergic females in LC-MS/MS. (Significant ratio > 2 or < 0.5 fold change).

Swiss-Prot entry	Entry Name	Protein names	Gene names	UP	Unique seq cov [%]	MW [kDa]	Ratio LFQ MS AM/NM	Ratio LFQ MS AF/NF	Ratio LFQ MS AM+AF
P21397; P27338	AOFA_HUMAN; AOFB_HUMAN	Amine oxidase [flavin-containing] A, Amine oxidase [flavin-containing] B	MAOA, MAOB	18	39.80	59.68	0.49	0.46	0.47
P21810	PGS1_HUMAN	Biglycan (Bone/cartilage proteoglycan I) (PG-S1)	BGN	12	54.10	34.88	2.39	2.39	2.39
P17980	PRS6A_HUMAN	26S protease regulatory subunit 6A	PSMC3	7	27.70	44.32	2.25	3.75	6.06
Q96CX2	KCD12_HUMAN	BTB/POZ domain-containing protein KCTD12 (Pfetin)	KCTD12	6	29.50	35.73	2.52	2.87	4.21
Q13492; O60641	PICAL_HUMAN; AP180_HUMAN	Phosphatidylinositol-binding clathrin assembly prot, Clathrin coat assembly protein AP180	PICALM, SNAP91	5	15.20	66.39	2.90	2.11	4.16
Q13724	MOGS_HUMAN	Mannosyl-oligosaccharide glucosidase	MOGS	5	9.40	91.92	2.18	2.95	4.40
P55786; A6NEC2	PSA_HUMAN; PSAL_HUMAN	Puromycin-sensitive aminopeptidase (PSA), Puromycin-sensitive aminopeptidase-like protein	NPEPPS, NPEPPSL1	3	4.40	92.87	2.63	0.49	0.45
P04196	HRG_HUMAN	Histidine-rich glycoprotein	HRG	3	7.40	59.51	2.36	2.86	3.31
Q3ZCM7	TBB8_HUMAN	Tubulin beta-8 chain	TUBB8	2	7.40	49.78	3.48	3.16	4.42
O95810	SDPR_HUMAN	Serum deprivation-response protein	SDPR	2	7.80	47.10	2.70	2.99	8.67
P05386	RLA1_HUMAN	60S acidic ribosomal protein P1	RPLP1	2	52.20	11.40	2.09	4.32	5.14
Q9Y4L1	HYOU1_HUMAN	Hypoxia up-regulated protein 1	HYOU1	13	25.70	94.20	1.68	3.25	3.04
P27487	DPP4_HUMAN	Dipeptidyl peptidase 4	DPP4	13	19.30	88.28	0.51	0.35	0.40
P15924	DESP_HUMAN	Desmoplakin (DP)	DSP	10	5.60	331.77	0.00	2.73	15.13
Q99798	ACON_HUMAN	Aconitate hydratase, mitochondrial (Aconitase)	ACO2	9	18.20	85.42	1.21	3.18	3.25
Q02218; Q9ULD0	ODO1_HUMAN; OGDHL_HUMAN	2-oxoglutarate dehydrogenase, mitochondrial, 2-oxoglutarate dehydrogenase-like, mitochondrial	OGDH, OGDHL	8	10.00	115.93	1.78	2.41	2.96
O95833	CLIC3_HUMAN	Chloride intracellular channel protein 3	CLIC3	8	49.20	26.65	1.64	2.80	2.52
Q53SF7	COBL1_HUMAN	Cordon-bleu protein-like 1	COBL1	7	8.80	131.79	1.83	2.81	4.79
Q9Y6N6	LAMC3_HUMAN	Laminin subunit gamma-3	LAMC3	7	5.70	171.23	0.60	0.41	0.20
P78371	TCPB_HUMAN	T-complex protein 1 subunit beta	CCT2	6	20.80	56.81	0.00	2.08	13.70

P09622	DLDH_HUMAN	Dihydrolipoyl dehydrogenase, mitochondrial	DLD	4	13.30	48.90	1.31	2.50	3.52
Q99832	TCPH_HUMAN	T-complex protein 1 subunit eta	CCT7	4	13.80	59.34	0.00	3.82	17.37
P54727	RD23B_HUMAN	UV excision repair protein RAD23 homolog B	RAD23B	4	12.40	42.31	0.00	2.55	19.02
Q14257	RCN2_HUMAN	Reticulocalbin-2 (Calcium-binding protein ERC-55)	RCN2	4	23.00	36.88	0.00	2.50	7.64
P55145	MANF_HUMAN	Mesencephalic astrocyte-derived neurotrophic factor	MANF	3	17.00	20.70	1.29	2.02	3.35
P05109	S10A8_HUMAN	Protein S100-A8 (Calgranulin-A)	S100A8	3	32.30	10.83	0.67	0.44	0.38
Q14956	GPNMB_HUMAN	Transmembrane glycoprotein NMB	GPNMB	3	7.50	50.58	0.00	6.00	23.92
P11279	LAMP1_HUMAN	Lysosome-associated membrane glycoprotein 1	LAMP1	3	8.10	42.59	0.00	3.45	21.96
P46783; Q9NQ39	RS10_HUMAN; RS10L_HUMAN	40S ribosomal protein S10, Putative 40S ribosomal protein S10-like	RPS10 DSP	3	24.20	18.90	0.00	3.09	5.36
Q99733	NP1L4_HUMAN	Nucleosome assembly protein 1-like 4	NAP1L4	3	13.30	42.82	0.00	2.13	11.75
O15260	SURF4_HUMAN	Surfeit locus protein 4	SURF4	2	16.70	21.13	0.00	12.07	12.07
Q16543	CDC37_HUMAN	Hsp90 co-chaperone Cdc37	CDC37	2	15.20	27.44	0.00	2.50	11.40
P68402	PA1B2_HUMAN	Platelet-activating factor acetylhydrolase IB subunit	PAFAH1B2	2	12.20	25.57	0.00	2.36	5.00
Q13642	FHL1_HUMAN	Four and a half LIM domains protein 1 (FHL-1)	FHL1	9	47.70	29.50	0.42	0.45	0.62
Q07960	RHG01_HUMAN	Rho GTPase-activating protein 1	ARHGAP1	5	17.1	50.44	2.13	1.34	2.27
Q9UL46	PSME2_HUMAN	Proteasome activator complex subunit 2	PSME2	5	31	27.40	0.49	0.00	2.20
P60953; P17081; Q9H4E5	CDC42_HUMAN; RHOQ_HUMAN; RHOJ_HUMAN	Cell division control protein 42 homolog Rho-related GTP-binding protein RhoQ Rho-related GTP-binding protein RhoJ	CDC42; RHOQ; RHOJ	4	30.9	21.26	2.22	1.61	3.72
O43390	HNRPR_HUMAN	Heterogeneous nuclear ribonucleoprotein R	HNRNPR	4	8.6	67.01	2.18	1.15	2.10
P22695	QCR2_HUMAN	Cytochrome b-c1 complex subunit 2, mitochondrial	UQCRC2	4	14.8	48.44	0.09	0.00	0.18
P84098	RL19_HUMAN	60S ribosomal protein L19	RPL19	2	15	20.82	0.36	0.00	0.36
D9YZU8	D9YZU8_HUMAN	Hemoglobin, gamma A	HBG1	2	7.5	16.13	0.19	0.00	0.42

° Abbreviations: UP represents unique peptides, Unique seq cov represents unique sequence coverage, LFQ MS represents label-free quantification mass spectrometry, AM represents allergic males, AF represents allergic females, AM+AF represents allergic males combined with allergic females, NM represents non-allergic males and NF represents non-allergic females.

° Bold number represents significant ratio (ratio > 2 or < 0.5 fold change).

Table 19. Summary of placental protein identification with significant label-free quantification (LFQ) ratios in specifically placentae of allergic males and allergic females in LC-MS/MS. (Significant ratio > 2 or < 0.5 fold change).

Swiss-Prot entry	Entry name	Protein names	Gene names	UP	Unique seq cov [%]	MW [kDa]	Ratio LFQ MS AM/NM	Ratio LFQ MS AF/NF
P04114	APOB_HUMAN	Apolipoprotein B-100 (Apo B-100)	APOB	21	7.2	515.55	2.48	1.22
P13645; Q7Z3Y7; Q7Z3Z0; Q7Z3Y8; Q2M2I5; Q7Z3Y9	K1C10_HUMAN; K1C28_HUMAN; K1C25_HUMAN; K1C27_HUMAN; K1C24_HUMAN; K1C26_HUMAN	Keratin, type I cytoskeletal 10; Keratin, type I cytoskeletal 28; Keratin, type I cytoskeletal 25; Keratin, type I cytoskeletal 27; Keratin, type I cytoskeletal 24; Keratin, type I cytoskeletal 26	KRT10; KRT28; KRT25; KRT27; KRT24; KRT26	17	38.7	58.83	0.42	0.80
P32119	PRDX2_HUMAN	Peroxiredoxin-2	PRDX2	14	53.6	20.11	0.48	1.04
Q96KP4	CNDP2_HUMAN	Cytosolic non-specific dipeptidase	CNDP2	11	35.4	51.50	3.76	1.00
P01019	ANGT_HUMAN	Angiotensinogen (Serpin A8)	AGT	10	28.1	51.06	2.04	1.60
P16152; O75828	CBR1_HUMAN; CBR3_HUMAN	Carbonyl reductase [NADPH] 1, Carbonyl reductase [NADPH] 3	CBR1, CBR3	9	48.0	30.38	2.60	1.03
P02792	FRIL_HUMAN	Ferritin light chain (Ferritin L subunit)	FTL	7	50.3	20.02	2.70	1.12
P16284	PECA1_HUMAN	Platelet endothelial cell adhesion molecule	PECAM1	7	13.6	81.78	2.53	0.00
Q9HDC9	APMAP_HUMAN	Adipocyte plasma membrane-associated protein	APMAP	7	25.5	46.48	0.47	0.85
P26641	EF1G_HUMAN	Elongation factor 1-gamma (EF-1-gamma)	EEF1G	7	19	50.12	0.32	0.59
P06744	G6PI_HUMAN	Glucose-6-phosphate isomerase (GPI)	GPI	6	16.8	59.99	2.61	1.30
P30048	PRDX3_HUMAN	Thioredoxin-dependent peroxide reductase	PRDX3	6	36.3	27.69	2.03	1.05
P35754	GLRX1_HUMAN	Glutaredoxin-1 (Thioltransferase-1)	GLRX	6	55.7	11.78	2.01	1.34
P35908	K22E_HUMAN	Keratin, type II cytoskeletal 2 epidermal	KRT2	6	16.6	65.43	0.41	0.88
Q9NZ08	ERAP1_HUMAN	Endoplasmic reticulum aminopeptidase 1	ERAP1	5	8.2	107.18	2.27	0.85
P62328	TYB4_HUMAN	Thymosin beta-4 (T beta-4)	TMSB4X	5	56.8	5.05	0.37	1.09
P27169	PON1_HUMAN	Serum paraoxonase/arylesterase 1 (PON 1)	PON1	2	8.5	39.73	2.35	0.00
P30049	ATPD_HUMAN	ATP synthase subunit delta	ATP5D	2	13.7	17.49	0.49	0.00
P69892	HBG2_HUMAN	Hemoglobin subunit gamma-2	HBG2	2	9.5	16.13	0.47	1.10

P01024	CO3_HUMAN	Complement C3	C3	68	52.4	187.15	0.82	0.46
P00738; P00739	HPT_HUMAN, HPTR_HUMAN	Haptoglobin (Zonulin), Haptoglobin-related protein	HP, HPR	24	50.5	45.21	1.73	0.46
Q14697	GANAB_HUMAN	Neutral alpha-glucosidase AB	GANAB	23	30.5	106.87	0.78	0.49
P05187; P09923; P10696	PPB1_HUMAN, PPBI_HUMAN, PPBN_HUMAN	Alkaline phosphatase, placental type, Intestinal-type alkaline phosphatase, Alkaline phosphatase, placental-like	ALPP; ALPI; ALPPL2	21	55.1	57.95	0.97	0.40
P05108	CP11A_HUMAN	Cholesterol side-chain cleavage enzyme, mitochondrial,	CYP11A1	18	39.9	60.10	0.66	0.44
P35527	K1C9_HUMAN	Keratin, type I cytoskeletal 9 (Cytokeratin-9)	KRT9	17	44.6	62.06	0.71	0.48
P13010	XRCC5_HUMAN	X-ray repair cross-complementing protein 5	XRCC5	13	24.5	82.70	0.87	3.43
P23526	SAHH_HUMAN	Adenosylhomocysteinase	AHCY	13	35.2	47.72	0.57	0.44
P16435	NCPR_HUMAN	NADPH--cytochrome P450 reductase (CPR)	POR	12	23.3	76.69	0.75	0.50
P00915	CAH1_HUMAN	Carbonic anhydrase 1 (Carbonate dehydratase I)	CA1	11	57.9	28.87	0.89	0.42
P27105	STOM_HUMAN	Erythrocyte band 7 integral membrane protein	STOM	10	56.5	25.96	0.62	0.47
P12110	CO6A2_HUMAN	Collagen alpha-2(VI) chain	COL6A2	9	10.7	108.58	0.83	0.48
P08195	4F2_HUMAN	4F2 cell-surface antigen heavy chain	SLC3A2	8	22.1	55.94	1.37	2.21
P02008	HBAZ_HUMAN	Hemoglobin subunit zeta (HBAZ)	HBZ	8	67.6	15.64	0.73	2.20
P21291	CSRP1_HUMAN	Cysteine and glycine-rich protein 1	CSRP1	8	66.3	20.04	0.61	0.49
Q9Y2B0	CNPY2_HUMAN	Protein canopy homolog 2	CNPY2	7	52.7	20.65	0.81	2.08
P35555; P35556	FBN1_HUMAN; FBN2_HUMAN	Fibrillin-1, Fibrillin-2	FBN1, FBN2	7	3.4	312.24	1.57	0.41
P02462; P29400	CO4A1_HUMAN, CO4A5_HUMAN	Collagen alpha-1(IV) chain, Collagen alpha-5(IV) chain	COL4A1, COL4A5	7	54.6	25.33	0.00	0.34
P01743	HV102_HUMAN	Ig heavy chain V-I region HG3		6	22.1	56.89	1.01	0.49
O60829	PAGE4_HUMAN	P antigen family member 4 (PAGE-4)	PAGE4	5	60.8	11.15	0.98	2.31
P05090	APOD_HUMAN	Apolipoprotein D (Apo-D)	APOD	4	22.8	21.28	0.69	0.48

° Abbreviations: UP represents unique peptides, Unique seq cov represents unique sequence coverage, LFQ MS represents label-free quantification mass spectrometry, AM represents allergic males, AF represents allergic females, AM+AF represents allergic males combined with allergic females, NM represents non-allergic males and NF represents non-allergic females.

° Bold number represents significant ratio (ratio > 2 or < 0.5 fold change).

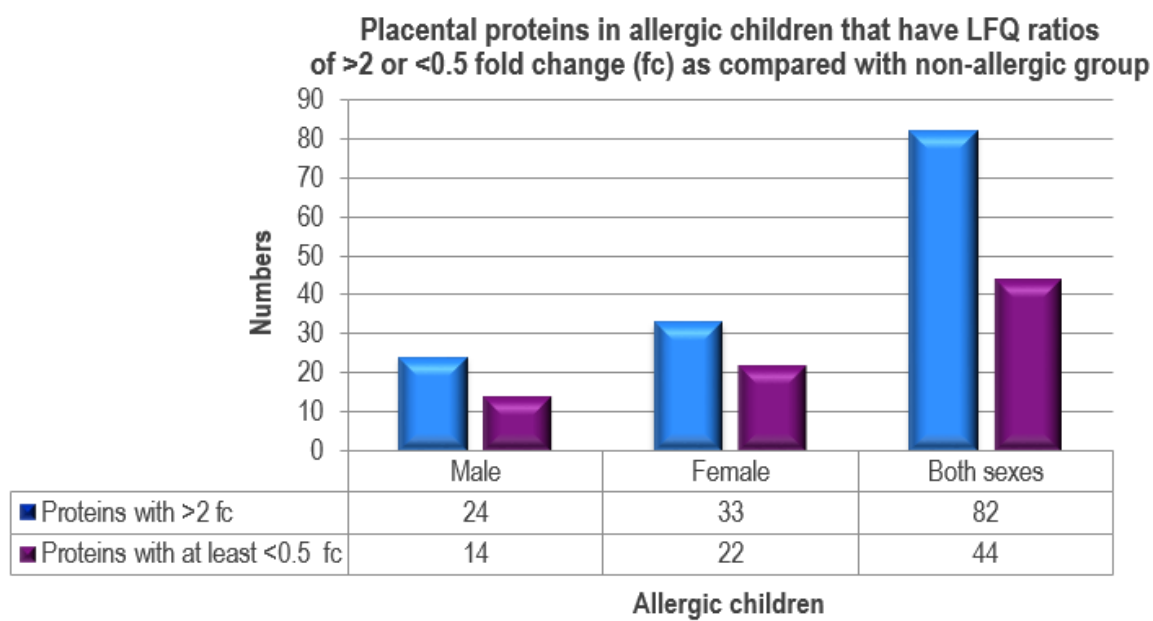


Figure 56. Placental proteins in allergic children that have LFQ ratios of >2 or <0.5 fold change (fc) as compared with non-allergic group (Max-LFQ protein analysis).



Figure 57. Venn diagram showing placental protein distribution with significant ratios of > 2 or < 0.5 fold change according to sex of allergic children as compared with non-allergic group in placental samples.

4.4 Validation study for placental samples with Western blot

4.4.1 Maternal characteristics during pregnancy and child characteristics in Western blot

Placental samples used in the validation studies were derived from the Asthma and Pregnancy Cohort study and retrospectively collected (refer Methodology section 2.1.1).

Maternal characteristics during pregnancy and child characteristics for validation studies with Western blot are detailed in Table 20. The mean age, BMI, gravida and parity of the mothers was 27.76 years, 27.62, 2.06 and 0.68, respectively while the mean gestational days and birthweight was 277.90 days and 3581.62 gram, respectively. There were no significance differences between males and females in maternal age, maternal BMI, gravida, parity, gestational age and birthweight. There were 40 placental samples from allergic children (males: n= 21, females: n= 19) and 28 placental samples from non-allergic children (males: n= 14, females: n= 14). The majority of specific allergic phenotypes was the AER group, which combined the allergies of asthma, eczema and rhinitis. This group consisted of 14 samples and the least was the eczema and rhinitis group that contributed to only 1 sample. Interestingly, the majority of children with and without an allergy had a mother with asthma (57.50 % and 78.57 %).

Table 20. Maternal characteristics during pregnancy and child characteristics for validation studies with Western blot. (Significant result was $P < 0.05$).

Maternal and child characteristics	Males	Females	Both sexes	* Significance differences between males and females
Maternal age in years (mean $\pm$ SE)	27.14 $\pm$ 0.89	28.42 $\pm$ 0.94	27.76 $\pm$ 0.65	p= 0.5924
Maternal BMI (mean $\pm$ SE)	26.90 $\pm$ 0.70	28.39 $\pm$ 0.98	27.62 $\pm$ 0.60	p= 0.3485
Gravida (mean $\pm$ SE)	1.97 $\pm$ 0.20	2.15 $\pm$ 0.21	2.06 $\pm$ 0.14	p= 0.5019
Parity (mean $\pm$ SE)	0.63 $\pm$ 0.18	0.73 $\pm$ 0.14	0.68 $\pm$ 0.11	p= 0.3495
Gestational age in days (mean $\pm$ SE)	278.57 $\pm$ 1.33	277.18 $\pm$ 1.80	277.90 $\pm$ 1.11	p= 0.8715
Birthweight in gram (mean $\pm$ SE)	3596.00 $\pm$ 63.64	3566.36 $\pm$ 90.04	3581.62 $\pm$ 54.64	p= 0.5118
Birthweight centile (mean $\pm$ SE)	50.99 $\pm$ 4.38	56.95 $\pm$ 5.16	53.88 $\pm$ 3.39	NA
Number of asthma (n)	3	4	7	NA
Number of eczema (n)	0	2	2	NA
Number of rhinitis (n)	0	2	2	NA
Number of asthma and eczema (n)	5	2	7	NA
Number of asthma and rhinitis (n)	4	3	7	NA
Number of eczema and rhinitis (n)	0	1	1	NA
Number of asthma, eczema and rhinitis (n)	9	5	14	NA
Number of asthma combination (n)	21	14	35	NA
Number of eczema combination (n)	14	10	24	NA
Number of rhinitis combination (n)	13	11	24	NA
Number of allergic children (n)	21	19	40	NA
Number of non-allergic children (n)	14	14	28	NA
% of asthmatic mothers (high risk of allergy) in allergic children	61.90	52.63	57.50	NA
% of asthmatic mothers (high risk of allergy) in non-allergic children	92.86	64.29	78.57	NA

% of non-asthmatic mothers (low risk of allergy) in allergic children	38.10	47.37	42.50	NA
% of non-asthmatic mothers (low risk of allergy) in non-allergic children	7.14	35.71	21.43	NA

* Data were analysed by Mann-Whitney test with two-tailed p-value.

° Abbreviations: NA represents non-applicable.

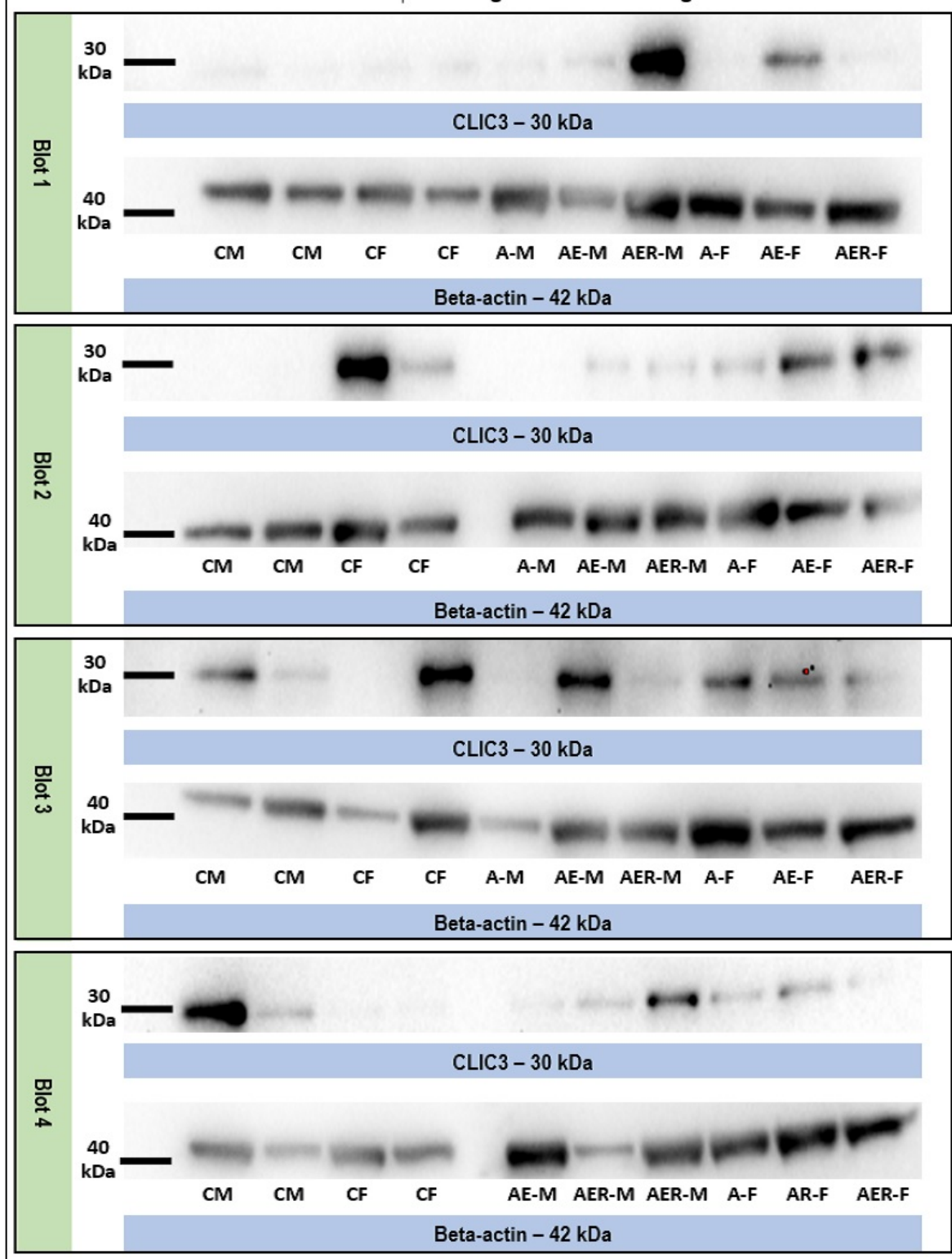
4.4.2 Western blot validation study using placental samples from children with and without an allergy

I validated and measured five placental proteins, including chloride intracellular channel protein 3 (CLIC3), peroxiredoxin-2 (PRDX2), complement C3 (C3), haptoglobin (Hp) and pan 14-3-3 in the placental samples of children with and without an allergy using Western blot. Pan 14-3-3 (K-19) antibody used in the Western blot is an antibody that can detect seven isoforms comprising this protein family of signaling intermediates, denoted 14-3-3 beta (β), gamma (γ), epsilon (ϵ), zeta (ζ), eta (η), theta (θ) and sigma (σ). Each of these proteins were normalised with the housekeeping protein, beta-actin, and band densitometry analyses were performed in relation to general allergy, the presence or absence of maternal asthma during pregnancy; child sex, and child allergy phenotypes (refer Methodology section 2.12.1 and Optimisation section 3.2.3). In the Western blot, eight micrograms of protein was loaded per well in each lane. However, β -actin band density was highly variable possibly due to variability between each individual.

4.4.2.1 Expression of chloride intracellular channel protein 3 (CLIC3) protein in the placental samples of children with and without an allergy

CLIC3 protein in the placental samples was not highly expressed in all of the allergic placentae and male allergic placentae using Western blot (Figure 58). There were no significant differences of CLIC3 in any group.

CLIC3: Ratio LFQ MS ↑ in allergic male and allergic children



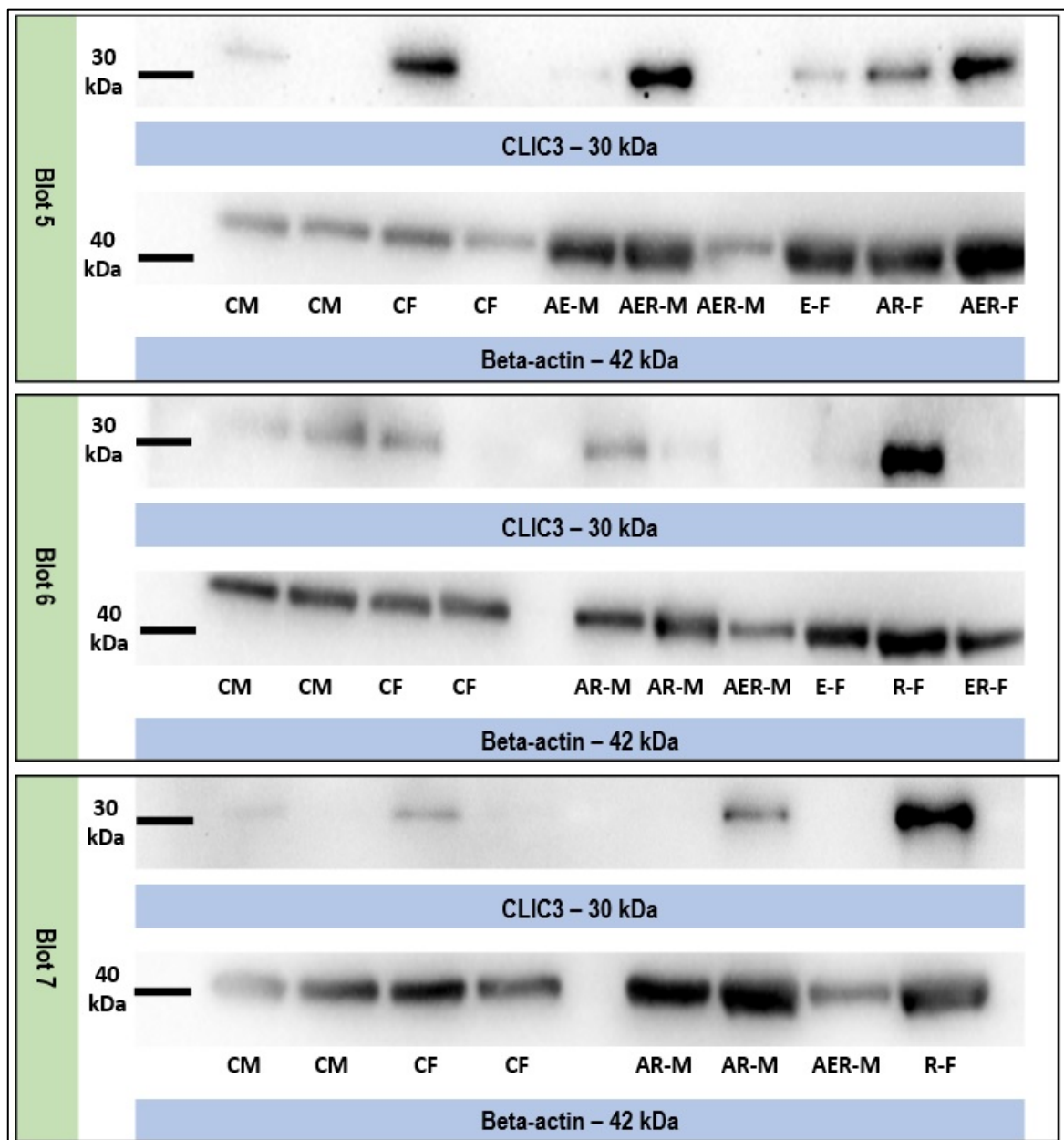
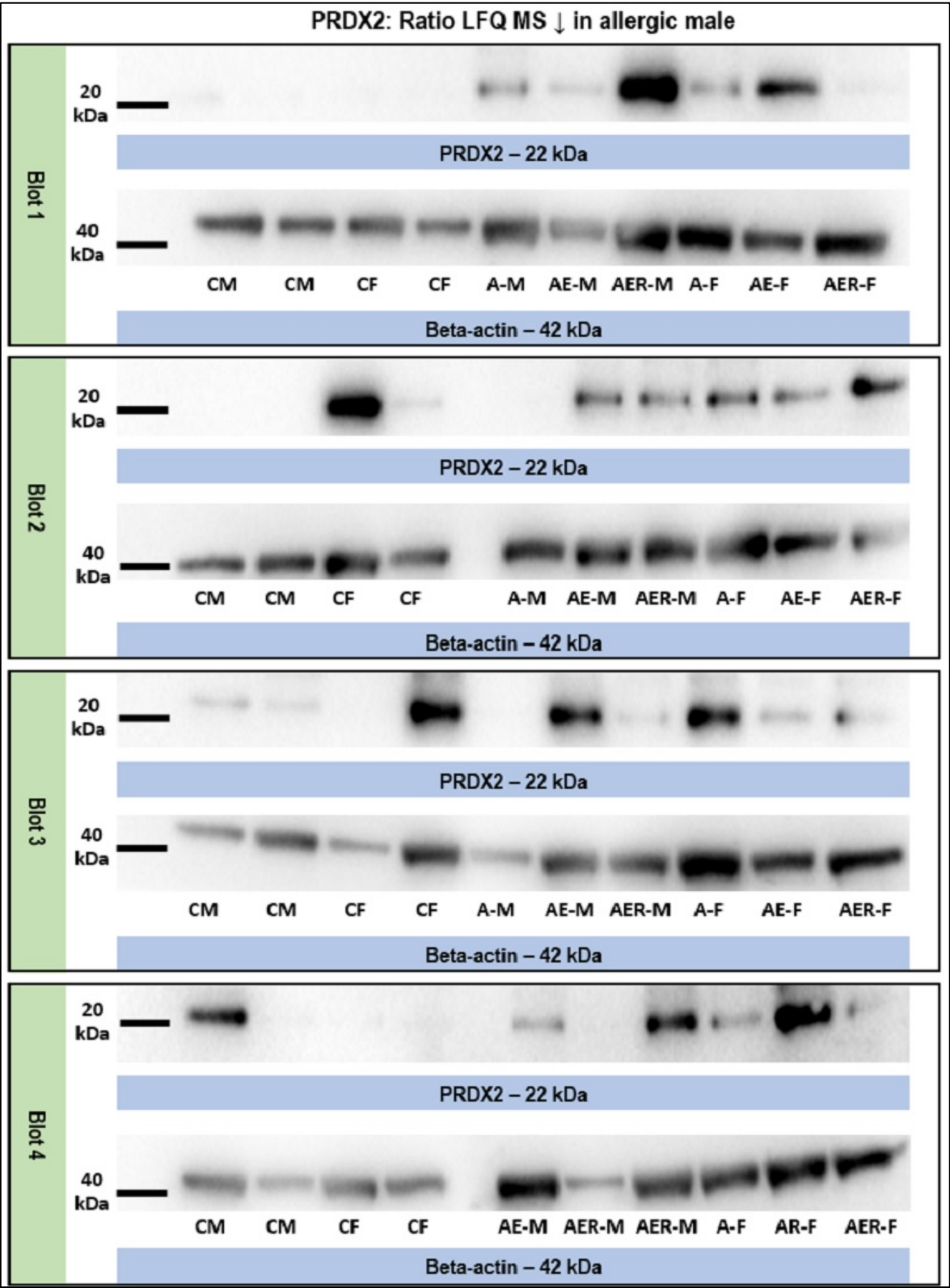


Figure 58. Representative Western blot of CLIC3 protein expression in the placental samples of children with (n= 40) and without (n= 28) an allergy. CLIC3 protein was detected at 30 kDa. (M represents male children, F represents female children, C represents control subjects who are children with no allergy, A represents children with asthma, E represents children with eczema and R represents children with rhinitis).

4.4.2.2 Expression of peroxiredoxin-2 (PRDX2) protein in the placental samples of children with and without an allergy

When comparing the difference in placental protein expression between children without an allergy to children with an allergy, PRDX2 (Mann Whitney U test, $P=0.005^{**}$ and $P=0.036^{*}$) was significantly increased in the allergic placentae regardless of sex and in male sex (Figures 60 and 64). Interestingly, the PRDX2 placental protein expression was influenced by both the presence (high risk of allergy) and the absence (low risk of allergy) of maternal asthma. PRDX2 (Mann Whitney U test, $P=0.020^{*}$ and $P=0.013^{*}$) was significantly increased in the allergic placentae of asthmatic mothers (high risk of allergy) regardless of sex and in male sex as compared to non-allergic placentae of asthmatic mothers (Figures 61 and 63). Moreover, allergic children with non-asthmatic mothers (low risk of allergy) had significant Peroxiredoxin-2 (Mann Whitney U test, $P=0.035^{*}$) placental protein expression regardless of sex, as compared to non-allergic placentae of non-asthmatic mothers (Figure 62). There was a significant increase in PRDX2 (Mann Whitney U test, $P=0.007^{**}$, $P=0.035^{*}$ and $P=0.021^{*}$) placental proteins in children with any asthma, eczema and rhinitis combination allergic phenotypes regardless of sex as compared to children without an allergy (Figure 65). There were no significant differences of placental PRDX2 protein expression in other groups.



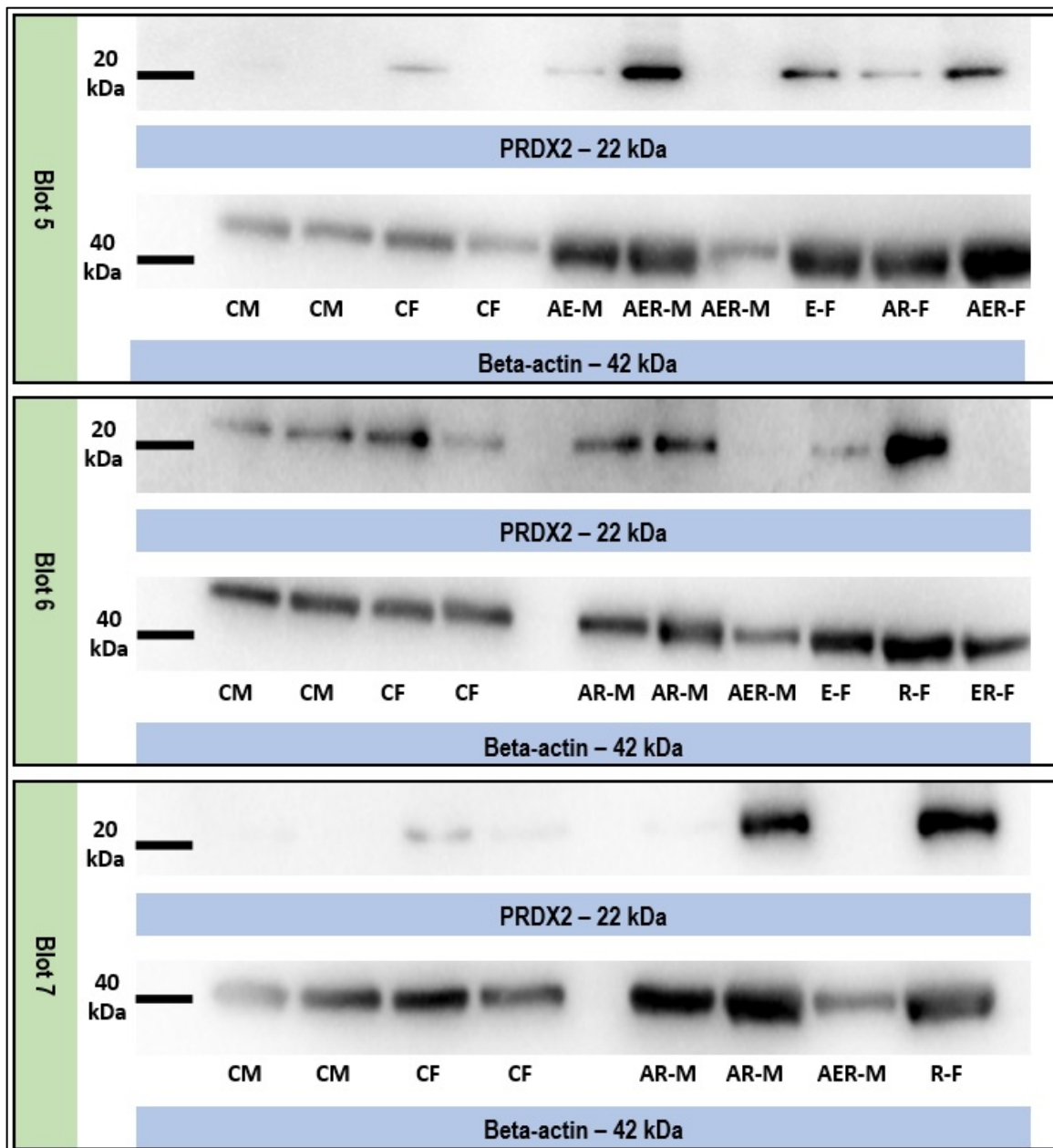


Figure 59. Representative Western blot of PRDX2 protein expression in the placental samples of children with (n= 40) and without (n= 28) an allergy. PRDX2 protein was detected at 22 kDa. (M represents male children, F represents female children, C represents control subjects who are children with no allergy, A represents children with asthma, E represents children with eczema and R represents children with rhinitis).

i. Children with allergy vs children with no allergy



Figure 60. PRDX2 protein expression in children with (n= 40) and without (n= 28) an allergy. Placental protein expression was examined using Western blot. Proteins were expressed relative to beta-actin. Data of PRDX2 protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

ii. The influence of the presence and absence of maternal asthma during pregnancy on placental protein expression and child allergy

a. Children of asthmatic mothers (high risk of allergy) with and without an allergy

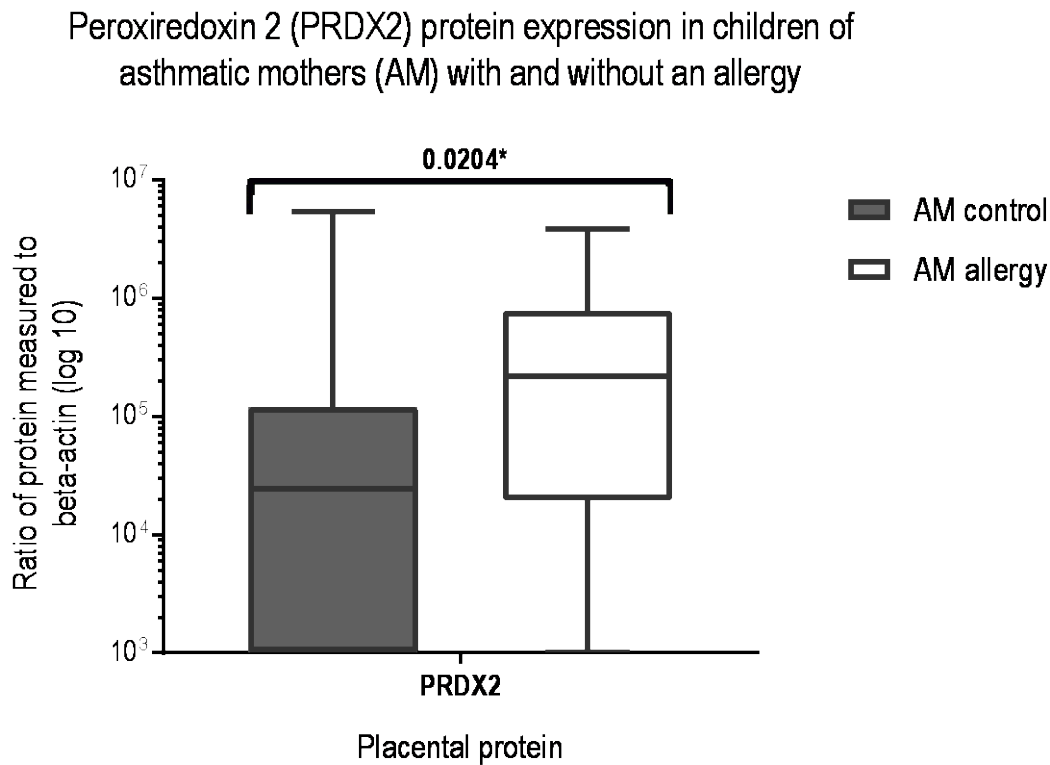


Figure 61. PRDX2 protein expression in children of asthmatic mothers (high risk of allergy) with (n= 24) and without (n= 22) an allergy. Placental protein expression was examined using Western blot. Proteins were expressed relative to beta-actin. Data of PRDX2 protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

b. Children of non-asthmatic mothers (low risk of allergy) with and without an allergy

Peroxiredoxin 2 (PRDX2) protein expression in children of non-asthmatic mothers (NAM) with and without an allergy

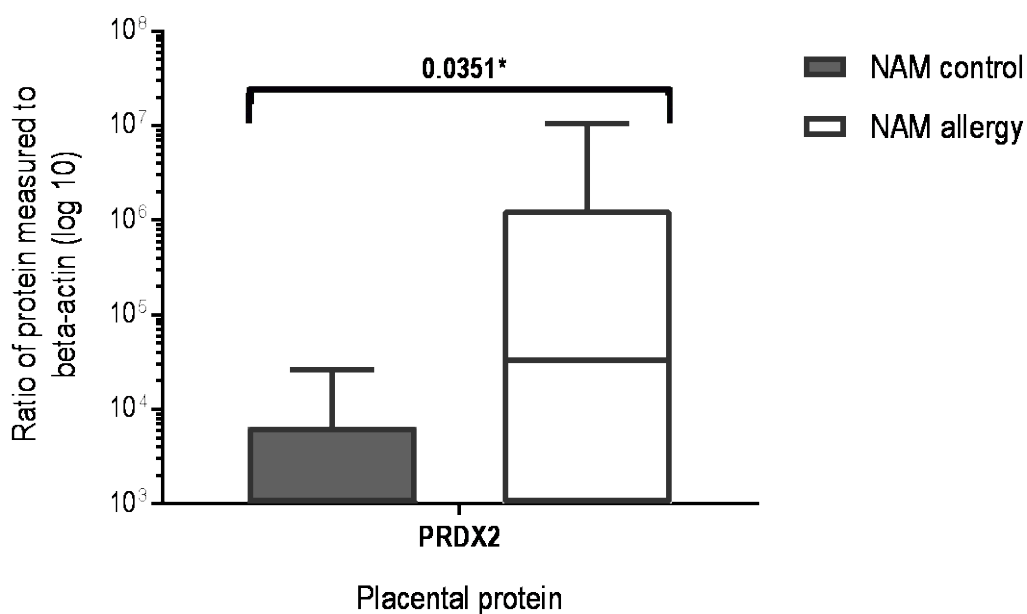


Figure 62. PRDX2 protein expression in children of non-asthmatic mothers (low risk of allergy) with (n= 16) and without (n= 6) an allergy. Placental protein expression was examined using Western blot. Proteins were expressed relative to beta-actin. Data of PRDX2 protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

c. The effect of sex and the presence and absence of maternal asthma on placental protein expression

Peroxiredoxin 2 (PRDX2) protein expression in children of asthmatic mothers (AM) with and without an allergy in male sex

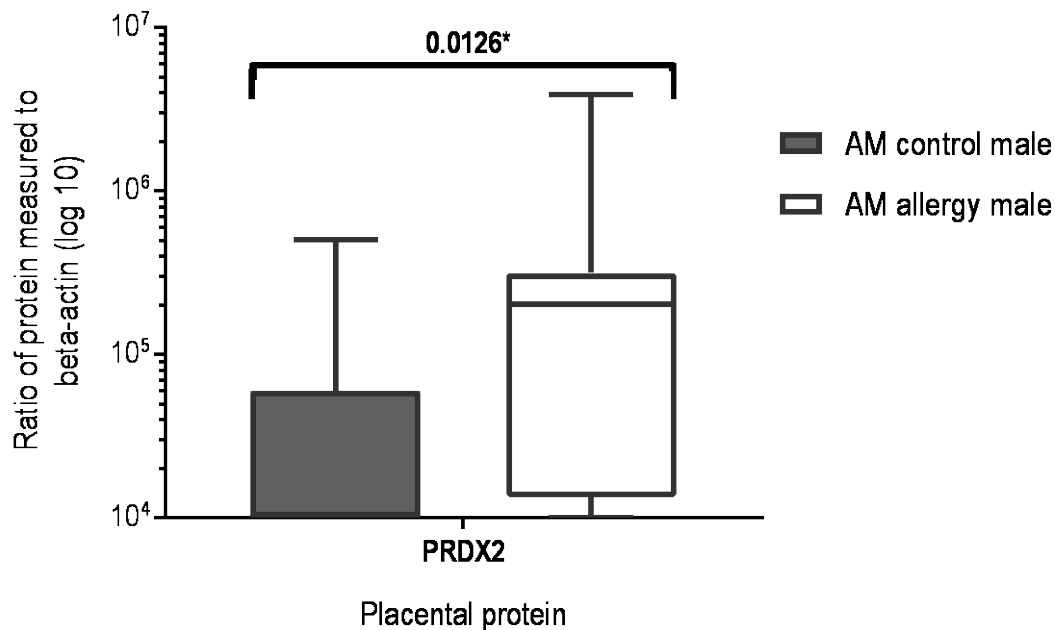


Figure 63. PRDX2 protein expression in children of asthmatic mother (high risk of allergy) with (n= 13) and without (n= 13) an allergy in male sex. Placental protein expression was examined using Western blot. Proteins were expressed relative to beta-actin. Data of PRDX2 protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

iii. Effect of sex on placental protein expression and child allergy

a. Children with allergy vs children with no allergy

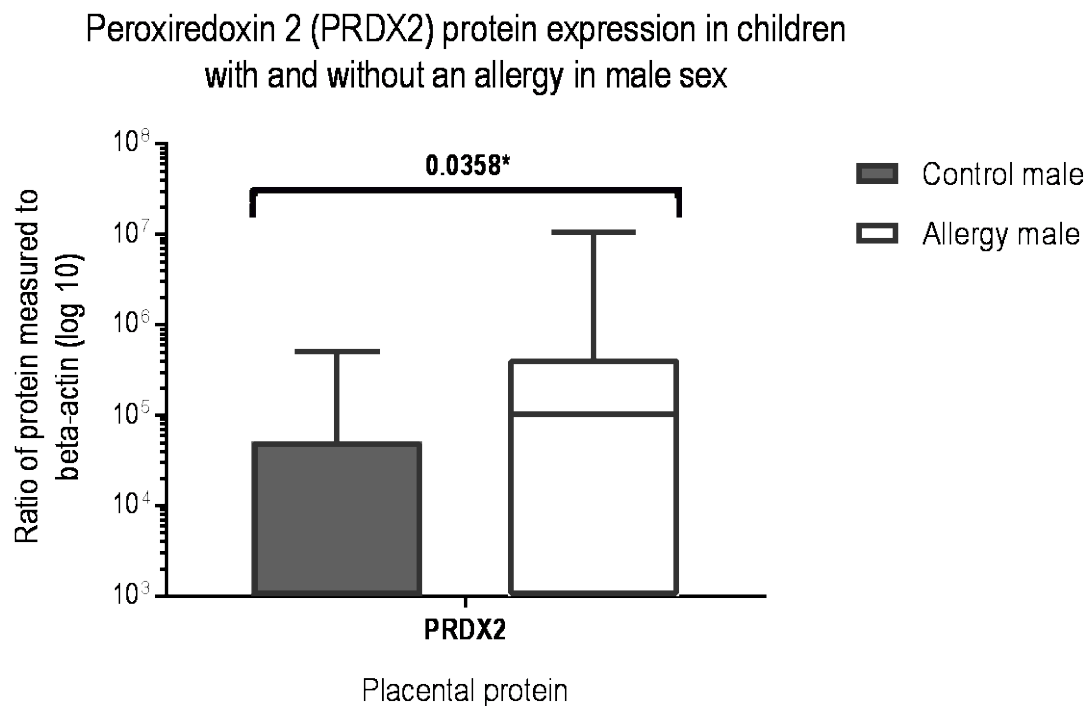


Figure 64. PRDX2 protein expression in children with (n= 21) and without (n= 14) an allergy in male sex. Placental protein expression was examined using Western blot. Proteins were expressed relative to beta-actin. Data PRDX2 protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

vi. Effect of specific allergic phenotypes on placental protein expression and child allergy

Peroxiredoxin 2 (PRDX2) protein expression in children with and without any asthma, eczema and rhinitis allergy combination

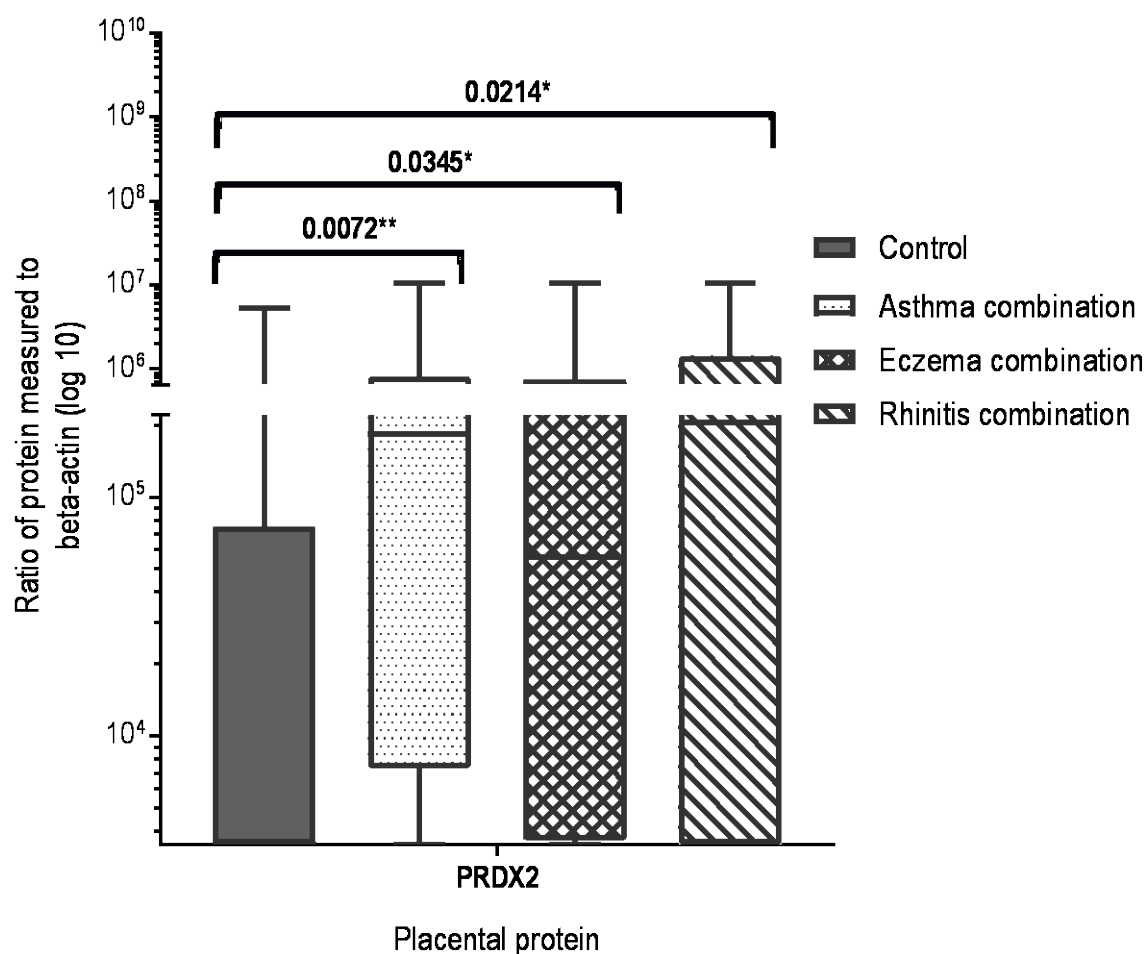
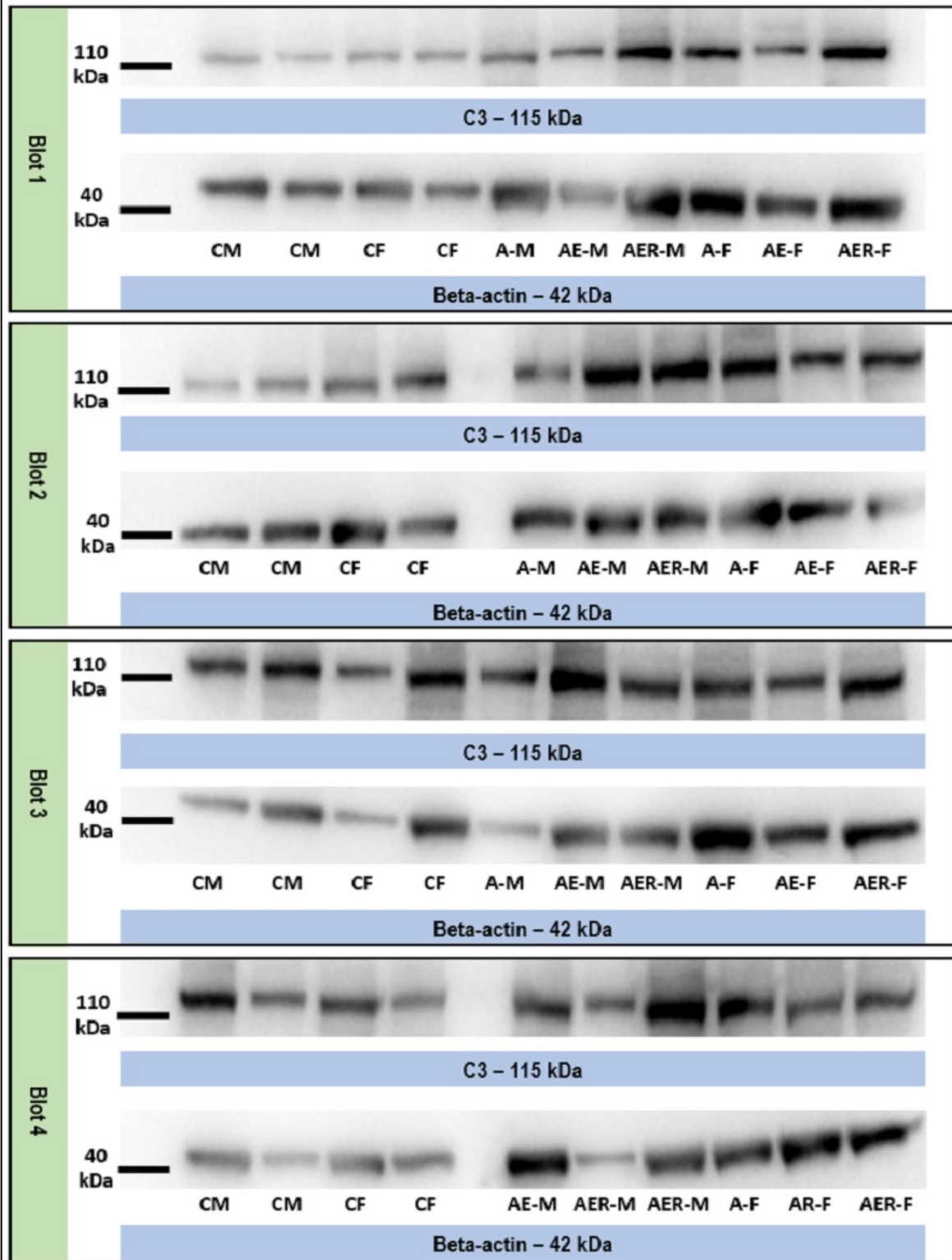


Figure 65. PRDX2 protein expression in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, n= 35; eczema combination, n= 25; rhinitis combination, n= 23; and non-allergic children, n= 28. Placental protein expression was examined using Western blot. Proteins were expressed relative to beta-actin. Data of PRDX2 protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$. A significant result was $P < 0.05$.

4.4.2.3 Expression of complement C3 (C3) protein in the placental samples of children with and without an allergy

When comparing the difference in protein expression of C3 between children without an allergy to children with an allergy of non-asthmatic mothers (low risk of allergy), C3 (Mann Whitney U test, $P=0.021^*$) was significantly increased in the placental samples of children with an allergy regardless of sex (Figure 67). Furthermore, placental C3 (Mann Whitney U test, $P=0.007^{**}$) was significantly increased in children with any rhinitis combination allergic phenotypes regardless of sex as compared to children without an allergy (Figure 68). It did not change significantly in any other groups.

C3: Ratio LFQ MS ↓ in allergic female



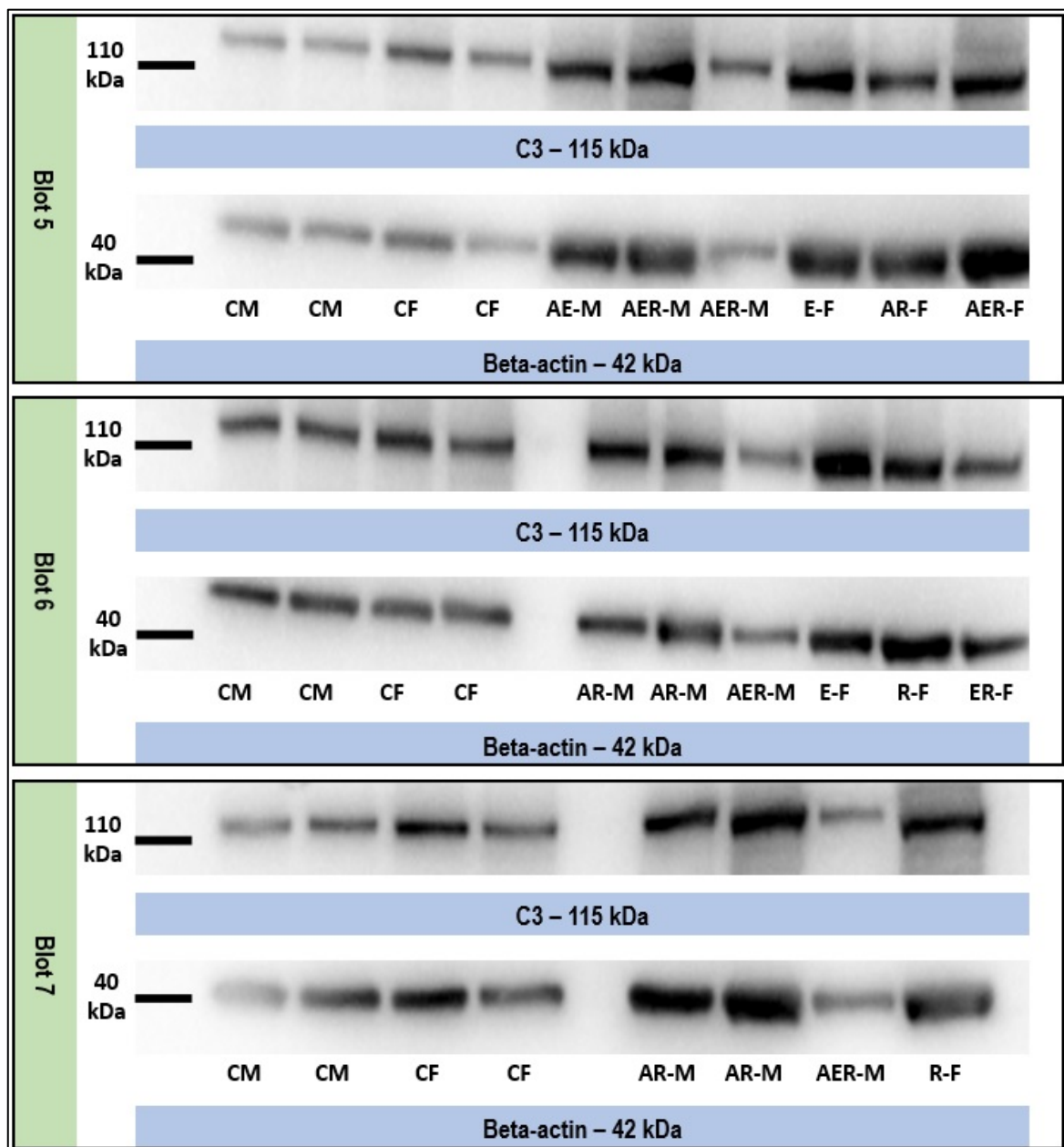


Figure 66. Representative Western blot of C3 protein expression in the placental samples of children with (n= 40) and without (n= 28) an allergy. C3 protein was detected at 115 kDa. (M represents male children, F represents female children, C represents control subjects who are children with no allergy, A represents children with asthma, E represents children with eczema and R represents children with rhinitis).

- i. The influence of the presence and absence of maternal asthma during pregnancy on placental protein expression and child allergy
 - a. Children of non-asthmatic mothers (low risk of allergy) with and without an allergy

Complement 3 (C3) protein expression in children of non-asthmatic mothers (NAM) with and without an allergy

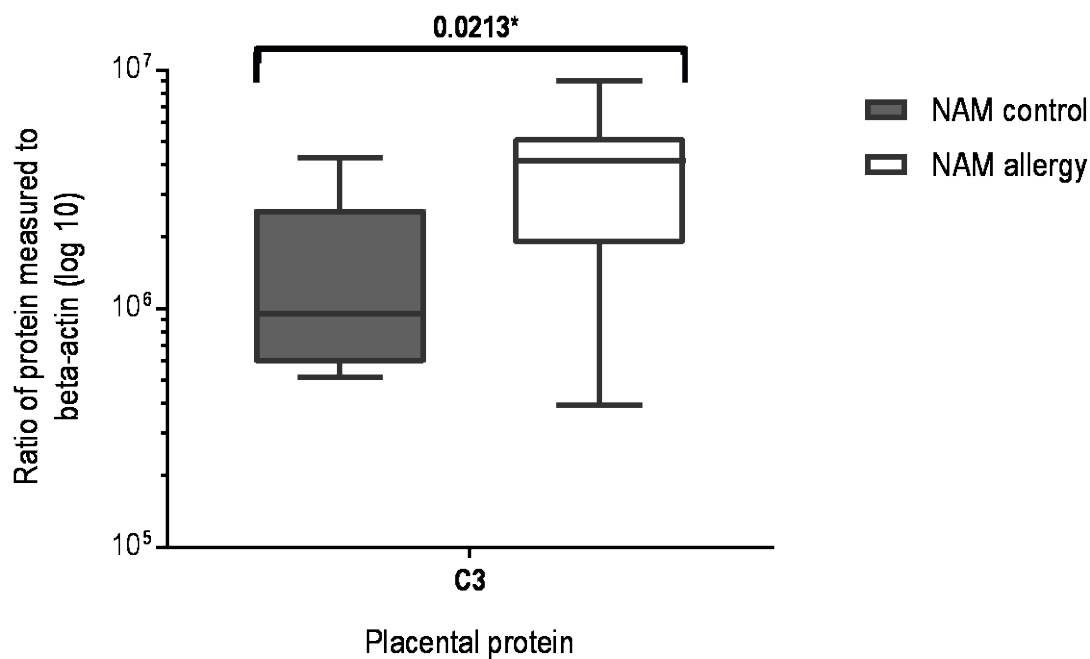


Figure 67. C3 protein expression in children of non-asthmatic mothers (low risk of allergy) with (n= 16) and without (n= 6) an allergy. Placental protein expression was examined using Western blot. Proteins were expressed relative to beta-actin. Data of C3 protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

ii. Effect of specific allergic phenotypes on placental protein expression and child allergy

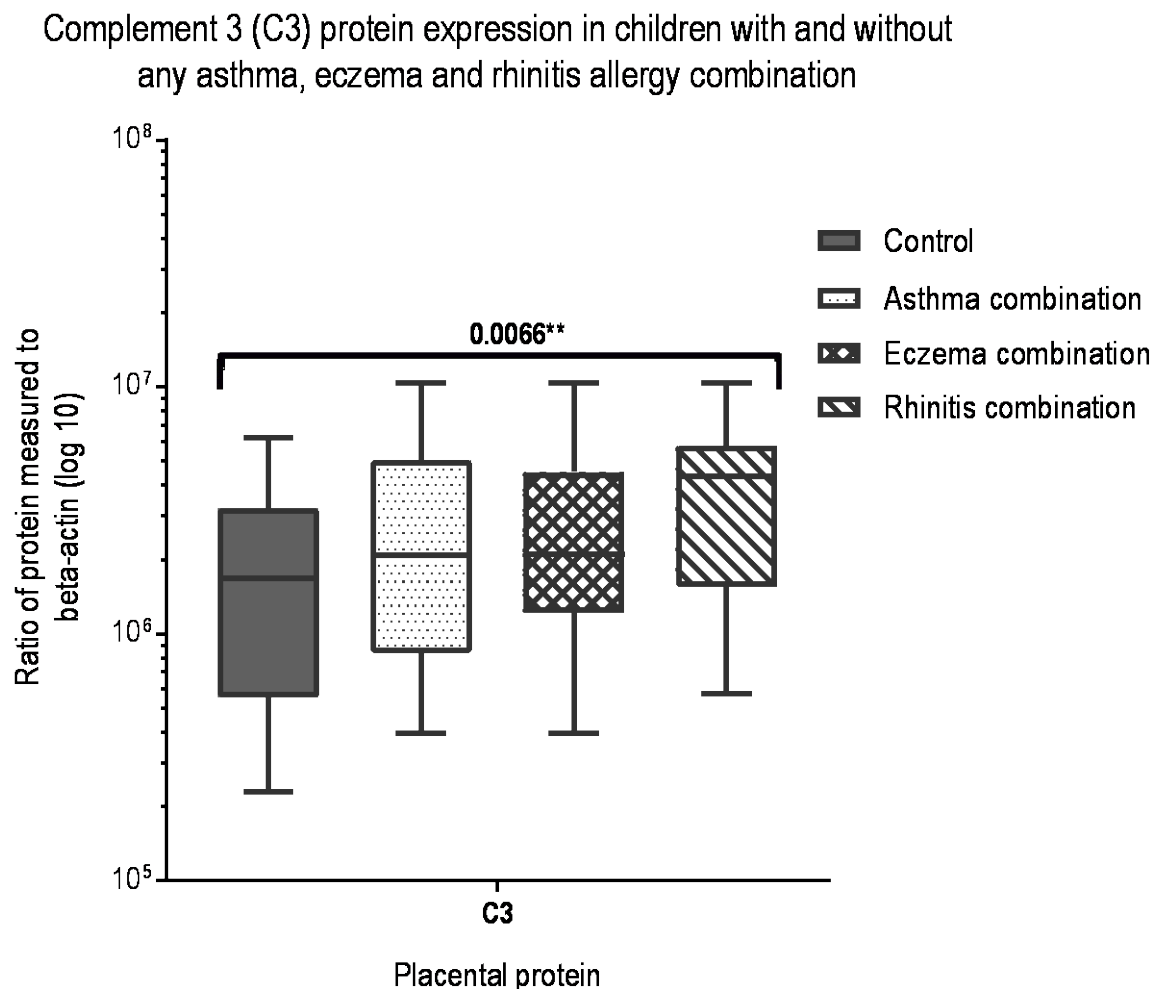
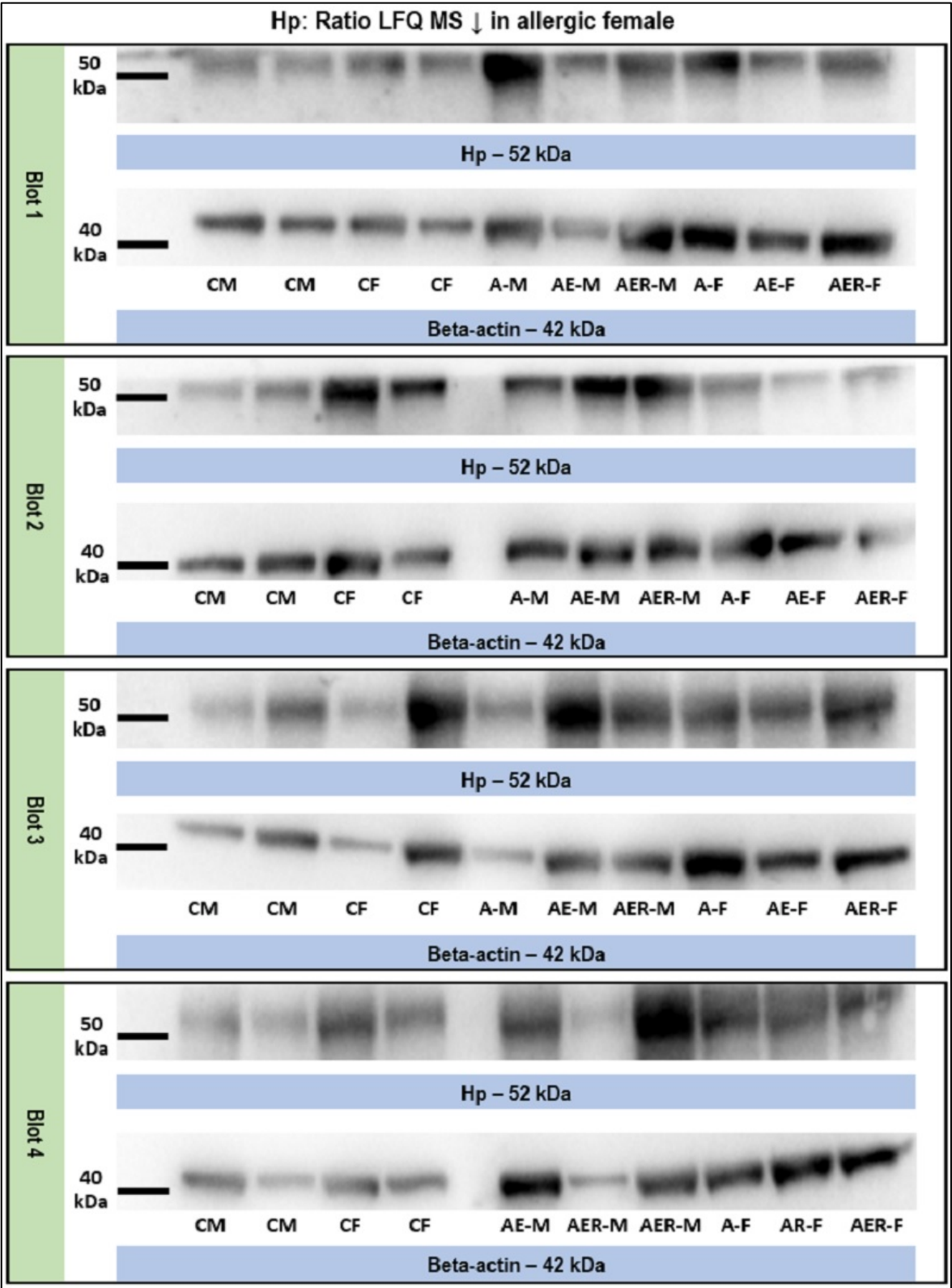


Figure 68. C3 protein expression in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, n= 35; eczema combination, n= 25; rhinitis combination, n= 23; and non-allergic children, n= 28. Placental protein expression was examined using Western blot. Proteins were expressed relative to beta-actin. Data of C3 protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

4.4.2.4 Expression of haptoglobin (Hp) protein in the placental samples of children with and without an allergy

Placental Hp was not identified in all female allergic placentae (AF) using Western blot (Figure 69). When comparing the differences in placental protein expression between children without an allergy to children with an allergy, Hp (Mann Whitney U test, $P= 0.015^*$ and $P= <0.0001^{****}$) was significantly increased in the placental samples of children with an allergy regardless of sex and in male sex (Figures 70 and 73). Placental Hp (Mann Whitney U test, $P= 0.006^{**}$ and $P= <0.0001^{****}$) was significantly increased in the allergic placentae of asthmatic mothers (high risk of allergy) regardless of sex and male sex, when compared to non-allergic placentae of asthmatic mothers (Figures 71 and 72). Moreover, there was significant increases in the Hp (Mann Whitney U test, $P= 0.032^*$) placental proteins expression in children with any asthma combination allergic phenotypes regardless of sex as compared to children without an allergy (Figure 74). It did not change significantly in any other groups.



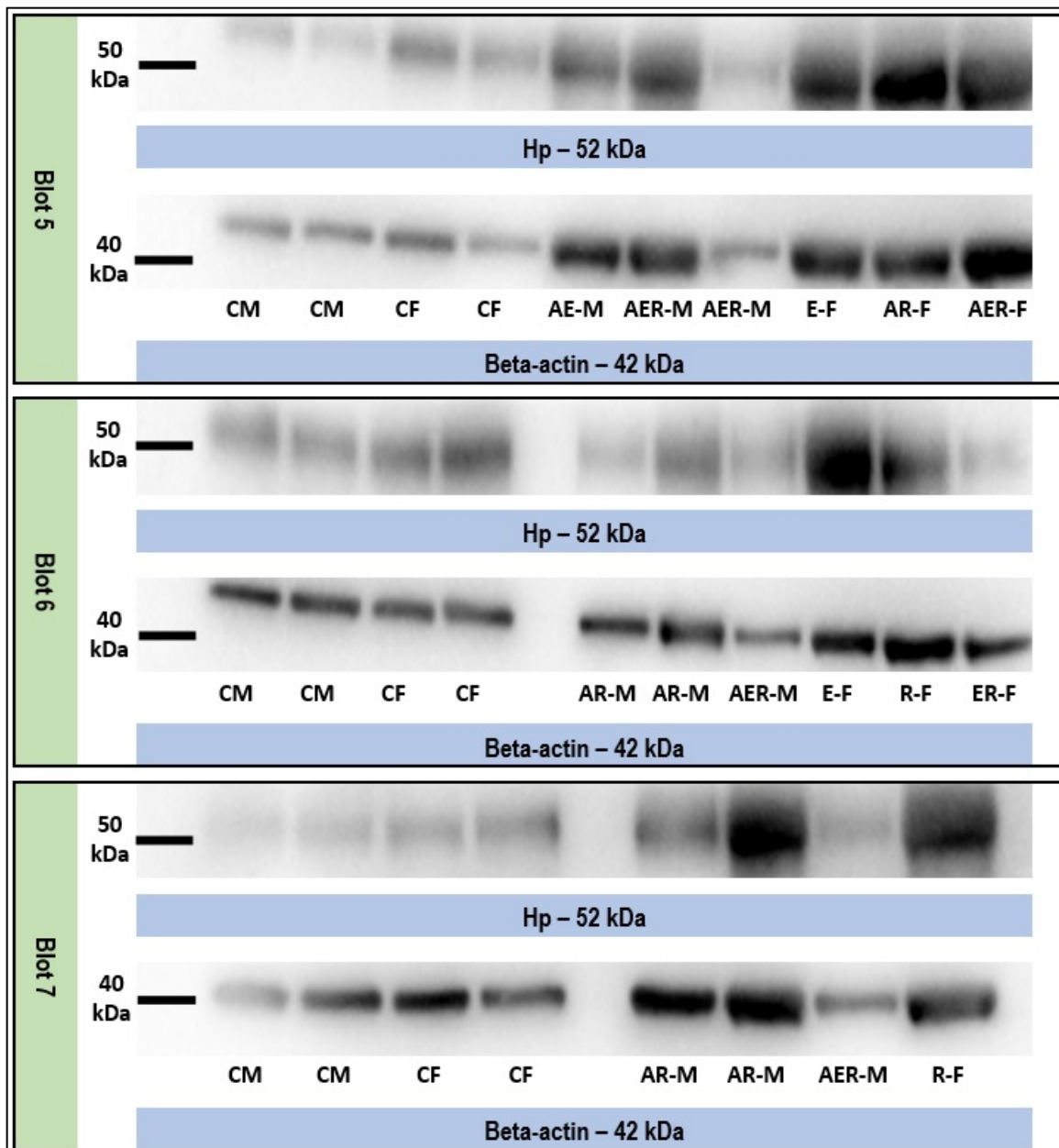


Figure 69. Representative Western blot of Hp protein expression in the placental samples of children with (n= 40) and without (n= 28) an allergy. Hp protein was detected at 52 kDa. (M represents male children, F represents female children, C represents control subjects who are children with no allergy, A represents children with asthma, E represents children with eczema and R represents children with rhinitis).

i. Children with allergy vs children with no allergy

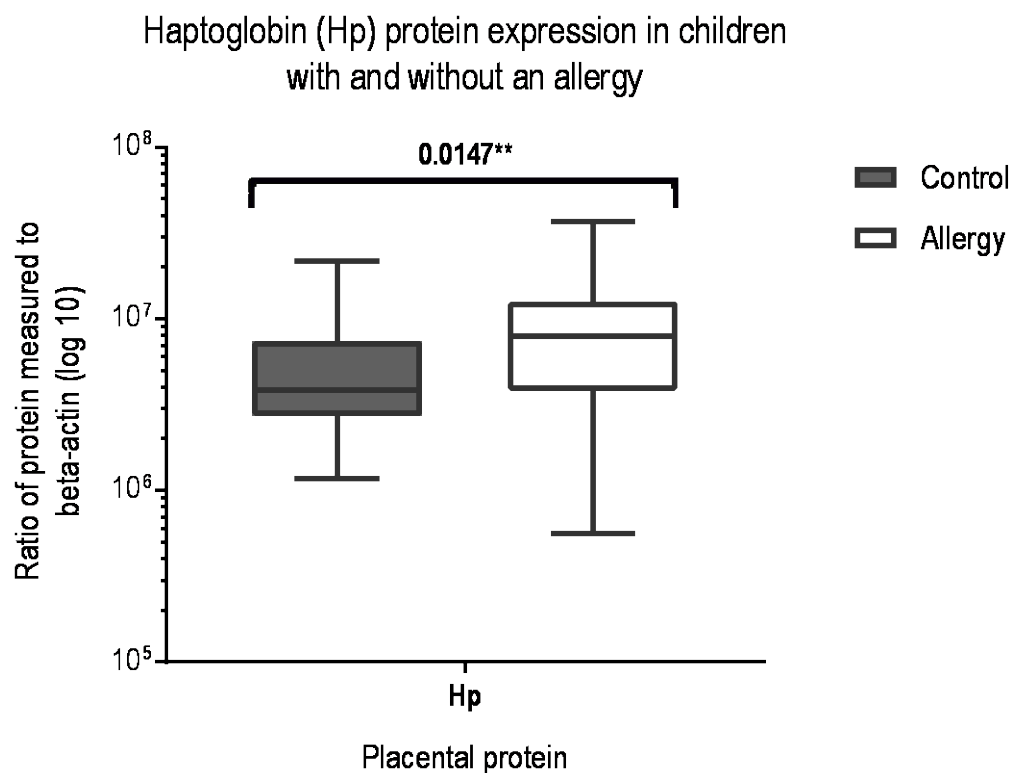


Figure 70. Hp protein expression in children with (n= 40) and without (n= 28) an allergy. Placental protein expression was examined using Western blot. Proteins were expressed relative to beta-actin. Data of Hp protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

ii. The influence of the presence and absence of maternal asthma during pregnancy on placental protein expression and child allergy

a. Children of asthmatic mothers (high risk of allergy) with and without an allergy

Haptoglobin (Hp) protein expression in children of asthmatic mothers (AM) with and without an allergy

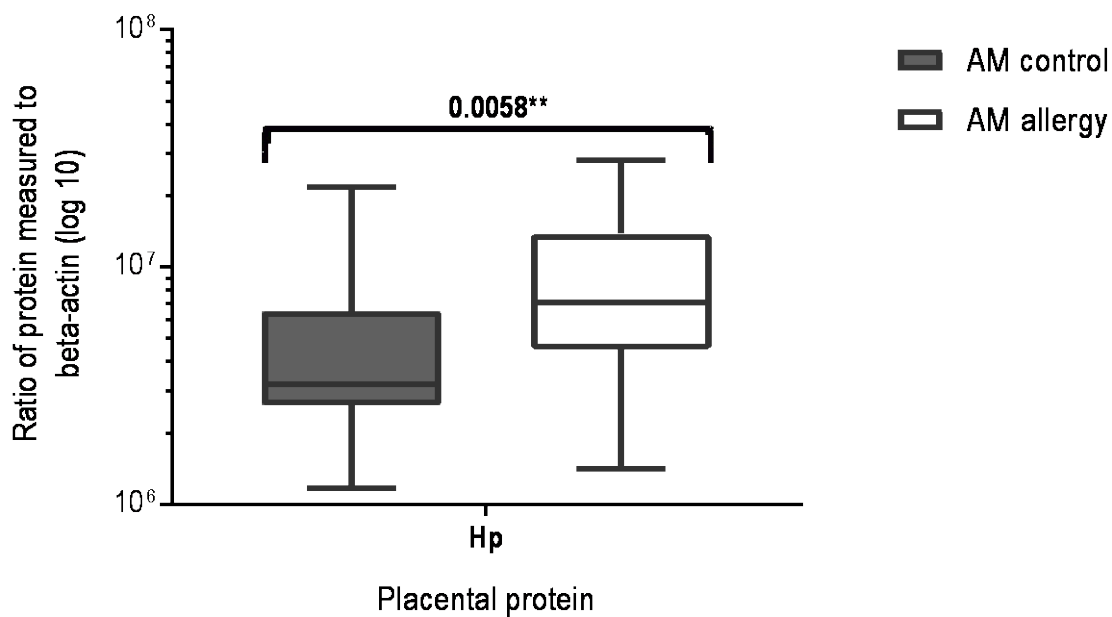


Figure 71. Hp protein expression in children of asthmatic mothers (high risk of allergy) with (n= 24) and without (n= 22) an allergy. Placental protein expression was examined using Western blot. Proteins were expressed relative to beta-actin. Data of Hp protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

b. The effect of sex and the presence and absence of maternal asthma on placental protein expression

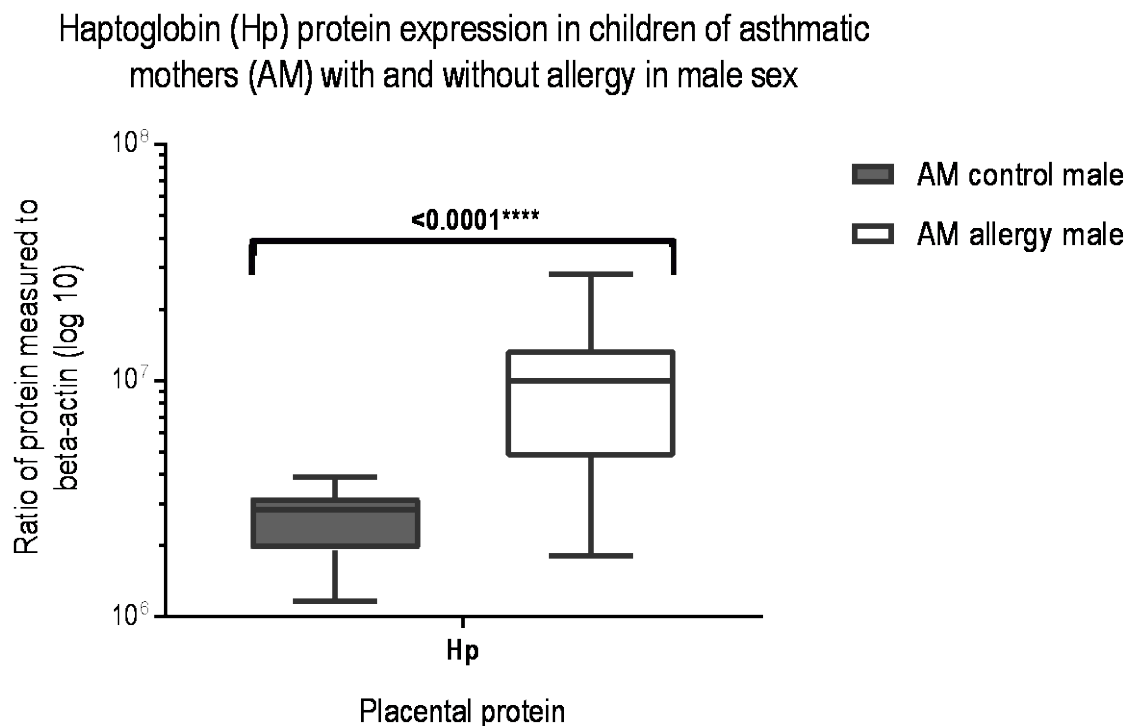


Figure 72. Hp protein expression in children of asthmatic mothers (high risk of allergy) with (n= 13) and without (n= 13) an allergy in male sex. Placental protein expression was examined using Western blot. Proteins were expressed relative to beta-actin. Data of Hp protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

iii. Effect of sex on placental protein expression and child allergy

a. Children with allergy vs children with no allergy

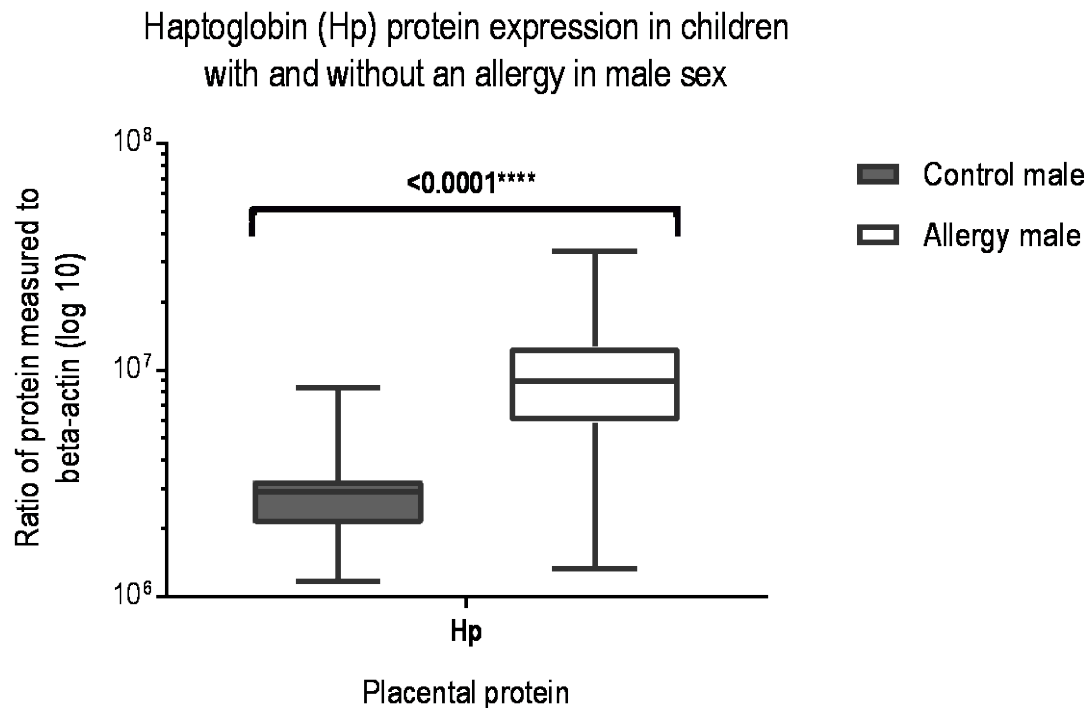


Figure 73. Hp protein expression in children with (n= 21) and without (n= 14) an allergy in male sex. Placental protein expression was examined using Western blot. Proteins were expressed relative to beta-actin. Data of Hp protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

iv. Effect of specific allergic phenotypes on placental protein expression and child allergy

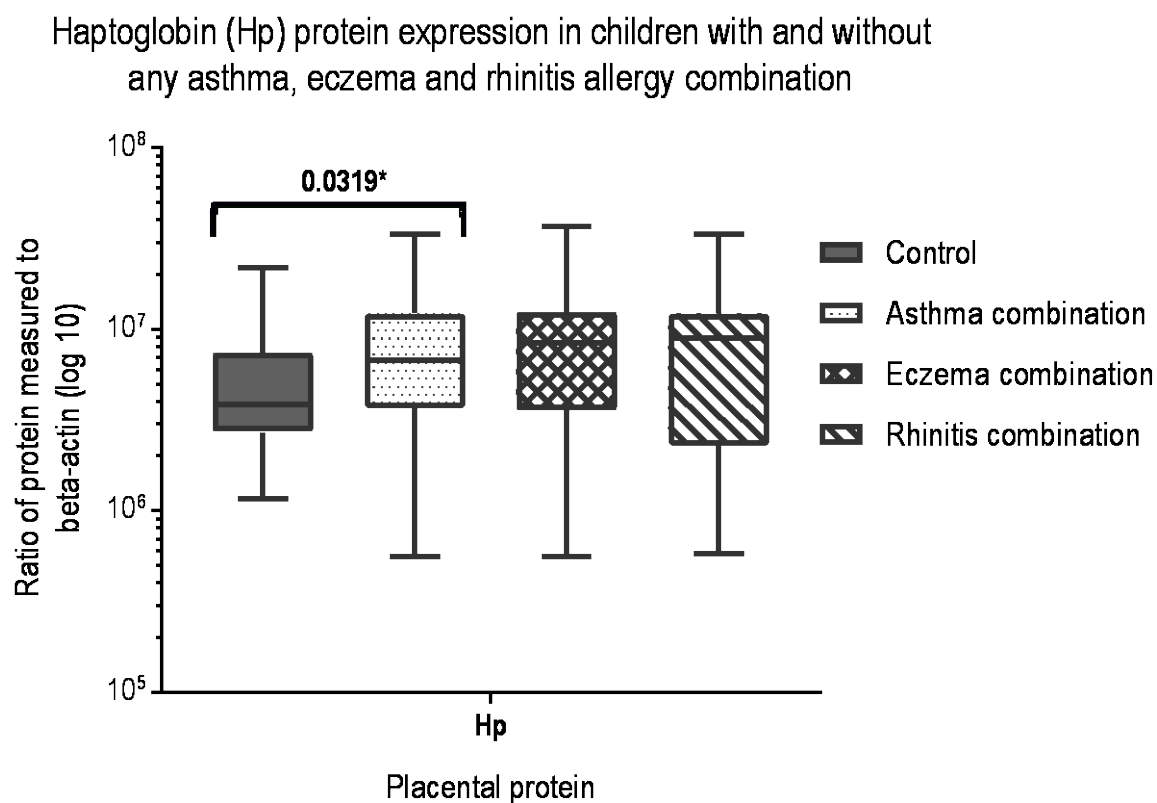


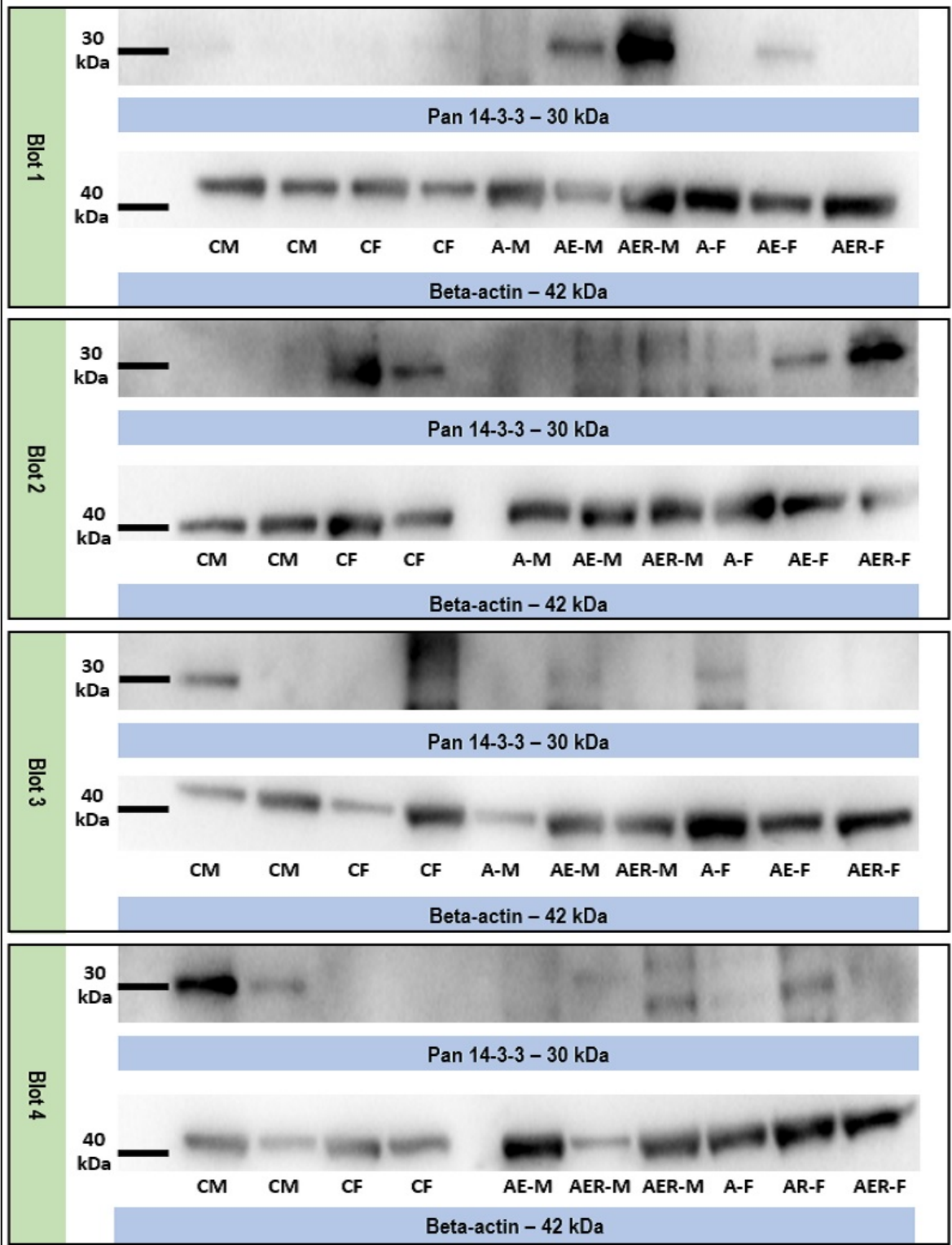
Figure 74. Hp protein expression in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, n= 35; eczema combination, n= 25; rhinitis combination, n= 23; and non-allergic children, n= 28. Placental protein expression was examined using Western blot. Proteins were expressed relative to beta-actin. Data of Hp protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

4.4.2.5 Expression of pan 14-3-3 protein in the placental samples of children with and without an allergy

Pan 14-3-3 protein had very high expression in allergic children and very low expression in the non-allergic group (Figure 75). There were no significant differences of pan 14-3-3 protein expression in any group.

The summary of statistical analyses performed on Western blot band densitometry of placental samples in relation to childhood allergy distribution and in relation to the presence (high risk of allergy) or the absence (low risk of allergy) of maternal asthma is depicted in Table 21.

Pan 14-3-3: Uncalculated LFQ MS ratio in allergic male and allergic female



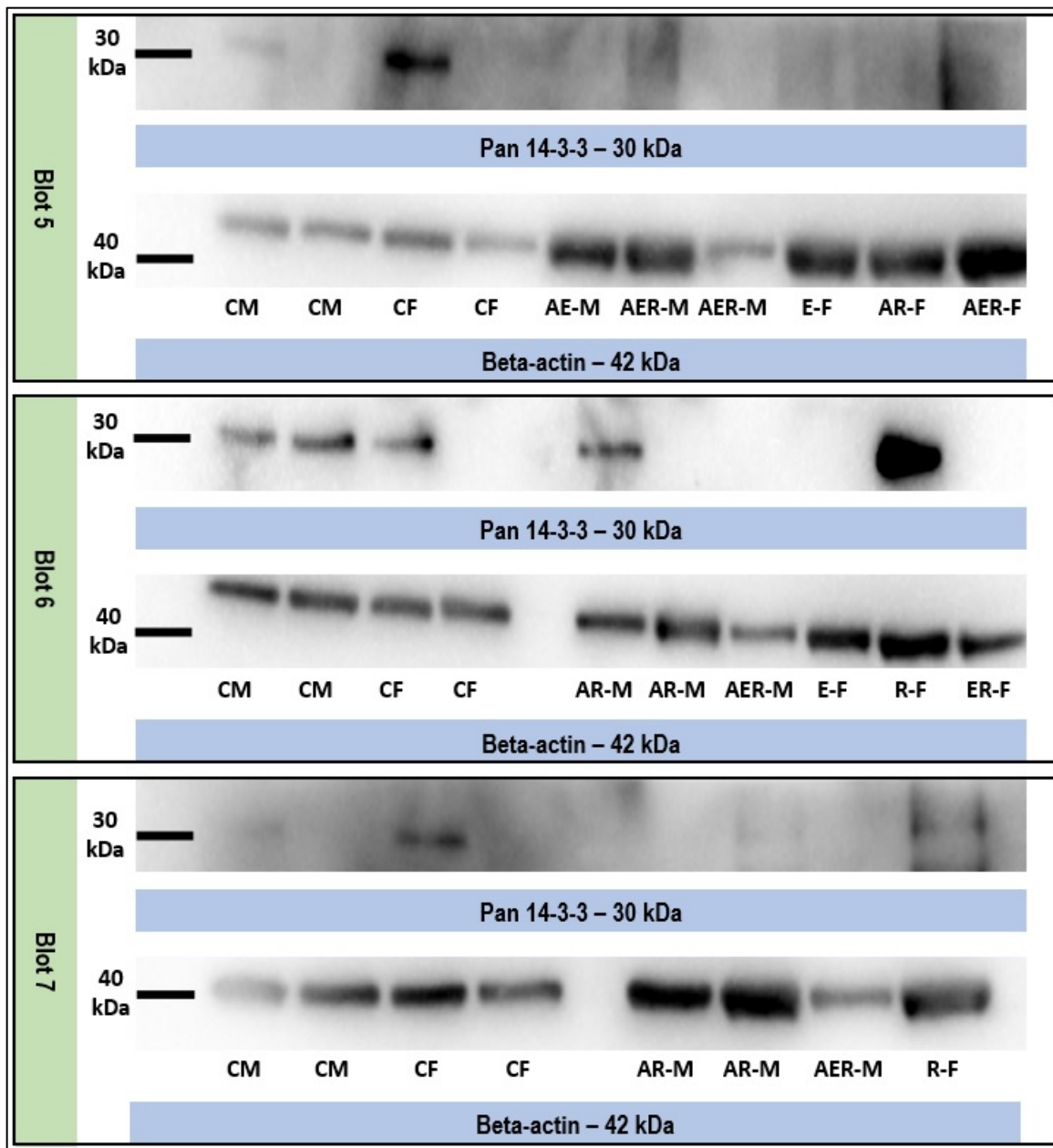


Figure 75. Representative Western blot of pan 14-3-3 protein expression in the placental samples of children with (n= 40) and without (n= 28) an allergy. Pan 14-3-3 protein was detected at 30 kDa. (M represents male children, F represents female children, C represents control subjects who are children with no allergy, A represents children with asthma, E represents children with eczema and R represents children with rhinitis).

Table 21. Significant statistical analyses of Western blot band densitometry of child saliva samples in relation to childhood allergy distribution and in relation the presence (high risk of allergy) or the absence (low risk of allergy) of maternal asthma. (Significant result was $P < 0.05$).

Placental proteins	Significant Western blot statistical analysis	Statistical analysis used	P values
Peroxiredoxin-2 (PRDX2)	Control vs allergy	MWU	0.0048**
	AM control vs AM allergy	MWU	0.0204*
	AM male control vs AM allergy male	MWU	0.0126*
	NAM control vs NAM allergy	MWU	0.0351*
	Control male vs allergy male	MWU	0.0358*
	Control vs specific allergy (asthma combination)	MWU	0.0072**
	Control vs specific allergy (eczema combination)	MWU	0.0345*
	Control vs specific allergy (rhinitis combination)	MWU	0.0214*
Complement C3 (C3)	Control vs allergy	MWU	0.0213*
	NAM control vs NAM allergy	MWU	0.0066**
Haptoglobin (Hp)	Control vs allergy	MWU	0.0147*
	AM control vs AM allergy	MWU	0.0058**
	AM male control vs AM allergy male	MWU	<0.0001****
	Control male vs allergy male	MWU	<0.0001****
	Control vs specific allergy (asthma combination)	MWU	0.0319*

° Abbreviations: AM represents children with asthmatic mothers (high risk of allergy), NAM represents children with non-asthmatic mothers (low risk of allergy) and MWU represents Mann Whitney U test with two-tailed p-value.

4.5 Discussion

The human placenta may program the fetal immune system in response to the maternal environment and lead to increased susceptibility for allergy in early life ⁽¹³¹⁾. Previous work has shown that placental inflammatory gene expression is involved in inflammation associated with development of specific childhood allergies supporting a programming role for the placenta ⁽¹³²⁾. Current findings suggest that placental protein expression varies *in utero* in children between the ages of six months to five years who subsequently develop an allergy. Furthermore, altered expression of placental proteins varies in a sex-specific manner, such as observed in Hp.

Our laboratory has shown that baseline mRNA expression of placental cytokines, such as *TNFα* and *ILs*, were altered in a sex-specific manner in pregnancies complicated by asthma ⁽¹³⁾. Interestingly, these changes only occurred in mothers pregnant with a female fetus and no changes were observed in placentae of male fetuses ⁽¹³⁾. This can be explained by the different mechanisms male and female fetuses use *in utero* to cope with multiple adverse conditions, including maternal asthma, via alteration in placental function, manifested by altered proteins ^(12, 13).

As a preliminary workflow, placental gene analysis (qRT-PCR) identified important genes, including *KLHDC7A*, *YWHAQ* (14-3-3 theta (θ) protein), *IL1B*, *IL1R2* and *KLHL13*. The protein of the *KLHDC7A* gene is called Kelch domain-containing protein 7A. Kelch domain-containing protein 7A is involved in pathway protein ubiquitination, which is part of protein modification ⁽³²⁰⁾. *KLHDC7A* is involved in Dupuytren's contracture, a disabling fibroproliferative condition in which an abnormal amount of collagen is produced. Dupuytren's contracture has family predisposition and is the most common connective tissue disorder of humans ⁽³²¹⁾. However, to date there is no reported literature on *KLHDC7A* involvement in immune system regulation and this may be the first study to find an association with *KLHDC7A* and immune function. Unfortunately, this protein was not detected in LC-MS/MS findings, thus no conclusions can be made about its protein expression and function.

14-3-3 theta (θ) protein (*YWHAQ* gene) is involved in pathogenesis of psoriasis, contact dermatitis and asthma ^(322, 323). 14-3-3 theta mRNA expression is increased in punch skin biopsies from patients with psoriasis and contact dermatitis ⁽³²²⁾. Moreover, 14-3-3 theta was markedly increased following T cell activation and suppressed protein kinase C theta-dependent IL-2 promoter activity by preventing its translocation to the membrane ^(324, 325). 14-3-3 proteins have been identified in placental samples associated with pregnancy complications, such as preeclampsia and GDM ^(326, 327). Recent study indicates that decreased *YWHAQ* and significant hypermethylation of the *YWHAQ* promoter was detected in preeclampsia placentae, as compared with control samples in CpG2-4, CpG9, CpG17 and

CpG19⁽³²⁶⁾. Both protein and mRNA levels of 14-3-3 proteins were also increased in placental villi of pregnant women complicated with GDM analysed with MALDI-TOF/TOF and Western blotting⁽³²⁷⁾.

As a discovery tool, placental LC-MS/MS analysis revealed interesting proteins that can exert a programming effect on the fetal immune system, hence are associated with allergic diseases. Amongst these proteins are apolipoproteins (apos) which include apoA-I, apo B-100 and apo-D, angiotensinogen and cholesterol side-chain cleavage enzyme. Interestingly LC-MS/MS data show that 14-3-3 protein eta (η), one of the isoforms of 14-3-3 proteins, was also detected in uncalculated LFQ MS ratio in the placentae of allergic children, as compared with non-allergic children. This data indicates that non-allergic children had almost no 14-3-3 eta protein present, while allergic children had an abundant volume of 14-3-3 eta protein.

ApoA-I was identified in an epigenetic study by Martino *et al.* In their findings, ApoA-I gene (*APOA1*) is one of the 136 CpG differentially methylated loci located at chromosome 11 in total CD4⁺ T lymphocytes in children with IgE-mediated FA⁽¹⁸⁷⁾. Of these 136 CpG, 44 were annotated to within 10 base pairs of common SNP variants and exhibited genetic effects. These loci were annotated to genes at *HLA-DQB1* and apparently were influenced by underlying variants within the *HLA*⁽¹⁸⁷⁾. Although apoA-I was identified with more than two unique peptides (UP) in the study, the protein was not present in significant LFQ MS ratio (0.79 fc) in placentae of allergic children, as compared with non-allergic placentae. However, for other classes of apolipoproteins, apo B-100 was found to be in significant LFQ MS ratio in allergic male (2.48 fc) and apo-D in allergic female (0.48 fc), as compared with placentae of non-allergic children (Refer Table 19, section 4.3.2). The stringent significant LFQ MS ratio (>2 or <0.5 fc) in this study, as compared to some other studies that used a less stringent significant ratio (>1.5 or <1 fc), apoA-I fall within the insignificant ratio of LFQ MS in this study.

Apolipoproteins are produced mainly in liver and intestine. They regulate lung development, homeostasis, adaptive immune responses, host defence and are also important inflammation modulators^(328, 329). High apoA-I is associated with childhood asthma and atopy⁽³³⁰⁾. ApoA-I and apoA-II downregulate neutrophil function, suppress bacterial growth and act as anti-inflammatory agents⁽⁷⁰⁾. In AR patients, apoA-I and apoA-II upregulation is associated with recurring inflammation of nasal mucosa⁽³³¹⁾. A recent study using nasal mucus of AR patients analysed by LC-MS/MS indicated that apoA-I and apoA-II were significantly increased in mucus of AR patients, as compared to non-AR subjects⁽³³¹⁾. Moreover, release of apoA-I and apoA-II was possibly triggered by local mechanisms as an anti-inflammatory response to allergens. Experimental murine models demonstrated that the apoA-I/ATP-binding cassette subfamily A, member 1 (*ABCA1*) pathway can reduce neutrophilic airway inflammation, predominantly by suppressing production of granulocyte-colony stimulating factor⁽³³²⁾. Murine models of allergic asthma administered with apoA-I mimetic peptides decreased neutrophilic

and eosinophilic airway inflammation, airway hyperresponsiveness (AHR) and mucous cell metaplasia, which suggest that the apoA-I/ABCA1 pathway may have a protective effect in asthma ⁽³³²⁾. In atopic and asthmatic patients, serum levels of apoA-I and high-density lipoprotein (HDL) cholesterol were positively correlated with FEV1 in less severe airflow obstruction, whereas serum triglycerides, low-density lipoprotein (LDL) cholesterol, apoB and apoB/apoA-I ratio were associated with more severe airflow obstruction ⁽³²⁹⁾. Thus, apolipoproteins are important drivers of allergic risk which increases childhood allergic susceptibility, especially in AR and asthma.

In placental LC-MS/MS analysis conducted in this research, angiotensinogen was identified. It has previously been associated with pathogenesis of bronchial asthma ⁽³³³⁾. Angiotensinogen (AGT gene), an essential component of the renin-angiotensin system (RAS), is a potent regulator of blood pressure, body fluid and electrolyte homeostasis. Angiotensinogen is expressed by the liver, secreted in plasma and can be converted to angiotensin 1-7, 2 and 3 ^(334, 335). Angiotensin-1 has an antithrombotic effect that involves MAS1-mediated release of nitric oxide from platelets ⁽³³⁴⁾. Angiotensin-2 AT1A, AT1B and AT2 receptors, angiotensinogen and angiotensin-converting enzyme were expressed in the lungs, trachea and main bronchi of rats ⁽³³³⁾. Angiotensin-2 induced bronchial smooth muscle hyperresponsiveness through activation of p42/44 ERK and may be an important mediator in pathogenesis of bronchial asthma ⁽³³³⁾.

Angiotensinogen and renin, a newly discovered constituent of mast cells, are involved in severe anaphylactic reactions to insect venom allergy (IVA) ⁽³³⁶⁻³³⁸⁾. IgE-mediated allergic hypersensitivity provokes release of enzymatically active renin protein from cultured and resident cardiac mast cells, a process likely to be exacerbated in a chronic inflammatory background ⁽³³⁸⁾. This process is inhibited by glucocorticoids and enhanced by the pro-inflammatory mediator, PGE2. Mast cells are known to play a major role in IgE-mediated allergic reactions and are widely distributed, as well as angiotensinogen and angiotensin-converting enzyme. Hence, anaphylactic shock may be elicited via renin release in immediate hypersensitivity reactions that could lead to local angiotensin-2 generation and multiorgan dysfunctions. Moreover, RAS dysfunction has been described as a potential factor in IVA ⁽³³⁶⁾. The frequency of angiotensin AGT *p.M235T* variant was significantly higher in IVA patients than in controls, thus may be responsible for severe anaphylactic reactions to IVA ⁽³³⁶⁾.

CYP11A1 gene (cholesterol side-chain cleavage enzyme) expression is increased in activated T cells ⁽¹²⁸⁾. In an animal study, the *CYP11A1* gene played an important role in development of peanut allergy, regulating peanut-induced allergic responses (intestinal anaphylaxis) through effects on steroidogenesis, an essential pathway in Th2 differentiation ⁽¹²⁸⁾. Cholesterol side-chain cleavage enzyme is the first rate-limiting enzyme in the steroidogenic pathway and the main function is to catalyse the side-chain cleavage reaction of cholesterol to pregnenolone ⁽³³⁹⁾. It is also known as

CYPXIA1 and cytochrome P450 (scc) protein, a member of cytochrome P450 (CYPs) superfamily. Another member of the CYP superfamily of enzymes, cytochrome P450 1A1 (*CYP1A1* gene) was previously identified in a study on maternal smoking effect in relation to epigenome-wide DNA methylation in 1062 newborn CB samples ⁽¹²⁷⁾. Findings revealed differential DNA methylation at epigenome-wide statistical significance for 26 CpGs mapped to 10 genes present at birth in children whose mothers smoked during pregnancy ⁽¹²⁷⁾. These include *CYP1A1* genes involved in the aryl hydrocarbon receptor signalling pathway which mediates detoxification of the components of tobacco smoke ⁽¹²⁷⁾. Hence, DNA methylation that occurs to *CYP1A1* due to *in utero* maternal smoking exposure can also occur to *CYP11A1* (cholesterol side-chain cleavage enzyme) which may influence immune system development, increasing risk of allergic disease susceptibility. However, due to close location of these three placental proteins on the MW ladder, angiotensinogen at 51.06 kDa, cholesterol side-chain cleavage enzyme at 60.10 kDa and Hp at 45.21 kDa, the latter was chosen to be further validated using Western blot.

Amongst LC-MS/MS identified proteins, six proteins, 14-3-3, CLIC3, C3, Hp, PRDX2 and PGS1, were chosen to be verified in Western blot. However, PGS1 was not able to be optimised using Western blot, hence was not used in the validation study. Further data analysis of Western blot findings demonstrated that of five placental proteins, only three proteins, C3, Hp and PRDX2, were most likely to play an important role in programming of the fetal immune system in childhood allergy development.

CLIC3 (*CLIC3* gene), one of the proteins validated in Western blot, is increased in allergic airway inflammation and remodelling ⁽³⁴⁰⁾. Mammalian CLIC (p64-related) proteins are widely expressed, due to unusual dual localisation, as soluble and integral membrane proteins ⁽³⁴¹⁾. The main function of CLIC3 is as an inducer of ion transport in the nucleus and plasma membrane, as well as a regulator of channel ions in the immune system ^(342, 343). CLIC3 is highly expressed in placenta, followed by lung and heart, whereas low expression was detected in skeletal muscle, kidney and pancreas ^(343, 344). CLIC3 in eosinophils is associated with superoxide generation and respiratory burst, both are characteristic features of allergic airway inflammation and remodelling ⁽³⁴⁰⁾. In peripheral blood and nasal lavage, higher and differential expression of novel transcript variants of *CLIC3* gene (*CLC3b* and *CLC3e*) were detected in mild-to-moderate and moderate-to-severe asthmatic eosinophils, as compared to healthy non-atopic subjects ⁽³⁴⁰⁾. Membranous and intracellular (granular) expressions of *CLIC3* in nasal lavage and peripheral blood eosinophils support their involvement in activation and migration of eosinophils in allergic asthma ⁽³⁴⁰⁾. Moreover, high expression of *CLIC3* mRNA was detected in trophoblast cells, amnion, choriodecidua, syncytiotrophoblast and villous cytotrophoblast cells during the first and completed third trimester of gestation ^(343, 344). Placental *CLIC3* facilitates regulation of cellular processes associated with movement of chloride in placental and fetal membrane

cells, which may possibly contribute to the programming effect on the fetal immune system and allergic diseases ⁽³⁴⁴⁾.

In Western blot analysis conducted in this research, placental C3 protein expression was increased in allergic children with any rhinitis combination allergic phenotypes, as compared to children without an allergy, consistent with findings of Tomazic *et al* ⁽⁷⁰⁾. These authors also reported that C3 protein was upregulated in nasal mucus of patients with AR, analysed by LC-MS/MS and confirmed with Western blotting ⁽⁷⁰⁾, the same method used in this study. In association with other complement proteins, C3 forms a major host mechanism for detection and clearance of potential pathogens ⁽³⁴⁵⁾. C3 protein plays an immunoregulatory role at the interface of innate and adaptive immune systems ⁽³⁴⁶⁾. At the interface of innate immunity, pro-inflammatory and immunoregulatory products from C3 play a crucial role in the processes of phagocytosis, respiratory burst and airways inflammation ⁽³⁴⁷⁾. C3 is also pivotal in adaptive immunity ⁽³⁴⁸⁾. In chronic inflammation, C3 acts as a chemoattractant for neutrophils and causes histamine release from mast cells and basophilic leukocytes ⁽³⁴⁹⁾.

In response to allergen challenge, C3-deficient mice exhibit diminished AHR and lung eosinophilia, reduced numbers of IL-4-producing cells and attenuated responses of Ag-specific IgE and IgG ⁽³⁴⁸⁾. These C3-deficient mice had impaired ability to produce Th2 cytokines, thus asthmatic features were significantly attenuated in the absence of C3 ⁽³⁴⁸⁾. C3 was significantly elevated in children with intermittent atopic asthma, compared with children without asthma ⁽³⁵⁰⁾. A case-controlled study demonstrated that serum C3 was significantly higher in asthmatic children, compared to non-allergic children, with positive correlation observed between serum C3 and asthma severity ⁽³⁵¹⁾. These studies suggest that serum C3 is a reliable biomarker for asthma diagnosis. The possible mechanism involved is that with increasing asthma severity, systemic pro-inflammatory cytokines, like TNF- α and IL-1, may exert a stimulatory effect on the liver to produce increased amounts of C3, thus simultaneously creating a viscous cycle that augments asthma severity ⁽³⁵¹⁾.

The critical role of C3a in mediated AHR and airway tissue remodelling was observed in mouse models of allergic asthma ⁽³⁵²⁾. Derived from proteolytic degradation of C3, C3a is an anaphylatoxin that stimulates mast cell degranulation at inflammation sites, such as the airway of active asthma patients ⁽³⁵³⁾. C3a is a mediator of the local inflammatory process via expression of its receptor in nasal epithelial cells. It induces smooth muscle contraction, increases vascular permeability and stimulates histamine release from mast cells and basophils, causing mucosal swelling and exudation ^(70, 353). Thus, C3a plays a critical role in asthma, predominantly by regulating mast cell-airway smooth muscle cell interaction ⁽³⁵⁴⁾. Interestingly, T cell-derived C3 activation fragments, C3a and C3b, are required for human Th1, but not Th2 responses ⁽³⁴⁹⁾. For example, engagement of the complement regulator CD46 by its ligand C3b generated upon TCR activation is required for IFN- γ production due to lack of Th1

responses in CD46-deficient patients ⁽³⁴⁹⁾. Asthma susceptibility may also be influenced by the intrinsic abnormality in complement activation and generation of C3a and C5a which alter cellular responses, thus resulting in Th2 predominance ⁽³⁵⁵⁾.

Polymorphisms of C3 and C5 genes affect susceptibility to bronchial asthma ⁽³⁴⁶⁾. C3 protein was significantly more abundant and C5 protein was newly identified in mucus from AR patients ⁽⁷⁰⁾. Both C3 and C5 are markers of plasma exudation in acute reactions to allergen challenge and participate in the inflammatory process of AR via activation of the complement system ⁽⁷⁰⁾. In asthmatic patients, increased C3a and C5a peptide levels in serum and bronchoalveolar lavage fluid (BALF) and increased expression of their respective receptors support the hypothesis of involvement in asthma pathogenesis ⁽³⁵²⁾.

Hp (*HP* gene), an acute phase protein, is associated with asthma and AD ^(356, 357). Hp is expressed by the liver and secreted in plasma ⁽³⁵⁸⁾. The main immunological function of Hp is as an antioxidant, bacteriostatic and anti-inflammatory, and modulates acute phase response of infection ⁽³⁵⁸⁾. Hp was significantly altered in serum of patients with occupational medicamentosa-like dermatitis induced by trichloroethylene (OMLDT) ⁽³⁵⁶⁾. In this study, Hp levels were increased in the acute-stage of OMLDT patients with severe liver damage and were restored to normal level after healing, indicating that Hp may play a role in repairing damaged liver cells ⁽³⁵⁶⁾. Similarly, this data correlates with the finding that demonstrated Hp placental protein expression was significantly increased in allergic children and in allergic children with asthma combination, as compared with non-allergic children.

In the LC-MS/MS study, the antioxidant enzyme, Thioredoxin reductase, also known as PRDX3, had significant LFQ MS ratio in allergic male (2.03 fc), as compared to placenta of non-allergic male children, however this protein was not validated by Western blot (Refer Table 19, section 4.3.2). PRDX3 has been reported to have an association with pregnancies complication by asthma ^(359, 360). Our previous studies have identified significant changes in antioxidant enzymes in placenta of pregnancy complicated by asthma which include PRDX3, glutathione peroxidase and superoxide dismutase ^(359, 360). We have also identified that some pathways change sex specifically under certain complications, such as in preterm delivery and maternal asthma ^(361, 362). These data suggest alterations in antioxidant pathways of PRDX may affect development of the fetal immune system and contribute to increased risk of childhood asthma and allergy.

PRDXs have been reported to be increased in asthma and play a critical role in controlling intracellular oxidative stress in pathogenesis of asthma ⁽³⁶³⁾. PRDXs are a family of thiol-specific intracellular antioxidant proteins ⁽³⁶⁴⁾. To date, six PRDX family members in humans have been identified: PRDX1, 2, 3, 4, 5 and 6 ^(365, 366). These proteins exert a protective antioxidant role in cells through peroxidase

activity, whereby PRDX2 reduces mainly H₂O₂ and alkyl hydroperoxides to water and alcohol ⁽³⁶⁷⁾. PRDX2 (*PRDX2* gene) plays an important role in regulating intracellular concentrations of peroxides generated during metabolism and participates in signalling cascades of growth factors and TNF- α . PRDX1 maintains a low level of intracellular H₂O₂, while permitting higher levels during signal transduction ^(364, 367). The ratios of hyperoxidised to total PRDX (PRDX-SO₃/PRDX) in PMBC were significantly higher in asthma patients than in normal subjects, which correlated with degree of disease severity, with the highest ratio observed in severe asthma ⁽³⁶³⁾. Interestingly, in this current study, a similar finding was observed where PRDX2 placental protein expression was significantly increased in allergic children with asthma combination, as compared with non-allergic children.

Although the mechanism of hyperoxidised PRDX in pathogenesis of asthma is not fully understood, it was indicated that hyperoxidation of PRDX2 could control structural transitions of PRDX2, which correlates with cell-cycle arrest ⁽³⁶⁷⁾. Hyperoxidation of PRDX6 has been reported to increase Ca²⁺-independent phospholipase A2 (iPLA2) activity in H₂O₂-induced cellular toxicity ^(367, 368). iPLA2 is involved in regulation of arachidonic acid release from membrane phospholipids which affects eicosanoid formation and generates acute inflammation onset. Thus, data suggest that hyperoxidation of PRDX in asthmatics may be implicated in the pathogenesis of asthma through the cell-cycle arrest and iPLA2 pathway ^(363, 369, 370).

Other than hyperoxidation, PRDXs may influence immune system development that leads to greater susceptibility to allergy via activation of damage-associated molecular pattern (DAMP) and anti-inflammatory action ^(371, 372). Helminth and mammalian-secreted PRDX have the capacity to activate DAMPs which interact with pattern recognition receptors (PRRs) leading to an inflammatory state ⁽³⁷²⁾. In addition, helminth-secreted PRDX can influence development of Th2-responses via a mechanism involving induction of alternatively activated macrophages via Ym1-expressing macrophages, which include alveolar, splenic and bone marrow macrophages, and microglia in mouse ⁽³⁷²⁾. However, humans lack Ym1, and its closest homolog is the eosinophil chemotactic cytokine by sequence identity that is not upregulated by IL-4 ⁽³⁷¹⁾. Animal studies have demonstrated that PRDX1 and PRDX2 have anti-inflammatory roles in a murine asthma model ^(363, 365). Exogenous PRDX1 administered to PRDX-null mice protects against allergen-related Th2-type airway inflammation and hyperresponsiveness, partly via suppression of lung expression of IL-2 and regulation of the Th1/Th2 balance and its anti-oxidative properties ^(363, 365). In a study using PRDX2-null mice, findings indicate that TLR4 proteins were decreased by PRDX2 deletion, indicating PRDX2 was protective against development of allergic airway inflammation through reduced reactive oxygen species (ROS) via suppression of NF- κ B activation by the action of PRDX hyperoxidation ⁽³⁷³⁾.

PRDX proteins are known to be epigenetically modified in other situations, particularly in carcinogenesis. Epigenetic modifications, such as DNA methylation and histone modifications, have been reported to modulate expression of PRDXs. The use of epigenetic regulators, such as HDAC, have been demonstrated to restore PRDX to normal levels ⁽³⁶⁶⁾. In drug-resistant, non-small cell lung carcinoma cells, *PRDX2* promoter was hypermethylated in A549 lung adenocarcinoma cells, where *PRDX2* was frequently downregulated ⁽³⁷⁴⁾. Interestingly, demethylation was observed in the same study in A549 gefitinib-resistant cells which highly expressed *PRDX2*, demonstrating that methylation status correlates to gene activity. In oligodendroglial tumours, *PRDX1*-associated CpG islands were frequently hypermethylated in 1p/19q-deleted primary tumours cells and expressed reduced *PRDX1* mRNA transcripts ^(366, 375). Furthermore, DNA hypermethylation and H3 histone hypoacetylation at *PRDX2* promoter was correlated with repressed tumour suppressor *PRDX2* expression in acute myeloid leukaemia ^(366, 376). These studies indicate that PRDX proteins can be epigenetically modified, thus similar epigenetic modifications may occur in the mechanism of childhood allergic susceptibility.

Based on current findings, the premise of this thesis is that placental proteins are interconnected and may influence immune function development in relation to allergy. Thus, the proposed model of placental protein mechanisms underlying increased susceptibility to allergy is as depicted in Figure 76. *In utero* exposure, such as maternal asthma, folate intake and nicotine exposure during pregnancy, are associated with epigenetic modifications and partly modulate fetal programming in modifying allergy risk. Placental proteins underlying increased susceptibility to allergy include 14-3-3, Hp, angiotensinogen, apolipoproteins, cholesterol side-chain cleavage enzyme, CLIC3, *PRDX2* and C3. Thus, placental proteins may be epigenetically modified via *in utero* exposures in regulating immune system development that leads to a greater allergy susceptibility.

PRDX2 and Hp placental proteins can be utilised to detect children with an allergy in the general population and allergic children with asthmatic mothers (high risk of allergy), as compared to non-allergic children. The data indicates there may be gene-specific mechanisms that drive allergic susceptibility which vary depending on maternal environment, such as presence or absence of maternal asthma ⁽¹⁹³⁾. The presence of maternal allergy, especially maternal asthma, is a significant risk factor that increases likelihood of offspring developing childhood allergy ⁽¹⁹³⁾. Furthermore, pregnancies complicated by asthma can have a significant effect on placental gene expression and potentially contribute to an atopic phenotype in offspring ⁽¹³²⁾.

A good and reliable protein biomarker would be able to detect allergic children from a population of non-allergic children in both sexes, thus must not be limited to a specific sex. Therefore, *PRDX2* and Hp placental proteins are good potential biomarkers that may detect allergic children regardless of child sex or genetic risk in a general population.

Based on a series of studies in placentae, this research identified gene and protein profiles of placentae from children with and without an allergy using a proteomic approach, identified novel proteins that vary in relation to allergy and validated the findings with several techniques in a larger population. Current findings support the first hypothesis that childhood susceptibility to allergy may be programmed *in utero* due to placental adaptation to maternal environment, and susceptibility to allergy may be identifiable through alterations in placental gene and proteomic profiles. Moreover, findings support that the altered placental gene-protein expression varies in presence or absence of maternal asthma and in a sex-specific manner.

The next step of this research is to investigate whether altered expression of specific placental proteins are detectable in saliva in early childhood.

5.0 EXAMINATION OF SALIVARY PROTEINS TO IDENTIFY ASSOCIATION WITH CHILDHOOD ALLERGY

5.1 Introduction

Saliva has great potential to replace serum as a biological fluid biomarker in diagnosing clinical allergy, especially in infants, toddlers, children, anxious and uncooperative patients ⁽²⁶⁰⁾. Saliva is an easily accessible, non-invasive, real time measurement of the ongoing human physiological system and consists of an abundance of biomarkers, such as genetic material and proteins ⁽²⁵⁷⁻²⁵⁹⁾.

5.1.1 Saliva as potential diagnostic fluid in childhood allergy

Previously, there has been controversy related to use of saliva as a diagnostic fluid due to its low concentration of analytes in comparison with blood. Nevertheless, technological advances, increased sensitivity of molecular methods and nanotechnologies, such as mass spectrometry (MS), have reduced these limitations ⁽²⁷¹⁾. The advantages of using saliva as diagnostic fluid are listed in Table 22 ⁽²⁶⁰⁾.

Approximately 2700 salivary proteins have been identified and this number is expected to increase dramatically in the future due to current advances in detection methods for markers in saliva ^(257, 377). Saliva proteins identified to date have been categorised according to their functions (Table 23). The main function is immunity that contributes 21 % of all known salivary proteins ⁽²⁶¹⁾. This suggests that this particular fluid is a suitable biological fluid for identifying a biomarker of allergic risk, which is the focus of this thesis.

Table 22. Advantages of salivary testing for diagnosis ⁽²⁶⁰⁾.

Advantages of salivary testing for diagnosis	
Non-invasive, easy to use, inexpensive	
Safer to administer than serum sampling (no needles)	
Real-time diagnostic values	
No need for trained medical staff	
Multiple samples can be obtained easily	
Collection and screening can be done at home	
Minimal risks of cross-contamination	
More economical sampling, shipping and storage compared to serum	
Requires less manipulation during diagnostic procedures compared to serum	
Commercial availability of screening assays	

Table 23. Saliva proteins identified according to functions. Table adapted from ⁽²⁶¹⁾.

Salivary protein percentage	Salivary functions
28.7%	Uncertain function
21.0%	Immunity
15.4%	Unknown function
9.7%	Signal transduction
7.1%	Cytoskeleton and endomembrane
5.2%	Metabolism
4.8%	Cell mobility and secretion
4.2%	Cell multiplication and cell cycle
2.3%	Transcription and ribosomes
1.6%	Protein replication and reparation

Most diseases, such as cancer and allergy, are likely to be treated efficiently if the disease can be diagnosed early. For example, ovarian cancer, the fifth most common cancer and cause of death in females, has a 25 % five-year-survival rate when detected at stage 4, in comparison to 93 % if diagnosed at stage 1 ⁽³⁷⁸⁾. Saliva has been used widely to detect a variety of diseases using proteomic approaches, including head and neck carcinoma (oral cavity, oropharynx, larynx and salivary glands), breast and gastric cancers, salivary gland function and disease, Sjögren syndrome, systemic sclerosis, dental and gingival pathology, preeclampsia and psychiatric and neurological diseases ^(257, 261-265) (Tables 24 and 25). Therefore, the hypothesis for this chapter was that childhood susceptibility to allergy can be detected in saliva. The purpose of this series of studies in saliva was to examine the protein profile of saliva in children with and without an allergy using a proteomic approach to identify novel proteins that vary in relation to allergy and validate findings with several techniques in a larger population.

The specific aims for this work were:

- 1) To optimise a proteomics method for identification of salivary proteins associated with allergic disease in childhood.
- 2) To determine if saliva is a biological fluid that can be used to detect allergy in susceptible children.
- 3) To determine whether proteomic profile varies with different allergic phenotypes.
- 4) To identify whether there is a common protein present for all allergic phenotypes.

Table 24. Salivary proteins involved in oncological pathologies ⁽²⁵⁷⁾.

Author, Year	Proteins involved	Proteins not involved	Site of tumour
Nakashima et al., 2006 ²⁶	Maspin Stathmin		Salivary gland
Contucci et al., 2005 ²⁷		Statherin	Salivary gland
An et al., 2004 ²⁸	Transketolase Dim1p v-Ha-ras oncogene type I collagen pro alpha Tumor necrosis factor pirin		Salivary gland with metastasis
Oshiro et al., 2007 ³²	Alpha-1-B-glycoprotein Complement factor B proteins	Cystatin S Parotid secretory factor Poly-4-hydrolase beta-subunit proteins	Head and neck
Dowling et al., 2008 ³³	Beta fibrin S100 calcium binding protein Transferrin IG heavy chain constant region γ Cofilin-1 Transthyretin		Head and neck
Mizukawa et al., 2001 ³⁵	Alpha-defensins Beta-defensins		Oral cavity
Pickering et al., 2007 ³⁶	Endothelins		Oral cavity
Contucci et al., 2005 ²⁷	Statherins		Oral cavity
Wong, 2006 ³⁷	Interleukin-8 (IL-8) Thioredoxin		Oral cavity
Streckfus et al., 2000 ⁴¹	c-erbB-2 protein CA15-3		Breast
Tabak, 2001 ⁴²	15-3 cancer antigen		Breast

Table 25. Salivary proteins involved in dental pathologies ⁽²⁵⁷⁾.

Author, Year	Proteins	Related pathology
Van Nieuw et al., 2004 ⁴⁹ ; Dowd, 1999 ⁵⁰	Mucins Proline-rich glycoprotein Statherin	Dental caries
Vitorino et al., 2006 ⁵³	Proline-rich proteins Lipocalin Cystatins Amylase Immunoglobulin a Lactoferrin	Dental caries
Rudney et al., 2009 ⁶⁰	Statherin Truncated cystatin S	Dental caries and other diseases
Ito et al., 2008 ⁵⁴	Cystatins Lysozyme	Periodontitis
Fábián et al., 2007 ⁵⁵ ; 2008 ⁵⁶	Immunoglobulin Molecular chaperone hsp70 Cystatin S Salivary amylase Calprotectin Histatins Lysozyme Lactoferrin Defensins Peroxidases Proline-rich proteins Mucins	Periodontitis
Kibayashi et al., 2007 ⁵⁷	Prostaglandin E(2) Lactoferrin Albumin Aspartate aminotransferase lactate dehydrogenase Alkaline phosphatase	Periodontitis
Nishida et al., 2006 ⁵⁸	IL-1 beta Albumin Aspartate aminotransferase	Periodontitis

5.2 Proteomic analysis of saliva in LC-MS/MS

5.2.1 Characteristics of the cohort children and maternal asthma status in LC-MS/MS

Samples of child saliva for LC-MS/MS were part of an existing prospective cohort from the Asthma and Pregnancy Cohort study that was designed to examine the effects of asthma during pregnancy on the mother, placenta and baby (refer Methodology section 2.1.1). The cohort consisted of women with and without asthma that were recruited at the first antenatal visit from the Lyell McEwin Hospital antenatal clinics between May 2009 and May 2012. They consented to take part in a non-interventional prospective asthma study evaluating the impact of maternal asthma on perinatal outcomes ⁽²⁷⁵⁻²⁷⁷⁾. The saliva of children from this cohort was collected at follow up visits between 6 months and 3 years of age. Children were followed up from 6 months of age and allergic status identified using a modified ISAAC questionnaire.

LC-MS/MS was performed using 18 child saliva samples. The mean age, weight and height for the population of children was 19.83 months, 10.22 kg and 75.68 cm, respectively. There was a significant difference between male and female children in relation to weight (Mann Whitney U test, $P = 0.036^*$) but not in height and age. There were 3 child saliva samples from each allergic phenotype of either asthma; eczema; and rhinitis in combination. Rhinitis often occurs as a co-morbidity with other allergies and rarely found to be present alone. Therefore, I selected rhinitis in combination with asthma and eczema, as this was the most common combination in young children. All 3 rhinitis in combination and 3 eczema phenotypes were male whilst all 3 asthma phenotypes are female. For control or non-allergic children, there were 5 males and 4 females. Allergy combination is the presence of a specific allergy, for example rhinitis, in one specific phenotype or in combination with other allergies. Therefore rhinitis combination consists of a group of children with rhinitis; asthma and rhinitis; eczema and rhinitis; and asthma, eczema and rhinitis. The majority of children with an allergy had a mother with asthma (high risk of allergy) (66.67 %), whereas most children without an allergy had a mother who was non-asthmatic (low risk of allergy) (100 %). Characteristics of the cohort children and maternal asthma status in LC-MS/MS are summarised in Table 26.

Table 26. Characteristics of the cohort children and maternal asthma status in LC-MS/MS. (Significant result was $P < 0.05$).

Child characteristics	Males	Females	Both sexes	* Significance differences between males and females
Age in months (mean $\pm$ SE)	20.64 $\pm$ 5.08	18.57 $\pm$ 4.93	19.83 $\pm$ 3.66	$p = 0.8731$
Weight in kg (mean $\pm$ SE)	11.5 $\pm$ 1.04	8.29 $\pm$ 0.57	10.22 $\pm$ 0.78	$p = 0.0360^*$
Height in cm (mean $\pm$ SE)	76.29 $\pm$ 2.15	74.63 $\pm$ 2.09	75.68 $\pm$ 1.72	$p = 0.6818$
Number of asthma (n)	0	3	3	NA
Number of eczema (n)	3	0	3	NA
Number of rhinitis (n)	0	0	0	NA
Number of asthma and eczema (n)	0	0	0	NA
Number of asthma and rhinitis (n)	2	0	2	NA
Number of eczema and rhinitis (n)	1	0	1	NA
Number of asthma, eczema and rhinitis (n)	0	0	0	NA
Number of asthma combination (n)	2	3	5	NA
Number of eczema combination (n)	4	0	4	NA
Number of rhinitis combination (n)	3	0	3	NA
Number of allergic children (n)	6	3	9	NA
Number of non-allergic children (n)	5	4	9	NA
% of asthmatic mothers (high risk of allergy) in allergic children	66.67	66.67	66.67	NA
% of asthmatic mothers (high risk of allergy) in non-allergic children	0	0	0	NA
% of non-asthmatic mothers (low risk of allergy) in allergic children	33.33	33.33	33.33	NA
% of non-asthmatic mothers (low risk of allergy) in non-allergic children	100	100	100	NA

* Data were analysed by Mann-Whitney test with two-tailed p-value.

° Abbreviations: NA represents non-applicable.

5.2.2 Identification of salivary proteins associated with allergy

From MaxLFQ and Mascot software analysis, 177 proteins were identified to be present in childhood saliva samples with allergic diseases including asthma, eczema and rhinitis in combination (Appendix 10, Table 3). Out of these 177 proteins, 111 proteins had more than two unique peptides (UP). A UP is defined as a peptide, irrespective of its length, that exists only in one protein of a proteome of interest, despite the fact that this peptide may appear more than once in the same protein ⁽³¹⁹⁾. Therefore, proteins that had more than 2 UP had a higher probability of being a genuine protein, and not a false positive result.

Using the label free quantification (LFQ) volume intensity in the MaxLFQ software, fold change (fc) ratio was calculated between LFQ intensity of allergic sample to LFQ intensity of non-allergic sample. However, LFQ intensity may be zero (0) when there is no calculated ratio existing from peptides in sample, hence generating the zero protein LFQ intensity and uncalculated ratio (refer Placenta section 4.3.2) ⁽³⁰¹⁾. The summary of LC-MS/MS results with significant LFQ ratios (ratio > 2 or < 0.5 fc) for saliva samples from the children are shown in Table 27 and the summary of LC-MS/MS result with uncalculated LFQ ratio for child saliva samples are shown in Table 28.

From the fc LFQ intensity ratio, there were 46 proteins identified with significant ratios (ratio > 2 or < 0.5 fc) when compared to non-allergic group in child saliva samples. The asthma group had the highest number of proteins with ratio of > 2 fc, while rhinitis group had the highest protein ratio of < 0.5 fc (Figure 77). There were three proteins that were identified to be present in all three allergic phenotypes of asthma, eczema and combination with rhinitis, including human mucin-5B and 5AC (ratio of >2 fc), human serum albumin and human serotransferrin (ratio <0.5 fold fc relative to non-allergic samples) (Table 27 and Figure 78). The saliva samples collected from children were not contaminated with blood. However, based on the selection criteria for proteins that are suitable candidates, human mucin-5B and 5AC and human serum albumin are commonly observed in saliva and are therefore not suitable candidates for salivary protein allergy biomarkers (refer Methodology section 2.3.2). Proteins found to be present in both asthma and eczema phenotype only, is cystatin-S and histatin-1 (Table 27). In addition, three proteins were identified to be present in rhinitis and eczema only: antileukoproteinase (ALP), peptidyl-prolyl cis-trans isomerase A and transthyretin (Table 27). From these 46 proteins, eight potential salivary proteins were chosen based on the selection criteria to be further validated as potential allergic biomarkers using Western blot in a larger sample size (n= 62).

Table 27. Summary of child salivary protein identification with the significant label-free quantification (LFQ) ratios in specific allergic phenotypes in LC-MS/MS.
(Significant ratio > 2 or < 0.5 fold change).

Swiss-Prot entry	Entry name	Protein names	Gene names	Sequence length (bp)	UP	Unique seq cov [%]	MW [kDa]	Ratio LFQ MS A/C	Ratio LFQ MS E/C	Ratio LFQ MS R/C
P02768	ALBU_HUMAN	Serum albumin	ALB	609	78	96.10	69.37	0.27	0.42	0.31
Q9HC84	MUC5B_HUMAN	Mucin-5B (Cervical mucin)	MUC5B	5762	47	12.50	596.33	3.11	3.12	3.25
P98088	MUC5A_HUMAN	Mucin-5AC (Gastric mucin)	MUC5AC	5654	47	12.50	596.33	3.11	3.12	3.25
P02787	TRFE_HUMAN	Serotransferrin (Transferrin)	TF	698	27	27.50	77.08	0.04	0.14	0.10
P15515	HIS1_HUMAN	Histatin-1	HTN1	57	4	43.90	6.96	8.42	5.18	1.04
P01036	CYTS_HUMAN	Cystatin-S	CST4	141	4	43.90	6.96	8.42	5.18	1.04
P02766	TTHY_HUMAN	Transthyretin	TTR	147	6	41.50	15.89	0.00	0.36	0.27
P62937	PPIA_HUMAN	Peptidyl-prolyl cis-trans isomerase A	PPIA	165	5	37.60	18.01	1.26	2.61	2.45
P03973	SLPI_HUMAN	Antileukoproteinase (ALP)	SLPI	132	5	32.60	14.33	1.62	0.25	0.34
Q9UBG3	CRNN_HUMAN	Cornulin (53 kDa putative calcium-binding protein)	CRNN	495	16	50.30	53.56	2.08	1.98	1.11
P09228	CYTT_HUMAN	Cystatin-SA	CST2	141	14	77.30	16.45	0.44	1.13	0.58
P31025	LCN1_HUMAN	Lipocalin-1	LCN1	176	9	43.80	19.25	5.69	0.00	0.00
Q8TAX7	MUC7_HUMAN	Mucin-7 (Salivary mucin-7)	MUC7	377	7	14.60	39.16	6.93	1.21	1.29
P04792	HSPB1_HUMAN	Heat shock protein beta-1	HSPB1	205	7	48.30	22.78	2.22	1.30	0.87
P06702	S10A9_HUMAN	Protein S100-A9 (Calgranulin-B)	S100A9	114	7	50.00	13.21	2.04	0.60	0.89
P23280	CAH6_HUMAN	Carbonic anhydrase 6	CA6	308	5	23.70	35.37	0.37	0.71	0.00
Q6MZM9	PRR27_HUMAN	Proline-rich protein 27	PRR27	219	3	23.70	22.72	4.41	0.67	0.00
P28325	CYTD_HUMAN	Cystatin-D (Cystatin-5)	CST5	142	3	38.70	16.08	2.49	1.31	0.00
P02808	STAT_HUMAN	Statherin	STATH	62	3	54.80	7.30	2.17	1.55	1.77
P27482	CALL3_HUMAN	Calmodulin-like protein 3 (CLP)	CALML3	149	2	31.50	16.89	2.16	0.00	0.00
Q504X8	Q504X8_HUMAN	SMR3B protein	SMR3B	106	2	65.80	8.19	2.07	1.62	0.74
P61626	LYSC_HUMAN	Lysozyme C	LYZ	148	15	77.00	16.54	1.49	0.32	0.53

P00738	HPT_HUMAN	Haptoglobin (Zonulin)	HP	406	8	19.60	44.93	0.00	0.07	0.00
A8K2U0	A2ML1_HUMAN	Alpha-2-macroglobulin-like protein 1	A2ML1	1454	7	6.10	161.10	0.00	0.29	0.00
P04217	A1BG_HUMAN	Alpha-1B-glycoprotein	A1BG	495	6	30.10	35.24	0.00	0.12	0.00
P18510	IL1RA_HUMAN	Interleukin-1 receptor antagonist protein	IL1RN	177	5	41.30	16.14	0.00	0.42	0.61
P19971	TYPH_HUMAN	Thymidine phosphorylase	TYMP	482	2	9.10	49.96	0.00	3.85	0.00
Q04917	1433F_HUMAN	14-3-3 protein eta	YWHAH	246	2	21.20	5.86	0.00	2.50	0.00
P02788	TRFL_HUMAN	Lactotransferrin (Lactoferrin)	LTF	710	37	59.50	78.38	1.29	0.63	0.11
P01037	CYTN_HUMAN	Cystatin-SN	CST1	141	15	70.20	16.39	0.74	0.58	0.46
P54108	CRIS3_HUMAN	Cysteine-rich secretory protein 3	CRISP3	245	6	23.30	27.63	1.47	1.03	0.35
Q6P5S2	LEG1H_HUMAN	Protein LEG1 homolog	LEG1	330	6	13.30	37.93	1.52	1.68	0.48
P07476	INVO_HUMAN	Involucrin	IVL	585	5	12.60	68.48	0.00	0.81	0.41

° Abbreviations: UP represents unique peptides, Unique seq cov represents unique sequence coverage, LFQ MS represents label-free quantification mass spectrometry, C represents control subjects who are children with no allergy, A represents children with asthma, E represents children with eczema and R represents children with rhinitis.

° Bold number represents significant ratio (ratio > 2 or < 0.5 fold change).

Table 28. Summary of child salivary protein identification with the uncalculated label-free quantification (LFQ) ratios in specific allergic phenotypes in LC-MS/MS.

Swiss-Prot entry	Entry name	Protein names	Gene names	Sequence length (bp)	UP	Unique seq cov [%]	MW [kDa]	Asthma	Eczema	Rhinitis
P14618	KPYM_HUMAN	Pyruvate kinase PKM	PKM	531	2	27.2	16.43	Yes	Yes	Yes
O15397	IPO8_HUMAN	Importin-8	IPO8	1037	2	3.3	119.94	Yes	–	–
P20061	TCO1_HUMAN	Transcobalamin-1	TCN1	433	2	11.3	48.21	Yes	–	–
P47929	LEG7_HUMAN	Galectin-7	LGALS7	136	2	30.9	15.08	Yes	–	–
Q02413	DSG1_HUMAN	Desmoglein-1 (Cadherin family member 4)	DSG1	1049	2	3.1	113.75	Yes	Yes	–
P47710	CASA1_HUMAN	Alpha-S1-casein	CSN1S1	185	3	14.6	21.67	–	Yes	–
P00709	LALBA_HUMAN	Alpha-lactalbumin	LALBA	142	3	37.3	16.23	–	Yes	–
Q6F113	H2A2A_HUMAN	Histone H2A type 2-A	HIST2H2AA3	130	2	29.5	14.10	–	Yes	–
Q16777	H2A2C_HUMAN	Histone H2A type 2-C	HIST2H2AC	129	2	29.5	13.99	–	Yes	–
Q08380	LG3BP_HUMAN	Galectin-3-binding protein (Basement membrane autoantigen p105)	LGALS3BP	585	2	14.8	46.42	–	Yes	–
P02750	A2GL_HUMAN	Leucine-rich alpha-2-glycoprotein	LRG1	347	2	10.6	36.49	–	Yes	–
Q8WZ42	TITIN_HUMAN	Titin	TTN	34350	2	0.1	3713.70	–	Yes	–
P06396	GELS_HUMAN	Gelsolin	GSN	782	2	9.5	52.37	–	Yes	–
Q6TDU7	CASC1_HUMAN	Protein CASC1	CASC1	716	2	9.5	83.16	–	Yes	–
P05814	CASB_HUMAN	Beta-casein	CSN2	226	2	11.9	25.38	–	Yes	–
P01593	KV125_HUMAN	Immunoglobulin kappa variable 1D-33	IGKV1D-33	117	2	42.1	12.85	–	Yes	Yes
P01599	KV118_HUMAN	Immunoglobulin kappa variable 1-17	IGKV1-17	117	2	42.1	12.78	–	Yes	Yes
P01597	KV108_HUMAN	Immunoglobulin kappa variable 1-39	IGKV1-39	117	2	42.1	12.74	–	Yes	Yes
Q16610	ECM1_HUMAN	Extracellular matrix protein 1	ECM1	540	4	13.3	60.67	–	–	Yes
P32926	DSG3_HUMAN	Desmoglein-3	DSG3	999	4	5.8	107.50	–	–	Yes
P04083	ANXA1_HUMAN	Annexin A1	ANXA1	346	3	45.2	12.64	–	–	Yes
P14174	MIF_HUMAN	Macrophage migration inhibitory factor (MIF)	MIF	115	2	11.3	12.48	–	–	Yes

Proteins in allergic phenotypes that have LFQ ratios of >2 or <0.5 fold change (fc) as compared with non-allergic group

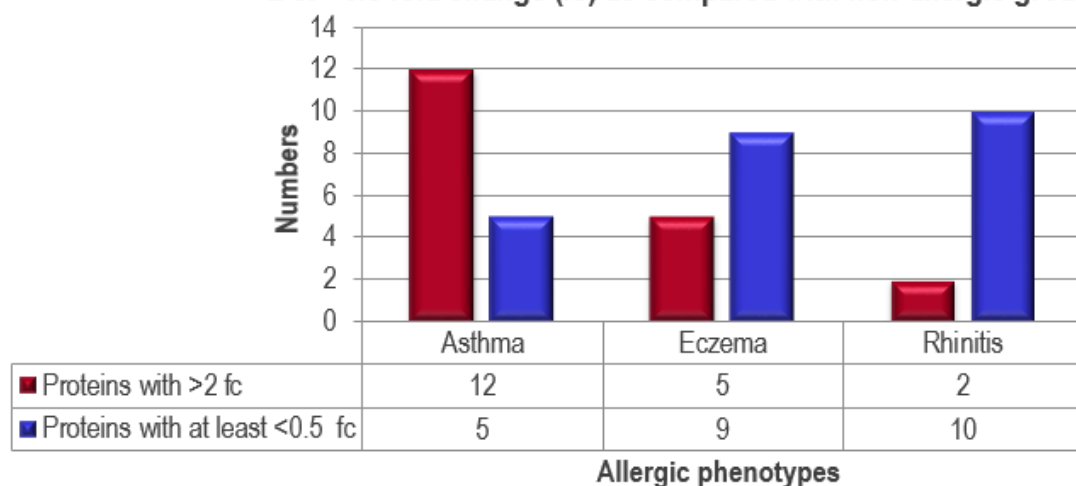


Figure 77. Salivary proteins in allergic phenotypes that have LFQ ratio of >2 or <0.5 fold change (fc) as compared with non-allergic group (Max-LFQ protein analysis). There were 46 proteins present in significant LFQ ratio (ratio > 2 or < 0.5 fc) when compared to non-allergic group.

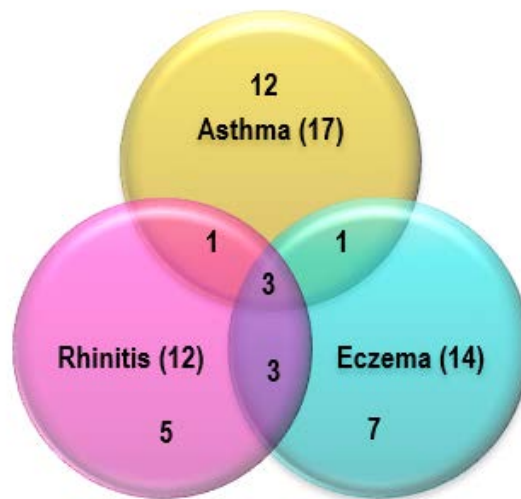


Figure 78. Venn diagram showing salivary protein distribution with significant ratios > 2 or < 0.5 fold change in relation to allergic phenotypes in child saliva samples.

5.3 Validation study for child saliva samples with Western blot

5.3.1 Characteristics of the cohort children and maternal asthma status in Western blot

Child saliva samples used in the validation studies were derived from the Asthma and Pregnancy Cohort study and prospectively collected (refer Methodology section 2.1.1).

There were 24 saliva samples from non-allergic children and 38 saliva samples from children with allergy (n= 62). The mean age, weight and height of the children was 21.56 months, 10.96 kg and 76.65 cm, respectively. Males were significantly heavier than females (Mann Whitney U test, $P= 0.021^*$) but not different in terms of height or age (Table 29). Characteristics of the children and maternal asthma status are summarised in Table 29.

Table 29. Cohort characteristics for validation studies with Western blot. (Significant result was $P < 0.05$).

Child characteristics	Males	Females	Both sexes	* Significance differences between males and females
Age in months (mean $\pm$ SE)	24.52 $\pm$ 3.22	20.55 $\pm$ 3.21	21.66 $\pm$ 2.29	p= 0.6302
Weight in kg (mean $\pm$ SE)	12.08 $\pm$ 0.73	9.62 $\pm$ 0.60	11.07 $\pm$ 0.53	p= 0.0208*
Height in cm (mean $\pm$ SE)	79.90 $\pm$ 3.08	73.50 $\pm$ 1.60	76.99 $\pm$ 1.86	p= 0.1465
Number of asthma (n)	4	8	12	NA
Number of eczema (n)	1	2	3	NA
Number of rhinitis (n)	0	1	1	NA
Number of asthma and eczema (n)	6	3	9	NA
Number of asthma and rhinitis (n)	2	0	2	NA
Number of eczema and rhinitis (n)	3	1	4	NA
Number of asthma, eczema and rhinitis (n)	3	4	7	NA
Number of asthma combination (n)	15	15	30	NA
Number of eczema combination (n)	13	10	23	NA
Number of rhinitis combination (n)	8	6	14	NA
Number of allergic children (n)	19	19	38	NA
Number of non-allergic children (n)	14	10	24	NA
% of asthmatic mothers (high risk of allergy) in allergic children	47.37	42.11	44.74	NA
% of asthmatic mothers (high risk of allergy) in non-allergic children	7.14	30.00	16.67	NA
% of non-asthmatic mothers (low risk of allergy) in allergic children	52.63	57.89	55.26	NA
% of non-asthmatic mothers (low risk of allergy) in non-allergic children	92.86	70.00	83.33	NA

* Data were analysed by Mann-Whitney test with two-tailed p-value.

° Abbreviations: NA represents non-applicable.

5.3.2 Western blot validation study using saliva samples from children with and without an allergy

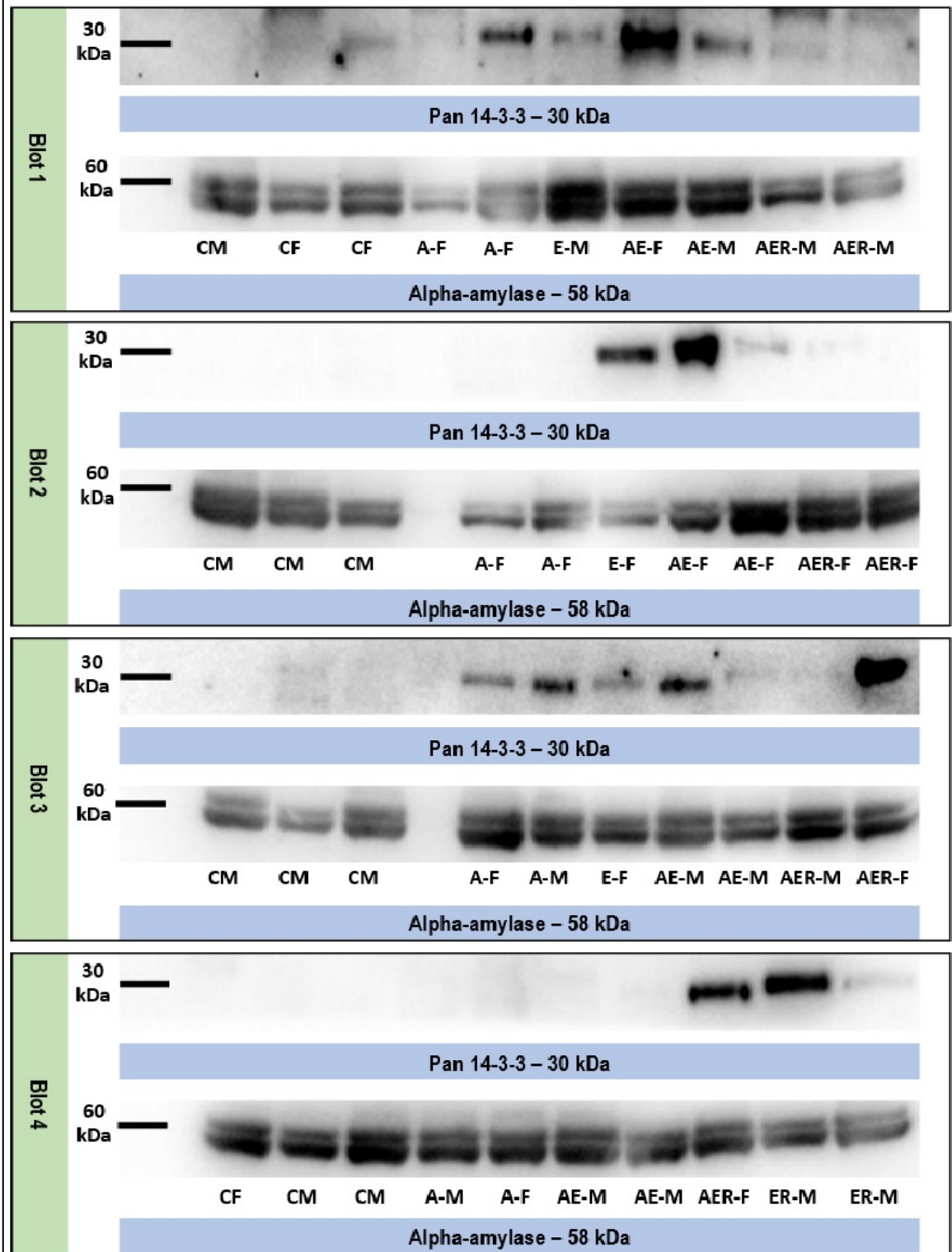
I validated and measured six salivary proteins that included pan 14-3-3, cornulin (CRNN), transferrin, involucrin, Hp and desmoglein 1 in saliva samples of children with and without an allergy using Western blot. Each of these proteins were normalised with the housekeeping protein, alpha amylase, and band densitometry analyses were performed in relation to general allergy, the presence or absence of maternal asthma during pregnancy; child sex, and child allergy phenotypes (refer Methodology section 2.12.1 and Optimisation section 3.3.3).

5.3.2.1 Expression of pan 14-3-3 protein in the saliva of children with and without an allergy

Salivary pan 14-3-3 protein was highly expressed in all three of the allergic samples with eczema using Western blot (Figure 79). When comparing the difference in protein expression between children without an allergy to children with allergies, pan 14-3-3 (Mann Whitney U test, $P = 0.001^{***}$ and $P = 0.006^{**}$) was significantly increased in child saliva with an allergy, regardless of sex and in female sex (Figures 80 and 83). Interestingly, the salivary pan 14-3-3 salivary protein expression was influenced by both the presence (high risk of allergy) and the absence (low risk of allergy) of maternal asthma. Pan 14-3-3 (Mann Whitney U test, $P = 0.030^{*}$) was significantly increased in the allergic child saliva of asthmatic mothers (high risk of allergy) regardless of sex as compared to non-allergic child saliva of asthmatic mothers (Figure 81). Moreover, allergic children with non-asthmatic mothers (low risk of allergy) had significant salivary pan 14-3-3 (Mann Whitney U test, $P = 0.019^{*}$) proteins expression regardless of sex as compared to non-allergic children with non-asthmatic mothers (Figure 82).

There was significant increases in the pan 14-3-3 (Mann Whitney U test, $P = 0.002^{**}$, $P = 0.002^{**}$ and $P = 0.001^{***}$) salivary proteins expression in children with any asthma, any eczema and any rhinitis combination regardless of sex as compared to children without an allergy (Figure 84). Allergy combination is the presence of a specific allergy, for example asthma, in one specific phenotype or in combination with other allergies. Thus, asthma combination consists of a group of children with asthma; asthma and eczema; asthma and rhinitis; and asthma, eczema and rhinitis. There was no significant differences of salivary pan 14-3-3 protein expression in other groups.

Pan 14-3-3: Ratio LFQ MS ↑ in eczema



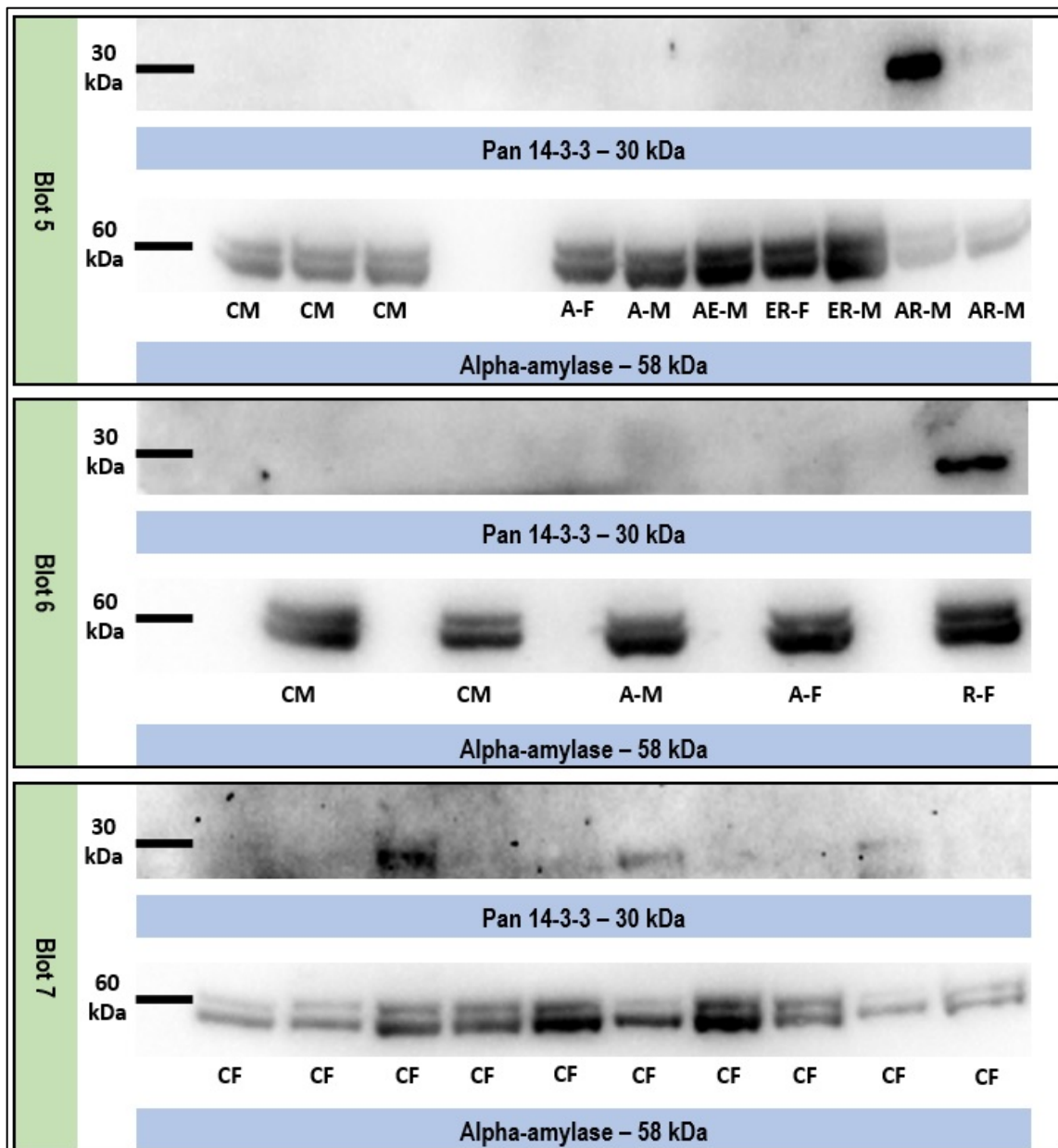


Figure 79. Representative Western blot of pan 14-3-3 protein expression in the saliva samples of children with ($n= 38$) and without ($n= 24$) an allergy. Pan 14-3-3 protein was detected at 30 kDa. (M represents male children, F represents female children, C represents control subjects who are children with no allergy, A represents children with asthma, E represents children with eczema and R represent children with rhinitis).

i. Children with allergy vs children with no allergy

Pan 14-3-3 protein expression in children with and without an allergy

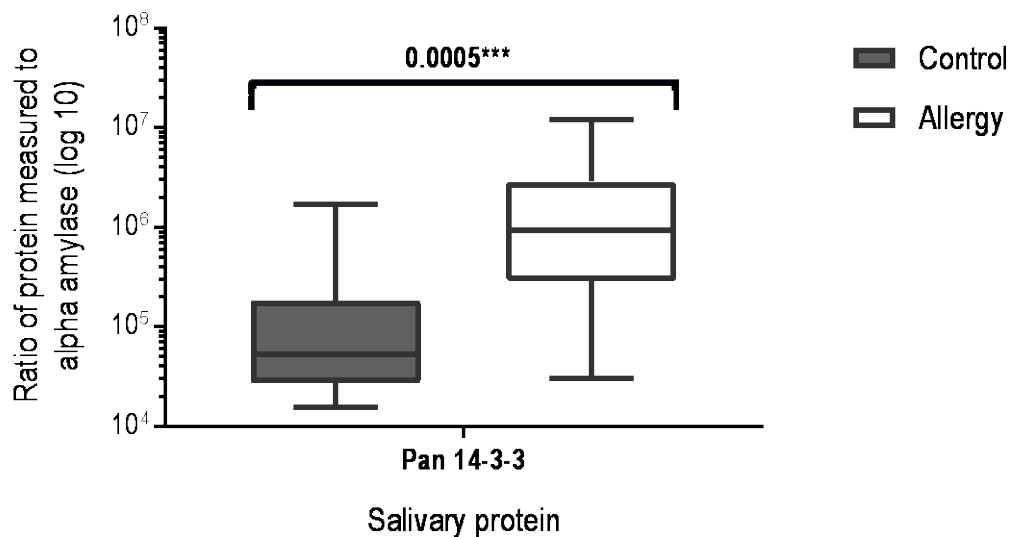


Figure 80. Pan 14-3-3 protein expression in children with (n= 23) and without (n= 9) an allergy. Protein expression was examined using Western blot. Proteins were expressed relative to alpha amylase. Data of pan 14-3-3 protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

ii. The influence of the presence and absence of maternal asthma during pregnancy on salivary protein expression and child allergy

a. Children of asthmatic mothers (high risk of allergy) with and without an allergy

Pan 14-3-3 protein expression in children of asthmatic mothers (AM) with and without an allergy

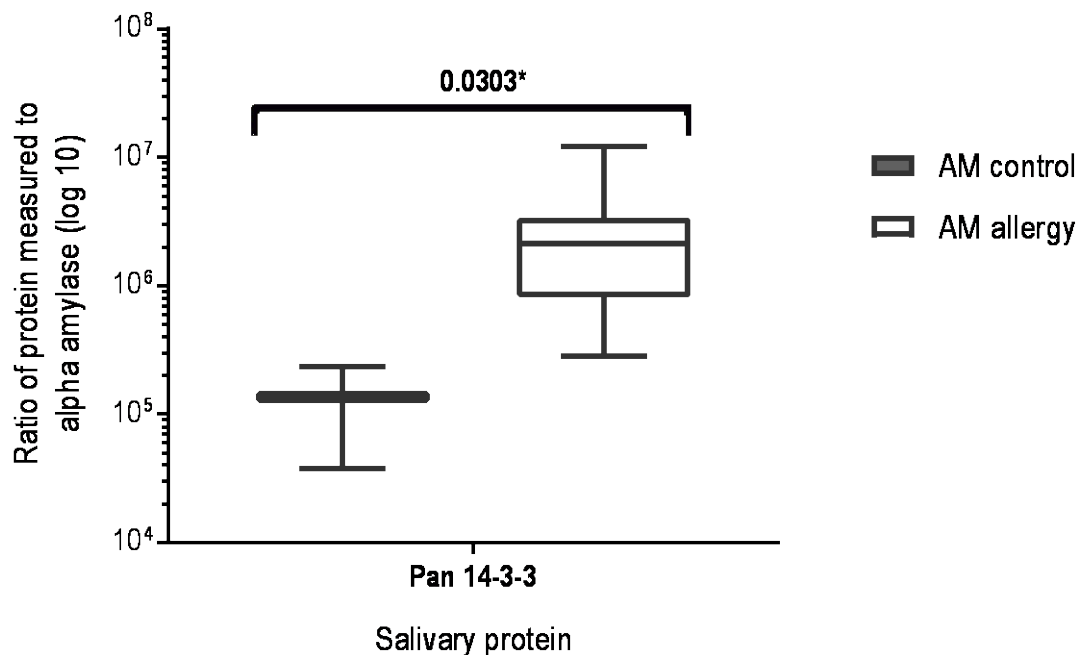


Figure 81. Pan 14-3-3 protein expression in children of asthmatic mothers (high risk of allergy) with (n= 10) and without (n= 3) an allergy. Protein expression was examined using Western blot. Proteins were expressed relative to alpha amylase. Data of pan 14-3-3 protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

b. Children of non-asthmatic mothers (low risk of allergy) with and without an allergy

Pan 14-3-3 protein expression in children of non-asthmatic mothers (NAM) with and without an allergy



Figure 82. Pan 14-3-3 protein expression in children of non-asthmatic mothers (low risk of allergy) with (n= 13) and without (n= 7) an allergy. Protein expression was examined using Western blot. Proteins were expressed relative to alpha amylase. Data of pan 14-3-3 protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

iii. Effect of sex on salivary protein expression and child allergy

a. Children with allergy vs children with no allergy

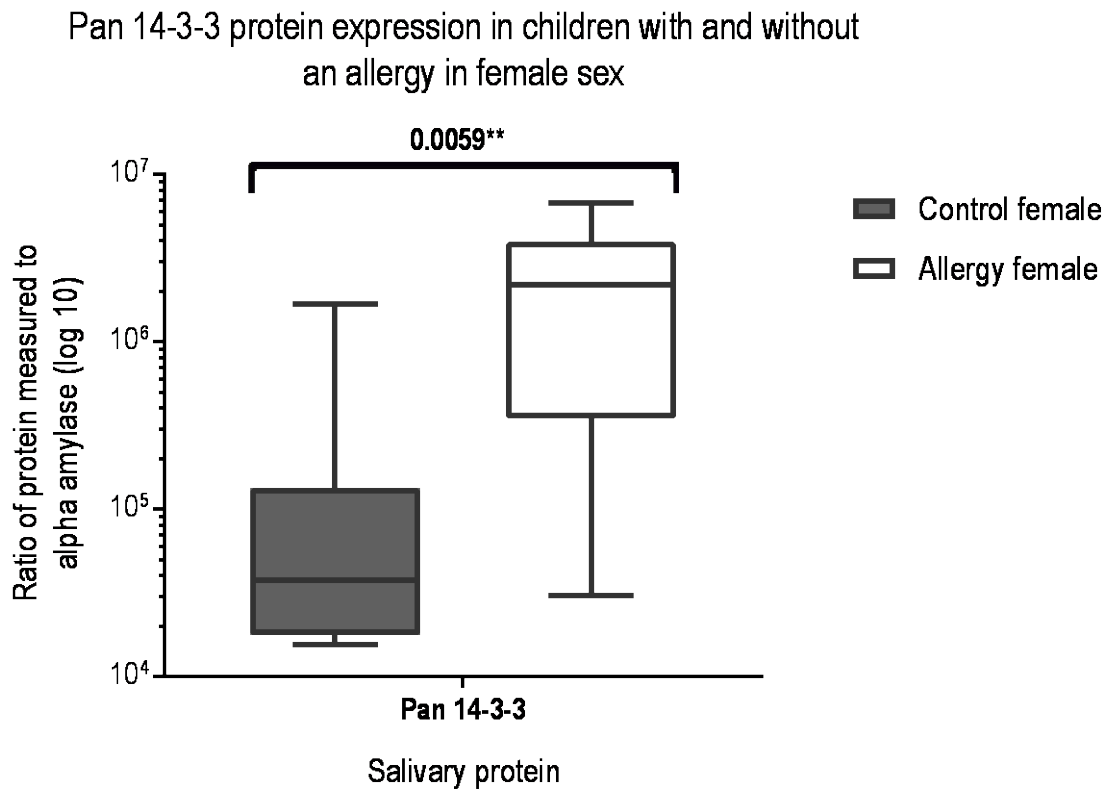


Figure 83. Pan 14-3-3 protein expression in children with (n= 11) and without (n= 7) an allergy in female sex. Protein expression was examined using Western blot. Proteins were expressed relative to alpha amylase. Data of pan 14-3-3 protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

iv. Effect of specific allergic phenotypes on salivary protein expression and child allergy

Pan 14-3-3 protein expression protein expression in children with and without any asthma, eczema and rhinitis allergy combination

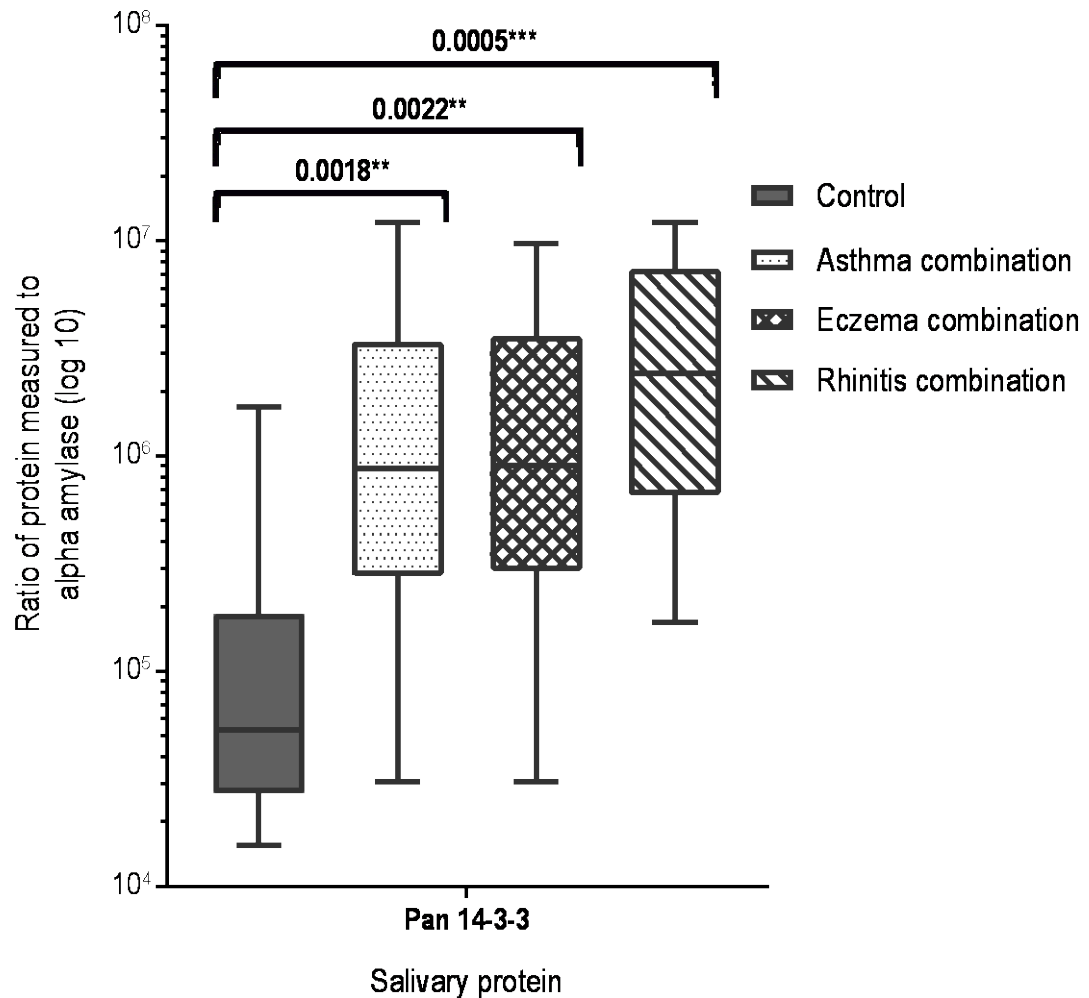
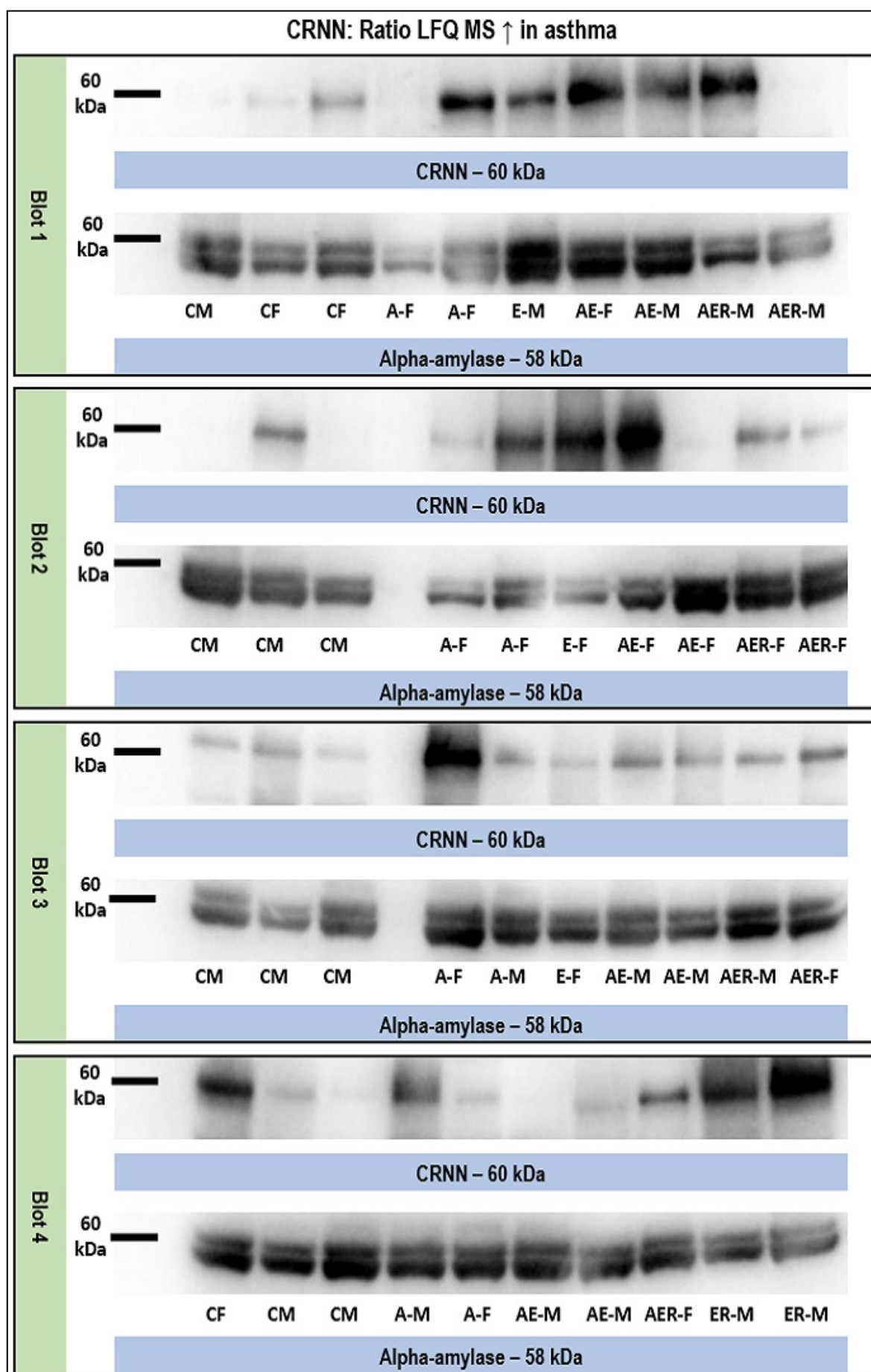


Figure 84. Pan 14-3-3 protein expression in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, n= 17; eczema combination, n= 16; rhinitis combination, n= 9; and non-allergic children, n= 9. Protein expression was examined using Western blot. Proteins were expressed relative to alpha amylase. Data of pan 14-3-3 protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

5.3.2.2 Expression of cornulin (CRNN) protein in the saliva of children with and without an allergy

For CRNN protein, the protein was not highly expressed in all of the allergic sample with asthma using Western blot (Figure 85). Salivary CRNN (Mann Whitney U test, $P= 0.047^*$) was significantly increased in the child saliva of non-asthmatic mothers (low risk of allergy) regardless of sex as compared to children of non-asthmatic mothers without an allergy (Figure 86). When comparing the difference in protein expression between children without an allergy to children with specific allergy, CRNN (Mann Whitney U test, $P= 0.042^*$) was significantly increased in the child saliva with rhinitis combination (Figure 87). It did not change significantly in any other groups.



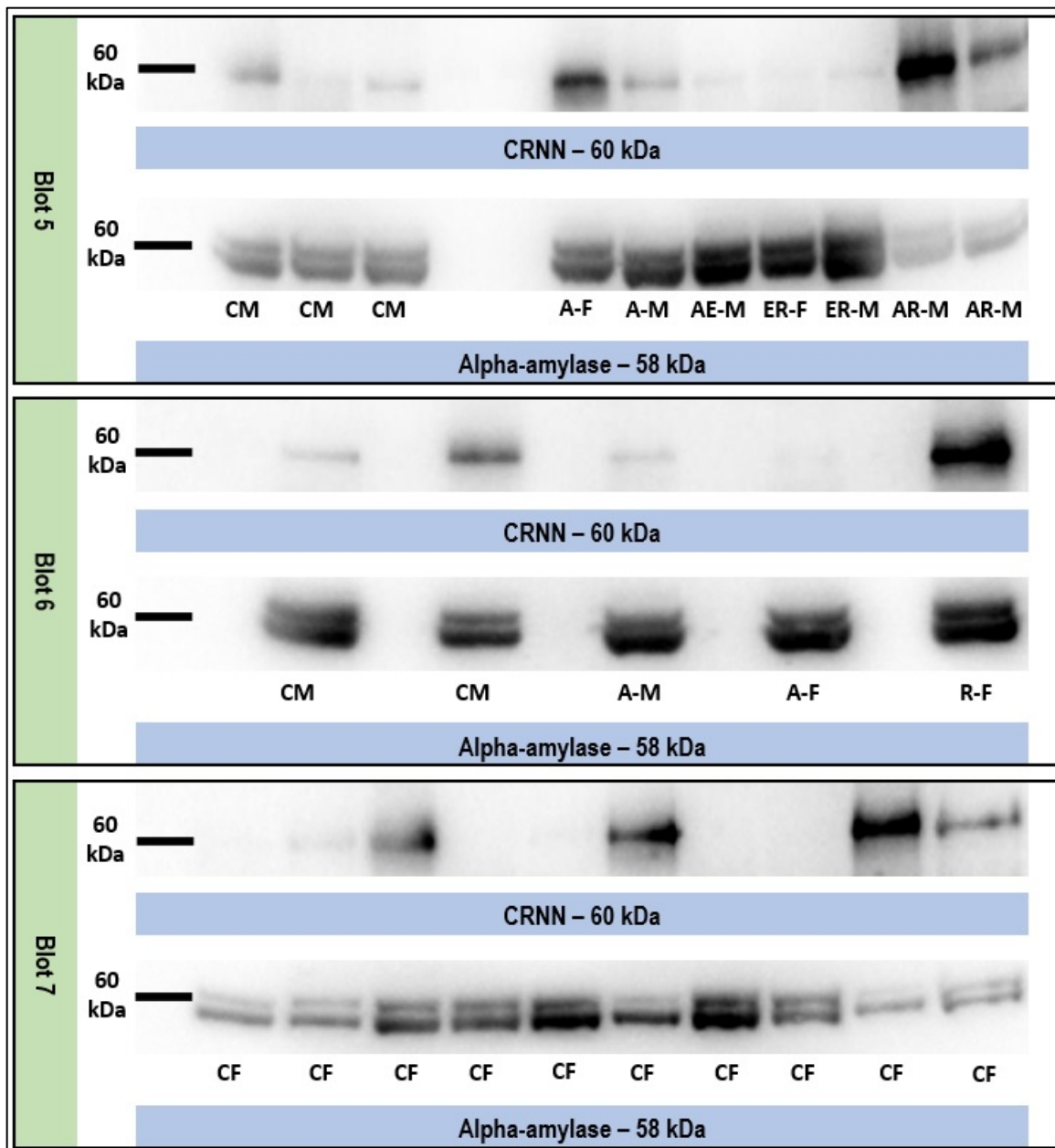


Figure 85. Representative Western blot of CRNN protein expression in the saliva samples of children with (n= 38) and without (n= 24) an allergy. CRNN protein was detected at 60 kDa. (M represents male children, F represents female children, C represents control subjects who are children with no allergy, A represents children with asthma, E represents children with eczema and R represents children with rhinitis).

i. The influence of the presence and absence of maternal asthma during pregnancy on salivary protein expression and child allergy

a. Children of non-asthmatic mothers (low risk of allergy) with and without an allergy

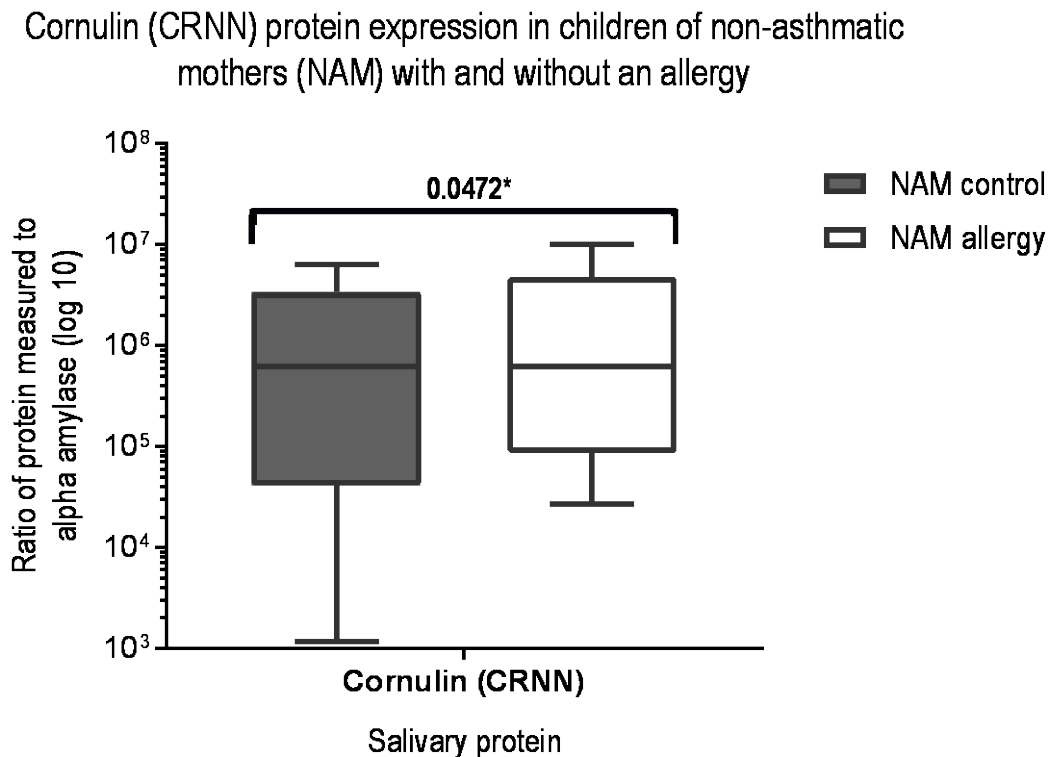


Figure 86. CRNN protein expression in children of non-asthmatic mothers (low risk of allergy) with (n= 16) and without (n= 14) an allergy. Protein expression was examined using Western blot. Proteins were expressed relative to alpha amylase. Data of CRNN protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

ii. Effect of specific allergic phenotypes on salivary protein expression and child allergy

Cornulin (CRNN) protein expression protein expression in children with and without any asthma, eczema and rhinitis allergy combination

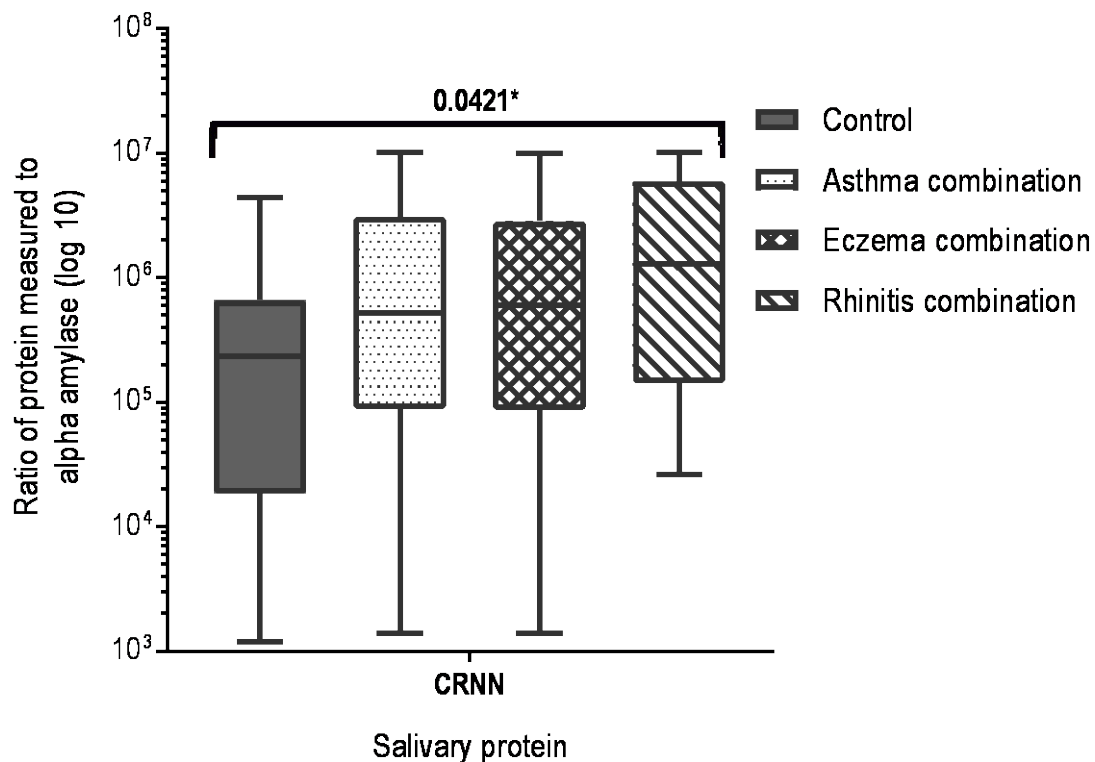
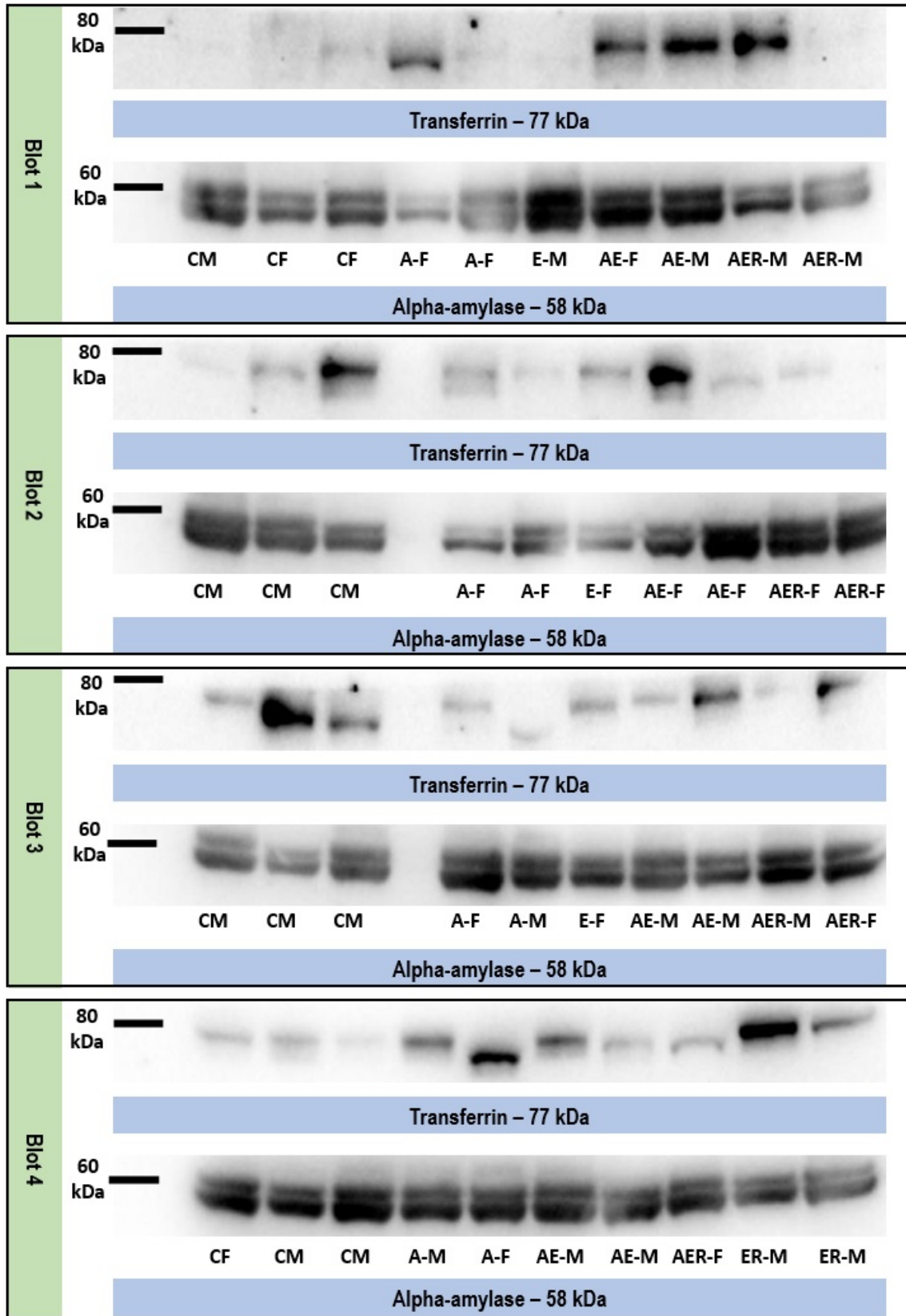


Figure 87. CRNN protein expression in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, $n=25$; eczema combination, $n=19$; rhinitis combination, $n=12$; and non-allergic children, $n=16$. Protein expression was examined using Western blot. Proteins were expressed relative to alpha amylase. Data of CRNN protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P<0.05$.

5.3.2.3 Expression of transferrin protein in the saliva of children with and without an allergy

Salivary transferrin protein was not highly expressed in non-allergic group as compared to the allergic samples that include asthma, eczema and rhinitis using Western blot (Figure 88). There were no significant differences of salivary transferrin protein expression in any groups.

Transferrin: Ratio LFQ MS ↑ in asthma, eczema and rhinitis



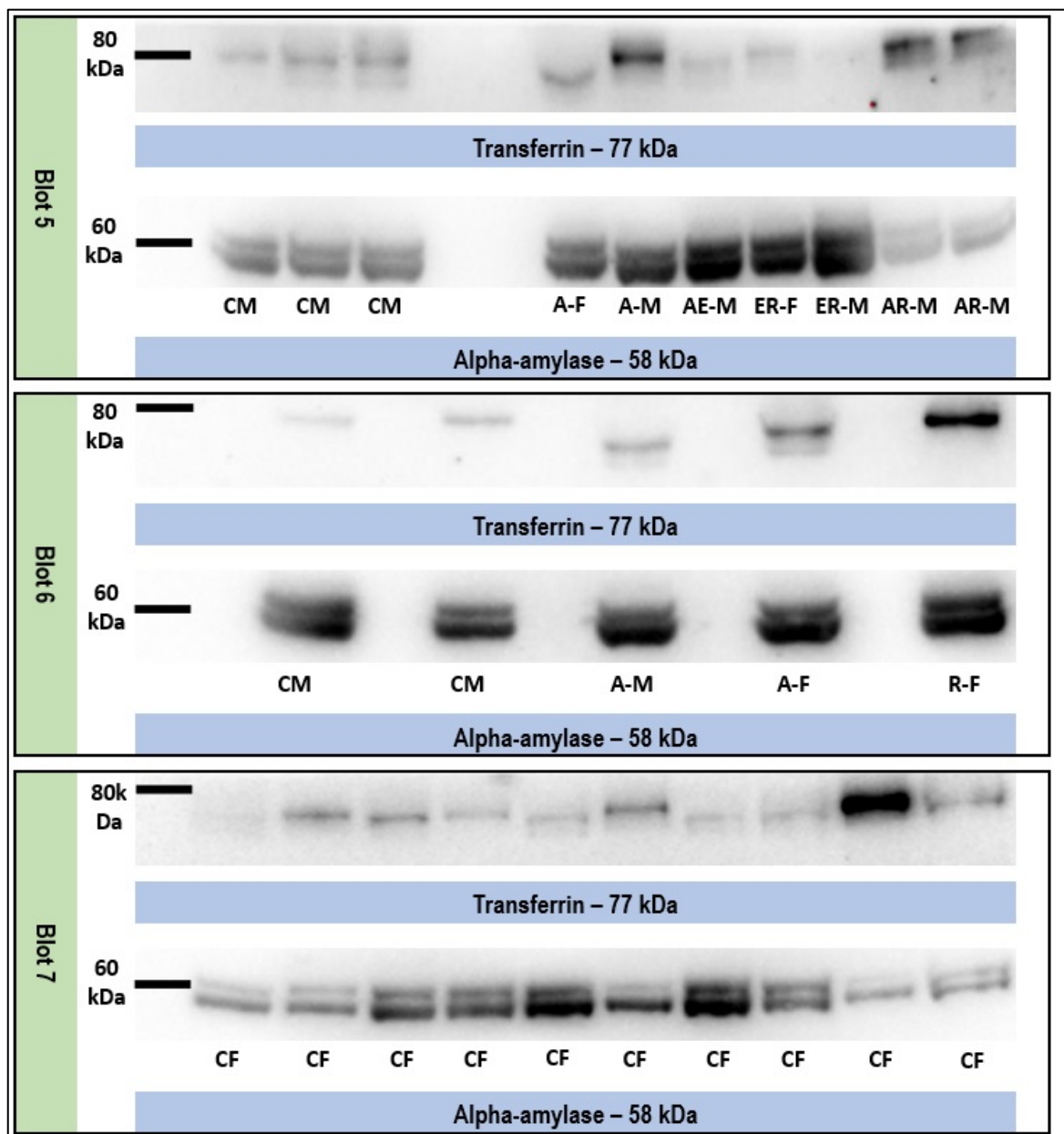
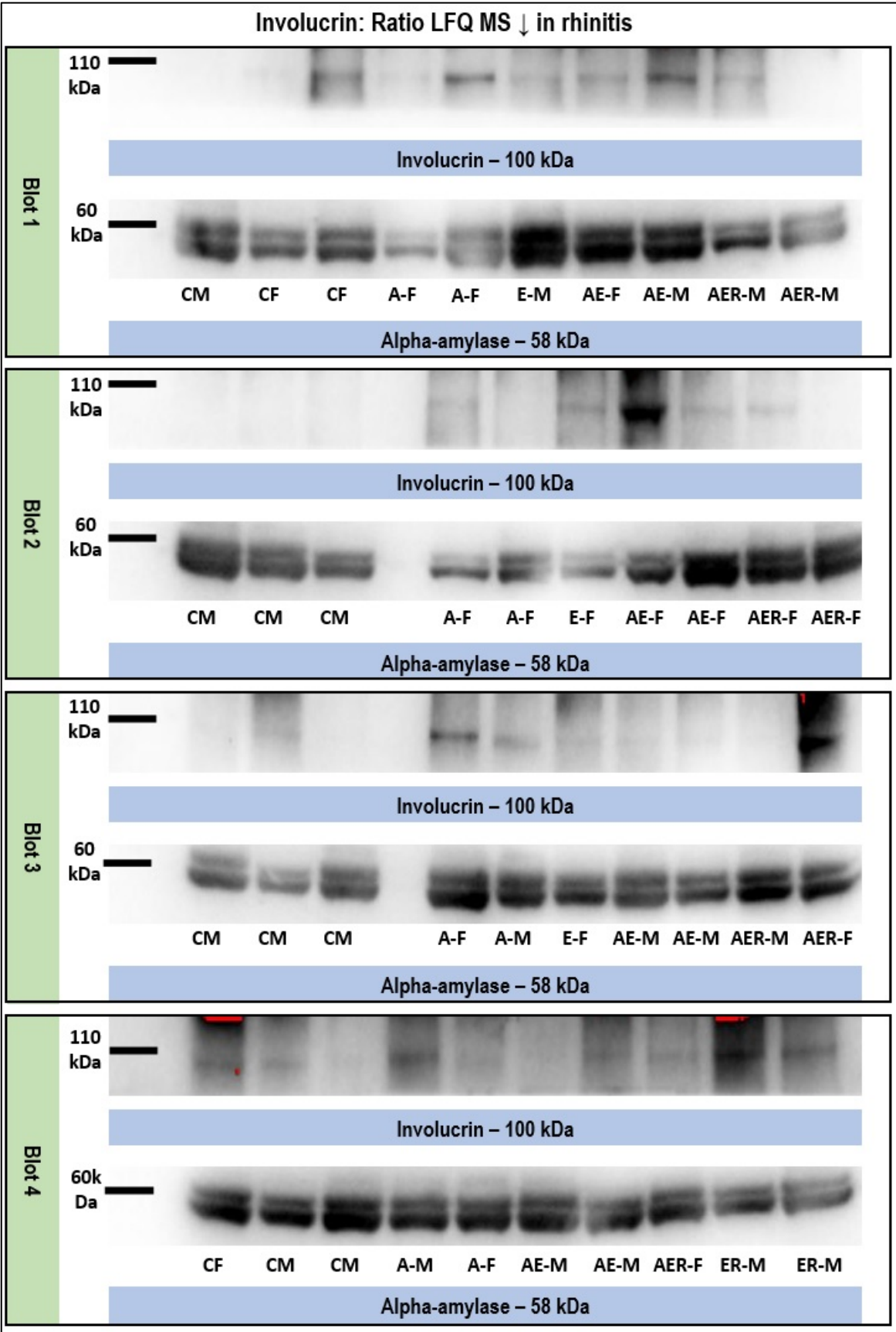


Figure 88. Representative Western blot of transferrin protein expression in the saliva samples of children with (n=38) and without (n=24) an allergy. Transferrin protein was detected at 77 kDa. (M represents male children, F represents female children, C represents control subjects who are children with no allergy, A represents children with asthma, E represents children with eczema and R represents children with rhinitis).

5.3.2.4 Expression of involucrin protein in the saliva of children with and without an allergy

In the Western blot of the child saliva samples, involucrin protein was not expressed in all of the allergic sample with rhinitis (Figure 89). Involucrin (Mann Whitney U test, $P=0.040^*$) protein expression was significantly increased in the saliva of allergic male children as compared to non-allergic male children (Figure 90). When comparing the difference in protein expression between children without an allergy to children with specific allergy, involucrin (Mann Whitney U test, $P=0.037^*$) was significantly increased in the child saliva with eczema combination (Figure 91). It did not change significantly in any other groups.



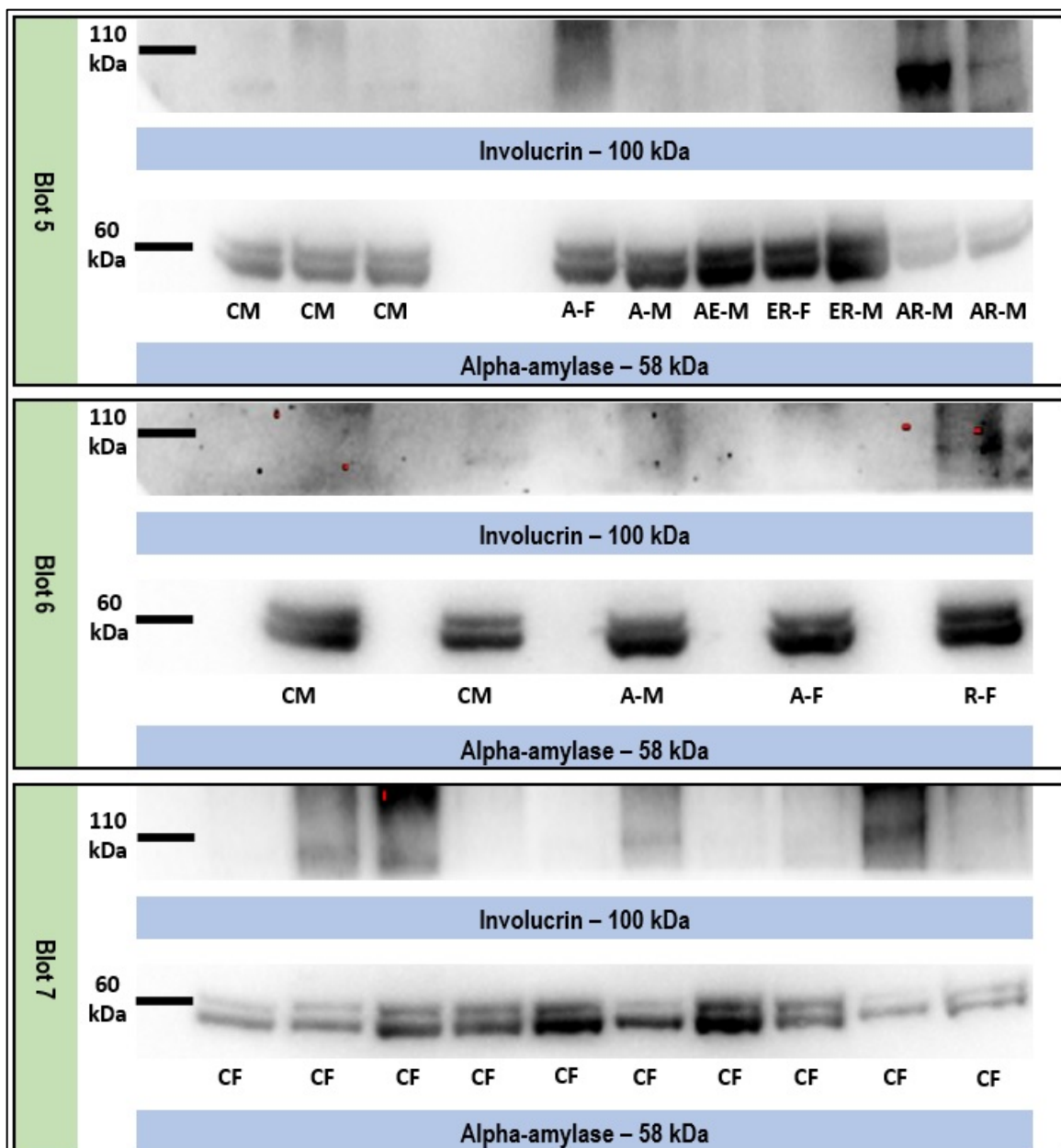


Figure 89. Representative Western blot of involucrin protein expression in the saliva samples of children with (n= 38) and without (n= 24) an allergy. Involucrin protein was detected at 100 kDa. (M represents male children, F represents female children, C represents control subjects who are children with no allergy, A represents children with asthma, E represents children with eczema and R represents children with rhinitis).

i. Effect of sex on salivary protein expression and child allergy

a. Children with allergy vs children with no allergy

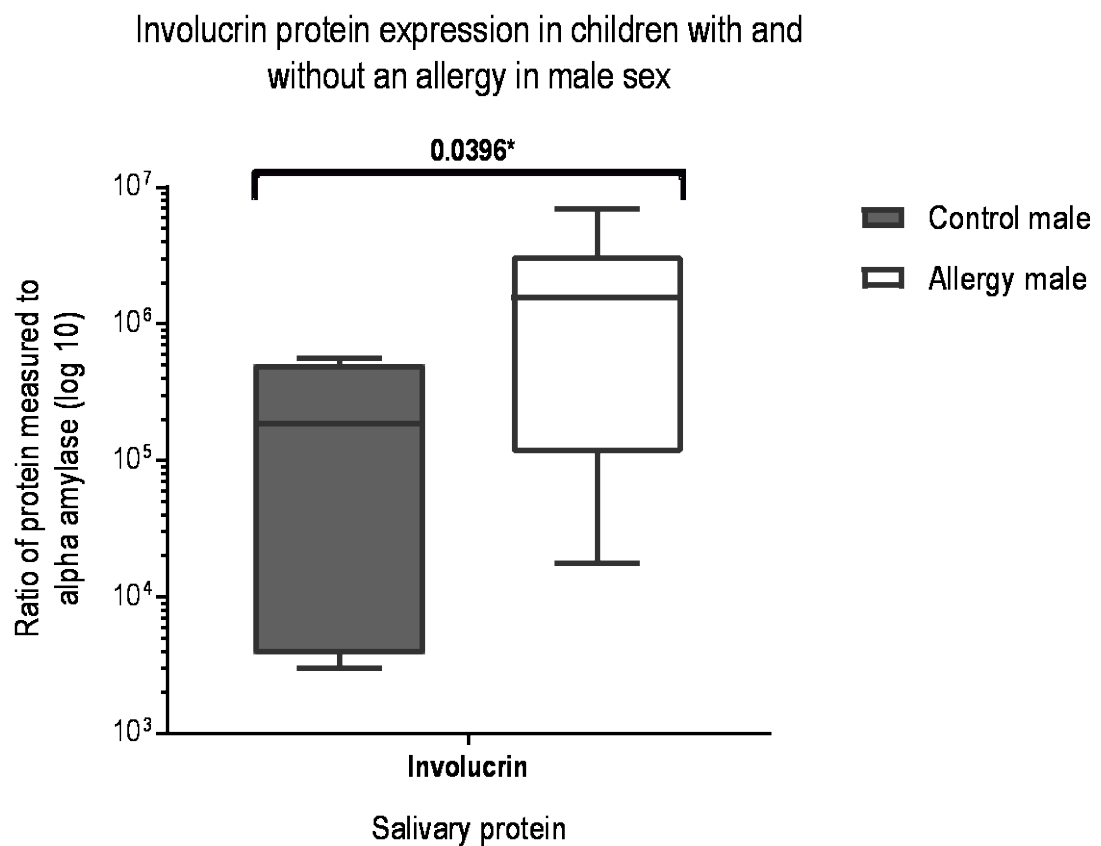


Figure 90. Involucrin protein expression in children with (n= 11) and without (n= 4) an allergy in male sex. Protein expression was examined using Western blot. Proteins were expressed relative to alpha amylase. Data of involucrin protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

ii. Effect of specific allergic phenotypes on salivary protein expression and child allergy

Involucrin protein expression protein expression in children with and without any asthma, eczema and rhinitis allergy combination

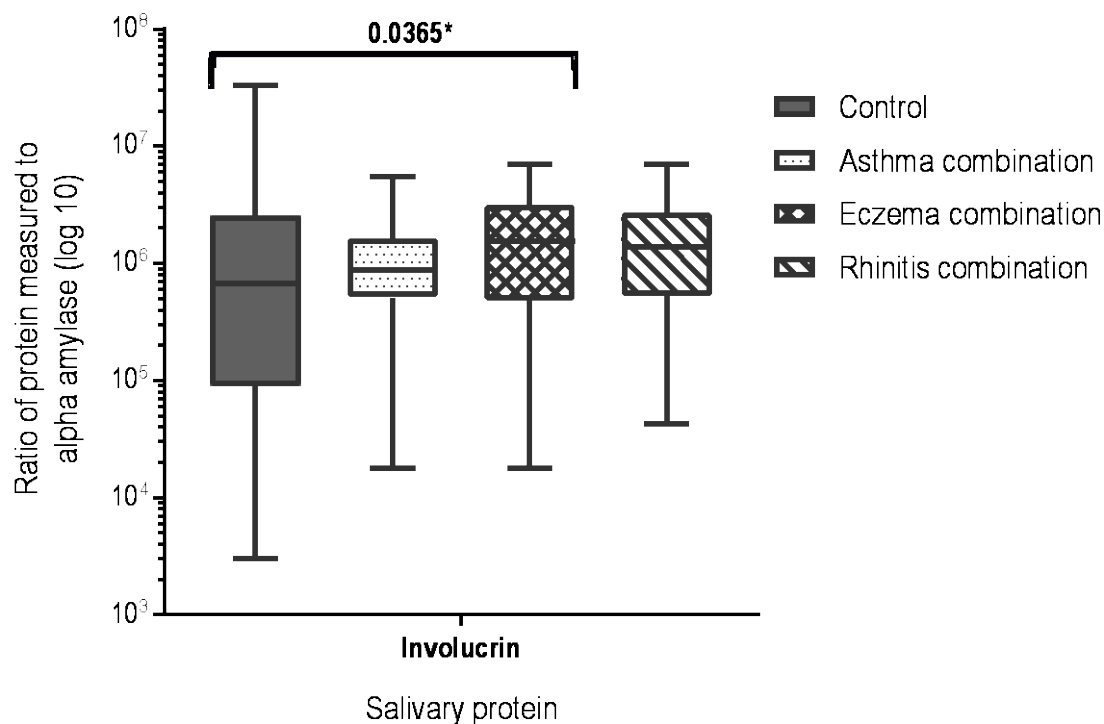
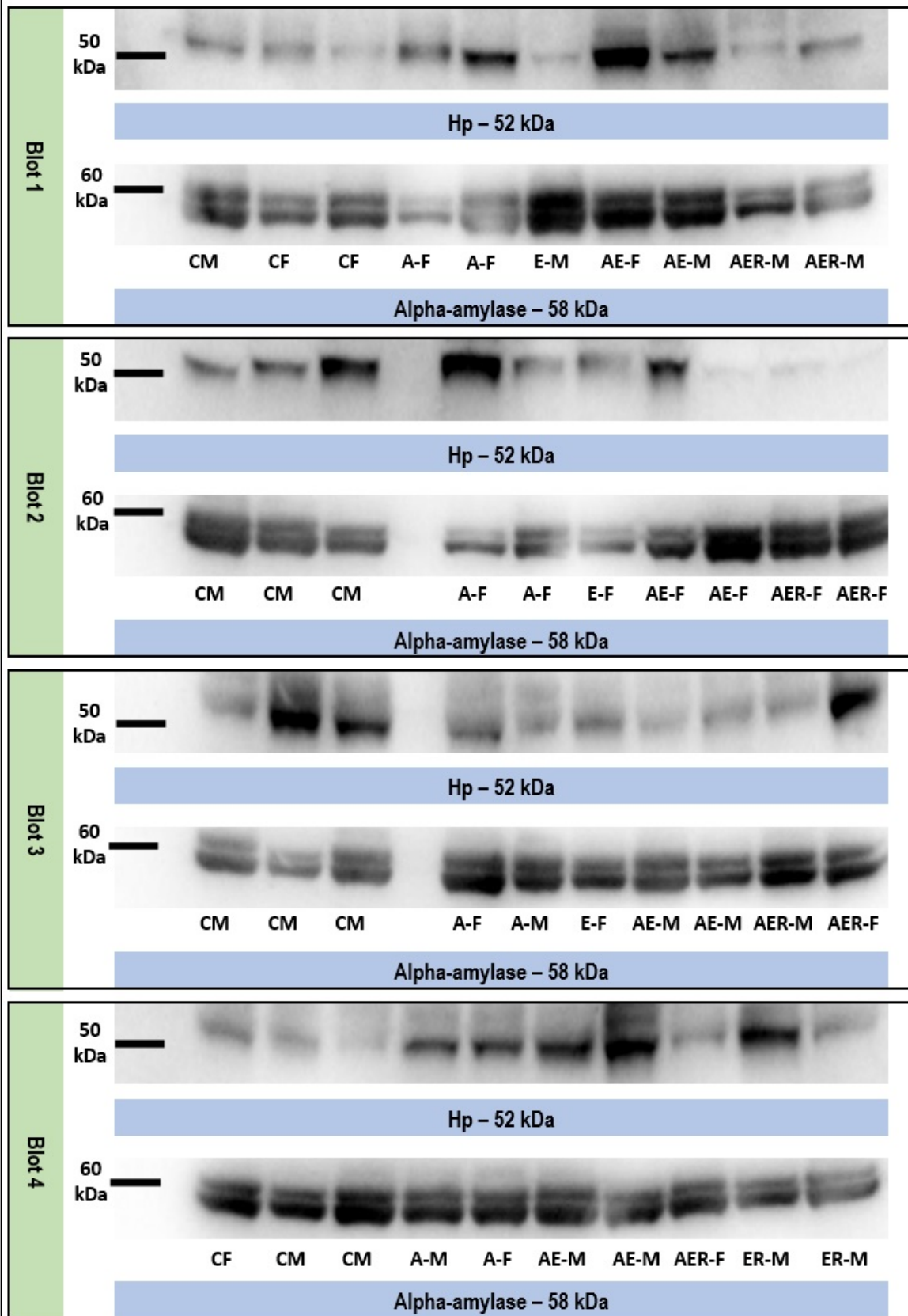


Figure 91. Involucrin protein expression in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, n= 20; eczema combination, n= 15; rhinitis combination, n= 9; and non-allergic children, n= 6. Protein expression was examined using Western blot. Proteins were expressed relative to alpha amylase. Data of involucrin protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

5.3.2.5 Expression of Haptoglobin (Hp) protein in the saliva of children with and without an allergy

For Hp protein, the protein was expressed not only in eczema group but also in other allergic group that include asthma and rhinitis; as compared to the non-allergic child saliva group, using Western blot (Figure 92). There were no significant differences of salivary Hp protein expression in any groups.

Hp: Ratio LFQ MS ↓ in eczema



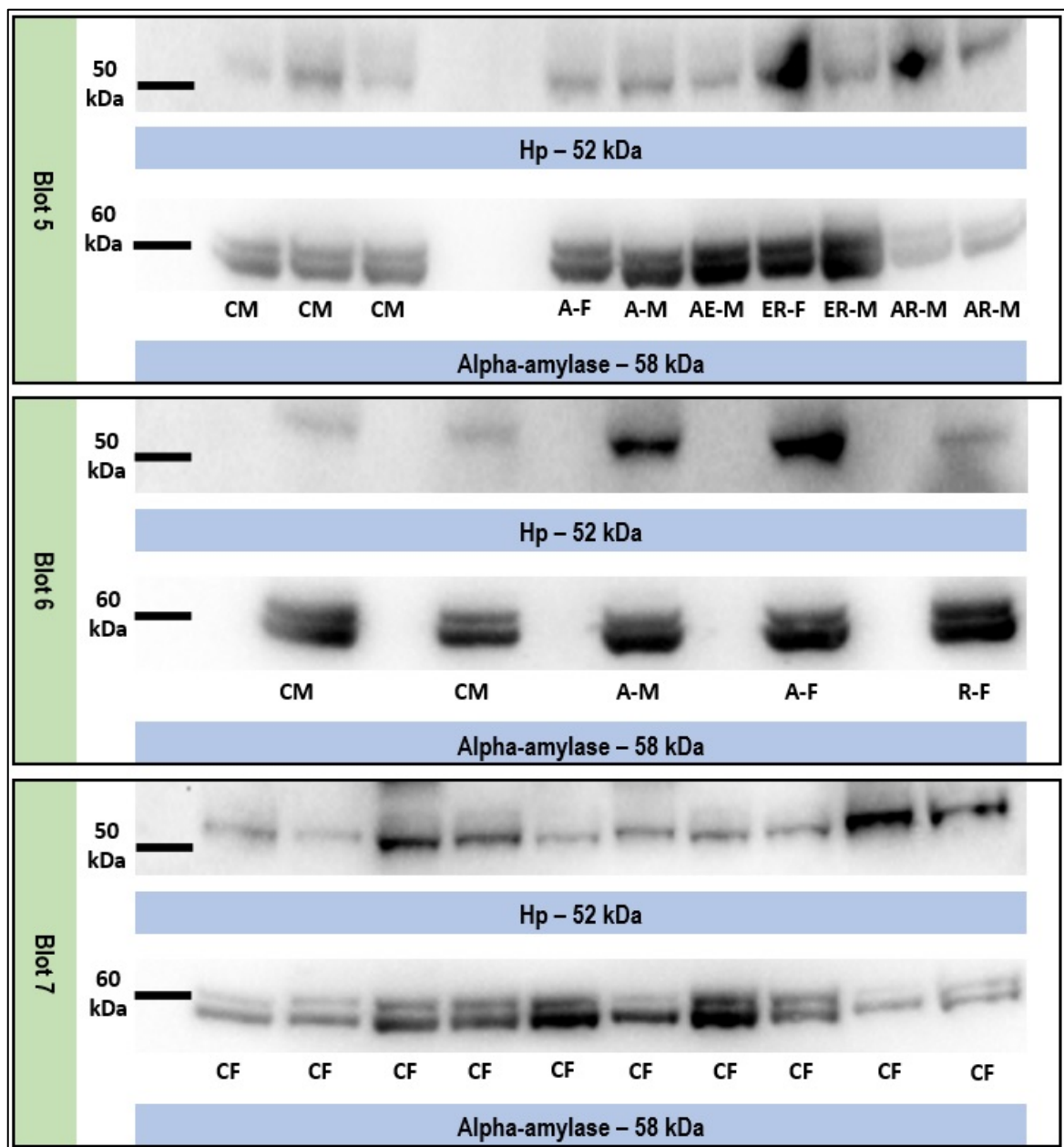


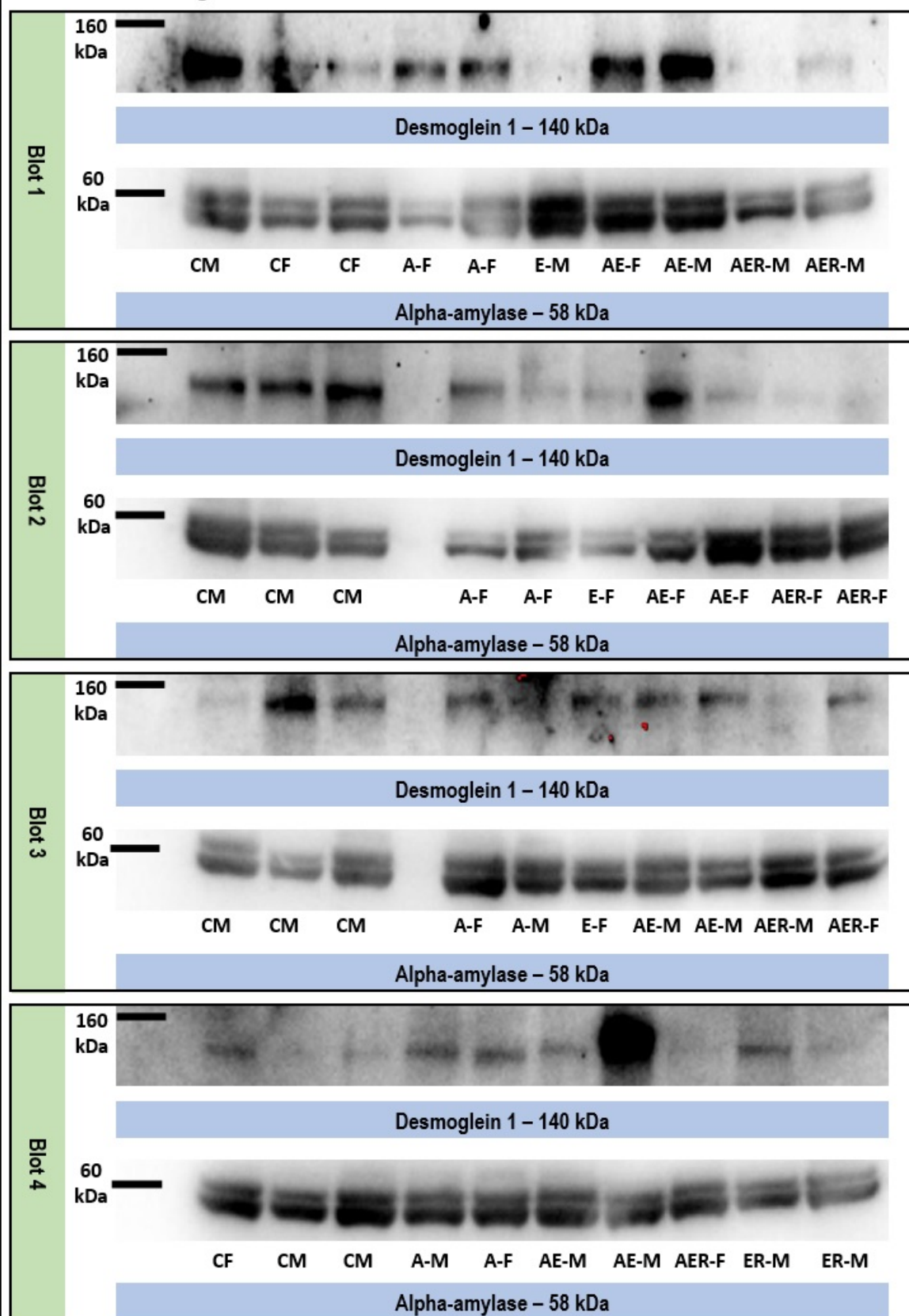
Figure 92. Representative Western blot of Hp protein expression in the saliva samples of children with (n= 38) and without (n= 24) an allergy. Hp protein was detected at 52 kDa. (M represents male children, F represents female children, C represents control subjects who are children with no allergy, A represents children with asthma, E represents children with eczema and R represents children with rhinitis).

5.3.2.6 Expression of desmoglein 1 protein in the saliva of children with and without an allergy

Salivary desmoglein 1 protein was not highly expressed in control group without allergy as compared with allergic sample with asthma and eczema using Western blot (Figure 93). Desmoglein 1 (Mann Whitney U test, $P= 0.002^{**}$) protein expression was significantly increased in the allergic female children of non-asthmatic mothers (low risk of allergy) as compared to non-allergic female children of non-asthmatic mothers (Figure 94). There was significant increases of desmoglein 1 (Mann Whitney U test, $P= 0.003^{**}$) protein expression in the saliva of allergic children in female sex as compared with non-allergic children in female sex (Figure 95). It did not change significantly in any other groups.

The summary of significant statistical analyses of Western blot band densitometry of child saliva samples in relation to childhood allergy distribution and to the presence (high risk of allergy) or the absence (low risk of allergy) of maternal asthma is listed in Table 30.

Desmoglein 1: Uncalculated LFQ MS ratio in asthma and eczema



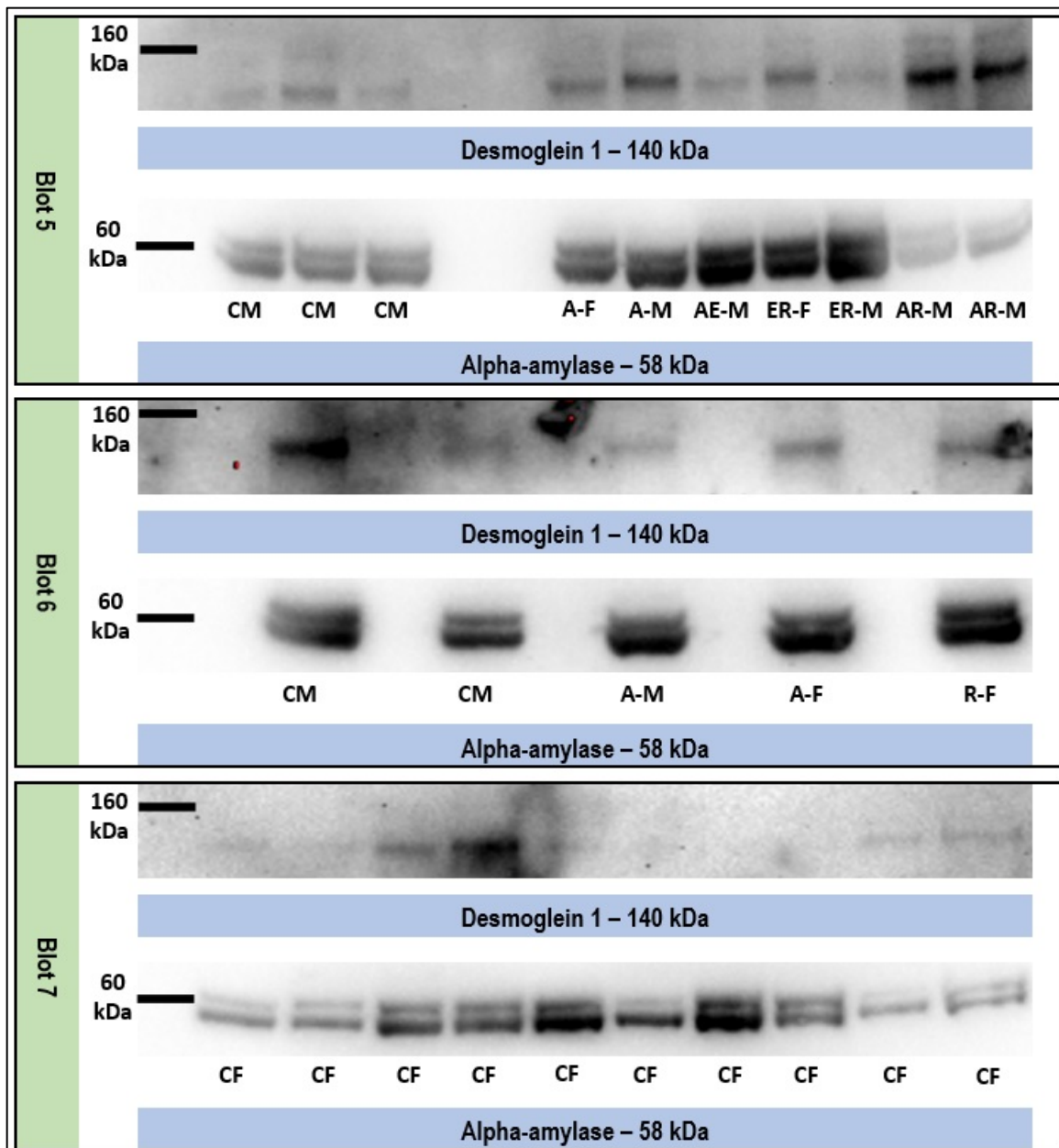


Figure 93. Representative Western blot of desmoglein 1 protein expression in the saliva samples of children with (n= 38) and without (n= 24) an allergy. Desmoglein 1 protein was detected at 140 kDa. (M represents male children, F represents female children, C represents control subjects who are children with no allergy, A represents children with asthma, E represents children with eczema and R represents children with rhinitis).

- i. The influence of the presence and absence of maternal asthma during pregnancy on salivary protein expression and child allergy
 - a. The effect of sex and the presence and absence of maternal asthma on salivary protein expression

Desmoglein 1 protein expression in children of non-asthmatic mothers (NAM) with and without an allergy in female sex

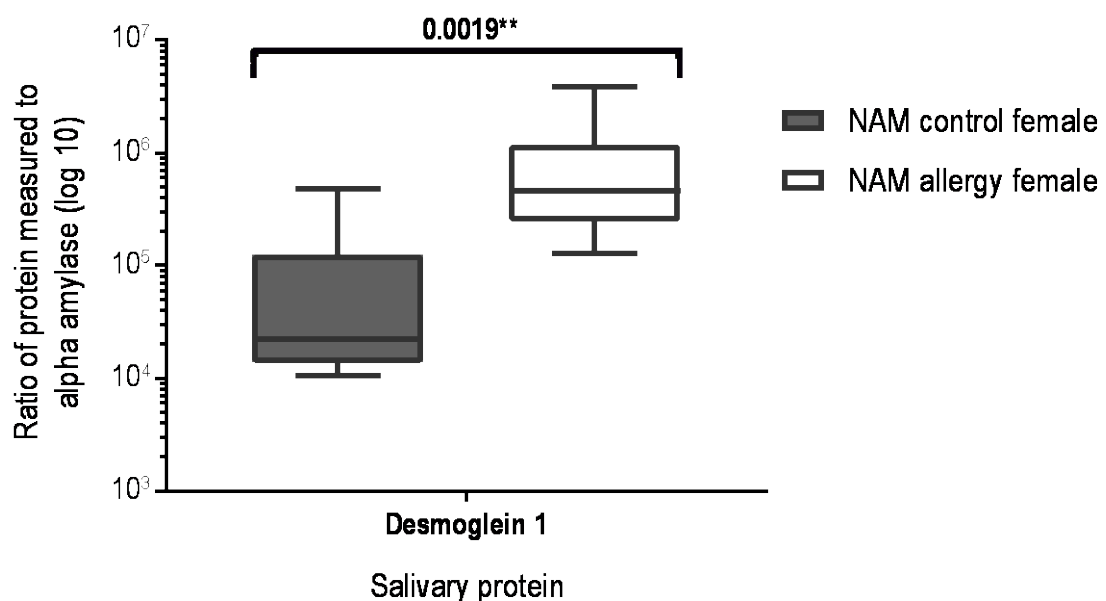


Figure 94. Desmoglein 1 protein expression in children of non-asthmatic mothers (low risk of allergy) with (n= 11) and without (n= 7) an allergy in female sex. Protein expression was examined using Western blot. Proteins were expressed relative to alpha amylase. Data of desmoglein 1 protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

ii. Effect of sex on salivary protein expression and child allergy

a. Children with allergy vs children with no allergy

Desmoglein 1 protein expression in children with and without an allergy in female sex



Figure 95. Desmoglein 1 protein expression in children with (n= 18) and without (n= 10) an allergy in female sex. Protein expression was examined using Western blot. Proteins were expressed relative to alpha amylase. Data of desmoglein 1 protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

Table 30. Significant statistical analyses of Western blot band densitometry of child saliva samples in relation to childhood allergy distribution and to the presence (high risk of allergy) or the absence (low risk of allergy) of maternal asthma. (Significant result was $P < 0.05$).

Salivary protein	Significant Western blot statistical analyses	Statistical analysis used	P values
Pan 14-3-3	Control vs allergy	MWU	0.0005***
	AM control vs AM allergy	MWU	0.0303*
	NAM control vs NAM allergy	MWU	0.0186*
	Control female vs allergy female	MWU	0.0059**
	Control vs specific allergy (asthma combination)	MWU	0.0018**
	Control vs specific allergy (eczema combination)	MWU	0.0022**
	Control vs specific allergy (rhinitis combination)	MWU	0.0005***
Cornulin (CRNN)	NAM control vs NAM allergy	MWU	0.0472*
	Control vs specific allergy (rhinitis combination)	MWU	0.0421*
Involucrin	Control male vs allergy male	MWU	0.0396*
	Control vs specific allergy (eczema combination)	MWU	0.0365*
Desmoglein 1	NAM control female vs NAM allergy female	MWU	0.0019**
	Control female vs allergy female	MWU	0.0030**

° Abbreviations: AR represent asthma and rhinitis, AM represents children with asthmatic mothers (high risk of allergy), NAM represents children with non-asthmatic mothers (low risk of allergy) and MWU represents Mann Whitney U test with two-tailed p-value.

5.4 Validation study for child saliva samples with Blitz system

5.4.1 Characteristics of the cohort children and maternal asthma status in Blitz system

Child saliva samples used in the validation studies were derived from the Asthma and Pregnancy Cohort study and prospectively collected. In addition to this Asthma and Pregnancy Cohort study, other samples of child saliva were collected from patients between 6 months to 5 years old that were receiving treatment in the Paediatric Clinic of Women's and Children's Hospital (refer Methodology section 2.1.1).

Blitz system was performed using 126 child saliva samples. The mean age, weight and height for the child population were 38.33 months, 15.34 kg and 94.66 cm, respectively. There were no significant differences between male and female in age, height and weight. There were 88 saliva samples from allergic children (males: n= 45, females: n= 43) and 38 saliva samples from non-allergic children (males: n= 21, females: n= 17). The majority of specific allergic phenotypes were asthma group which consisted of 23 samples; and the least was the rhinitis group, which contributed to only 3 samples. As expected, the majority of children with an allergy had a mother with asthma (57.95 %), whereas most children without an allergy had a mother who was non-asthmatic (low risk of allergy) (76.32 %). Characteristics of the cohort children and asthma status of their mother for this chapter are summarised in Table 31.

Table 31. Characteristics of the cohort children and maternal asthma status in Blitz system. (Significant result was $P < 0.05$).

Child characteristics	Males	Females	Both sexes	* Significance differences between males and females
Age in months (mean $\pm$ SE)	34.58 $\pm$ 2.60	42.47 $\pm$ 2.71	38.33 $\pm$ 1.87	p= 0.0513
Weight in kg (mean $\pm$ SE)	14.59 $\pm$ 0.66	16.15 $\pm$ 0.71	15.34 $\pm$ 0.49	p= 0.0864
Height in cm (mean $\pm$ SE)	91.11 $\pm$ 2.51	98.09 $\pm$ 2.11	94.66 $\pm$ 1.67	p= 0.0623
Number of asthma (n)	10	13	23	NA
Number of eczema (n)	4	4	8	NA
Number of rhinitis (n)	2	1	3	NA
Number of asthma and eczema (n)	13	6	19	NA
Number of asthma and rhinitis (n)	4	0	4	NA
Number of eczema and rhinitis (n)	2	7	9	NA
Number of asthma, eczema and rhinitis (n)	10	12	22	NA
Number of asthma combination (n)	37	31	68	NA
Number of eczema combination (n)	29	29	58	NA
Number of rhinitis combination (n)	18	20	38	NA
Number of allergic children (n)	45	43	88	NA
Number of non-allergic children (n)	21	17	38	NA
% of asthmatic mothers (high risk of allergy) in allergic children	60.00	55.81	57.95	NA
% of asthmatic mothers (high risk of allergy) in non-allergic children	19.05	29.41	23.68	NA
% of non-asthmatic mothers (low risk of allergy) in allergic children	40.00	44.19	42.05	NA
% of non-asthmatic mothers (low risk of allergy) in non-allergic children	80.95	70.59	76.32	NA

* Data were analysed by Mann-Whitney test with two-tailed p-value.

° Abbreviations: NA represents non-applicable.

5.4.2 Blitz system validation study using placental samples from children with and without an allergy

I validated and measured three salivary proteins that included 14-3-3 zeta (an isomer of pan 14-3-3), involucrin and CRNN in saliva samples of children with and without an allergy using Blitz system. Pan 14-3-3 (K-19) antibody used in the Western blot is an antibody that can detect seven isoforms comprising this protein family of signaling intermediates, denoted 14-3-3 beta (β), gamma (γ), epsilon (ϵ), zeta (ζ), eta (η), theta (θ) and sigma (σ). The analyses of the estimated proteins concentrations of these three proteins were performed in relation to general allergy, the presence or absence of maternal asthma during pregnancy, child sex and child allergy phenotypes (refer Methodology section 2.12.1).

5.4.2.1 Concentration of 14-3-3 zeta, involucrin and cornulin (CRNN) protein in the saliva of children with and without an allergy

i. Children with allergy vs children with no allergy

When comparing the difference in protein expression between children without an allergy to children with an allergy, out of the three salivary proteins, only CRNN (Mann Whitney U test, $P= 0.049^*$) was significantly decreased in the saliva of children with an allergy (Figure 96). There were no significant protein differences in 14-3-3 zeta and involucrin (Table 32).

14-3-3 zeta, involucrin and cornulin (CRNN) protein concentrations in the saliva of children with and without an allergy

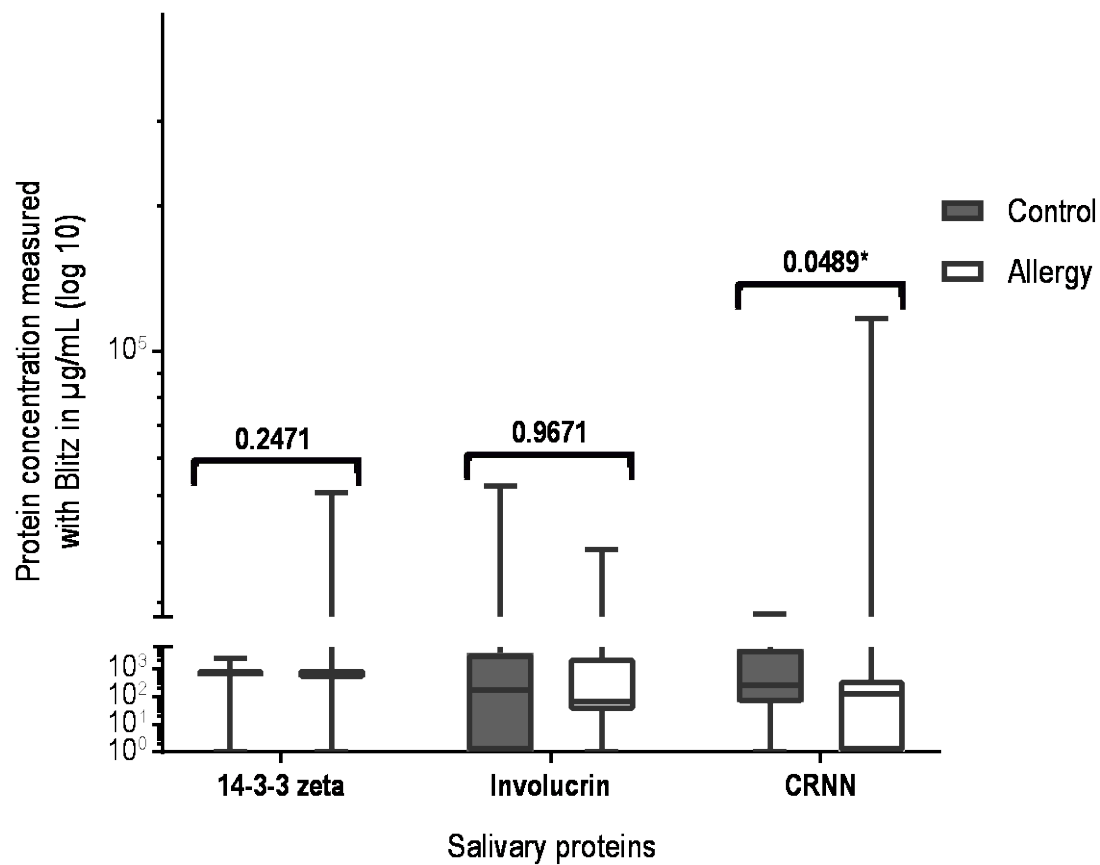


Figure 96. 14-3-3 zeta, involucrin and CRNN proteins concentrations in the saliva of children with (n= 88) and without (n= 38) an allergy. Proteins concentrations was measured using Blitz system. Data of 14-3-3 zeta, involucrin and CRNN proteins concentrations were expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was P<0.05.

Table 32. 14-3-3 zeta and involucrin salivary protein that did not change significantly in the child saliva samples of children with an allergy as compared to children without an allergy.

Blitz salivary proteins	Proteins concentrations measured with Blitz in children with no allergy		Proteins concentrations measured with Blitz in children with allergy	
	Medians	Interquartile range	Medians	Interquartile range
14-3-3 zeta	683.4	207.5	635.2	56.4
Involucrin	172	3736	65.99	2469.63

5.4.2.2 Concentration of 14-3-3 zeta protein in the saliva of children with and without an allergy

There was a significant decreased of 14-3-3 zeta (Mann Whitney U test, $P=0.008^{**}$) protein expression in the allergic child saliva of asthmatic mothers (high risk of allergy) regardless of sex, as compared to non-allergic child saliva of asthmatic mothers (Figure 97). When comparing the difference in protein expression between children without an allergy to children with specific allergy, 14-3-3 zeta (Mann Whitney U test, $P=0.012^{*}$) was significantly decreased in the child saliva with rhinitis combination (Figure 98). It did not change significantly in any other groups.

- i. The influence of the presence and absence of maternal asthma during pregnancy on salivary protein expression and child allergy
 - a. Children of asthmatic mothers (high risk of allergy) with and without an allergy

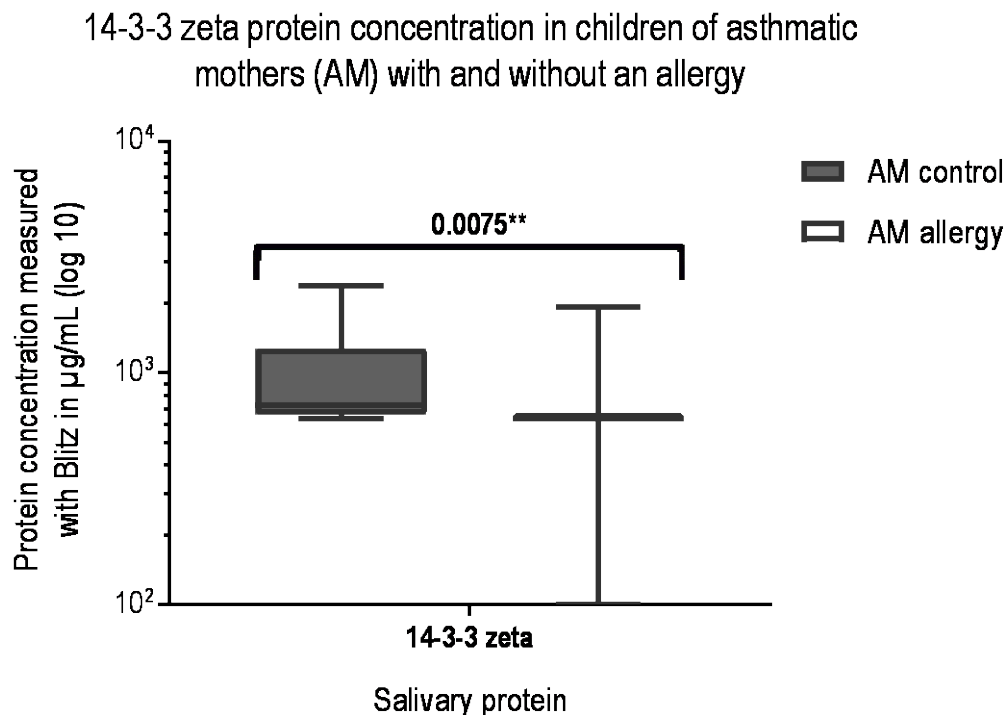


Figure 97. 14-3-3 zeta protein concentration in children of asthmatic mothers (high risk of allergy) with (n= 51) and without (n= 9) an allergy. Protein concentration was measured using Blitz system. Data of 14-3-3 zeta protein concentration was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

ii. Effect of specific allergic phenotypes on salivary protein expression and child allergy

14-3-3 zeta protein concentration in children with and without any asthma, eczema and rhinitis allergy combination

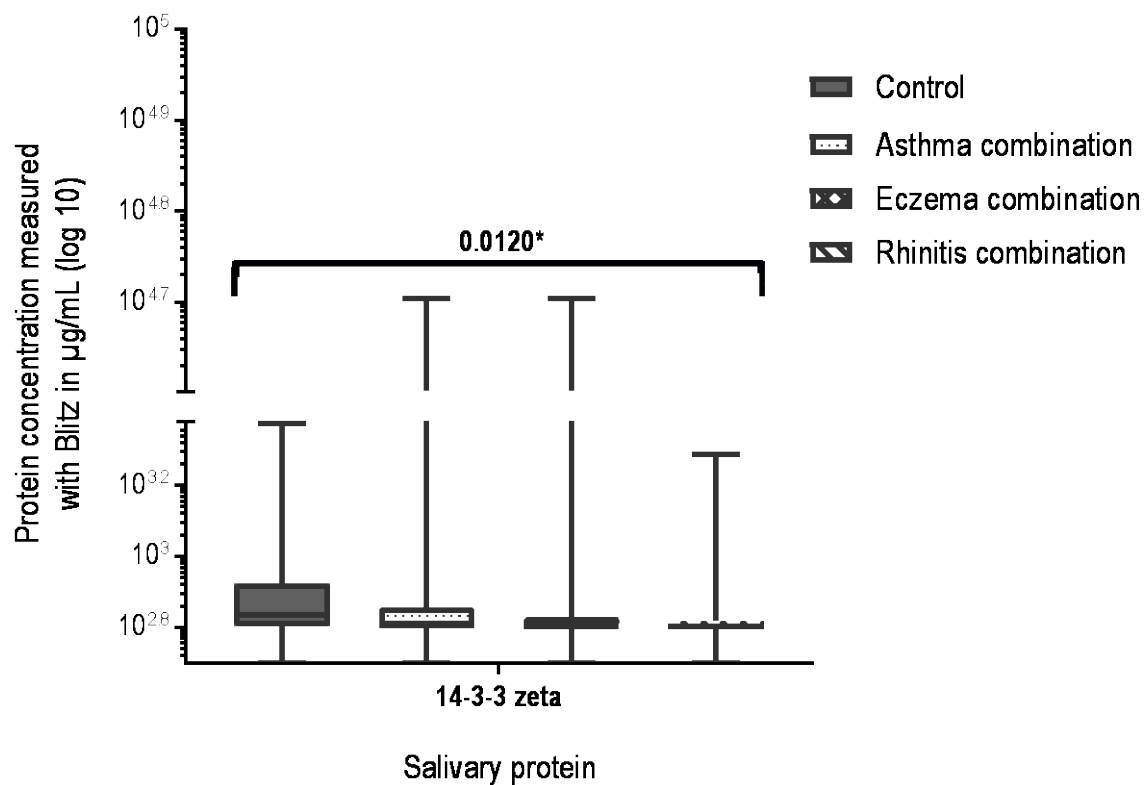


Figure 98. 14-3-3 zeta protein concentration in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, $n = 68$; eczema combination, $n = 58$; rhinitis combination, $n = 38$; and non-allergic children, $n = 38$. Protein expression was examined using Western blot. Proteins were expressed relative to alpha amylase. Data of 14-3-3 zeta protein volume intensity was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

5.4.2.3 Concentration of involucrin protein in the saliva of children with and without an allergy

Interestingly, there were influences of both the presence and the absence of maternal asthma in salivary involucrin protein expression using Blitz system. Involucrin (Mann Whitney U test, $P=0.034^*$) protein was significantly decreased in the child saliva of asthmatic mothers (high risk of allergy) with an allergy regardless of sex, as compared to children of asthmatic mothers without an allergy (Figure 99). In children of non-asthmatic mothers (low risk of allergy), involucrin (Mann Whitney U test, $P=0.021^*$) was significantly increased in the saliva of male allergic children as compared to male non-allergic children (Figure 100). There were no significant differences in any other groups.

- i. The influence of the presence and absence of maternal asthma during pregnancy on salivary protein expression and child allergy
 - a. Children of asthmatic mothers (high risk of allergy) with and without an allergy

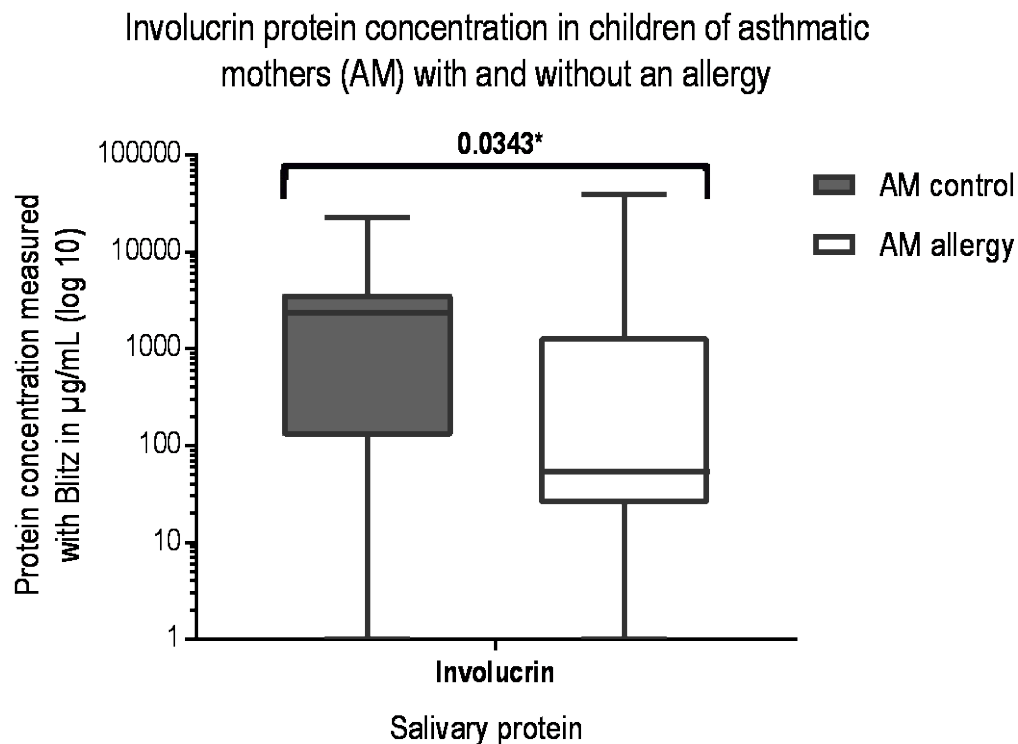


Figure 99. Involucrin protein concentration in children of asthmatic mothers (high risk of allergy) with ($n= 51$) and without ($n= 9$) an allergy. Protein concentration was measured using Blitz system. Data of involucrin protein concentration was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P<0.05$.

b. The effect of sex and the presence and absence of maternal asthma on salivary protein expression

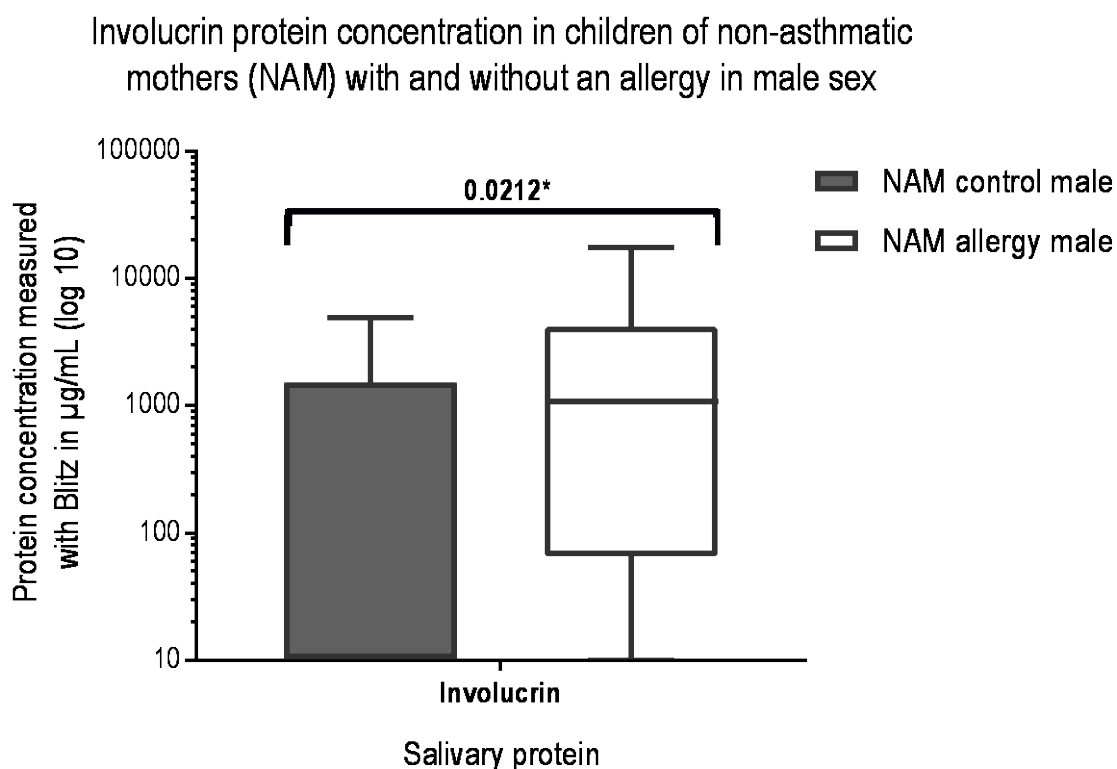


Figure 100. Involucrin protein concentration in children of non-asthmatic mothers (low risk of allergy) with ($n= 18$) and without ($n= 17$) an allergy in male sex. Protein concentration was measured using Blitz system. Data of involucrin protein concentration was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P<0.05$.

5.4.2.4 Concentration of cornulin (CRNN) protein in the saliva of children with and without an allergy

When comparing the difference in salivary protein expression between children without an allergy to children with allergies, CRNN (Mann Whitney U test, $P=0.049^*$) was significantly decreased in the children with an allergy regardless of sex (Figure 101). Salivary CRNN (Mann Whitney U test, $P=0.0002^{***}$, $P=0.0005^{***}$ and $P=0.038^*$) protein expression was significantly decreased in the allergic children of asthmatic mothers (high risk of allergy) regardless of sex, in male sex and in female sex as compared to non-allergic children of asthmatic mothers (Figures 102, 103 and 104). Furthermore, there was significant decreases in the CRNN (Mann Whitney U test, $P=0.005^{**}$ and $P=0.003^{**}$) salivary protein expression in children with any eczema and any rhinitis combination regardless of sex, as compared to children without specific allergy (Figure 105). It did not change significantly in any other groups.

The summary of the significant statistical analyses on estimated proteins concentrations for these three proteins on the Blitz system is listed in Table 33.

i. Children with allergy vs children with no allergy

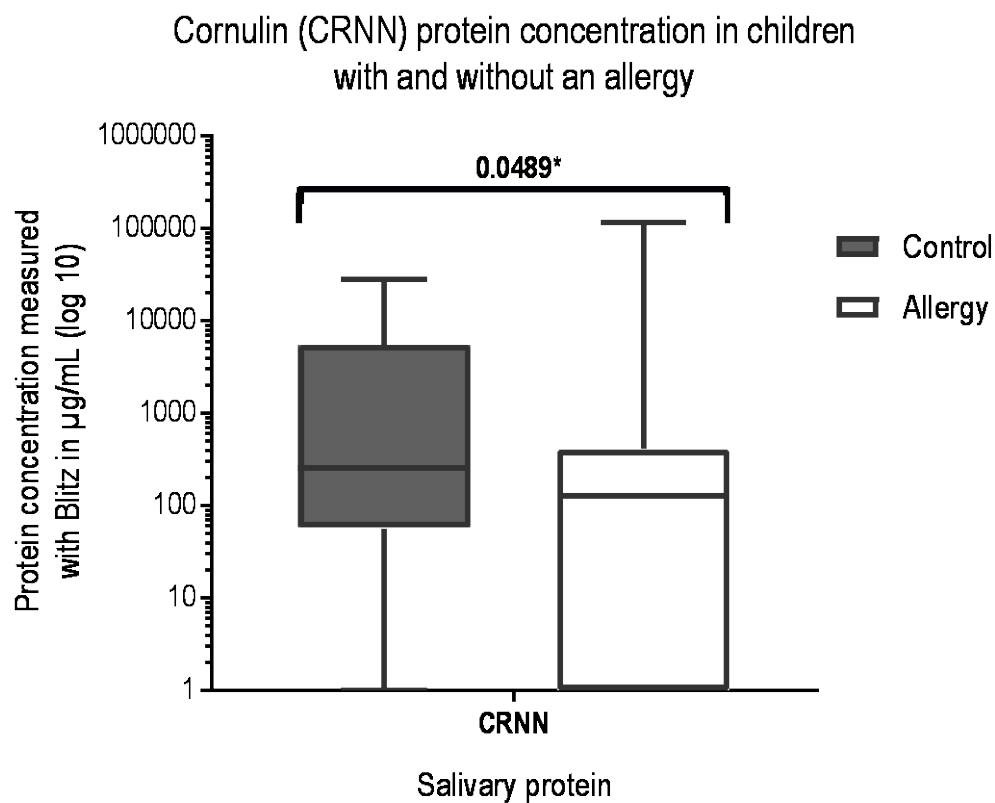


Figure 101. CRNN protein concentration in children with (n= 65) and without (n= 31) an allergy. Protein concentration was measured using Blitz system. Data of CRNN protein concentration was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

ii. The influence of the presence and absence of maternal asthma during pregnancy on salivary protein expression and child allergy

a. Children of asthmatic mothers (high risk of allergy) with and without an allergy

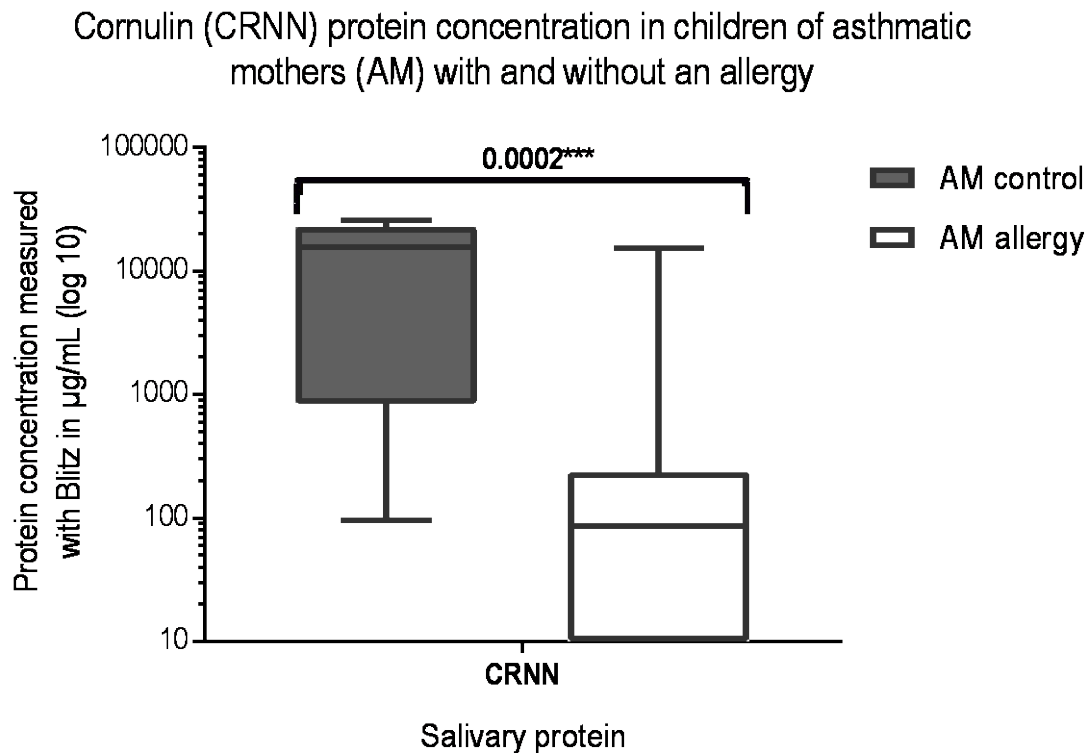


Figure 102. CRNN protein concentration in children of asthmatic mothers (high risk of allergy) with ($n=37$) and without ($n=7$) an allergy. Protein concentration was measured using Blitz system. Data of CRNN protein concentration was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P<0.05$.

b. The effect of sex and the presence and absence of maternal asthma on salivary protein expression

Cornulin (CRNN) protein concentration in children of asthmatic mothers (AM) with and without an allergy in male sex

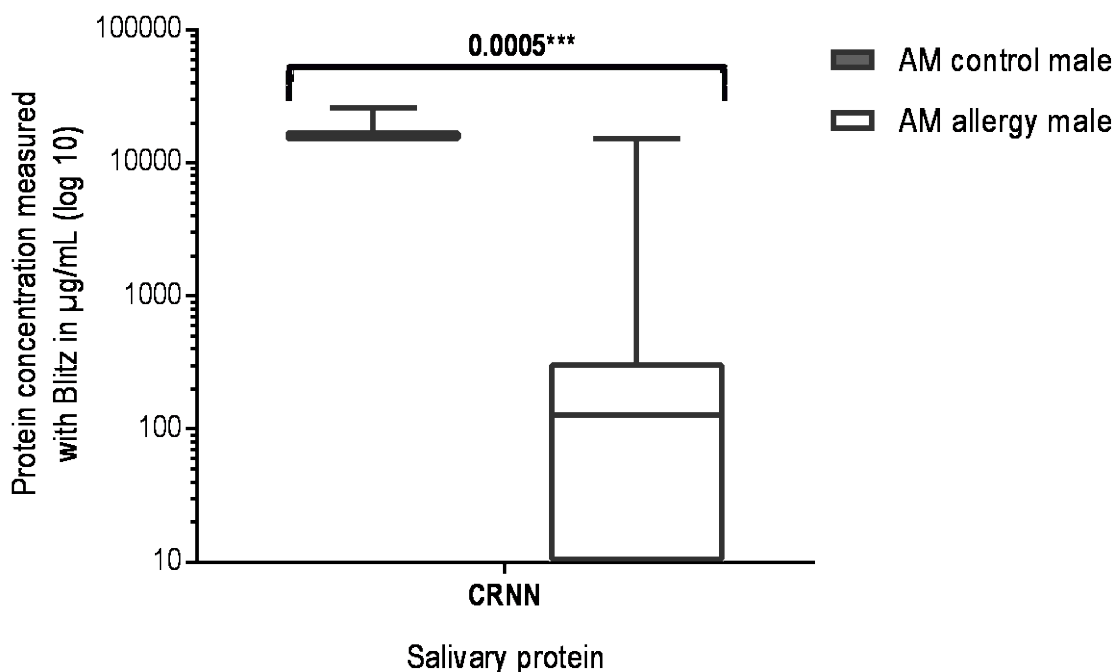


Figure 103. CRNN protein concentration in children of asthmatic mothers (high risk of allergy) with (n= 21) and without (n= 3) an allergy in male sex. Protein concentration was measured using Blitz system. Data of CRNN protein concentration was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

Cornulin (CRNN) protein concentration in children of asthmatic mothers (AM) with and without an allergy in female sex

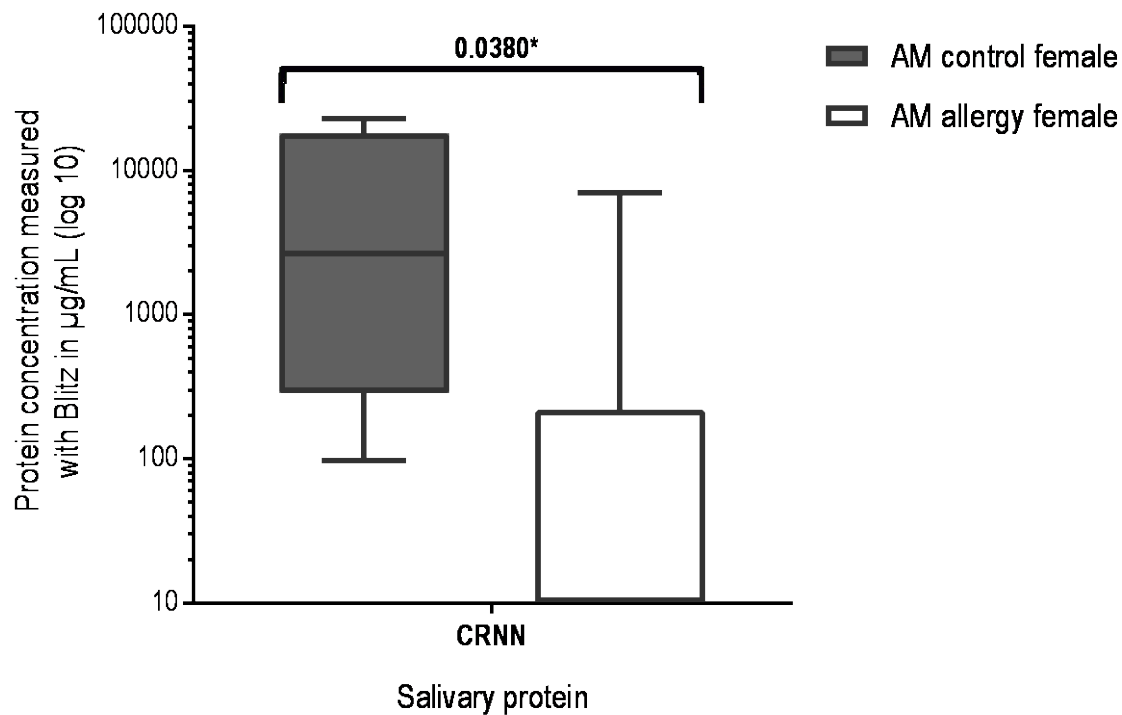


Figure 104. CRNN protein concentration in children of asthmatic mothers (high risk of allergy) with (n= 16) and without (n= 4) an allergy in female sex. Protein concentration was measured using Blitz system. Data of CRNN protein concentration was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

iii. Effect of specific allergic phenotypes on salivary protein expression and child allergy

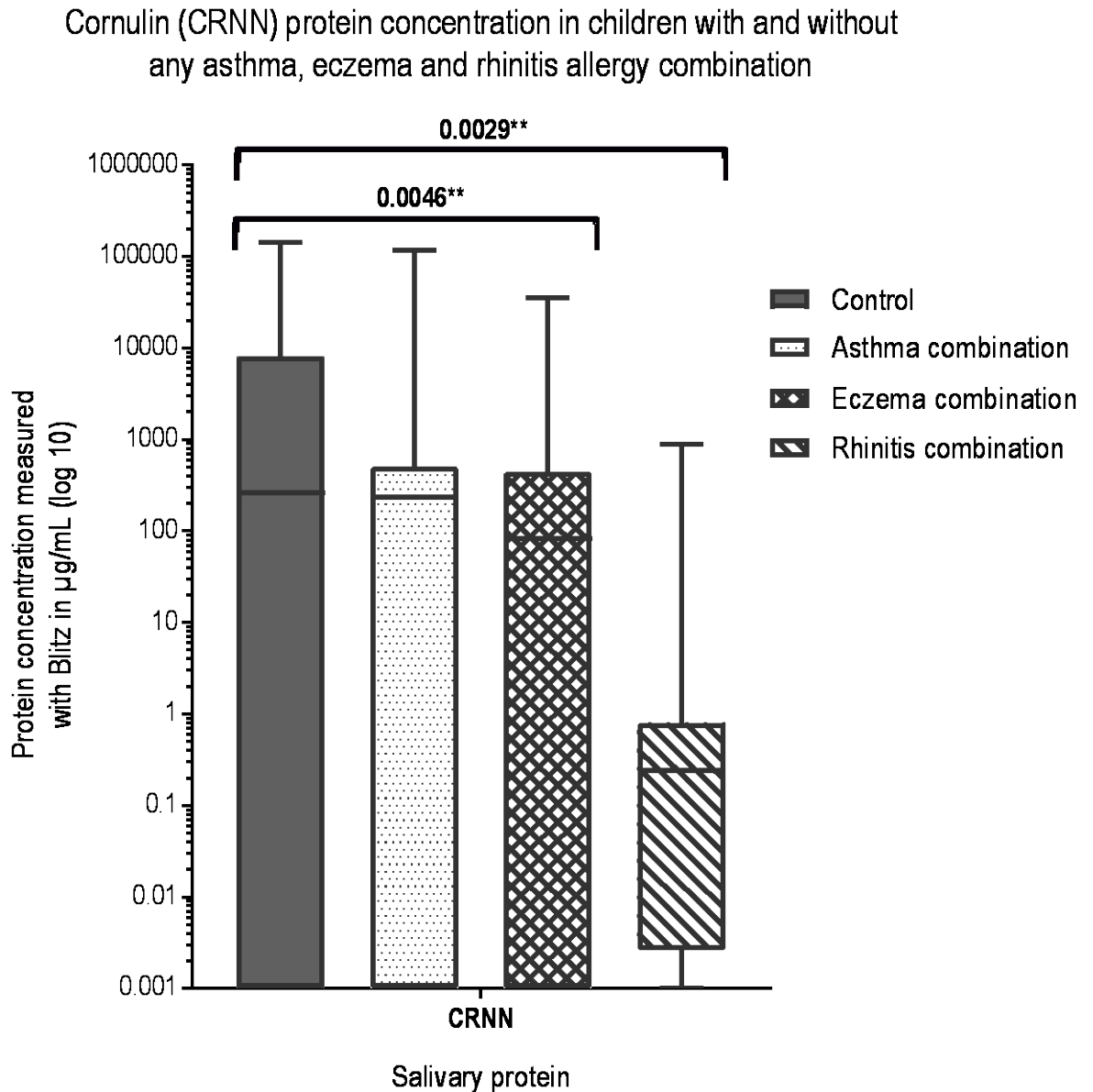


Figure 105. CRNN protein concentration in children with and without any asthma, eczema and rhinitis allergy combination. Asthma combination, $n = 52$; eczema combination, $n = 43$; rhinitis combination, $n = 27$; and non-allergic children, $n = 31$. Protein concentration was measured using Blitz system. Data of CRNN protein concentration was expressed as box plot with the minimum and maximum values plotted. Data were analysed by Mann-Whitney U test with two-tailed p-value. A significant result was $P < 0.05$.

Table 33. Significant statistical analyses of estimated proteins concentrations on the Blitz system in relation to childhood allergy distribution and to the presence (high risk of allergy) or the absence (low risk of allergy) of maternal asthma. (Significant result was $P < 0.05$).

Salivary protein	Significant Blitz system statistical analyses	Statistical analysis used	P values
14-3-3 zeta	AM control vs AM allergy	MWU	0.0075**
	Control vs specific allergy (rhinitis combination)	MWU	0.0120*
Involucrin	AM control vs AM allergy	MWU	0.0343*
	NAM control male vs NAM allergy male	MWU	0.0212*
Cornulin (CRNN)	Control vs allergy	MWU	0.0489*
	AM control vs AM allergy	MWU	0.0002***
	AM control male vs AM allergy male	MWU	0.0005***
	AM control female vs AM allergy female	MWU	0.0380*
	Control vs specific allergy (eczema combination)	MWU	0.0046**
	Control vs specific allergy (rhinitis combination)	MWU	0.0029**

° Abbreviations: AM represents children with asthmatic mothers (high risk of allergy), NAM represents children with non-asthmatic mothers (low risk of allergy) and MWU represents Mann Whitney U test with two-tailed p-value.

5.4.3 Blitz blind test of 14-3-3 zeta and cornulin (CRNN) salivary proteins using saliva samples from children with and without an allergy

To verify the findings in the Blitz experiments, I conducted a Blitz blind test on 14-3-3 zeta and CRNN salivary proteins. A total of 50 child saliva samples (Control n= 26, Allergy n= 24) were randomized and the allergy status was kept confidential and unknown to the researcher prior to conducting the Blitz blind test. Similarly as in Blitz validation test, analyses of the estimated protein concentrations of these two proteins were performed in relation to child allergy phenotype, child sex, and the presence or absence of maternal asthma during pregnancy (refer Methodology section 2.12.1).

From the Blitz blind test result, none of the protein had significant difference in child saliva with allergy as compared to child saliva with no allergy. Moreover, the receiver operating characteristic (ROC) for salivary 14-3-3 zeta protein indicate area under the ROC curve of 0.528 with p value= 0.734, which was far less than the perfect value of 1.0 (data not shown). For salivary CRNN protein, there is no obvious difference of protein binding rate between allergic child saliva and non-allergic child saliva on Blitz blind test and this data was not statistically significant. Furthermore, the area under the ROC curve for salivary CRNN protein was 0.521 with p value= 0.834. On further analysis, combining 14-3-3 zeta and CRNN salivary proteins gave higher area under ROC which was 0.565 with p value= 0.734, however the differences between area under ROC was still not significant. These data indicate that the Blitz blind test did not discriminate between allergic children and non-allergic children which possibly be due to the low number of child saliva samples used (n= 49) in the test.

5.5 Discussion

Current findings suggest that salivary protein expression can be altered in children between the ages of six months to five years who subsequently develop an allergy. Altered expression of salivary proteins varies in relation to presence (high risk of allergy) or absence (low risk of allergy) of maternal asthma, including increased 14-3-3 protein expression altered in both conditions and increased CRNN expression only in absence of maternal asthma (low risk of allergy), identified with Western blot.

As a discovery tool, LC-MS/MS analysis revealed interesting proteins that can exert a programming effect on the fetal immune system, hence are associated with allergic diseases. These include interleukin-1 receptor antagonist protein (IL-1RN), calmodulin-like protein 3, histatin-1, cystatin-SA, lactotransferrin, 14-3-3 eta (η) and Hp. Interestingly, both 14-3-3 eta (an isomer of the 14-3-3 proteins) and Hp proteins identified in placental samples were also present in saliva samples.

There are several lines of evidence showing that IL-1RN protein (*IL1RN* gene) is associated with inflammation and allergic diseases ⁽³⁷⁹⁻³⁸²⁾. The main function of IL-1RN is to inhibit activity of IL-1 by binding to receptor *IL1R1*, thus preventing its association with the co-receptor IL-1 receptor accessory protein (IL-1RAcP) for signalling ⁽³⁸³⁾. IL-1RN is an effective anti-inflammatory treatment in several chronic inflammatory diseases including rheumatoid arthritis and periodontitis ^(383, 384). Variations of the *IL1RN* gene are associated with microvascular complications of diabetes 4 (MVCD4) ⁽³⁷⁹⁾. The *IL1RN* gene is a potential genetic marker for inflammatory complication severity in MVCD4 which include diabetic retinopathy, diabetic nephropathy leading to end-stage renal disease and diabetic neuropathy ⁽³⁷⁹⁾. Furthermore, IL-1RN is a potential therapeutic candidate for treatment of asthma with neutrophil predominance by effectively reducing airway neutrophilic inflammation ⁽³⁸⁰⁾. In allergic contact dermatitis, there is enhanced expression of the IL-1 family, including IL-1B, IL-1RN, IL-36 and IL-33, implicated to pathogenesis of this disease ⁽³⁸²⁾. Moreover, children with acute spontaneous urticaria have increased levels of IL-1RN and IL-18 than healthy subjects which correlates with CRP, D-dimer and IL-1 β levels ⁽³⁸¹⁾. These findings suggest that alteration of IL-1RN protein expression is associated with allergic diseases. However, because IL-1RN is altered in inflammatory responses in a nonspecific manner, it was not chosen to be further validated by Western blot in this research.

Calmodulin-like protein 5, also known as calmodulin-like skin protein (CLSP), is increased in skin samples and vernix of AD patients ^(56, 385). It plays a role in modulating the function of calcium-dependent proteins involved in skin barrier formation and is significantly increased in the epidermis of psoriatic patients ⁽³⁸⁵⁾. Increased calmodulin-like protein 5 expression was observed in the epidermis of patients with acute exacerbated AD, as compared to patients with non-exacerbated AD and control

subjects ⁽³⁸⁵⁾. Other than skin samples, vernix collected at birth is used to detect expression level of calmodulin-like protein 5 to predict development of AD in early childhood ⁽⁵⁶⁾. Moreover, strong negative correlation was observed between calmodulin-like protein 5 and polyubiquitin-C to children with AD ⁽⁵⁶⁾. Calmodulin-like protein 3 (*CALML3* gene) is expressed in normal mammary, prostate, cervical and epidermal tissues, but is greatly reduced or undetectable in transformed cells ⁽³⁸⁶⁾. The main function of calmodulin-like protein 3 is as a specific light chain of unconventional myosin-10 (Myo10) by enhancing Myo10 translation, possibly by acting as a chaperone for the emerging Myo10 heavy chain protein ⁽³⁸⁷⁾.

Amongst the proteins identified in LC-MS/MS, eight potential salivary protein biomarkers for allergy, Imp8, calmodulin-like protein 3, transferrin, desmoglein 1, 14-3-3, involucrin, CRNN and Hp, were further chosen to be validated in Western blot. However, Imp8 and calmodulin-like protein 3 were not able to be optimised in Western blot, hence were not used in the validation study. Western blot data validation analysis demonstrated that of six salivary proteins, only four (desmoglein 1, 14-3-3, involucrin and CRNN) were possibly involved in the mechanism of childhood allergy susceptibility. Some altered expression of these four salivary proteins vary in a sex-specific manner, as previously observed in placental samples, such as increased 14-3-3 and desmoglein 1 expression in female allergic children and increased involucrin expression in male allergic children.

Increased serum transferrin level during pregnancy is associated with decreased maternal iron status, which is adversely associated with childhood wheeze, lung function and atopic sensitisation ⁽³⁸⁸⁾. Transferrin protein (*TF* gene) is expressed by the liver and secreted in plasma. Among the main functions of transferrin protein are: (1) as an iron binding transport protein; (2) transport of iron from sites of absorption and heme degradation; and (3) plays a role in stimulating cell proliferation ⁽³⁸⁹⁾. Transferrin protein is found in very high concentrations in serum (>1000 mg/L), but low concentrations in saliva (<5 mg/L) under normal circumstances ⁽³⁹⁰⁾. Its large size (77 kDa) prevents transferrin from being passively or actively transported from general circulation to saliva ⁽³⁹¹⁾. Interestingly, in placenta LC-MS/MS data analysis of this study, transferrin protein had low expression (ratio <0.5) in allergic child saliva, as compared to non-allergic children. As inflammation occurs, transferrin protein level is decreased via a mechanism associated with the innate immune system ⁽³⁹²⁾. Moreover, transferrin protein is usually low to low-normal in anaemia of chronic disease, such as chronic asthma ⁽³⁹³⁾. This hypothesis is supported by a study that demonstrated school children with poorly controlled asthma had decreased vitamin E and transferrin protein levels ⁽³⁹⁴⁾. Hence, factors such as inflammation process and chronic asthma may possibly explain low expression level of transferrin protein in LC-MS/MS data analysis of this study.

Desmoglein 1 protein (*DSG1* gene) plays an important role in various human cutaneous disorders, including AD, complex autoimmune diseases, such as pemphigus vulgaris and pemphigus foliaceus, and chronic rhinosinusitis ^(395, 396). Desmoglein 1 protein is a member of the cadherin superfamily of calcium dependent cell adhesion molecules and is expressed in the epidermis as keratinocytes transit out of the basal human epidermis layer ^(395, 397, 398). Desmoglein 1 forms a component of intercellular desmosome junctions and is involved in the interaction between plaque proteins and intermediate filaments mediating cell-cell adhesion ⁽³⁹⁶⁾. It is also expressed in the tongue, tonsil and oesophagus ⁽³⁹⁶⁾. Diminished expression of desmoglein 1 that leads to mRNA decay may be caused by homozygous nonsense mutation of the *DSG1* gene ⁽³⁹⁵⁾. Moreover, biallelic mutations of the *DSG1* gene are associated with severe dermatitis, multiple allergies and metabolic wasting ⁽³⁹⁵⁾. The *DSG1* gene is reduced in oesophageal biopsies from patients with eosinophilic esophagitis, an allergic disorder characterised by chronic inflammation within the oesophageal mucosa ⁽³⁹⁹⁾. In this condition, *DSG1* gene silencing weakens oesophageal epithelial integrity, induces cell separation and impairs barrier function, despite high levels of desmoglein 3. These data suggest that *DSG1* gene loss may potentiate allergic inflammation through induction of pro-inflammatory mediators, such as periostin ⁽³⁹⁹⁾. In addition, altered *DSG2* gene expression is associated with chronic inflammatory conditions, such as Crohn's disease, and carcinogenesis, such as non-small cell lung cancer (NSCLC) and prostate cancer ^(397, 398, 400).

From placental Western blot data analysis, three potential salivary protein biomarkers for allergy that include 14-3-3, involucrin and CRNN were chosen to be further validated. The next step was to prove concept of these salivary proteins in the second stage of validation study using Blitz system with a larger sample size of child saliva (n= 126) (refer Methodology section 2.11.3.1).

The first salivary protein validated using Blitz system was 14-3-3. Other isomers of 14-3-3 proteins, 14-3-3 *sigma* mRNA expression, have been shown to be increased in punch skin biopsies from patients with psoriasis and contact dermatitis ⁽³²²⁾. The 14-3-3 proteins are a highly conserved protein family that are ubiquitously expressed in various mammalian tissues ^(401, 402). To date, there are seven isoforms of 14-3-3 encoded in humans: beta (β), gamma (γ), epsilon (ϵ), zeta (ζ), eta (η), theta (θ or τ) and sigma (σ) ⁽⁴⁰²⁾. 14-3-3 proteins are involved in a number of cellular processes, including cell signalling, cell cycle regulation and gene transcription, as well as in immune function ^(322, 403). In adult saliva samples, 14-3-3 proteins were decreased in subjects with generalised aggressive periodontitis and were differentially expressed in rheumatoid arthritis patients ^(404, 405). 14-3-3 sigma released from keratinocytes acts on the dermal fibroblast and modulates its production of extracellular matrix proteins ⁽⁴⁰⁶⁾. The epithelial cell-derived extracellular form of 14-3-3 sigma protein is an important inducer of *MMP1* gene expression in skin fibroblasts and in the respiratory airway ⁽³²³⁾. Furthermore, *MMP1* gene

is needed to modulate production or breakdown of collagen by lung fibroblasts during airway remodelling in asthma ⁽³²³⁾. Therefore, 14-3-3 proteins are most likely to be involved in the mechanism of allergic susceptibility, especially in asthma and contact dermatitis.

Involucrin is the second salivary protein validated using the Blitz system. Involucrin protein (*IVL* gene) is associated with AD, most possibly via skin barrier regulation ⁽⁵⁴⁾. Involucrin is a protein precursor of the cross-linked cornified envelope (CE) which eventually becomes an early differentiation marker of epidermal keratinocytes ^(407, 408). Insoluble CE of stratified squamous epithelium in the outermost epidermal layers is assembled from transglutaminase cross-linked proteins and lipids ⁽⁵⁴⁾. Involucrin is amongst the proteins that form the protein scaffold on which the CE assembles ^(66, 409). CE proteins are encoded in the EDC located on chromosome 1q21, and are divided into four families. Of the four families, involucrin and loricrin are among the members of late CE (LCE) proteins of which profilaggrin (pro-FLG), FLG-2 and CRNN belong to S100-fused type proteins ⁽⁴¹⁰⁾.

The *IVL* gene is involved in pathogenesis of AD and asthma ^(66, 409, 411). Using microarray data, functional enrichment analysis revealed that the *IVL* gene is among differentially expressed genes significantly enriched for proteins related to epidermis development associated with AD ⁽⁴¹²⁾. Involucrin is suppressed in human AD and its expression in keratinocytes is downregulated by IL-4 post transcriptionally, probably via miRNA regulation ⁽⁴⁰⁸⁾. Other than AD, involucrin is associated with other skin pathologies, including psoriasis and skin squamous cell carcinoma (SCC) ^(407, 413). In psoriasis patients, there was increased expression of involucrin which leads to abnormal keratinocyte differentiation and hence to formation of psoriatic plaques ⁽⁴¹³⁾. In contrast, in poorly differentiated skin SCC with psoriasis condition, low or no quantity of involucrin was detected ⁽⁴⁰⁷⁾. This data suggests that *IVL* may also act as a tumour suppressor gene.

There are contradicting results regarding up or downregulation of *IVL* gene expression in AD skin in human studies ^(62, 66, 414). A large scale microarray analysis study by Sugiura *et al.* observed increased *IVL* gene expression in lesional AD skin ⁽⁶²⁾. Consistent with this study, Jarzab *et al.* demonstrated significant upregulation of *IVL* RNA in chronic AD skin lesions using qRT-PCR method ⁽⁶⁶⁾. Contrary to the findings of Sugiura *et al.*, Jensen *et al.* indicate decreased *IVL* expression analysed with immunohistochemistry and Western blotting ⁽⁴¹⁴⁾. Discrepancies of these contradicting results may be explained by difference in experimental design ⁽⁴¹⁵⁾. During skin sample collection via method of punch biopsy, onset of acute or chronic AD predominantly influence up or downregulation of *IVL* expression which affects overall study findings. This is because AD lesions are biphasic, with acute atopic lesions having more Th2 cytokine milieu and chronic atopic lesions having more Th1 cytokine milieu ^(415, 416). AD onset during skin sample collection was not clearly characterised in the previous studies, except by Jarzab *et al.* that used chronic AD.

In this research, child saliva samples were used which were not predominantly influenced by biphasic skin lesion of AD and mostly were collected when the child was not in an acute stage of AD. Furthermore, biological fluids, such as saliva, are more reliable because saliva has less cells compared to skin samples, therefore is less likely to be influenced by the changes in Th1/Th2 cytokines milieu. With regards to involucrin level in AD patients, results of this research were consistent with those of Sugiura *et al.* and Jarzab *et al.*, demonstrating increased involucrin protein expression in allergic children ^(62, 66).

Involucrin influences immune system development that leads to a greater susceptibility to allergy via mechanisms involving IL-13 and IL-4 via STAT6 signalling pathway ^(417, 418). IL-13, which is secreted by activated T cells, shares many biologic responses with IL-4, including signalling through IL-4 receptor to activate the STAT6 signalling pathway to down-regulate important components of the innate immune response ^(417, 418). Importance of the STAT6 signalling pathway was observed in response to IL-4 in STAT6-deficit mice. The study demonstrated: (1) reduced expression of CD23 and MHC class II in resting B-cells, (2) abolished B-cell proliferation costimulated by anti-IgM antibody and (3) reduced T cell proliferative response ⁽⁴¹⁸⁾. Moreover, production of Th2 cytokines (IL-4 and IL-13) from T cells, along with IgE and IgG1 responses, were extremely reduced ⁽⁴¹⁸⁾. In primary keratinocytes, *IVL* gene expression was downregulated by IL-4 and IL-13, possibly due to overexpression of Th2 cytokines in acute AD skin ⁽⁴¹⁵⁾. Skin biopsies from STAT6 transgenic mice exhibited significantly lower levels of *IVL*, as compared to their wild-type controls, which support that IL-4 and IL-13 modulation of *IVL* is mediated by STAT6 activation ⁽⁴¹⁵⁾. Moreover, decreased expression of *IVL* in AD is partly caused by overexpression of Th2 cytokines that downregulate *IVL* expression during differentiation into CE ⁽⁴¹⁵⁾.

IVL is known to be epigenetically modified in keratinocyte differentiation. A previous study investigated regulation of *EDC* gene expression reporting that treatment of keratinocytes with DNA methyltransferase inhibitor, 5-azacytidine, increased involucrin expression ⁽⁴¹⁹⁾. *IVL* expression in keratinocyte differentiation can be silenced by histone methylation mediated by signalling of protein arginine methyltransferase 5 and methylosome protein 50 (PRMT5/MEP50) ⁽⁴²⁰⁾. This was demonstrated in a recent study that investigated the relationship between protein kinase C δ and p38 δ , key proteins that stimulate keratinocyte differentiation and PRMT5/MEP50 signalling ⁽⁴²⁰⁾. PRMT5/MEP50 symmetrically dimethylates arginine 8 of histone 3 and arginine 3 of histone 4 to silence *IVL* gene expression. MEP50 suppresses *IVL* mRNA level and promoter activity by increasing arginine dimethylation of *IVL* gene-associated H3/H4 ⁽⁴²⁰⁾.

The third salivary protein validated using Blitz system was CRNN (*CRNN* gene). *CRNN* gene has been identified as a new differentially expressed gene in AD ⁽⁴²¹⁾. This gene comprises three exons and encodes a protein of 495 amino acids with an EF-hand calcium binding motif, implying that *CRNN* may

bind to calcium ^(422, 423). This gene is predominantly expressed in differentiated squamous epithelium with highest expression in granular and lower cornified cell layers of scalp epidermis and foreskin and in calcium-induced differentiated cultured keratinocytes with the highest levels found in oesophagus and skin ^(421, 424). CRNN, a CE protein also encoded in the EDC, is among of S100-fused type protein members ^(410, 421, 424). The main functions of CRNN protein is as a survival factor that participates in the clonogenicity of squamous oesophageal epithelium cell lines, attenuating deoxycholic acid (DCA)-induced apoptotic cell death and in release of calcium ^(422, 423). Reduced expression of *CRNN* may be an initial part of pathogenesis leading to epithelial barrier dysfunction in AD which is characterised by increased keratinocyte proliferation, impaired differentiation and increased apoptosis of keratinocytes ^(54, 421). Thus, it is possible that after AD is established, CRNN protein expression may increase to compensate for epithelial barrier dysfunction.

AD susceptibility is increased with genetic variation at the *CRNN* gene locus ^(410, 421). In a gene expression study, Lieden *et al.* demonstrated that *CRNN* mRNA expression is significantly decreased in eczematous skin, as compared to controls ⁽⁴²¹⁾. The findings revealed that Der p 2 (house dust mite allergen) patched mice developed localised eczema with downregulation of *CRNN* mRNA, as well as a Th2 skewed systemic response. Moreover, *CRNN* polymorphism, rs941934, was significantly associated with atopic eczema, though only as part of an extended haplotype including a known associated variant (2282del4) in *FLG* ⁽⁴²¹⁾. Recently, Trzeciak *et al.* analysed the SNP of *CRNN* (rs941934) using TaqMan genotyping assay and PCR-restriction fragment length polymorphism (RFLP) in blood samples of 159 AD patients ⁽⁴¹⁰⁾. Data demonstrated that *CRNN* rs941934 (A allele) was associated with AD, a severe course of disease, elevated IgE levels, eosinophilia and concomitant asthma. In addition, mRNA level of *CRNN* was significantly decreased in AD skin ⁽⁴¹⁰⁾. Both studies were consistent with findings of reduced *CRNN* mRNA level in lesional AD skin, as compared to non lesional AD skin, and skin from healthy controls ^(410, 421). However, the possibility of whether *CRNN* mRNA reduction in AD skin is influenced by confounding factors, such as presence of 2282del4 variant in *FLG* whose expression is diminished by Th2 cytokines, was not fully addressed in both studies ⁽⁴²⁵⁾.

In addition to allergic diseases, CRNN is linked to other cancer pathologies, such as oesophageal SCC, oral SCC and malignant progression of oral leukoplakia ⁽⁴²⁶⁻⁴²⁸⁾. CRNN was frequently downregulated in primary oesophageal SCC at mRNA and protein level, which was significantly associated with lymph node metastases, advanced clinical stage and overall survival rate ⁽⁴²²⁾. As a tumour suppressor gene, *CRNN* helps maintain barrier function in squamous epithelium in response to injury, which may play a role in skin barrier dysfunction in AD ^(422, 429). To our current knowledge, *CRNN* gene has not been linked to epigenetic modifications as yet.

Both involucrin and CRNN are CE proteins encoded in the EDC on chromosome 1q21, markers of epidermal differentiation and are reduced in AD ^(414, 415, 421). Thus, these salivary proteins are interconnected and may possibly be epigenetically modified in regulating immune system that increases risk of allergy susceptibility. The proposed mechanistic model of salivary proteins that include involucrin and CRNN underlying increased susceptibility to allergy is as depicted in Figure 106. *In utero* exposure, such as maternal asthma, folate intake and nicotine exposure during pregnancy, are associated with epigenetic modifications and partly modulate fetal programming in modifying allergy risk. Salivary proteins, including 14-3-3, Hp, IL1-RN, calmodulin like-protein, desmoglein 1, transferrin, involucrin and CRNN, are predominantly influenced by postnatal exposures, such as breastfeeding, and early exposure to allergens and microbials, that are also associated with epigenetic modifications. Thus, salivary proteins may possibly be epigenetically modified via *in utero* and postnatal exposures in regulating immune system development that leads to greater allergy susceptibility.

The Blitz system provides practical use of protein biomarkers in a clinical setting. The advantages of the Blitz system are that it is easy to use, more feasible and expertise skill is not required. In contrast, Western blot is laborious and relies on experience and expertise skill. In addition, initial processing to denature proteins in Western blot usually takes a long time, and more equipment and reagents, which is not cost effective. Overall, some salivary protein findings in Western blot and Blitz system show unequivocal and consistent data with each other. In Western blot and Blitz results, CRNN and 14-3-3 protein expression was increased in allergic children with rhinitis combination, as compared to non-allergic children. Furthermore, in both validation studies, pan 14-3-3 had significant protein difference in allergic children with an asthmatic mother (high risk of allergy). This suggests that a certain immune mechanism is altered in the saliva sample by presence of maternal asthma and not associated with gene alterations associated with allergy susceptibility in early childhood ⁽¹³²⁾.

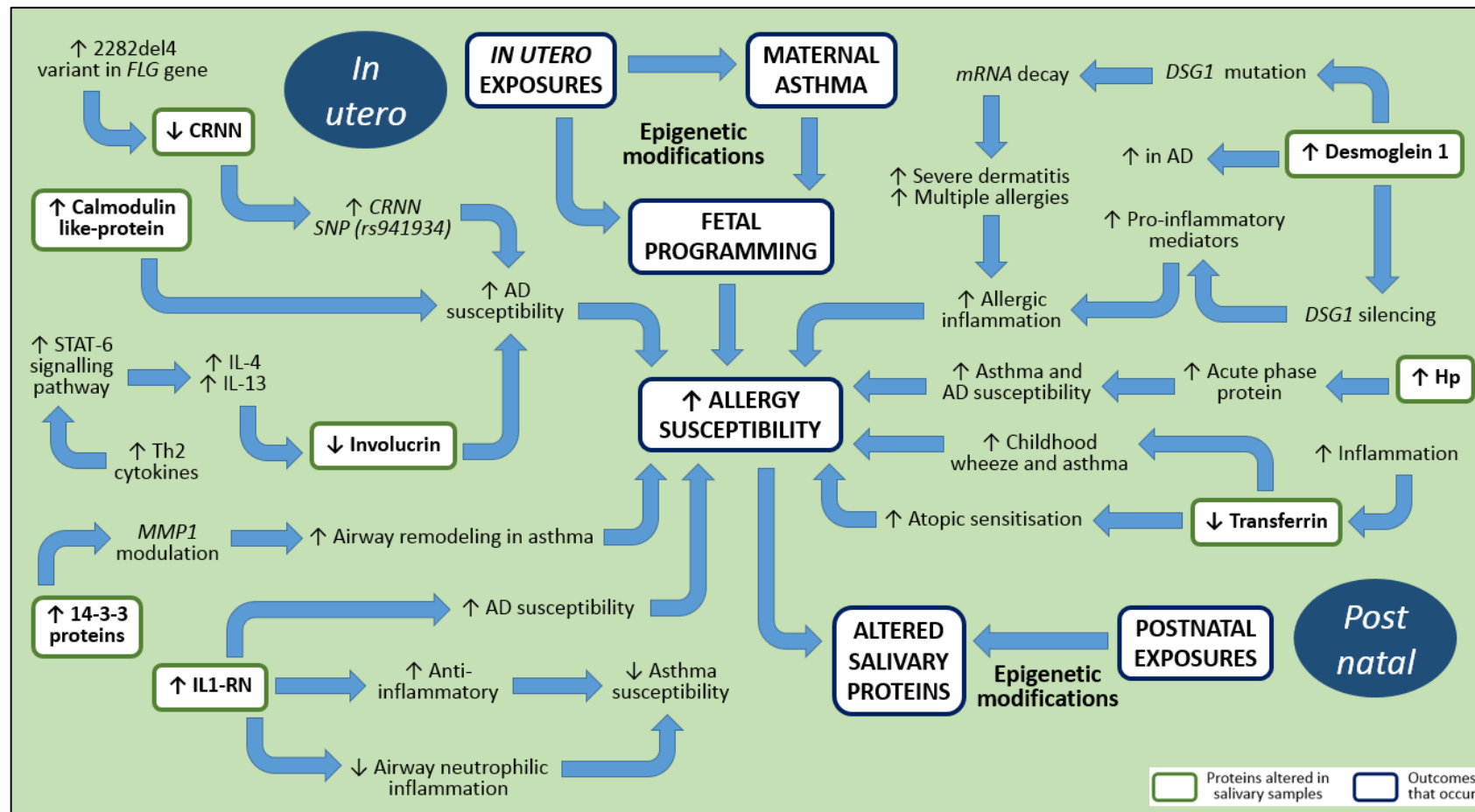


Figure 106. Proposed model of salivary proteins mechanism underlying increased susceptibility to allergy. (Hp represents haptoglobin, CRNN represents cornulin, IL-1RN represents interleukin-1 receptor antagonist protein, AD represents atopic dermatitis, STAT-6 represents signal transducer and activator of transcription, *DSG1* represents desmoglein 1 gene and *FLG* represents filaggrin gene).

Despite both are reliable validation methods, differences were observed between Western blot and Blitz system data. The main difference in terms of data analyses was that the Blitz system used a larger sample size as compared with Western blot (Western blot saliva n: 62, Blitz saliva n: 126). Larger sample size usually provides better study power, whereas small sample size significantly decreases statistical power and decreases flexibility of detecting effect size ^(430, 431). The advantages of having larger sample sizes are: (1) the chances of detecting significant effects are increased drastically, (2) ability to detect small and large effect sizes, regardless of their respective variances, (3) allows for flexibility in detecting allergy effects, and (4) increases flexibility by being able to detect a wide spectrum of effect sizes (small, large, homogeneous and heterogeneous) ^(431, 432). Power of a study is based on total number of study participants. For example, in the Western blot subgroup analysis for allergic susceptibility according to sex, the total sample size for allergic children was 38, and the sample size for male allergic children was 19. Therefore, subgroup analysis performed within the study may not have adequate statistical power because the number of people in subgroups was lower than the total sample size of the study ⁽⁴³⁰⁾.

The second difference is that protein expression level varies between these two validation studies. In Western blot, increased protein expression was observed in allergic children, whereas using the Blitz system decreased protein expression was seen in allergic children, as compared with non-allergic children. Although protein expression level differs, statistical analyses still demonstrated significant protein differences between allergic children and non-allergic children in Western blot and Blitz system. These significant protein differences indicate that protein expression levels were vastly distinctive between these two groups, either in native or denatured state of protein. Interestingly, similar findings were seen in placental qRT-PCR data where significant gene expression was decreased in allergic children, as compared with non-allergic children. The Blitz system and qRT-PCR were not using denatured protein, but native protein and complementary DNA (cDNA).

Different protein expressions level between these two validation studies is most likely due to nature of the protein. The Blitz system used native and crude child saliva samples, whereas Western blot used denatured protein. Denaturation of the protein that occurs in Western blot destroys its tertiary structure and leads to random-coiled aggregation and decreased solubility ⁽³¹²⁾. Moreover, denatured proteins vary from their native precursors in properties such as chain conformational entropies, hydrophobicity, electrostatic forces and solubility ⁽⁴³³⁾. The structure of a native protein plays an important role for IgE-binding conformational epitopes ⁽⁴³⁴⁾. For example, although native ovalbumin (OVA) is a soluble protein, urea-denatured OVA (CM-OVA) may be highly aggregated into the particulate form ^(435, 436). OVA denaturation leads to 10-fold higher frequency of IFN- γ -producing cells than native OVA. The particulate antigen may be predominantly localised in the draining lymph nodes and, therefore, more

efficiently processed by APC ⁽⁴³⁷⁾. Protein denaturation increased IgE-binding conformational epitopes ⁽³¹²⁾. A recent study examined the effect of sublingual administration of a native or denatured allergen alone, or plus adjuvant, on systemic Th2 responses and mucosal immunity in mice ⁽³¹²⁾. Findings revealed that sublingual vaccination with OVA, or urea-denatured OVA plus adjuvant cholera toxin or CpG oligodeoxynucleotides, can induce systemic and mucosal immunity, characterised by strong serum IgG and saliva secretory IgA antibody levels. Urea-denatured OVA plus CpG oligodeoxynucleotides had the best prophylactic and therapeutic effects on IgE antibody production ⁽³¹²⁾. These data may explain findings in this study of increased protein expression in allergic children using denatured protein in Western blot as opposed to decreased level of protein expression using native protein in the Blitz system.

A good and reliable salivary protein biomarker would be able to detect allergic children from the population of non-allergic children regardless of other confounding factors, including presence of maternal asthma. Therefore, salivary CRNN is a good potential biomarker as it can discriminate allergic children regardless of child sex and genetic risk, whereas salivary 14-3-3 protein can only detect allergic children with presence of maternal asthma (high risk of allergy). Nevertheless, a combination of CRNN and 14-3-3 protein may make good and reliable salivary protein biomarkers for allergic diseases in children.

This chapter examined the protein profile of saliva in children with and without an allergy using a proteomic approach, identified novel proteins that vary in relation to allergy and validated findings with several techniques in a larger population. The current findings support the second hypothesis that childhood susceptibility to allergy can be detected in saliva. Moreover, findings indicate that saliva is a biological fluid that can be used to easily detect allergy in susceptible children, and the salivary proteomic profile varies with different allergic phenotypes. In addition, findings support that altered expression of these salivary proteins varies in presence or absence of maternal asthma. The chapter investigates whether changes in placenta proteomic profiles are reflected in saliva in early childhood.

6.0 COMPARISON OF PROTEINS ASSOCIATED WITH ALLERGY BETWEEN PLACENTA AND SALIVA SAMPLES

6.1 Introduction

Environmental exposures during critical stages of early human development can alter gene expression and disease predisposition through epigenetic mechanisms ^(102, 103). These alterations *in utero* may induce permanent changes in many physiological processes and increase the risk of chronic disease susceptibility in postnatal life, including type 2 DM, obesity, hypertension, coronary heart disease, chronic lung disease, chronic kidney disease, metabolic and immune function disorders that include allergic diseases ^(7, 19, 103-107). From the Barker hypothesis, it was proposed that LBW caused by intrauterine nutrient restriction was a major determinant for cardio-metabolic disease in adulthood ⁽⁴³⁸⁾. Mechanisms involved in allergic disease susceptibility include *in utero* programming of organ structure and permanent changes in gene expression through epigenetic modifications ^(17, 19).

To date, changes that occur *in utero* that are measurable in early life are limited. Some examples include protein biomarkers of fetal lung hypoplasia and kidney function ^(439, 440). The degree of fetal lung hypoplasia *in utero* is measurable in premature infants using amniotic fluid concentrations of SP-B and SP-C ^(227, 440). Surfactant proteins may also be applied as biomarkers for alveolar permeability and continue to be different through life as long as disease persists ⁽⁴⁴¹⁾. Recent evidence suggests that serum SP-B is the most reliable biological marker of alveolar capillary membrane function in heart failure ⁽⁴⁴²⁾. For kidney function, cystatin-C originates almost exclusively in the neonate and is not affected by maternal values ^(443, 444). Cystatin-C values reflect the maturity level of renal function which is inversely correlated with GFR in the fetus and in postnatal life ⁽⁴⁴⁴⁾. Moreover, serum cystatin-C is a better diagnostic marker of renal function in detection of early acute kidney injury after perinatal hypoxia/asphyxia compared with creatinine, especially in the first three days after birth ^(439, 445). These data suggest events that influence programming of organ development *in utero* and result in alterations of gene or protein functions can persist into early life.

The placenta mediates the effects in the offspring directly. The *in utero* alterations which occur in the placenta can be detected in the postnatal life and through life as long as disease persists ^(441, 444). The hypothesis for this particular analysis was that changes in placenta proteomic profiles are reflected in

saliva in early childhood. Therefore, the purpose of this series of studies in placenta and saliva was to compare proteins present between placenta and saliva samples in children with and without an allergy.

The specific aims for this work were:

- 1) To determine proteins with similar expression profile in placenta and saliva samples of children with allergic diseases.
- 2) To determine whether differential expression of proteins that are altered in placenta can also be detected in saliva of children with allergic diseases.

LC-MS/MS findings on placental and saliva samples discussed in this chapter are depicted in Figure 107.

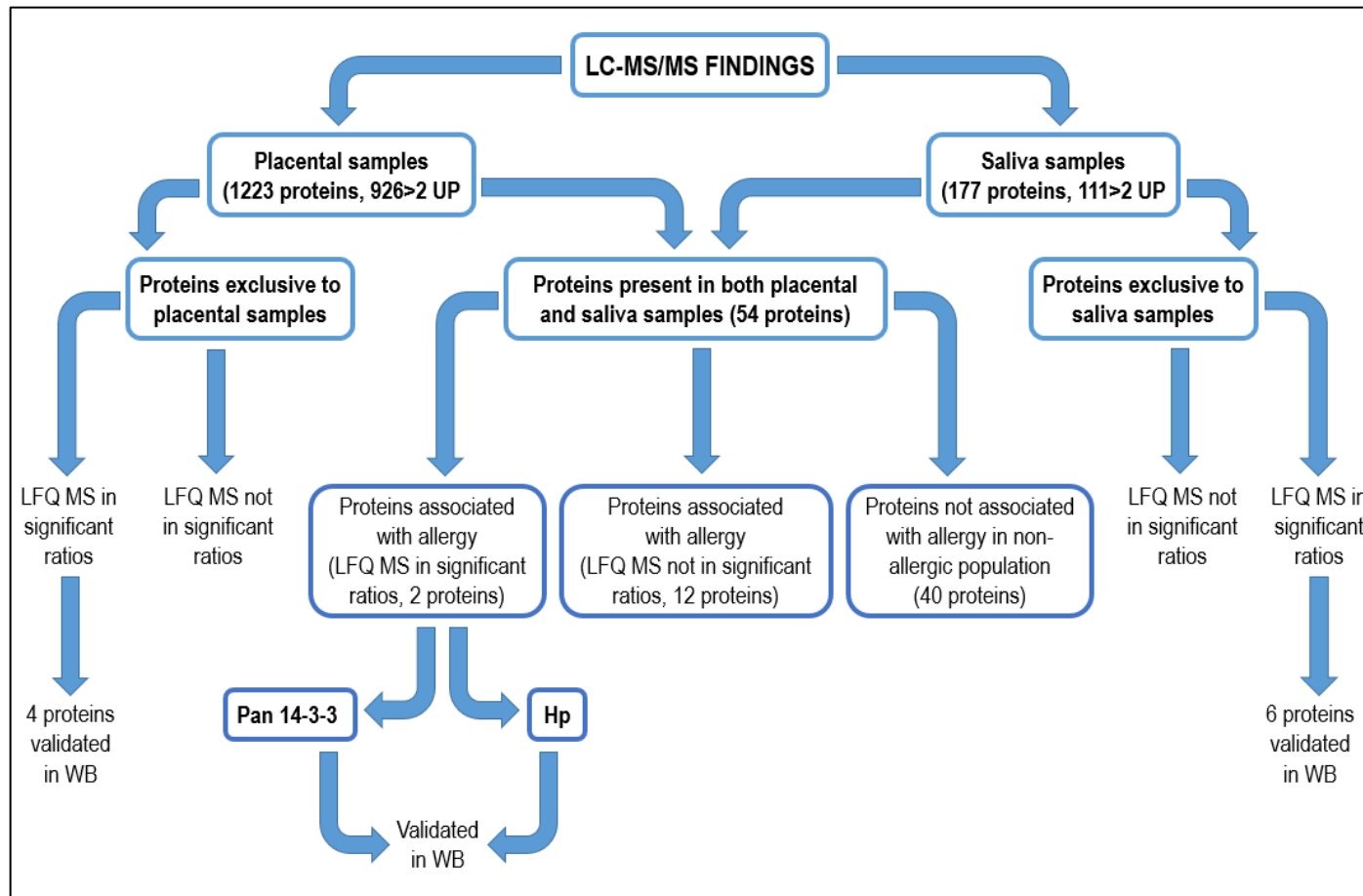


Figure 107. LC-MS/MS findings on both the placental and saliva samples. (Significant ratio > 2 or < 0.5 fc, LC-MS/MS represents liquid chromatography-tandem mass spectrometry, UP represents unique peptides, LFQ MS represents label-free quantification mass spectrometry, Hp represents haptoglobin and WB represents Western blot).

6.2 Proteins identified in placenta and saliva samples

There were 54 similar proteins identified by LC-MS/MS in placenta and saliva samples, regardless of whether these proteins had significant LFQ MS ratios or not in both the allergic children and non-allergic children (Table 34). Amongst proteins that were found in both saliva and placenta regardless of presence or absence of allergy were: Thioredoxin; Immunoglobulin kappa variable 3-20; Protein S100-A9; Transthyretin, PRDX5; Triosephosphate isomerase; 14-3-3 protein sigma; Hp; Immunoglobulin heavy constant alpha 1; Serum albumin; Serotransferrin; Myeloperoxidase; Alpha-2-macroglobulin, C3, and Titin (Table 34).

The UP and unique sequence coverage for the identical protein differed between placental and saliva samples. UP are the total number of peptides specifically associated with only one protein group and these peptides are not shared with another protein group. Unique sequence coverage is the percentage of the sequence that is covered by the identified unique peptides of the protein sequence contained in the protein group.

For placenta samples, UP varied from 0 to 68 whereas for saliva samples, UP varied from 0 to 78. Unique sequence coverage for placenta samples varied from 0 % to 85.7 % whereas for saliva samples, they varied from 0 % to 96.10 %. The high unique sequence coverage does not depend completely on the number of unique peptides but also on the sequence length of the peptides and the protein the peptide is matching ⁽³⁰¹⁾. Thus, proteins with the highest UP did not necessarily have the highest unique sequence coverage.

The highest number of UP for the placental samples was C3 protein and the highest number of UP for saliva samples was serum albumin protein (bold and highlighted with blue colour in Table 34). C3 protein did not have a significant LFQ MS ratio in the saliva of allergic children as demonstrated by LC-MS/MS although, it did have a significant LFQ MS ratio in the placenta of allergic females as compared to non-allergic children. Moreover, it was one of the five placental proteins selected in the Western blot validation study. Serum albumin had a significant LFQ MS ratio in the allergic (asthma, eczema and rhinitis) saliva samples analysed by LC-MS/MS as compared to non-allergic children. However, serum albumin was not selected because it is commonly observed in saliva and hence is not a suitable candidate for salivary protein biomarkers of allergy.

Table 34. LC-MS/MS comparison of proteins that were identified in both placenta and saliva samples regardless their expression in allergy.

Swiss-Prot entry	Entry Name	Protein names	Gene names	Sequence length (bp)	MW [kDa]	Placenta LC-MS/MS		Saliva LC-MS/MS	
						Unique peptides	Unique sequence coverage [%]	Unique peptides	Unique sequence coverage [%]
Q9BPY8	HOP_HUMAN	Homeodomain-only protein	HOPX	73	8.26	2	35.6	4	19.2
P07108	ACBP_HUMAN	Acyl-CoA-binding protein	DBI	87	10.04	3	52.9	2	18.4
P01717	LV403_HUMAN	Immunoglobulin lambda variable 3-25	IGLV3-25	96	10.35	1	19.8	1	19.8
P05109	S10A8_HUMAN	Protein S100-A8	S100A8	93	10.83	3	32.3	3	29.0
P04080	CYTB_HUMAN	Cystatin-B	CSTB	98	11.14	2	33.7	11	84.7
P05386	RLA1_HUMAN	60S acidic ribosomal protein P1	RPLP1	113	11.40	2	52.2	1	28.3
P10599	THIO_HUMAN	Thioredoxin	TXN	105	11.74	6	51.4	6	65.7
B1N7B8	B1N7B8_HUMAN	Cryocryoglobulin CC1 kappa light chain variable region	N/A	107	11.96	1	15.3	1	22.4
P08493	MGP_HUMAN	Matrix Gla protein	MGP	103	12.35	2	23.3	1	10.7
P14174	MIF_HUMAN	Macrophage migration inhibitory factor (MIF)	MIF	115	12.48	3	35.7	2	11.3
P01619	KV320_HUMAN	Immunoglobulin kappa variable 3-20	IGKV3-20	116	12.56	1	9.0	1	9.0
P04433	KV309_HUMAN	Immunoglobulin kappa variable 3-11	IGKV3-11	115	12.58	0	0.0	1	15.8
P01764	HV323_HUMAN	Immunoglobulin heavy variable 3-23	IGHV3-23	117	12.58	1	8.0	1	8.0
P01625	KV402_HUMAN	Immunoglobulin kappa variable 4-1	IGKV4-1	114	12.64	1	15.8	7	50.0
A0N5G5	A0N5G5_HUMAN	Rheumatoid factor D5 light chain	V<kappa>3	118	12.77	1	15.3	3	46.1
P01593	KV101_HUMAN	Immunoglobulin kappa variable 1D-33	IGKV1D-33	117	12.85	2	25.0	1	14.5
P01617	KV204_HUMAN	Immunoglobulin kappa variable 2D-28	IGKV2D-28	120	12.96	1	23.5	3	46.1
P06702	S10A9_HUMAN	Protein S100-A9	S100A9	114	13.21	6	51.8	7	49.7
P01614	KV201_HUMAN	Immunoglobulin kappa variable 2D-40	IGKV2D-40	121	13.31	0	0.0	3	32.1

Q9UL88	Q9UL88_HUMAN	Myosin-reactive immunoglobulin heavy chain variable region	N/A	131	14.14	1	14.5	5	37.6
Q01469	FABP5_HUMAN	Fatty acid-binding protein, epidermal	FABP5	135	15.16	7	64.4	4	42.8
P02766	TTHY_HUMAN	Transthyretin	TTR	147	15.89	6	49.0	2	13.4
P68871	HBB_HUMAN	Hemoglobin subunit beta	HBB	147	16.00	0	0.0	2	13.4
P30044	PRDX5_HUMAN	Peroxiredoxin-5, mitochondrial	PRDX5	162	17.03	9	73.5	0	0.0
P62937	PPIA_HUMAN	Peptidyl-prolyl cis-trans isomerase A	PPIA	165	18.01	10	66.7	0	0.0
P30086	PEBP1_HUMAN	Phosphatidylethanolamine-binding protein 1	PEBP1	187	21.06	8	69.0	0	0.0
P02763	A1AG1_HUMAN	Alpha-1-acid glycoprotein 1	ORM1	201	23.51	5	25.4	1	8.0
P19652	A1AG2_HUMAN	Alpha-1-acid glycoprotein 2	ORM2	201	23.51	4	18.4	1	8.0
P01700	LV147_HUMAN	Immunoglobulin lambda variable 1-47	IGLV1-47	235	24.82	1	5.5	1	8.0
P01834	IGKC_HUMAN	Immunoglobulin kappa constant	IGKC	231	25.14	0	0.0	1	9.0
V9HW34	V9HW34_HUMAN	Epididymis luminal protein 213	HEL-213	235	25.65	0	0.0	0	0.0
Q6P5S8	Q6P5S8_HUMAN	IGK@ protein	IGK@	236	25.77	0	0.0	0	0.0
P60174	TPIS_HUMAN	Triosephosphate isomerase	TPI1	249	26.67	17	78.7	7	52.6
P31947	1433S_HUMAN	14-3-3 protein sigma	SFN	248	27.77	1	3.2	10	51.6
P25311	ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	AZGP1	298	34.26	5	22.1	24	56.7
P02750	A2GL_HUMAN	Leucine-rich alpha-2-glycoprotein	LRG1	330	36.49	4	15.5	2	10.6
P30740	ILEU_HUMAN	Leukocyte elastase inhibitor	SERPINB1	341	38.69	2	6.2	1	4.4
P02765	FETUA_HUMAN	Alpha-2-HS-glycoprotein	AHSG	367	39.32	6	16.6	3	19.1
P04075	ALDOA_HUMAN	Fructose-bisphosphate aldolase A	ALDOA	364	39.42	19	67.6	4	18.7
P80303	NUCB2_HUMAN	Nucleobindin-2	NUCB2	338	40.37	3	14.2	2	10.1
Q562R1	ACTBL_HUMAN	Beta-actin-like protein 2	ACTBL2	376	42.00	1	4.8	1	4.8
P00738	HPT_HUMAN	Haptoglobin	HP	406	45.21	24	50.5	8	19.6
P06733	ENOA_HUMAN	Alpha-enolase	ENO1	434	47.17	22	59.9	28	61.8
P19971	TYPH_HUMAN	Thymidine phosphorylase	TYMP	482	49.96	3	10.8	2	9.1

P02774	VTDB_HUMAN	Vitamin D-binding protein	GC	474	52.96	1	5.5	2	11.4
P01876	IGHA1_HUMAN	Immunoglobulin heavy constant alpha 1	IGHA1	494	53.09	1	6.5	2	9.5
Q6ZW64	Q6ZW64_HUMAN	cDNA FLJ41552 fis, clone COLON2004478, highly similar to Protein Tro alpha1 H	N/A	494	53.32	1	6.5	1	6.5
P02768	ALBU_HUMAN	Serum albumin	ALB	609	69.37	66	85.7	78	96.1
P11021	GRP78_HUMAN	78 kDa glucose-regulated protein	HSPA5	654	72.33	33	52.0	1	2.8
P02787	TRFE_HUMAN	Serotransferrin	TF	698	77.08	0	0.0	27	27.5
P05164	PERM_HUMAN	Myeloperoxidase	MPO	745	83.87	1	1.9	2	4.0
P01023	A2MG_HUMAN	Alpha-2-macroglobulin	A2M	1474	163.29	49	47.4	4	3.9
P01024	CO3_HUMAN	Complement C3	C3	1663	187.15	68	52.4	3	2.5
Q8WZ42	TITIN_HUMAN	Titin	TTN	33423	3713.70	8	0.3	2	0.1

6.3 Proteins with differential expression associated with allergy identified in placenta and saliva samples

There were 14 same proteins associated with allergy present in both placental and saliva samples identified with LC-MS/MS (Table 35). Out of these 14 proteins, only 2 proteins that were present in LFQ MS significant ratios: pan 14-3-3 and Hp proteins (bold and highlighted with blue colour in Table 35).

Pan 14-3-3 (consists of seven 14-3-3 isoforms) was significantly altered in both the placental and child saliva samples. In allergic females, placental *YWHAQ* mRNA (14-3-3 theta (θ) protein) was significantly decreased (Mann Whitney U test, $P= 0.023^*$) as identified by qRT-PCR whereas salivary pan 14-3-3 was significantly increased (Mann Whitney U test, $P= 0.006^{**}$) as compared to non-allergic female as demonstrated by Western blot (Table 36).

Interestingly, the findings of 14-3-3 protein isomers were observed throughout the study in both placental and saliva samples. Using LC-MS/MS, 14-3-3 eta (η) was in uncalculated LFQ MS ratio in the placentae of allergic children; and in significant LFQ MS ratio (2.50 fc) in saliva samples of allergic children with eczema. Moreover, in the saliva samples, there were some interesting findings with regards to allergic children with asthmatic mothers (high risk of allergy). In this group, salivary pan 14-3-3 was significantly increased (Mann Whitney U test, $P= 0.031^*$) in Western blot whereas salivary 14-3-3 zeta (ζ) was decreased (Mann Whitney U test, $P= 0.008^{**}$) in Blitz system as compared to non-allergic children with asthmatic mothers (Table 36). Thus, these current study findings indicate that 14-3-3 proteins may have an important role in the mechanism of childhood allergy susceptibility.

Using LC-MS/MS, Hp protein had significant LFQ MS ratios in both samples; 0.46 fc in female allergic placentae; and 0.07 fc in child saliva samples with eczema as compared with non-allergic children. However, using Western blot validation, Hp protein expression only changed significantly in placental samples but not in the saliva samples. In the placental samples, Hp protein expression was significantly different in allergic children and in males with an allergy (Mann Whitney U test, $P= 0.015^*$ and $P= <0.0001^{****}$); allergic children with asthmatic mothers (high risk of allergy) and in males of asthmatic mothers (Mann Whitney U test, $P= 0.006^{**}$ and $P= <0.0001^{****}$), and in children with asthma combined with other allergic phenotypes (Mann Whitney U test, $P= 0.032^*$).

Table 35. LC-MS/MS comparison of proteins with differential expression associated with allergy identified in placenta and saliva samples.

Swiss-Prot entry	Entry Name	Protein names	Gene names	Sequence length (bp)	MW [kDa]	Placenta LC-MS/MS		Saliva LC-MS/MS	
						Unique peptides	Unique sequence coverage [%]	Unique peptides	Unique sequence coverage [%]
P05109	S10A8_HUMAN	Protein S100-A8	S100A8	93	10.83	3	32.3	3	29.0
P05386	RLA1_HUMAN	60S acidic ribosomal protein P1	RPLP1	113	11.40	2	52.2	1	28.3
P14174	MIF_HUMAN	Macrophage migration inhibitory factor (MIF)	MIF	115	12.48	3	35.7	2	11.3
P06702	S10A9_HUMAN	Protein S100-A9	S100A9	114	13.21	6	51.8	7	49.7
P02766	TTHY_HUMAN	Transthyretin	TTR	147	15.89	6	49.0	2	13.4
P62937	PPIA_HUMAN	Peptidyl-prolyl cis-trans isomerase A	PPIA	165	18.01	10	66.7	0	0.0
P30086	PEBP1_HUMAN	Phosphatidylethanolamine-binding protein 1	PEBP1	187	21.06	8	69.0	0	0.0
P31947	1433S_HUMAN	14-3-3 protein sigma	SFN	248	27.77	1	3.2	10	51.6
P00738	HPT_HUMAN	Haptoglobin	HP	406	45.21	24	50.5	8	19.6
P19971	TYPH_HUMAN	Thymidine phosphorylase	TYMP	482	49.96	3	10.8	2	9.1
P02768	ALBU_HUMAN	Serum albumin	ALB	609	69.37	66	85.7	78	96.1
P02787	TRFE_HUMAN	Serotransferrin	TF	698	77.08	0	0.0	27	27.5
P01024	CO3_HUMAN	Complement C3	C3	1663	187.15	68	52.4	3	2.5
Q8WZ42	TITIN_HUMAN	Titin	TTN	33423	3713.70	8	0.3	2	0.1

Table 36. The summary of statistical significant protein expression changes related to allergy in placental and child saliva samples analysed by qRT-PCR, LC-MS/MS, Western blot and Blitz system. (Significant result was P<0.05).

PROTEINS	ALLERGIC DISEASES RELATED	TYPES OF SAMPLES	METHODS	RATIO LFQ MS	GROUPS OF STATISTICAL ANALYSES												
					Control vs allergy#	Influence of <i>in utero</i> exposure						Effect of sex		Effect of specific allergic phenotypes			
						Asthmatic mother (AM) (high risk for allergy)			Non-asthmatic mother (NAM) (low risk for allergy)								
						AM control vs AM allergy#	AM control male vs AM allergy male#	AM control female vs AM allergy female#	NAM control vs NAM allergy#	NAM control male vs NAM allergy male#	NAM control female vs NAM allergy female#	Control male vs allergy male#	Control female vs allergy female#	Asthma combination #	Eczema combination #	Rhinitis combination #	
Pan 14-3-3	Contact dermatitis, asthma	Placenta	qRT-PCR (YWHAQ gene)	Uncalculated ratio of 14-3-3 eta in AM and AF	NS	NS	NS	NS	NS	NS	NS	NS	0.0234*	NS	NS	NS	
			WB		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		
		Saliva	WB	E/C= 2.50 (14-3-3 eta)	0.0005***	0.0303*	NS	NS	0.0186*	NS	NS	NS	NS	0.0059**	0.0018**	0.0022**	0.0005***
			Blitz		NS	0.0075**	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.0120*	
Hp	Asthma, rhinitis	Placenta	WB	AF/NF= 0.46	0.0147*	0.0058**	<0.0001****	NS	NS	NS	NS	<0.0001****	NS	0.0319*	NS	NS	
		Saliva		E/C= 0.07	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
CRNN	Eczema	Saliva	WB	A/C= 2.08	NS	NS	NS	NS	0.0472*	NS	NS	NS	NS	NS	NS	0.0421*	
			Blitz		0.0489*	0.0002***	0.0005***	0.0380*	NS	NS	NS	NS	NS	NS	0.0046**	0.0029**	
Involucrin	Atopic dermatitis	Saliva	WB	R/C= 0.41	NS	NS	NS	NS	NS	NS	NS	0.0396*	NS	NS	0.0365*	NS	
			Blitz		NS	0.0343*	NS	NS	NS	0.0212*	NS	NS	NS	NS	NS	NS	

Data were analysed by Mann Whitney U test with two-tailed p-value.

° Abbreviations: LFQ MS represents label-free quantification mass spectrometry, AM represents children with asthmatic mothers (high risk of allergy), NAM represents children with non-asthmatic mothers (low risk of allergy), C represents control subjects who are children with no allergy, A represents children with asthma, E represents children with eczema, R represents children with rhinitis, AM represents allergic male, AF represents allergic female, NF represents non-allergic female, Hp represents Haptoglobin protein, CRNN represents cornulin protein, WB represents Western blot and NS represents not significant.

6.4 Proteins that were identified in the non-allergic population in placenta and saliva samples

There were 40 proteins that were identified in the non-allergic population of children in both placenta and saliva samples analysed by LC-MS/MS (Table 37). These included Thioredoxin; Immunoglobulin kappa variable 3-20; Fatty acid-binding protein, epidermal; Hemoglobin subunit beta; PRDX5; Triosephosphate isomerase; Leukocyte elastase inhibitor; Beta-actin-like protein 2; Immunoglobulin heavy constant alpha 1; Myeloperoxidase and Alpha-2-macroglobulin.

Table 37. LC-MS/MS comparison of proteins that were identified in the non-allergic population in both placenta and saliva samples.

Swiss-Prot entry	Entry Name	Protein names	Gene names	Sequence length (bp)	MW [kDa]	Placenta LC-MS/MS		Saliva LC-MS/MS	
						Unique peptides	Unique sequence coverage [%]	Unique peptides	Unique sequence coverage [%]
Q9BPY8	HOP_HUMAN	Homeodomain-only protein	HOPX	73	8.26	2	35.6	4	19.2
P07108	ACBP_HUMAN	Acyl-CoA-binding protein	DBI	87	10.04	3	52.9	2	18.4
P01717	LV403_HUMAN	Immunoglobulin lambda variable 3-25	IGLV3-25	96	10.35	1	19.8	1	19.8
P04080	CYTB_HUMAN	Cystatin-B	CSTB	98	11.14	2	33.7	11	84.7
P10599	THIO_HUMAN	Thioredoxin	TXN	105	11.74	6	51.4	6	65.7
B1N7B8	B1N7B8_HUMAN	Cryoglobulin CC1 kappa light chain variable region	N/A	107	11.96	1	15.3	1	22.4
P08493	MGP_HUMAN	Matrix Gla protein	MGP	103	12.35	2	23.3	1	10.7
P01619	KV320_HUMAN	Immunoglobulin kappa variable 3-20	IGKV3-20	116	12.56	1	9.0	1	9.0
P04433	KV309_HUMAN	Immunoglobulin kappa variable 3-11	IGKV3-11	115	12.58	0	0.0	1	15.8
P01764	HV323_HUMAN	Immunoglobulin heavy variable 3-23	IGHV3-23	117	12.58	1	8.0	1	8.0
P01625	KV402_HUMAN	Immunoglobulin kappa variable 4-1	IGKV4-1	114	12.64	1	15.8	7	50.0
A0N5G5	A0N5G5_HUMAN	Rheumatoid factor D5 light chain	V<kappa>3	118	12.77	1	15.3	3	46.1
P01593	KV101_HUMAN	Immunoglobulin kappa variable 1D-33	IGKV1D-33	117	12.85	2	25.0	1	14.5
P01617	KV204_HUMAN	Immunoglobulin kappa variable 2D-28	IGKV2D-28	120	12.96	1	23.5	3	46.1
P01614	KV201_HUMAN	Immunoglobulin kappa variable 2D-40	IGKV2D-40	121	13.31	0	0.0	3	32.1
Q9UL88	Q9UL88_HUMAN	Myosin-reactive immunoglobulin heavy chain variable region	N/A	131	14.14	1	14.5	5	37.6
Q01469	FABP5_HUMAN	Fatty acid-binding protein, epidermal	FABP5	135	15.16	7	64.4	4	42.8
P68871	HBB_HUMAN	Hemoglobin subunit beta	HBB	147	16.00	0	0.0	2	13.4
P30044	PRDX5_HUMAN	Peroxisomal oxidoreductase, mitochondrial	PRDX5	162	17.03	9	73.5	0	0.0

P30086	PEBP1_HUMAN	Phosphatidylethanolamine-binding protein 1	PEBP1	187	21.06	8	69.0	0	0.0
P02763	A1AG1_HUMAN	Alpha-1-acid glycoprotein 1	ORM1	201	23.51	5	25.4	1	8.0
P19652	A1AG2_HUMAN	Alpha-1-acid glycoprotein 2	ORM2	201	23.51	4	18.4	1	8.0
P01700	LV147_HUMAN	Immunoglobulin lambda variable 1-47	IGLV1-47	235	24.82	1	5.5	1	8.0
P01834	IGKC_HUMAN	Immunoglobulin kappa constant	IGKC	231	25.14	0	0.0	1	9.0
V9HW34	V9HW34_HUMAN	Epididymis luminal protein 213	HEL-213	235	25.65	0	0.0	0	0.0
Q6P5S8	Q6P5S8_HUMAN	IGK@ protein	IGK@	236	25.77	0	0.0	0	0.0
P60174	TPIS_HUMAN	Triosephosphate isomerase	TPI1	249	26.67	17	78.7	7	52.6
P25311	ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	AZGP1	298	34.26	5	22.1	24	56.7
P30740	ILEU_HUMAN	Leukocyte elastase inhibitor	SERPINB1	341	38.69	2	6.2	1	4.4
P02765	FETUA_HUMAN	Alpha-2-HS-glycoprotein	AHSG	367	39.32	6	16.6	3	19.1
P04075	ALDOA_HUMAN	Fructose-bisphosphate aldolase A	ALDOA	364	39.42	19	67.6	4	18.7
P80303	NUCB2_HUMAN	Nucleobindin-2	NUCB2	338	40.37	3	14.2	2	10.1
Q562R1	ACTBL_HUMAN	Beta-actin-like protein 2	ACTBL2	376	42.00	1	4.8	1	4.8
P06733	ENOA_HUMAN	Alpha-enolase	ENO1	434	47.17	22	59.9	28	61.8
P02774	VTDB_HUMAN	Vitamin D-binding protein	GC	474	52.96	1	5.5	2	11.4
P01876	IGHA1_HUMAN	Immunoglobulin heavy constant alpha 1	IGHA1	494	53.09	1	6.5	2	9.5
Q6ZW64	Q6ZW64_HUMAN	cDNA FLJ41552 fis, clone COLON2004478, highly similar to Protein Tro alpha1 H	N/A	494	53.32	1	6.5	1	6.5
P11021	GRP78_HUMAN	78 kDa glucose-regulated protein	HSPA5	654	72.33	33	52.0	1	2.8
P05164	PERM_HUMAN	Myeloperoxidase	MPO	745	83.87	1	1.9	2	4.0
P01023	A2MG_HUMAN	Alpha-2-macroglobulin	A2M	1474	163.29	49	47.4	4	3.9

6.5 Correlations of proteins associated with allergy between placenta and saliva samples

Spearman correlation coefficients were calculated to determine whether there was any association between proteins expressed in the placenta relative to the saliva (Table 38). A strong positive correlation was found between placental PRDX2 and salivary pan 14-3-3 (Spearman correlation coefficient= 0.487**, p value= 0.005). Moreover, there was a moderate positive correlation between placental PRDX2 and salivary CRNN (Spearman correlation coefficient= 0.322*, p value= 0.026). A weak positive correlation was found between placental C3 and salivary CRNN (Spearman correlation coefficient= 0.291*, p value= 0.045).

Table 38. Spearman correlation coefficients for each placental protein versus each salivary protein according to Western blot data.

Spearman correlations coefficients				Pan 14-3-3	Involucrin	Transferrin	CRNN	Desmoglein 1	Hpt
Salivary proteins									
Spearman's rho (ρ)	Placental protein	C3	Correlation coefficient	0.234	-0.182	0.123	0.291*	0.122	0.104
			P value	NS	NS	NS	0.045	NS	NS
		Hp	Correlation coefficient	-0.164	0.069	0.026	0.036	0.099	0.040
			P value	NS	NS	NS	NS	NS	NS
		PRDX2	Correlation coefficient	0.487**	0.303	0.203	0.322*	0.131	0.092
			P value	0.005	NS	NS	0.026	NS	NS
		CLIC3	Correlation coefficient	0.068	0.174	-0.009	0.124	0.108	0.067
			P value	NS	NS	NS	NS	NS	NS
		Pan 14-3-3	Correlation coefficient	0.100	0.131	0.120	0.176	0.160	0.049
			P value	NS	NS	NS	NS	NS	NS

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

° Abbreviations: C3 represents Complement C3, Hp represents Haptoglobin, PRDX2 represents Peroxiredoxin-2, CLIC3 represents Chloride intracellular channel protein 3 and NS represents not significant.

6.6 Discussion of the protein comparisons associated with allergy between placenta and saliva samples

Current findings of proteomic profiles in placental and saliva samples suggest that placental protein expression is altered *in utero* in children who subsequently develop an allergy between the ages of six months to five years and some of these proteins are detectable in saliva in early life. Two proteins associated with allergy present in placental and saliva samples that had significant LFQ MS ratios were pan 14-3-3 and Hp proteins. Furthermore, altered expression of some placental and salivary proteins varies in presence or absence of maternal asthma and in a sex-specific manner.

A strong positive correlation relationship between PRDX and 14-3-3 proteins was demonstrated in a study conducted for characterising the proteomic profile on the regenerative function of human embryonic stem cells (hESCs), human mesenchymal stem cells (hMSCs) and marrow isolated adult multilineage inducible (MIAMI) cells as delivery systems of therapeutic molecules ⁽⁴⁴⁶⁾. Comparative analysis of protein expression indicate that MIAMI cells appear to have more proteins in common with hESCs than hMSCs. Interestingly, in these three stem cell populations, PRDXs (PRDX1, PRDX2 and PRDX6) and 14-3-3 zeta were identified. 14-3-3 zeta was expressed at higher levels in MIAMI cells compared to MSCs and ESCs, whereas PRDX1 expressed at higher levels in hESCs compared to hMSCs and MIAMI cells ⁽⁴⁴⁶⁾. This data suggest that relationship between PRDX and 14-3-3 proteins is biologically relevant. However, to the author's knowledge, there are no reported literature reviews on the relationships between placental PRDX2 and salivary CRNN, and placental C3 and salivary CRNN.

6.6.1 14-3-3 proteins and allergy susceptibility

To date, seven different isoforms of 14-3-3 have been identified in human: 14-3-3 beta (β), gamma (γ), epsilon (ϵ), zeta (ζ), eta (η), theta (θ) and sigma (σ) ⁽³²²⁾. 14-3-3 proteins are present in human biological fluids, including colostrum milk, cervicovaginal fluid and saliva samples, and human tissue such as placenta and skin samples ^(327, 447, 448). 14-3-3 theta and sigma are expressed in T cells and epithelial cells ⁽⁴⁴⁹⁾.

14-3-3 proteins are increased in asthma and AD ^(322, 323). Airway remodelling in obstructive lung diseases which include asthma involves various mediators modulating production or breakdown of collagen by lung fibroblasts, including the *MMP1* gene, modulated by 14-3-3 beta ⁽³²³⁾. Moreover, 14-3-3 sigma protein in an epithelial cell-derived extracellular form is an important inducer of *MMP1* gene expression in skin fibroblasts and in the respiratory airway ⁽³²³⁾. Surprisingly, there are not many studies

that emphasise the role of 14-3-3 isoforms in skin diseases ^(322, 450). Raaby *et al.* investigated expression of the seven 14-3-3 isoforms in four common skin diseases using qRT-PCR and Western blot, validated with immunofluorescence ⁽³²²⁾. The four common skin diseases were psoriasis, a benign Th-1 and Th-17 driven chronic inflammatory hyperproliferating skin disorder, AD, acute, allergic nickel induced contact dermatitis, and basal cell carcinoma (BCC), a malignant hyperproliferating skin disorder. Increased 14-3-3 *theta* and *sigma* mRNA levels were upregulated in psoriasis and allergic contact dermatitis, whereas in BCC, particularly, 14-3-3 *theta* mRNA expression level was increased ⁽³²²⁾. In addition, these findings supported a previous study by Lodygin *et al.* that reported high levels of 14-3-3 sigma protein expression in psoriasis ⁽⁴⁵⁰⁾.

14-3-3 sigma gene (*SFN*) can also be inactivated by epigenetic silencing via CpG methylation of the promoter region, which is seen in different types of carcinomas, including BCC ^(322, 450). Lodygin *et al.* demonstrated that keratinocytes transformed to BCC had partial, or complete, loss of 14-3-3 sigma protein expression, and no *SFN* CpG methylation of p16(INK4A), which is usually seen in BCC. This data suggests that silencing of *SFN* may contribute to remission in BCC ⁽⁴⁵⁰⁾. The p53-regulated *SFN* encodes an inhibitor of cell cycle progression essential for senescence and clonal evolution of keratinocytes *in vitro* ⁽⁴⁵⁰⁾. Increased expression of 14-3-3 sigma in cells after DNA damage concomitantly with p53 plays a critical role in the G2/M checkpoint in the cell cycle ⁽⁴⁵¹⁾. Via G2/M checkpoint activation in cell cycles, maintenance or increment of 14-3-3 sigma protein expression in hyperproliferative skin disorders, which include psoriasis and allergic contact dermatitis, can prevent malignant transformation to BCC ^(322, 451).

The actions of 14-3-3 protein have been associated with immune system regulation. 14-3-3 proteins have been involved in immune system regulation of TLR signalling, where stimulation of the murine macrophage cell line (RAW cells) with LPS led to protein kinase C (PKC) phosphorylation and its association with 14-3-3 beta in a MyD88-dependent manner ⁽⁴⁵²⁾. 14-3-3 protein (14-3-3 epsilon and 14-3-3 sigma) has been reported to inhibit TLR-mediated pro-inflammatory cytokine induction ⁽⁴⁵³⁾. In addition, 14-3-3 theta was markedly increased following T cell activation and suppressed protein kinase C theta-dependent IL-2 promoter activity by preventing its translocation to the membrane ^(324, 325).

The mechanism in which 14-3-3 protein influences development of the immune system towards greater susceptibility to allergy is closely related to tumorigenesis ⁽⁴⁰²⁾. A recent study by Han *et al.* on over-expression and modulation of tumour inflammation by 14-3-3 zeta (ζ) in oral squamous cell carcinoma (OSCC) cell lines and cancer tissues provides more information on the possible mechanism of 14-3-3 in allergy ⁽⁴⁰²⁾. Han *et al.* demonstrated that 14-3-3 zeta knockdown caused elevated pro-inflammatory cytokine expression that include TNF- α , IL-6, IFN- β and RANTES in OSCC cells ⁽⁴⁰²⁾. In contrast, 14-3-3 zeta introduction attenuated cytokine expression in human normal keratinocytes and fibroblasts

stimulated with IFN- γ and LPS ⁽⁴⁰²⁾. Supernatants from 14-3-3 zeta knockdown OSCC cells up-regulated cytokine synthesis of peritoneal macrophages, significantly augmented IL-12 and MHC class II expression in dendritic cells, and promoted proliferation and IL-2 and IFN- γ expression of T cells ⁽⁴⁰²⁾. This supports interactions mediated by 14-3-3 zeta between OSCC and the immune system. The interaction possibly caused via an alternative mechanism that enables established tumours to evade immune recognition through either failing to enter lymph nodes, or walling themselves off from T cells in lymph nodes ⁽⁴⁰²⁾. Interestingly, STAT3 was found to directly interact with 14-3-3 zeta, and its disruption relieved inhibition induced by 14-3-3 zeta in tumour inflammation including OSCC ⁽⁴⁰²⁾. A previous study demonstrated that STAT3 can mediate immune evasion by blocking production and sensing of inflammatory signals by multiple components of the immune system ⁽⁴⁵⁴⁾.

Based on the above data, the possible mechanism of allergy with regards to increased 14-3-3 proteins is depicted in Figure 108 and involves: (1) inhibitory effect of pro-inflammatory cytokine induction; (2) negatively regulates innate immune and adaptive immune response; (3) augment IL-12 and MHC class II expression in dendritic cells; (4) promote proliferation and the IL-2 and IFN- γ expression of T cells; and (5) with STAT3 agonist effect, also inhibit inflammatory cytokine expression. Thus, 14-3-3 proteins influence immune system development and lead to greater susceptibility to allergy by redirecting Th1 responses in favour of allergic Th2 responses, as manifested in allergic individuals.

6.6.2 Haptoglobin (Hp) protein and allergy susceptibility

Haptoglobin (Hp) is a plasma α_2 -glycoprotein consisting of an alpha (α) and a beta (β) polypeptide chain, linked by disulfide bonds ^(455, 456). Hp, a major positive acute-phase protein, increases in plasma during inflammation, infection, trauma, tissue damage and malignant proliferation ⁽⁴⁵⁷⁾. The acute-phase response is an antigen-nonspecific, innate response that eliminates microbes to prevent infection. The innate immune response involves recruitment and activation of macrophages and leukocytes that release inflammatory cytokines on recognition of a pathogen-associated molecular pattern (PAMP) ⁽⁴⁵⁸⁾. *Hp* gene is expressed in hepatocytes and in several hepatoma cell lines where it is upregulated by several cytokines, particularly by the IL-6-like cytokine family ⁽⁴⁵⁹⁾. Moreover, elevated serum concentration of Hp occurs in plasma of pregnant women at term, suggesting it may have a role during pregnancy, but the exact mechanism is unclear ^(456, 460).

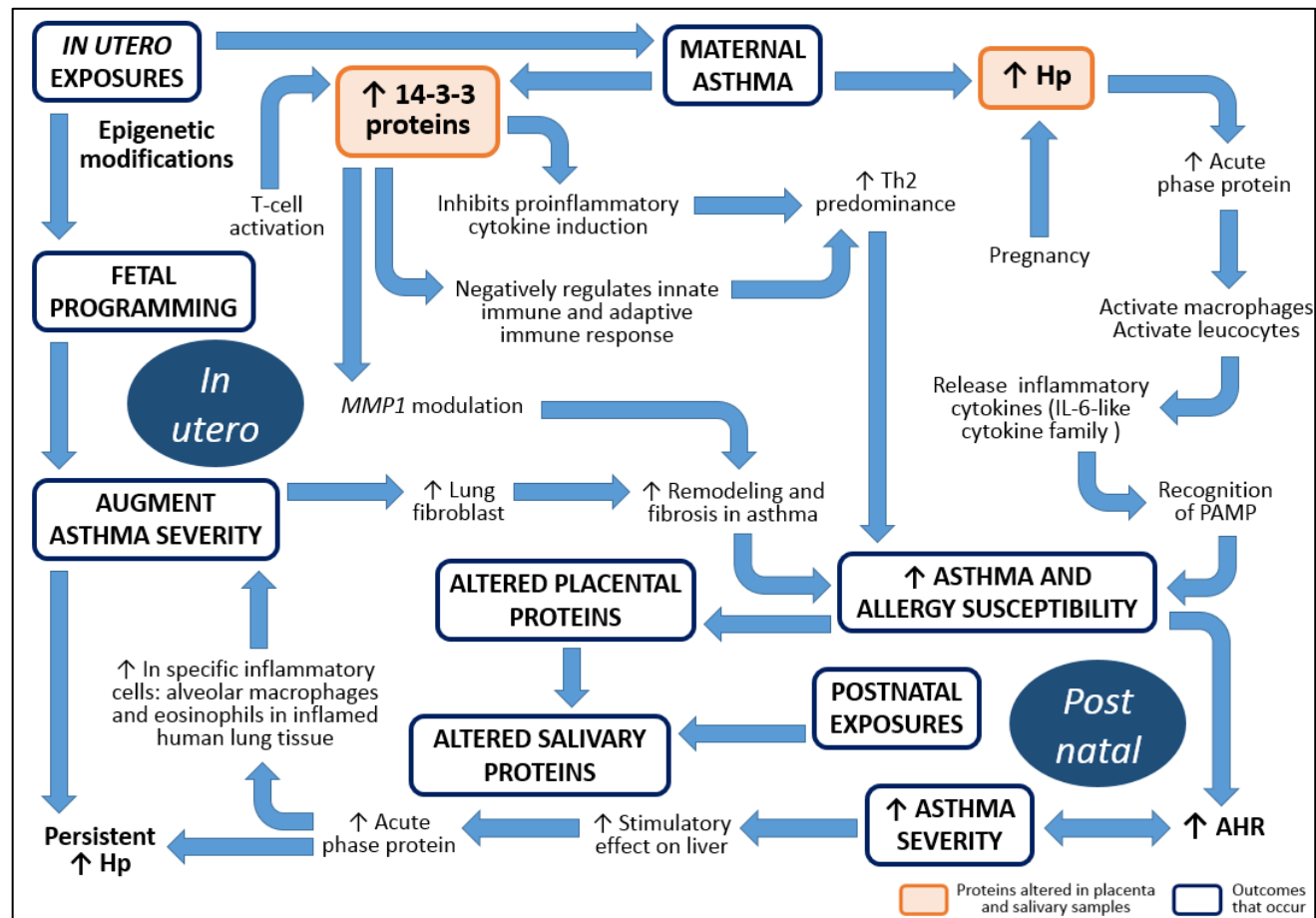


Figure 108. Proposed model of 14-3-3 and Hp proteins mechanism underlying increased susceptibility to allergy in placental samples. (Hp represents haptoglobin, *MMP1* represents matrix metalloproteinase-1, AHR represents airway hyperresponsiveness and PAMP represents pathogen-associated molecular pattern).

Hb is increased in asthma because of its important function in antioxidant activity, angiogenesis, tissue repair function and immune response modulation ^(456, 457, 461-466). The proposed model of Hp protein mechanism underlying increased susceptibility to allergy in placental samples is shown in Figure 108. With regards to Hp antioxidant activity and angiogenesis, Hp has been correlated with FEV₁ in asthma patients ⁽⁴⁶¹⁾. An animal study using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and LC-MS/MS revealed that the Hp alpha-subunit was significantly increased in BALF from OVA-challenged mice, as compared to a control group ⁽⁴⁶⁷⁾. In a human study using LC-MS/MS and two dimensional electrophoresis (2-DE) to determine asthma-specific proteins in BALF, increased Hp-beta was observed in asthmatic patients, as compared with non-asthmatic patients ⁽⁴⁶³⁾. Moreover, Hp protein can discriminate between clinical groups of patients with asthma, patients with COPD and control subjects ⁽⁴⁵⁸⁾.

Hp acts as an immune system natural antagonist for receptor-ligand activation and modulates immune responses to infection and inflammation ^(457, 464). Consistent with its role in modulating immune responses, Hp at high levels is related to AHR in asthma ⁽⁴⁶¹⁻⁴⁶³⁾. The increment of Hp correlates with degree of airway inflammation during acute exacerbation of asthma in patients that respond poorly to initial bronchodilator therapy ⁽⁴⁶²⁾. Hp is only expressed at high levels in specific inflammatory cells, including alveolar macrophages, and eosinophils in diseased or inflamed human lung tissues ⁽⁴⁶⁴⁾.

Hp has a tissue repair function by inducing fibroblast progenitor cell differentiation into lung fibroblasts, implicating Hp in remodelling and fibrosis in asthma and COPD ⁽⁴⁶⁶⁾. In a human study, elevated levels of Hp expression in the BALF proteome with differentiating fibroblast progenitor cells, termed fibrocytes, derived from the airway lumen, was observed in asthmatic patients ⁽⁴⁶⁶⁾. Increased Hp expression and increased eosinophils in BALF fibroblasts suggests that an acute and specific inflammatory state facilitates differentiation of fibroblast progenitor cells into activated fibroblasts in airway lumen in mild-asthmatic patients ^(463, 466).

Interestingly, Hp protein was reported to be epigenetically modified in an animal study that investigated expression of immune-related genes on dairy cows fed a high concentrate (HC) diet known to trigger systemic inflammation ⁽⁴⁶⁸⁾. Elevation of acute phase proteins, including Hp, was observed in livers of cows fed a HC diet. In addition, demethylation at the promoter region of *HP* gene was demonstrated, accompanied by chromatin decompaction. After HC diet feeding, LPS derived from the digestive tract translocated to the liver via the portal vein, enhancing hepatic immune gene expression. Upregulation of immune genes, including *HP*, was mediated by epigenetic mechanisms, which involve DNA methylation and chromatin remodelling ⁽⁴⁶⁸⁾. To this author's knowledge, no human study has yet been conducted in relation to Hp and epigenetic modifications.

In Western blot placental findings of this study, Hp protein was present in allergic males in a sex-specific manner, a finding supported by data from previous studies ^(13, 469-471). Hp and sex difference was observed in a recent study on Laron Syndrome, GH-insensitive individuals, using growth hormone receptor gene-disrupted (GHR-/-) mice, which are dwarf, insulin sensitive and long lived, despite being obese ⁽⁴⁷⁰⁾. Characteristics associated with their increased longevity and age-related plasma proteomic changes in these GHR-/- mice were investigated, showing increased Hp levels and sex differences in male GHR-/- mice ⁽⁴⁷⁰⁾. In contrast, female GHR-/- mice showed decreased inflammatory cytokines, including IL-1 β and monocyte chemoattractant protein-1. Moreover, Hp, one of the 15 metabolic syndrome-related parameters, was significantly associated with leptin concentration predominantly in males, thus providing further evidence of sex-related differences mediated by leptin in inflammatory mechanisms ⁽⁴⁷¹⁾. In addition, *HP* genetic polymorphism, *HP* 1/1 has been associated with higher risk for invasive cervical cancer and sex steroid hormone ingestion, which include contraceptives and replacement therapy that contain estrogen and progesterone ⁽⁴⁶⁹⁾. The above studies indicate that Hp and sex difference in male possibly involves a mechanism related to growth hormone, serum leptin and sex hormones; however, the exact mechanism is not fully understood.

Placental Hp presence was significantly different in allergic children, as compared to non-allergic children, regardless of asthmatic status of mothers (Table 36). Thus, using Hp protein, newborns can be screened for allergic diseases immediately after birth using the placenta. However, because placental tissue is used, the Blitz system is not suitable and the best method to identify this protein expression is with Western blot using denatured protein. Other than using Western blot, Hp protein placental samples can be tested with more the reliable MS-based methods which provide higher sensitivity and accuracy for mass measurement ⁽²⁷²⁾. These methods include a variety of MS techniques, such as LC-MS/MS, MALDI-TOF/MS and SELDI-TOF/MS, that have been extensively applied to identification of biomarkers in saliva (refer Introduction section 1.6.4) ⁽²⁷²⁾. Although the allergy screening method using placental tissue is more time consuming and relatively more expensive, diagnosis of allergic diseases can be established in newborns and children less than six months of age. Early diagnosis of allergic disease will enable early interventions, reduce morbidity and mortality, as well as avoid implications of severe childhood allergic diseases that may include fatal anaphylactic shock.

6.6.3 Allergy susceptibility in children with asthmatic mother

Th2-skewed maternal immune responses are predominantly caused by pregnancy state and maternal asthma. Interestingly, in Western blot and Blitz analysis, this study found that salivary pan 14-3-3 protein was only altered in allergic children with high risk of allergy and was not altered in allergic children with low risk of allergy (Table 36). Moreover, this study found that salivary involucrin was present in significant protein differences in male allergic children with a non-asthmatic mother in Blitz analysis (Table 36). These data suggest that certain immune mechanisms that are altered in the fetal-placental unit by presence of maternal asthma are not associated with gene alterations associated with allergy susceptibility in children without maternal asthma ⁽¹³²⁾. Findings of this study are supported by previous work performed by our team that demonstrated pregnancies complicated by maternal asthma were associated with alterations in placental function, such as decreased placental 11 β -HSD2 accompanied by reduced female fetal growth ⁽⁴⁷²⁾. Altered placental immune genes identified in presence of maternal asthma, which include *TNF- α* and *IL* including *IL-1 β* , *IL-6*, *IL-8* and *IL-5*, were not altered in placentae of allergic children ⁽¹³⁾. Presence of maternal asthma may program allergic susceptibility *in utero*, whereas children with non-asthmatic mothers that develop an allergy may be influenced by postnatal exposures. Therefore, certain immune mechanisms that are altered in the fetal-placental unit by presence of maternal asthma are not associated with gene alterations associated with allergy susceptibility in the absence of maternal asthma.

Mechanisms underlying changes of placental and salivary proteins in relation to allergy susceptibility in children with an asthmatic mother are still unknown, however epigenetic modifications are a plausible mechanism. The proposed model of mechanism underlying increased allergy susceptibility in children with an asthmatic mother is depicted in Figure 109.

Using a murine model, maternal allergy has been demonstrated to alter DNA methylation of the *IL4* gene promoter in offspring of mothers with peanut allergy (O-PAM mice) ⁽⁴⁷³⁾. After peanut sensitisation challenge, O-PAM mice had significantly higher peanut-specific IgE levels, histamine levels and anaphylactic symptom scores than offspring of naive mothers (O-NM mice). These findings were supported by increased Th2 cytokines in cultured splenocytes and mediastinal lymph nodes ⁽⁴⁷³⁾. Moreover, in cells from O-PAM mice, significant reduction of DNA methylation at CpG sites of the *IL4* gene promoter was evidence of neonatal splenocyte hypomethylation and was inversely correlated with IL-4 and IgE production. The study indicated that increased susceptibility to peanut allergy in O-PAM mice was associated with hypomethylation of the *IL4* gene promoter ⁽⁴⁷³⁾. Thus, DNA methylation is involved in the mechanism of allergy susceptibility in children with asthmatic mothers.

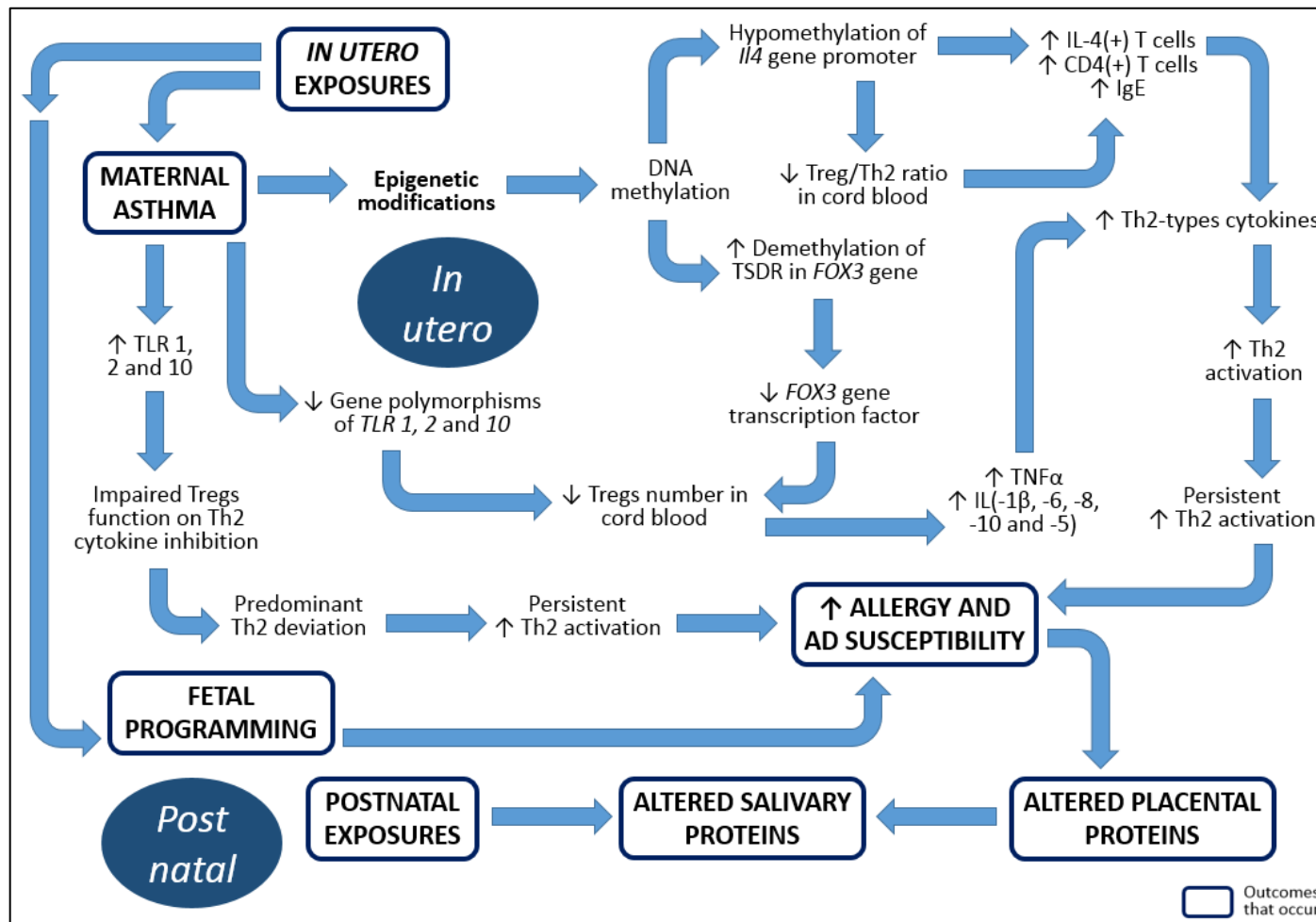


Figure 109. Proposed model of mechanism underlying increased allergy susceptibility in children with asthmatic mother. (*IL4* represents IL-4 gene, TSDR represents Treg-specific demethylation region, *FOX3* represents forkhead box protein P3 and AD represents atopic dermatitis).

Tregs are associated with epigenetic modifications in the mechanism of AD and FA in children with asthmatic mothers ⁽⁴⁷⁴⁾. Tregs with stable *FOXP3* expression are characterised by a specific demethylated region in the *FOXP3* gene (Treg-specific demethylated region, TSDR) ⁽⁴⁷⁴⁾. Using CB, another study measured Tregs numbers by analysing methylation state of the TSDR in the *FOXP3* gene, which are an exclusive, specific and reliable marker for stable Tregs ⁽⁴⁷⁴⁾. Lower numbers of Treg in CB were related to sex of offspring and *in utero* exposures, including parental atopy or allergy, maternal smoking and maternal cytokines, which were consecutively associated with higher risk of developing AD and food allergen sensitisation during the first year of life ⁽⁴⁷⁴⁾.

Based on the studies cited above, findings of this study may play a role in supporting the hypothesis of the particular immune mechanism manifested in altered child salivary protein predominantly occurs in children with asthmatic mothers (high risk of allergy) ⁽⁴⁷⁴⁾. Furthermore, allergic disease mechanism of children with low risk of allergy is not influenced by *in utero* events, but is predominantly influenced by postnatal events, including epigenetic and environmental exposures, such as breastfeeding and early microbial exposure ^(77, 242, 244, 245). To the author's knowledge, no human study has yet investigated the role of maternal asthma in epigenetic modifications involved in childhood allergy susceptibility using placental or saliva samples.

Using data previously collected in validation studies, this study compared proteins present in placenta and saliva samples in children with and without an allergy. Findings support the third hypothesis that several changes in placental proteomic profiles are reflected in a similar change in saliva in early childhood. Findings support the theory that altered protein present in the placenta is different and detectable in saliva of children with allergic disease.

7.0 FINAL DISCUSSION AND CONCLUSION

This study has shown that placental protein expression can be altered *in utero* in children who subsequently develop an allergy, and some altered proteins are detectable in saliva in early childhood. These data suggest that *in utero* events may permanently affect production of proteins, possibly through epigenetic modifications associated with development of chronic disease, such as allergy. Furthermore, study data suggests presence or absence of maternal asthma in pregnancy influences the protein profile in placenta and saliva, which may be influential in fetal immune system development and increase risk of childhood allergy. Interestingly, some differences were sex-specific, suggesting sex may be a determinant of allergic risk. Based on these findings, it is possible that placenta and saliva are biological sources of biomarkers for allergic risk.

Several studies have identified that placental gene expression varies in children who subsequently develop an allergy ^(15, 132, 475). Placental samples examined in this study were derived from an asthma and pregnancy cohort. Therefore, a high percentage of children from this cohort developed an allergy in early childhood (>87 %). Previous studies using placental samples from allergic children have not examined the protein profile, but this study identified that PRDX2, C3 and Hp proteins were significantly altered, possibly via epigenetic modifications (refer Comparison section 6.6.2). Apolipoproteins (apos), including apoB-100, apo-D and cholesterol side-chain cleavage enzyme, had significant LFQ MS ratio in allergic placentae, but were not validated using Western blot. However, these proteins are also involved in the mechanistic pathway of how changes in placenta may program the immune system and increase susceptibility to allergy.

Previous studies have shown that PRDX2 (*PRDX* gene) levels correlate with degree of asthma disease severity ⁽³⁶³⁾. PRDXs, a family of thiol-specific intracellular antioxidant proteins, are different to conventional antioxidants ⁽³⁶⁴⁾. They are unique due to the ability to transform into irreversible hyperoxidised forms (PRDX-SO₃) in the presence of excess hydrogen peroxide (H₂O₂) that produces functionally inactive molecules ^(364, 367). Ratios of hyperoxidised to total PRDX (PRDX-SO₃/PRDX) in PBMC were reported to be significantly higher in asthma patients than in non-asthmatic subjects ⁽³⁶³⁾. In asthma pathogenesis, hyperoxidation of PRDX2 was correlated with cell-cycle arrest, Ca²⁺- iPLA2 activity in H₂O₂-induced cellular toxicity and activation of DAMP that interacted with PRRs inducing alternative immune cell activation leading to an inflammatory state ^(363, 367-370). In addition, helminth-secreted PRDX can influence development of Th2-responses via a mechanism involving induction of alternatively activated macrophages via Ym1-expressing macrophages in mouse ⁽³⁷²⁾. In a study using PRDX2-null mice, findings indicate that TLR4 proteins were decreased by PRDX2 deletion, indicating

PRDX2 was protective against development of allergic airway inflammation through reduced ROS via suppression of NF- κ B activation by the action of PRDX hyperoxidation ⁽³⁷³⁾.

Epigenetic modifications, including DNA methylation and histone modifications, have been reported to modulate expression of PRDXs, particularly in carcinogenesis. Furthermore, HDAC, an epigenetic regulator, has been demonstrated to restore PRDX to normal levels ⁽³⁶⁶⁾. Interestingly, hyper- and demethylation of *PRDX2* promoter was observed in a study on lung adenocarcinoma cells ⁽³⁷⁴⁾. In this study, hypermethylation of *PRDX2* promoter was observed in A549 lung adenocarcinoma cells, with demethylation in A549 gefitinib-resistant cells, demonstrating that methylation status correlates to gene activity. In oligodendroglial tumours, *PRDX1*-associated CpG islands were frequently hypermethylated in 1p/19q-deleted primary tumours cells and expression reduced *PRDX1* mRNA transcripts ^(366, 375). In acute myeloid leukaemia, DNA hypermethylation and H3 histone hypoacetylation at the *PRDX2* promoter correlated with repressed tumour suppressor PRDX2 expression ^(366, 376). These studies indicate that PRDX proteins can be epigenetically modified. Thus, similar epigenetic modification may occur *in utero* to increase childhood susceptibility to allergy.

Cholesterol side-chain cleavage enzyme (*CYP11A1 gene*), a member of the CYP superfamily, may program the immune system via the placenta and influence susceptibility to allergy. In an animal study of peanut allergy, *CYP11A1* gene regulated peanut-induced allergic responses (intestinal anaphylaxis) through effects on steroidogenesis, an essential pathway in Th2 differentiation ⁽¹²⁸⁾. Interestingly, another member of the CYP superfamily of enzymes, *CYP1A1* gene, was previously identified as a differential DNA methylation site at 26 CpGs mapped to 10 genes present at birth in a study on the effects of maternal smoking on epigenome-wide DNA methylation ⁽¹²⁷⁾. Thus, DNA methylation may be one mechanism that influences expression of *CYP11A1* (cholesterol side-chain cleavage enzyme) which may influence immune system development that leads to greater susceptibility to allergy.

Alteration of apolipoproteins *in utero* may be central to fetal immune system programming and increased allergic risk in childhood. Upregulation of apolipoproteins is associated with recurring inflammation of nasal mucosa in AR patients ⁽³³¹⁾. Moreover, experimental murine models demonstrated that the apoA-I/ABCA1 pathway may have a protective effect in asthma ⁽³³²⁾. In atopic and asthmatic patients, serum levels of apoA-I and high-density lipoprotein cholesterol were positively correlated with FEV1 in less severe airflow obstruction, whereas serum triglycerides, low-density lipoprotein cholesterol, apoB and apoB/apoA-I ratio were associated with more severe airflow obstruction ⁽³²⁹⁾. Thus, it is likely that apolipoproteins are important drivers of allergic risk and may be modified *in utero* to increase allergic susceptibility in childhood, especially in relation to mucosal allergies, such as AR and asthma.

C3 (C3 gene), together with C3a, have been reported as increased in asthma and AR patients ^(70, 349, 352). C3 plays an immunoregulatory role at the interface of innate and adaptive immune systems in the complement system ⁽³⁴⁶⁾. At the interface of innate immunity, pro-inflammatory and immunoregulatory products from C3 play an essential role in processes of phagocytosis, respiratory burst and airway inflammation ⁽³⁴⁷⁾. Most importantly, C3 is also pivotal in adaptive immunity ⁽³⁴⁸⁾. In response to allergen challenge, C3-deficient mice exhibit diminished AHR and lung eosinophilia; reduced numbers of IL-4-producing cells; and attenuated responses of Ag-specific IgE and IgG ⁽³⁴⁸⁾. All of these findings indicate that these mice-deficient C3 had impaired ability to produce Th2 cytokines, thus indicating that asthmatic features were significantly attenuated in the absence of C3 ⁽³⁴⁸⁾. Moreover, C3 was significantly elevated in children with intermittent atopic asthma when compared with children without asthma ⁽³⁵⁰⁾. A case-controlled study demonstrated that serum C3 was significantly higher in asthmatic children, with a positive correlation between serum C3 and the severity of asthma ⁽³⁵¹⁾. Hence, all of these studies suggest that C3 is a reliable biomarker for diagnosis of asthma.

C3a, an anaphylatoxin derived from proteolytic degradation of C3, is a mediator of the local inflammatory process via expression of its receptor in nasal epithelial cells, thus explaining its role in AR ^(70, 353). It induces smooth muscle contraction, increases vascular permeability, and stimulates histamine release from mast cells and basophils, causing mucosal swelling and exudation ^(70, 353). C3a plays a critical role in asthma predominantly by regulating mast cell-airway smooth muscle cell interaction ⁽³⁵⁴⁾. Increased asthma susceptibility may be influenced by the complement system via an intrinsic abnormality in complement activation and generation of C3a and C5a that can alter cellular responses, thus resulting in Th2 predominance ⁽³⁵⁵⁾. In addition, with increasing asthma severity, systemic pro-inflammatory cytokines like TNF- α and IL-1 might exert a stimulatory effect on the liver to produce increased amounts of C3, thus simultaneously creating a viscous cycle that can augment the severity of asthma ⁽³⁵¹⁾. Thus, with regards to C3 immunoregulatory role, it may predominantly influence the development of the immune system that leads to a greater susceptibility to allergy.

C3 has networks with other placental proteins identified in my LC-MS/MS data which includes Serum albumin, apolipoproteins and Hp. Tomazic *et al.* 2014 conducted a study to investigate AR mucus proteome and function ⁽⁷⁰⁾. Hp, C3 and apolipoproteins were amongst 267 proteins identified in AR mucus by using LC-MS/MS and Western blot. ApoA-2, apoA-1 and C3 were significantly upregulated in AR patients whereas Hp was downregulated as compared with levels seen in healthy control subjects ⁽⁷⁰⁾. Other studies have reported that C3, Serum albumin and apoA-I proteins were identified to be altered in the plasma proteome of normal healthy subjects after increased daily synthetic folic acid of 1.2 mg administration over a 12 week period ^(476, 477). The plasma proteome was assessed by 2-DE, and proteins involved in immune function were identified by LC-MS/MS. Albumin and C3 were

upregulated while apoA-I was downregulated ^(476, 477). This data suggested that the association of C3, Serum albumin and apoA-I was probably via epigenetic modifications that may occur in relation with dietary folate.

Maternal dietary folate, a methyl donor, is associated with changes in gene expression and allergic disease susceptibility through gene hypermethylation ⁽³⁾. An animal study had reported that increased intake of maternal dietary folate during pregnancy is associated with allergic airway disease susceptibility in the offspring through gene hypermethylation in mice ⁽⁴⁷⁸⁾. Moreover, this finding is supported by a human study which identified increased *IGF2* gene differentially methylation region in 120 children whose mothers had 400 µg synthetic folic acid, the recommended dosage for folic acid antenatal supplement ^(477, 479). All of these studies indicate that C3, Hp, and apolipoproteins may be interrelated most possibly via epigenetic modification in the immune system development which will influence susceptibility to allergy.

Based on these data, on the basis of epigenetic modifications via antioxidant and anti-inflammatory action of PRDXs, cholesterol side-chain cleavage enzyme and apolipoproteins, as well as C3 immunoregulatory role, I believe that these placental proteins are interconnected and play major roles in regulating the development of the immune system that leads to a greater susceptibility to allergy.

To date, there are no studies conducted to detect salivary proteins in association with childhood allergy susceptibility. In my study, both of involucrin and CRNN proteins were exclusively present in saliva samples in children with allergic diseases but were not found in the placental samples.

Previous studies have shown that gene and protein expression of involucrin was decreased in the skin from AD patients ^(408, 414, 415). Involucrin (*IVL* gene) is a member of late cornified envelope (CE) proteins family within the human epidermal differentiation complex (EDC) located on chromosome 1q21 ^(66, 409, 411). The mechanism by which involucrin may be associated with allergy is most possibly via the regulation of skin barrier as *IVL* is regarded as an early human keratinocyte epidermal differentiation marker ^(54, 407). Variants in both *FLG* and *IVL* genes within the EDC are involved in the pathogenesis of AD and asthma ^(66, 409, 411).

Involucrin may influence the development of the immune system which leads to a greater susceptibility to allergy via mechanisms involving IL-13 and IL-4 via STAT6 signalling pathway ^(417, 418). The importance of STAT6 signalling pathway was observed in response to IL-4 in STAT6-deficit mice ⁽⁴¹⁸⁾. There were reduced expression of CD23 and MHC class II in resting B-cells, abolished B-cell proliferation, reduced T cell proliferative response and reduced production of Th2 cytokines (IL-4 and IL-13) from T cells along with IgE and IgG1 responses ⁽⁴¹⁸⁾. In primary keratinocytes, *IVL* gene

expression was downregulated by IL-4 and IL-13, possibly due to the over-expression of Th2 cytokines in acute AD skin ⁽⁴¹⁵⁾. Skin biopsies from STAT6 transgenic mice exhibited significantly lower levels of *IVL* as compared to their wild-type controls, which support that IL-4 and IL-13 modulation of *IVL* is mediated by STAT6 activation ⁽⁴¹⁵⁾. In addition, decreased expression of *IVL* in AD is partly caused by the over-expression of Th2 cytokines that downregulate *IVL* expression during the differentiation into CE ⁽⁴¹⁵⁾.

IVL gene is epigenetically modified in keratinocyte differentiation. Treatment of keratinocytes with DNA methyltransferase inhibitor, 5-azacytidine, increases involucrin expression ⁽⁴¹⁹⁾. Moreover, *IVL* gene expression in keratinocyte differentiation can be silenced by histone methylation mediated by signalling of protein arginine methyltransferase 5 and methylome protein 50 ⁽⁴²⁰⁾. Hence, it is possible that *IVL* epigenetic modification occurring in keratinocyte differentiation may also be involved in the mechanism of allergy. Findings of the above mentioned studies suggest that childhood allergy susceptibility may be influenced by *IVL* downregulation, implicating IL-13 and IL-4 via STAT6 signalling pathway and over-expression of Th2 cytokines, with possible involvement of epigenetic modification.

CRNN (*CRNN* gene), a CE protein encoded in the EDC, similar to involucrin, is among members of S100-fused type protein ^(410, 421, 424). *CRNN* gene is a marker of late epidermal differentiation and is downregulated in AD ⁽⁴²¹⁾. Reduced expression of *CRNN* gene may be an initial part of pathogenesis leading to epithelial barrier dysfunction that characterises AD ⁽⁴²¹⁾.

There may be genetic inheritance of AD via variations in the *CRNN* gene. AD susceptibility was increased with genetic variation at the associated variant (2282del4) in *FLG* and SNP of *CRNN* (rs941934) ^(410, 421). Lieden *et al.* demonstrated that *CRNN* mRNA expression is significantly decreased in eczematous skin, as compared to controls, and SNP of *CRNN* (rs941934) was significantly associated with atopic eczema ⁽⁴²¹⁾. A recent study by Trzeciak *et al.* reported that *CRNN* mRNA levels were significantly decreased in AD skin and *CRNN* rs941934 (A allele) was associated with AD, severe course of disease, elevated IgE levels, eosinophilia and concomitant asthma ⁽⁴¹⁰⁾. Data findings in both studies were consistent with reduced *CRNN* mRNA levels in lesional AD skin, as compared to non lesional AD skin, and skin from healthy controls ^(410, 421). To this author's knowledge, *CRNN* gene has not been linked to epigenetic modifications.

As a tumour suppressor gene, *CRNN* may help maintain barrier function in squamous epithelium in response to injury ^(422, 429). However, *CRNN* is downregulated in AD patients with reduced *FLG* gene, co-existence of genetic variation at the associated variant (2282del4) in *FLG* and having SNP of *CRNN* (rs941934) ^(410, 421). As *CRNN* expression reduced, the regulatory mechanism to maintain barrier function in squamous epithelium is compromised, causing skin barrier dysfunction which progressively

augments AD severity by allowing allergen and human pathogen penetration through the damaged skin barrier, which in turn perpetuates the AD viscous cycle.

Involucrin and CRNN proteins are CE proteins encoded in the EDC, which are reduced in AD and are markers of epidermal differentiation ^(414, 415, 421). Both salivary proteins are interconnected and may possibly be epigenetically modified in relation to regulating immune system development that leads to greater susceptibility to allergy. The proposed model of placental and salivary protein mechanisms, including 14-3-3 and Hp proteins, underlying increased susceptibility to allergy is depicted in Figure 110. Mechanisms of these proteins that lead to increased allergy susceptibility originate *in utero*. *In utero* exposure, such as maternal asthma and folate intake during pregnancy, are associated with epigenetic modifications and partly modulate fetal programming in modifying allergy risk. Salivary proteins are predominantly influenced by postnatal exposures, such as breastfeeding and early exposure to allergens and microbes associated with epigenetic modifications. 14-3-3 and Hp proteins (highlighted with red colour in Figure 110) were altered in placenta and salivary samples. Thus, placental and salivary proteins may be epigenetically modified via *in utero* and postnatal exposures in regulating immune system development that leads to greater susceptibility to allergy.

In this study, the population of children can be divided into three main groups: (1) low risk of allergic disease in children of non-asthmatic mothers; (2) high risk of allergic disease in children of asthmatic mothers; and (3) the general population whose maternal asthma status was unknown (Figure 111). This study identified that salivary 14-3-3 and CRNN proteins were significantly altered in children who developed an allergy and whose mothers were non asthmatic, suggesting these could be markers for allergic susceptibility in low risk populations. In a population of children with high allergy risk, three proteins: salivary 14-3-3, CRNN and involucrin were significantly altered in children with an allergy. For the general population of children, salivary 14-3-3 and CRNN could identify allergic children from non-allergic children. In addition, involucrin may be of use in identifying males at low risk of allergy as this protein was sex-specifically expressed in saliva (Figure 111). Overall, salivary 14-3-3 and CRNN may be used to detect allergy susceptibility in all populations of children, regardless of child sex or genetic risk.

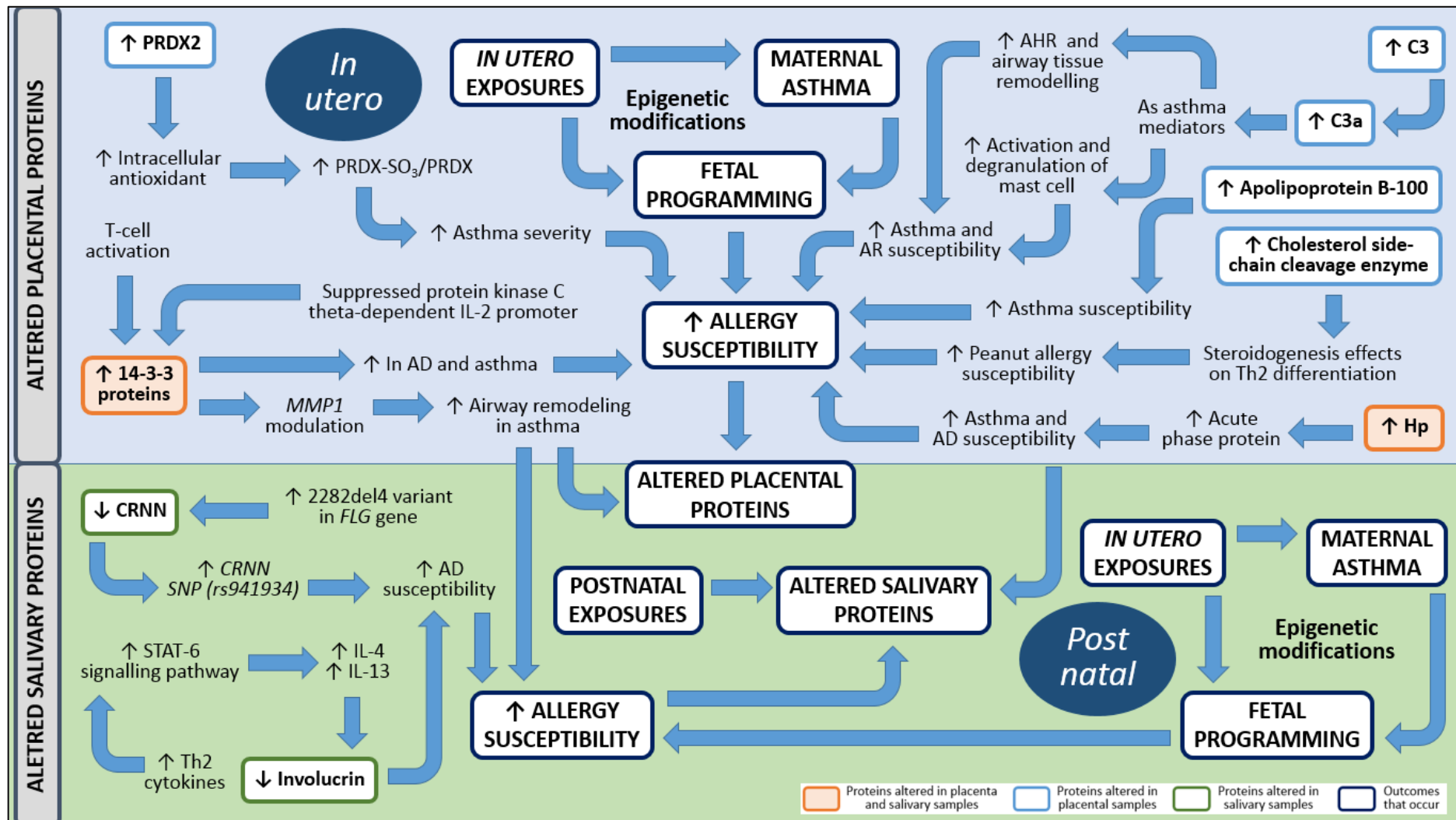


Figure 110. Proposed model of placental and salivary proteins mechanism underlying increased susceptibility to allergy. *In utero* exposure, such as maternal asthma and folate intake during pregnancy, are associated with epigenetic modifications and partly modulate fetal programming in modifying allergy risk. Regulation of these placental proteins occur via antioxidant and anti-inflammatory action of PRDXs, cholesterol side-chain cleavage enzyme and apolipoproteins B-100, as well as C3 immunoregulatory role. Salivary proteins, involucrin and CRNN are involved in AD, allergy and AD susceptibility, particularly via regulation of skin barrier. They are predominantly influenced by postnatal exposures, such as breastfeeding and early exposure to allergens and microbials associated with epigenetic modifications. 14-3-3 and Hp proteins (highlighted with red colour) were altered in placenta and salivary samples. 14-3-3 protein expression is increased in response to T cell activation and suppressed protein kinase C theta-dependent IL-2 promoter. Increased 14-3-3 protein expression may aggravate airway remodelling in asthma via *MMP1* modulation and further increase allergy susceptibility, as evidenced by altered protein level in placenta and saliva samples. For Hp, increment of its expression level stimulates production of acute phase protein that leads to greater susceptibility of asthma, AD and allergy, which can also manifest as altered protein in placenta and saliva samples. Thus, placental and salivary proteins may be epigenetically modified via *in utero* and postnatal exposures in regulating immune system development that leads to greater susceptibility to allergy. (PRDX represents peroxiredoxin, C3 represents complement 3, CRNN represents cornulin, AHR represents airway hyperresponsiveness, AD represents atopic dermatitis, STAT-6 represents signal transducer and activator of transcription and FLG represents filaggrin gene).

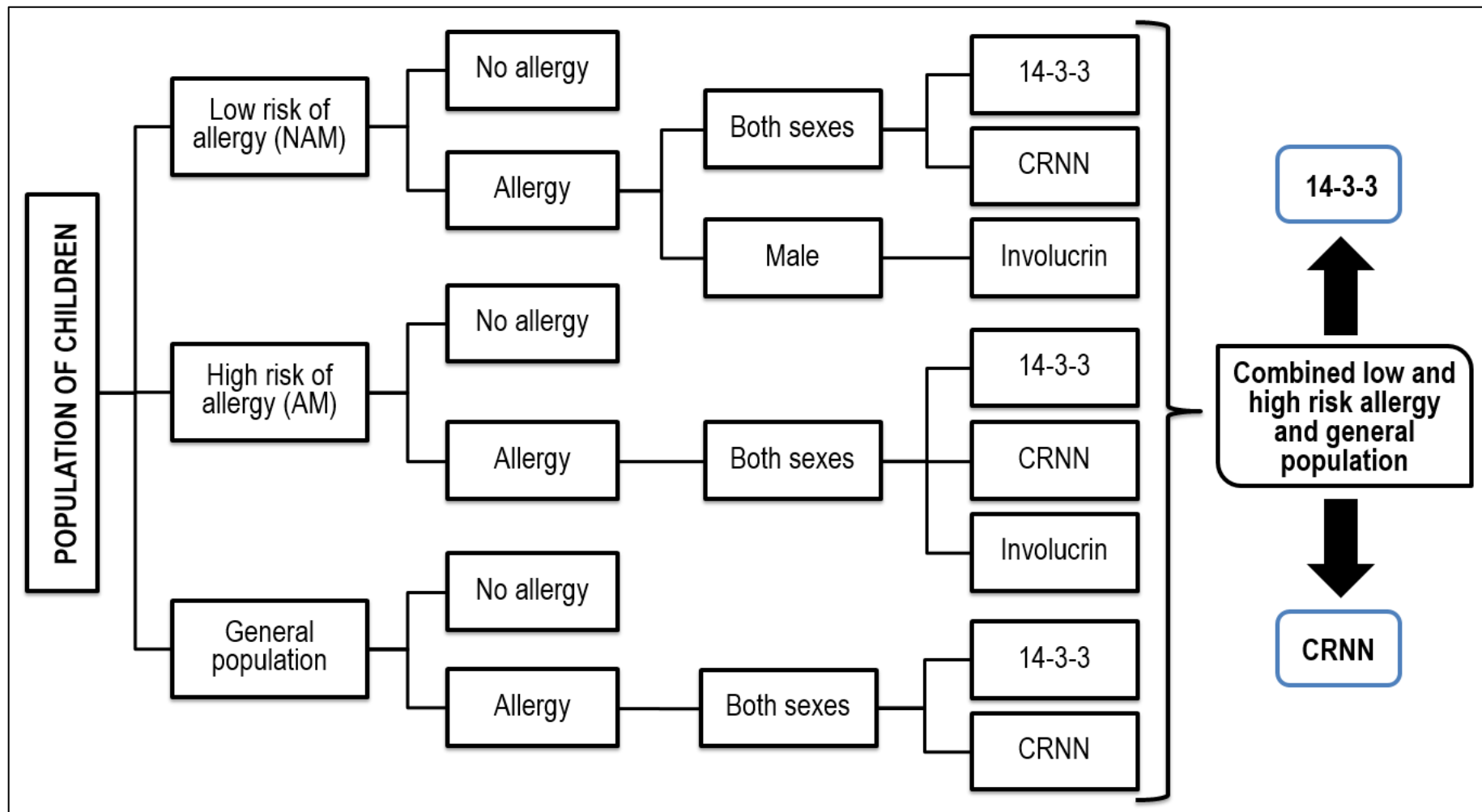


Figure 111. Summary of data analysis on potential salivary protein biomarkers for allergy. (NAM represents non-asthmatic mothers, AM represents asthmatic mothers and CRNN represents cornulin).

Previous data from our group has shown that baseline mRNA expression of placental cytokines, such as *TNFα* and *ILs*, are sex-specifically regulated in pregnancies complicated by asthma⁽¹³⁾. Interestingly, these alterations were more prevalent in mild asthma compared to severe asthma and these changes only occurred in mothers' pregnant with a female fetus, with no changes observed in placentae of male fetuses⁽¹³⁾. We also identified that some pathways change sex-specifically under certain adverse conditions during pregnancy, such as preterm delivery and maternal asthma^(361, 362). This can be explained by different mechanisms males and female fetuses use *in utero* to cope with adverse conditions via alteration in the placental function, manifested by altered proteins^(12, 13). Furthermore, maternal asthma can alter placental gene expression in a sex-specific manner which may influence fetal immune system development and possibly contribute to development of childhood allergy.

The main limitation of this study was that it is more common for a child to have multiple allergic phenotypes, as compared to having one specific allergy. Therefore, it is impossible to identify allergic risk for just one phenotype of allergy and more advantageous to examine proteins altered in all allergic diseases. The combination of allergic diseases occurs due to the 'atopic march', which is a pattern of progression through different allergic disorders in early childhood, where individuals who first present with AD in early childhood later develop AR and then atopic asthma⁽²⁸⁾. Hence, the study on one specific allergic phenotype for example, rhinitis, was not feasible as the sample size for this was very small in placental (n= 2 in qRT-PCR, n= 1 in Western blot) and saliva samples (n= 1 in Western blot, n= 3 in Blitz system). For this reason, most of data analyses performed were more reliable when conducted as general allergy, or allergy combination of asthma, eczema or rhinitis, as compared with one specific allergic phenotype.

The second limitation of the study was small sample sizes of placental and saliva samples analysed with LC-MS/MS due to expensive cost of the LC-MS/MS method. In LC-MS/MS, the sample size used in placental samples was 16 (control: 8, allergy: 8) and for saliva samples was 18 (control: 9, allergy: 9). Small sample size was compensated using a much larger sample size of placental and child saliva samples in validation studies of Western blot (n placenta= 68, n saliva= 62) and Blitz system (n saliva= 126). This compensatory method of using larger sample size in validation studies after running a small sample size in preliminary MS has been applied in previous established studies on salivary biomarker identification⁽²⁷²⁾.

Some differences were observed between Western blot validation and LC-MS/MS preliminary investigations for placenta and saliva samples which may be explained by small sample size. Regardless, LC-MS/MS serves as an excellent established screening method to identify placental and salivary protein biomarkers in children with allergy⁽²⁷²⁾. In addition, statistical analyses performed between LC-MS/MS and Western blot used the populations of different allergic groups. In LC-MS/MS,

using saliva samples LFQ MS ratios were calculated according to one specific allergic phenotype (asthma, eczema or rhinitis), whereas in placental samples LFQ MS ratios were calculated according to allergy and sex (allergic children, allergic male and allergic female). This method was appropriate, as when LC-MS/MS was performed for placental samples there were not enough samples of allergic placentae with one specific allergic phenotype (four allergic placentae for each allergy), as previously conducted with saliva samples.

The third study limitation was that in LC-MS/MS analysis there was a stringent range of significant LFQ MS ratio for protein identification. In this study, significant ratio varied between >2 or <0.5 fc, as compared to other studies that used less a stringent significant ratio of >1.5 or <1 fc. Thus, there may possibly be other potential salivary and placental proteins in LC-MS/MS analysis within >1.5 or <1 fc significant ratios that were not fully investigated and validated in this study.

Another limitation was differences in protein concentrations in saliva validation studies. The main experimental difference between the two validation studies were that the Blitz system ($n=126$) used a larger sample size, as compared with Western blot ($n=62$), and the nature of the saliva samples. Study power is higher with larger sample size and lower when subgroup analysis is performed ^(430, 431). For example, in Western blot subgroup analysis for allergic susceptibility according to sex, total sample size for allergic children regardless of sex was 38, whereas sample size for male allergic children was 19. Thus, as number of subjects in subgroups is lower than total sample size, the analysis may not have adequate statistical power ⁽⁴³⁰⁾. The Blitz system used native and crude child saliva samples, whereas Western blot used the denatured protein form of saliva. Denaturation of the protein destroys tertiary structure, leads to random-coiled aggregation and decreased solubility, which may affect IgE-binding conformational epitopes ^(312, 434). This may explain differences in protein expression in allergic children which were increased using denatured protein in Western blot, as opposed to decreased expression using native protein in the Blitz system. Although these differences were present, protein expression between allergic children and non-allergic children in Western blot and the Blitz system were statistically significant and biologically plausible. The significance of protein expression indicates that salivary protein expressions were vastly distinctive between the two groups, either in the native or denatured state.

Based on findings of this study, salivary 14-3-3 and CRNN are the most suitable potential salivary protein biomarkers for childhood allergy as they can detect allergic children from non-allergic children in random sampling, regardless of child sex or genetic risk (Figure 111). These two potential salivary protein biomarkers can be utilised to develop an assay for detection of allergic risk in children. Moreover, salivary 14-3-3 and CRNN can also be used as target proteins for interventions that prevent allergy onset. These tools may be used as justification for targeted interventions for allergy prevention

in early childhood. However, sensitivity, specificity and normal ranges of 14-3-3 and CRNN proteins have not yet been established.

To be a useful diagnostic tool, a biomarker must be sensitive and detect disease with 90 % specificity. For a biomarker to be of clinical value, the other key quality is specificity, that is, probability that a positive signal is a true sign of disease and not an error of at least 90 % ⁽⁴⁸⁰⁾. In many situations, including in proteomics, after lengthy and costly effort, many biomarker candidates do not achieve desired sensitivity and specificity, despite development of technologies ⁽²⁷⁹⁾. To find biomarkers that fulfil required sensitivity and specificity is challenging and many potential candidates, including protein markers, do not meet such criteria. However, disease biomarkers constitute another group that can be informative regarding underlying molecular mechanisms of disease or treatment, even if they do not necessarily meet criteria of diagnostic specificity and sensitivity. An example is disease biomarkers for neurodegenerative disorders ⁽²⁷⁹⁾. These can be indicative of neuroinflammation, although not specific for a particular disease of the central nervous system. In regards to this, the same condition may apply to potential salivary protein biomarkers for childhood allergy: 14-3-3 and CRNN proteins. Other than overcoming the challenges of sensitivity and specificity, a biomarker must be capable of being reproducibly quantified in independent laboratories by independent personnel ⁽²⁷⁹⁾.

The Blitz system has potential as a rapid detection method of allergic risk using native and crude child saliva samples. The Blitz system with BLI principle allows rapid identification, quantitation and characterisation of expressed protein from as little as four microlitres of sample (refer Methodology section 2.11.3.1) ⁽³⁰⁴⁾. The SAX biosensors immobilised with pan 14-3-3 and CRNN antibodies can be regenerated up to 10 times for optimum results without compromising antibody-protein binding, provided valid regeneration protocol is established ⁽³¹⁸⁾. Therefore, the Blitz method is relatively cost effective. Furthermore, the Blitz system has advantages of not requiring expertise skill, is easy to use, more feasible, is a rapid test (approximately three minutes) and applicable for use in clinical settings.

Future investigations to confirm validity of the saliva protein candidates identified in this thesis are required. A prospective cohort study of children at six months of age is required, with saliva collected and tested, to determine sensitivity and specificity of potential salivary biomarkers for detection allergic susceptibility. In this study, we predicted allergy susceptibility using salivary protein biomarkers 14-3-3 and CRNN and these need to be validated further.

In this study, statistical analyses were used to determine significance of 14-3-3 and CRNN protein concentrations using the Blitz system in a large number of child saliva samples (n= 126). However, in the clinical setting, it is more feasible if normal ranges of 14-3-3 and CRNN proteins are established prior to quantification of child saliva sample concentrations. For example, normal ranges of serum Hb

distribution varies with sex and age, ethnicity and physiological status, including pregnancy state or athletic lifestyle ⁽⁴⁸¹⁾. Although salivary 14-3-3 and CRNN proteins are not known to change with sex and age, it will be more beneficial if normal ranges for these salivary proteins for allergic and non-allergic children are validated according to these two variables.

In conclusion, this study has demonstrated that placental proteins were altered *in utero* due to changes in placental adaptation to maternal environment in children who subsequently developed an allergy, most possibly via mechanisms involving epigenetic modifications. Potential mechanisms driving increased susceptibility to allergy via these altered placental and salivary proteins are illustrated in proposed models (Figure 111). Based on these models, it is proposed that altered expression of 14-3-3 and CRNN proteins cause bias towards a Th2 immune profile in fetal circulation with increased secretion of anti-inflammatory cytokines and decreased Tregs activity, as previously indicated by reduced placental *FOXP3* expression ⁽¹⁵⁾. As a result, altered programming of placental and fetal immune function may result in increased susceptibility of childhood allergic disease that can be identified using biomarkers in placenta.

Several changes in placenta proteomic profiles are reflected in similar change in saliva in early childhood, demonstrated by similar alterations of 14-3-3 and Hp proteins in placenta and child saliva samples. Thus, findings of this study support the hypothesis that the particular immune mechanism manifested in altered child salivary protein predominantly occurs in children with asthmatic mothers (high risk of allergy), possibly via a mechanism involving epigenetic modifications.

Allergic disease mechanisms of children with low risk of allergy appeared not to be influenced by *in utero* events, but were most likely influenced by postnatal events that include environmental exposures, such as breastfeeding and early microbial exposure ^(77, 242, 244, 245). Study findings support the hypothesis that altered proteins present in the placenta are also detectable in saliva of children with allergic diseases. Therefore, data indicate that placenta and saliva samples are reliable biological sources of biomarkers for detection of allergic risk in children.

Appendices



Government of South Australia
SA Health

Human Research Ethics Committee (TQEH/LMH/MH)
Basil Hetzel Institute DX465101
The Queen Elizabeth Hospital
28 Woodville Road
Woodville South SA 5011
Telephone: 08 8222 6641

14th October 2013

A/Prof Vicki Clifton
Robinson Institute
Lyell McEwin Hospital

Dear A/Professor Clifton

HREC reference number: 2009045

Project title: *"Effect of asthma during pregnancy on maternal health, placental function, fetal growth and childhood development"*

Re: 2009045 – Ethics Extension Approval

The following documents have been reviewed and approved:

- Notification of Amendment, dated 10 July 2013
- Annual Review Form dated 5th July 2013
- LMH Participant Information – sheet and Consent Form, version 3, dated 5th July 2013

Approval Status: **FINAL**

Period of Approval: **19th August 2013 – 19th August 2014**

Previous approval for this study ran out on 17th May 2012

Research conducted under this protocol in the interval between the date that previous approval expired and the date on this letter must be considered to have been conducted without cover of HREC approval. Approval has now been granted from the date the chairman approved the annual review report.

***Please note the terms under which Ethical approval is extended:**

1. Researchers are required to immediately report to this HREC anything which might warrant review of ethical approval of the protocol, including:
 - a. serious or unexpected adverse effects on participants;
 - b. proposed changes in the protocol; and
 - c. unforeseen events that might affect continued ethical acceptability of the project.
2. Studies are approved for up to twelve months only and a report is required at the end of the study or 12 month period. Extensions will not be granted without a report to this HREC.
3. All research subjects shall be provided with a Participant Information Sheet and Consent Form, unless otherwise approved by the Committee.
4. The Participant Information Sheet and Consent Form shall be printed on the relevant site letterhead stating the contact details for the researchers.
5. A report and a copy of any published material should be forwarded to the Committee at the completion of the project.

A copy of this letter should be provided to all relevant Research Governance Officers for the institutions where this study is running.

Yours sincerely

A/Professor Timothy Mathew
Chairman, Human Research Ethics Committee (TQEH/LMH/MH)

TM:cc

Appendix 1. Lyell McEwin Hospital's ethical approval.

Modified ISAAC Questionnaire

DATE OF SCREENING _____
DOB _____

POSTCODE _____

GENDER Male []
Female []

MATERNAL ASTHMA _____

1. Has your child had wheezing or whistling in the chest in the last 12 months?

Yes []
No []

IF YOU HAVE ANSWERED “NO” PLEASE SKIP TO QUESTION 4

2. How many attacks of wheezing has your child had in the last 12 months?

1 to 3 []
4 to 12 []
More than 12 []

3. In the last 12 months how often, on average, has your child’s sleep been disturbed due to wheezing?

Never woken with wheezing []
Less than one night per week []
One or more nights per week []

4. In the last 12 months, has your child had a dry cough at night, apart from a cough associated with a cold or chest infection?

Yes []
No []

5. In the last 12 months, has your child ever had wheezing in association with a cold/ flu?

Yes []
No []

6. Has a doctor ever told you that your child had asthma?

Yes []
No []

7. In the last 12 months, has your child had a problem with sneezing, or a runny, or a blocked nose when he/ she DID NOT have a cold or the flu?

Yes []
No []

IF YOU ANSWERED “NO” PLEASE SKIP TO QUESTION 9

8. In the last 12 months, has this nose problem been accompanied by itchy-watery eyes?

Yes []
No []

9. Has your child ever had hay fever?

Yes []
No []

10. In the last 12 months, has your child had a dry itchy rash at any time?

Yes []
No []

IF YOU ANSWERED “NO” PLEASE SKIP TO QUESTION 13

11. Has this dry itchy rash at any time affected any of the following places; the folds of the elbows, behind the knees, in front of the ankles, under the buttocks, or around the neck, ears or eyes?

Yes []
No []

12. In the last 12 months, how often, on average, has your child been kept awake at night by this itchy rash?

Never in the last 6 months []

Less than one night per week	[]
One or more nights per week	[]

13. Has your child ever had eczema?

Yes	[]
No	[]

14. Did you breast feed or bottle feed your child in the first 12 months?

Breastfeed	[]
Bottlefeed	[]
Both	[]

a) If breastfed, for how long

0-3 months	[]
3-6 months	[]
6-9 months	[]
9-12 months	[]
Over 12 months	[]

15. Does anyone smoke regularly inside your house?

Yes	[]
No	[]

16. Has your child ever been diagnosed with any other illnesses in the last 12 months?

Ear infections	Yes	[]
	No	[]
Chest infections (pneumonia)	Yes	[]
	No	[]
Gastroenteritis	Yes	[]
	No	[]
Urinary tract infections	Yes	[]
	No	[]

Other

17. Has your child been prescribed asthma medication (e.g. puffers such as relievers or preventers) in the last 12 months?

Relievers (Ventolin/ Asmol/ Bricanyl)	Yes	[]
	No	[]

Preventers (Flixotide/ QVAR/ Singulair/ Seretide)	Yes	[]
	No	[]

Nurse to enter the following details for the child:

Weight (kg):

Height (cm):

Appendix 2. Modified ISAAC questionnaire.

18th June 2015



Research Secretariat
Level 2, Samuel Way Building
72 King William Road
Tel 08 8161 6390
Tel 08 8161 6521
www.wch.sa.gov.au

Dr Andrew Tai
Paediatric Respiratory and Sleep Medicine
WCHN

Dear Andrew

Re: Screening for paediatric sensitisation to allergic disease with new biosensor technology. HREC/15/WCHN/29. Expiry date: 30th June 2018

Lead HREC for the above study for the following institutions/sites:

Women's and Children's Health Network

I refer to your letter dated 23rd April 2015 and email dated 5th June 2015 in which you responded to matters raised by the WCHN Human Research Ethics Committee at its March 2015 meeting. I am pleased to advise that your protocol has been granted full ethics approval and meets the requirements of the *National Statement on Ethical Conduct in Human Research*.

Specifically, the following documents have been noted/approved:

Document	Version	Date
Patient Information Sheet/Consent Form: Consent form	1	17 February 2015
Questionnaire/s: Modified ISAAC questionnaire	1	17 February 2015
Patient Information Sheet/Consent Form: Parent information	1	17 February 2015
Protocol: Saliva collection protocol	1	17 February 2015
Application		
Response to Request for Further Information: Response Letter		23 April 2015
Site Specific Participant Information Sheet: Patient Information Sheet	1.5	31 May 2015

This letter constitutes advice on ethical consideration only. You must not commence this research project at a site until you have obtained separate research governance approval from the site concerned. A copy of this letter should be forwarded to all site investigators for submission to the relevant Research Governance Officer.

At the WCHN, or any other SA Health site, separate authorisation from the Chief Executive or delegate of that site must be obtained through a Site Specific Assessment (SSA) request. For information on this process at the WCHN, please contact the A/WCHN Research Governance Officer, Ms Katherine McPhail (telephone 8161 6688, email katherine.mcphail@health.sa.gov.au).

I remind you approval is given subject to:

- immediate notification of any serious or unexpected adverse events to participants;
- immediate notification of any unforeseen events that might affect continued ethical acceptability of the project;
- submission of any proposed changes to the original protocol. Changes must be approved by the Committee before they are implemented;
- immediate advice, giving reasons, if the protocol is discontinued before its completion;
- submission of an annual report on the progress of the study, and a final report when it is completed to the WCHN Research Governance Officer. It is your responsibility to provide these reports, without reminder. The proforma for the report may be found on the WCHN Research Governance and Ethics website.

Approval is given for three years only. If the study is more prolonged than this, an extension request should be submitted unless there are significant modifications, in which case a new submission may be required. Please note the expiry date in the title above and include it in any future communications.

Yours sincerely

TAMARA ZUTLEVICS (DR)
CHAIR
WCHN HUMAN RESEARCH ETHICS COMMITTEE

WOMEN'S & CHILDREN'S HEALTH NETWORK (WCHN) HUMAN RESEARCH ETHICS COMMITTEE (HREC)

Parent information sheet Version 1.5, 31 May 2015

Lay title of study	Early screening for allergic diseases
Scientific title of study	Screening for paediatric sensitisation to allergic diseases with new biosensor technology
Study Number	HREC/15/WCHN/29
Protocol Number	
Principal Investigator	Dr Andrew Tai
Institution	Women's and Children's Hospital Network (WCHN)
Phone Number	(08) 8161 7234 or (08) 8161 7000 (24-hr emergency)

Your child is being invited to participate in this research study because he/she has either allergic diseases or no history of allergic diseases at all.

- This Information Sheet explains this research study and your child's participation in the study.
- Please read it carefully and take as much time as you need.
- Ask questions about anything that you do not understand now, or when you think of them later.
- Your child's participation is voluntary. If you enrol your child in the study and change your mind later, you may withdraw at any time without prejudice to your child's future treatment or relationship with the health service.
- Whilst your child is in the study, the study team will keep you informed of any new information that could affect your decision to keep your child in the study.
- Once you have read the Information Sheet and all of your questions have been answered and you understand the research study, and you agree to have your child take part, you will be asked to sign the consent form when you meet with the study team for your initial visit. You will be given a copy of the signed consent form for your records at that time.

Why are we doing the study?

Allergic diseases occur when the people's immune system reacts to things in the environment like pollen, house dust mite, pet dander, bee venom or different food types.

The ranges of allergic diseases include diseases asthma, hay fever, food allergy and eczema. The severity of childhood allergies varies from person to person and can range from minor skin irritation or, in an extreme case, severe anaphylaxis - a potentially life-threatening emergency.

The purpose of this study is to screen for allergic diseases by the collection of saliva. The contents of the saliva will be measured to identify chemicals (called biomarkers) which may differ in children with allergy as compared to children without allergy. By detecting allergy early in children, it may allow us to provide early intervention to reduce the complications.

This study will also form part of PhD project for Nurul Zainal, PhD student from The University of Adelaide.

Does my child have to take part?

No. It is your choice about having your child in the study. If you agree to have your child involved you are free to change your mind at any time. Nothing will happen if you choose to not have your child involved or if you drop out of the study. Your child will still be able to attend the clinic and receive the same treatment care.

What does my child need to do to be in the study?

At the initial visit we will ask you about your child's health to see if he/she indeed has an allergic disease. If your child does have an allergic disease, we will also be asking you about your child's overall allergic disease symptoms with a questionnaire. You will also be asked about breastfeeding and smoking in the questionnaires. This assessment may take 10 to 15 minutes to complete.

We will then endeavour to obtain **one** saliva sample from your child using a painless method with a tube called a salivette. It can be done by you or if you prefer, by the study staff. The saliva should be collected prior to brushing teeth in the morning and after rinsing the mouth with water OR during the day, saliva should be collected no earlier than 30 minutes after eating or drinking. There are 2 methods to collect the saliva based on your child's age (Figure 1).

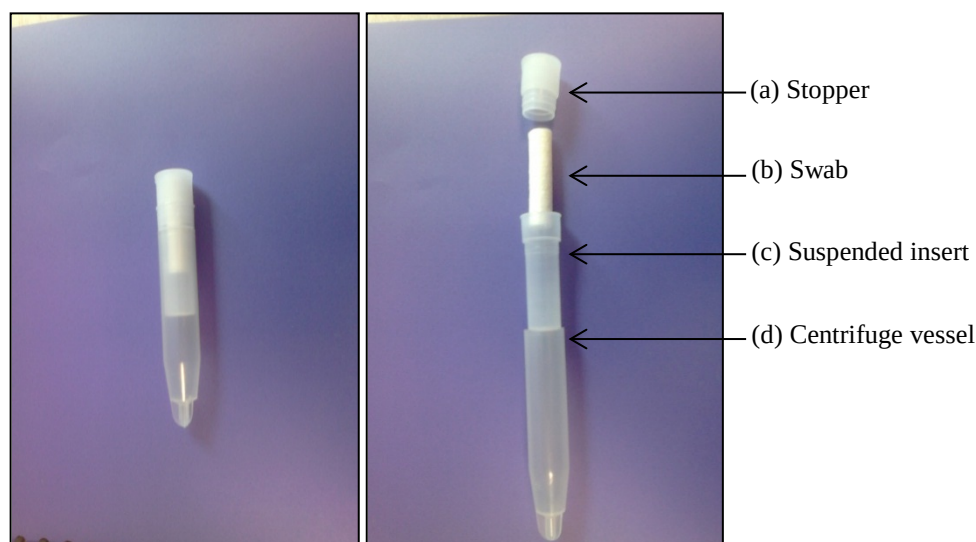


Figure 1: Salivette

If the child is less than 3 years old,

- Hold the salivette at the rim of the suspended insert and remove the stopper by slightly pushing it to the side.
- Remove the swab from the salivette
- Place the tube at the tip of your child's mouth
- Ask your child to spit inside the tube, or you can also collect the drooling saliva from his/her mouth (saliva volumes ~ 1 mL).
- Close the salivette firmly with the stopper (a).

If the child is more than 3 years old ,

- Get your child to gently chew the swab for 1 minute. Keep the swab in your child's mouth until he/she feels that he/she can no longer prevent themselves from swallowing the saliva produced.
- Return the saturated swab (b) to the suspended insert (c)
- Close the salivette firmly with the stopper (a).

What happens to the data that are collected?

The information we collect will be entered into a database by the research team at The University of Adelaide. At all times your child's confidentiality will be protected and the results we share will not be labelled with your child's name. More information on privacy and confidentiality can be found below. Your child's medical records may also be reviewed by the study doctor and team as required to confirm the diagnosis of allergy and to assess the progression of allergy.

Is there likely to be a benefit to my child?

There may be no direct benefit to your child in short term but this study may help to improve the management of children with allergic diseases in the near future.

Is there likely to be a benefit to other people in the future?

The findings in this study may enable early screening of allergic diseases in children and possible early intervention can be implemented. Therefore complication of allergic disease in children may be reduced in the future.

What are the possible risks and/or side effects?

Participation in this study will involve a small collection of saliva into a tube (see above) which is generally painless.

Are there any changes to the standard care of my child if they are in the study?

Yes. Your child will have an extra procedure which includes a painless saliva sample collection and a questionnaire completed to assess the spectrum of allergic diseases.

Can I withdraw my child from the study?

You may decide not to let your child take part or you may want to withdraw your child from this study at any time. If you decide to withdraw your child from this study please notify the study doctor. If you decide to withdraw your child from this study, there is no penalty or loss of benefits in your child's routine medical care or any other benefit(s) that your child would otherwise be entitled to receive.

What are the reasons that the researchers may withdraw my child from the study?

Your child may be removed from the study without your consent at any time. Reasons why he/she may be removed from the study include, but are not limited to, the following:

- Your treating doctor determines that it is in your child's best interest not to continue
- The study is terminated by the Hospital or The University of Adelaide

Will I receive payment for having my child be involved in the study?

You will not receive payment for having your child take part in this study.

Where is your child's information kept?

During the study the study doctor and study staff will collect and record information about your child. This information will be transferred to a secure password protected electronic database so that researchers can analyse it. Identifying information such as your child's name or address will not be stored in the database. Your child will be identified by a code that is assigned by the study. Electronic data will be stored at The University of Adelaide for a period of 30 years from the time when the last patient completes the study.

What about your child's privacy?

By signing the informed consent form, you are giving your permission for authorised representatives of the study sponsor monitor(s), auditor(s) and the ethics committee to be granted direct access to your child's original medical records and other source data to the extent permitted by the applicable laws and regulations pertaining to this study. Your child's information will remain confidential except in the case of a legal requirement to pass on personal information to authorised third parties. This requirement is standard and applies to information collected both in research and non-research situations. Such requests to access information are rare; however we have an obligation to inform you of this possibility.

Who has approved the study?

This study has been approved by the Human Research Ethics Committee at Women's and Children's Health Network.

Will I be informed of the results when the research study is finished?

Most probably you will not be informed as the result of the study will not affect your child treatment. Furthermore, there are still much more analysis to be done in relation to the study.

Who to contact for more information about this study:

If you would like any more information about this study, please do not hesitate to contact one contact one of the research team. They are very happy to answer your questions.

Name

Dr Andrew Tai

Contact Number

(08) 8161 7234

Position

Principal Investigator

Who do I contact if I have concerns about the organisation or running of the study?

If you have any concerns or complaints regarding this study, you can contact the Secretary of the Human Research Ethics Committee (Ms Brenda Penny, Research Secretariat, ph (08) 81616521).

WOMEN'S & CHILDREN'S HEALTH NETWORK (WCHN) HUMAN RESEARCH ETHICS COMMITTEE (HREC)

CONSENT FORM

Screening for paediatric sensitisation to allergic disease with new biosensor technology

Early screening for allergic diseases

I _____

**hereby consent to my child's involvement in the research project entitled:
Screening for paediatric sensitisation to allergic disease with new biosensor technology**

1. The nature and purpose of the research project described on the attached Information Sheet has been explained to me. I understand it and agree to my child taking part.
2. I understand that my child may not directly benefit by taking part in this study.
3. I acknowledge that the possible risks and/or side effects, discomforts and inconveniences, as outlined in the Information Sheet, have been explained to me.
4. I understand that I can withdraw my child from the study at any stage and that this will not affect medical care or any other aspects of my child's relationship with this healthcare service.
5. I understand that there will be no payment to my child for taking part in this study.
6. I have had the opportunity to discuss taking part in this research project with a family member or friend, and/or have had the opportunity to have a family member or friend present whilst the research project was being explained by the researcher.
7. I am aware that I should retain a copy of the Consent Form, when completed, and the Information Sheet.
8. I consent to saliva specimen being taken from my child and being used in the above project.
9. I agree to the accessing of my child's medical records for the purpose of this study.
10. I understand that my child's information will be kept confidential as explained in the information sheet except where there is a requirement by law for it to be divulged.

Signed:

Relationship to Patient:

Full name of patient:

Dated:

I certify that I have explained the study to the parent and/or child and consider that he/she understands what is involved.

Signed: Title:

Dated:

Appendix 5. Consent form.

Appendix 6. Supplementary table. List of chemicals, solutions, enzyme, peptide and equipment.

Chemicals/Solutions	Grade/Purity	Manufacturer	Catalogue number
Protease inhibitor cocktail (PIC)	-	Sigma	P8340
Ammonium hydrogen carbonate	Analytical grade	Merck	103025E
Guanidine hydrochloride (GdnHCL)	Analytical grade	Sigma	G4630-1KG
Dithiothreitol (DTT)	Electrophoresis grade	Sigma	D9163-5G
Iodoacetamide (IAA)	Analytical grade	GE Healthcare	RPN6302OL/AG
Trifluoroacetic acid (TFA)	Protein sequencer grade	AB applied biosystems	400003
Formic acid (FA)	LC-MS grade	Fluka	56302-50ML-F
Acetonitrile (ACN)	Gradient grade for LC	Merck	1.00030.2500
Methanol	Gradient grade for LC	Merck	1.06018.2500
Acetic acid glacial	Analytical grade	Merck	1.00063.2511
Alpha-cyano-4-hydroxycinnamic acid (CHCA)	MALDI-MS grade	Bruker Daltonics	203072
Sodium dodecyl sulfate (SDS)	-	TSU MLS	
Commercial kits	Grade/Purity	Manufacturer	Catalogue number
ReadyPrep™ 2-D Cleanup kit	-	Bio-Rad	163-2130
EZQ® protein assay	-	Invitrogen	R-33200
Vivacon 500 (Membrane: 10 000 MWCO HY)	-	Sartorius	VNO1 HO1
Enzyme	Grade/Purity	Manufacturer	Catalogue number
Trypsin enzyme from porcine	Sequencing grade	Promega	V5111
Peptide	Grade/Purity	Manufacturer	Catalogue number
MassPREP Peptide Mixture	MALDI-MS grade	Waters	186002337
Equipment	Grade/Purity	Manufacturer	Catalogue number/Model
Vacuum centrifuge, SpeedVac	-	Thermo Fisher Scientific	Savant SVC100
ZipTip® Pipette Tips	-	Merck Milipore	ZTC18S096
Amersham ECL Semi-Dry Blotters	-	GE Healthcare	TE 77

SALIVA COLLECTION PROTOCOL

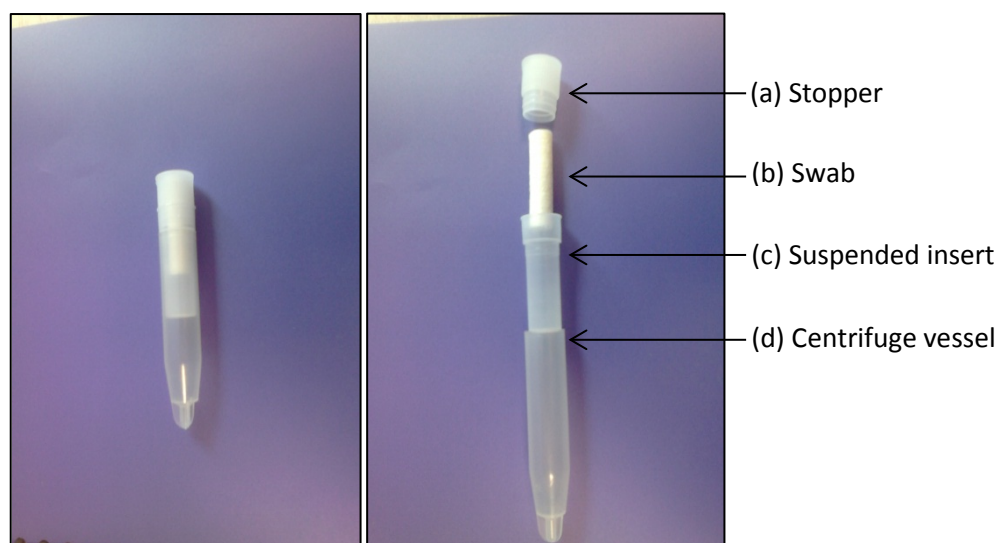


Figure 1 : Salivette

- 1) Saliva should be collected prior to brushing teeth in the morning and after rinsing the mouth with water. During the day, saliva should be collected no earlier than 30 minutes after eating or drinking.
- 2) There are 2 methods to collect the saliva based on your child's age.

Method A (children < 3 years old)	Method B (children > 3 years old)
➤ Hold the salivette at the rim of the suspended insert (c) and remove the stopper (a) by slightly pushing it to the side. ➤ Remove the swab (b) from the salivette. ➤ Place the tube at the tip of your child's mouth ➤ Collect the drooling saliva from his/her mouth (saliva volumes ~ 1 mL). ➤ Put the swab back to the suspended insert and close the salivette firmly with the stopper (a).	➤ Gently chew the swab for 1 minute. Keep the swab in your child's mouth until he/she feels that he/she can no longer prevent themselves from swallowing the saliva produced. ➤ Return the saturated swab (b) to the suspended insert (c). ➤ Put the swab back to the suspended insert and close the salivette firmly with the stopper (a).

- 3) The samples should be kept on ice or -20 degrees prior and after the collection procedure because the salivette had been added with 10 µl protease inhibitor.

Appendix 7. Saliva collection by parents.

Appendix 8. Table 1. In-solution protein digestion calculation used for saliva samples in LC-MS/MS.

Reduction of the S-S linkage				Alkylation of the –SH group		In-solution digestion with Trypsin				Extraction of peptides	
RN	Saliva conc (µg/µL)	MiliQ water (µL)	Volume of sample for 5 µg protein digestion	Volume of 0.5 M DTT (µL)	55 mM IAA (2 µL) + 100 mM NH ₄ HCO ₃ (8 µL)	ACN (µL)	100 mM NH ₄ HCO ₃ (µL)	Trypsin (µL)	Final volume (µL)	10 % TFA (µL) (0.5 % final volume)	Volume after TFA (µL)
134b	0.69	2.37	7.24	0.4	10	8.68	57.14	1.0	86.82	4.34	91.17
211b	1.17	5.34	4.26	0.4	10	5.11	25.01	1.0	51.12	2.56	53.68
279	1.98	7.07	2.53	0.4	10	3.04	6.32	1.0	30.36	1.52	31.87
282	0.65	1.94	7.66	0.4	10	9.20	61.77	1.0	91.97	4.60	96.57
295	0.60	1.24	8.37	0.4	10	10.04	69.34	1.0	100.38	5.02	105.39
320	1.62	6.51	3.09	0.4	10	3.71	12.38	1.0	37.09	1.86	38.95
355	0.87	3.87	5.73	0.4	10	6.88	40.91	1.0	68.79	3.44	72.23
356	1.22	5.50	4.10	0.4	10	4.92	23.26	1.0	49.17	2.46	51.63
404	0.69	2.37	7.23	0.4	10	8.68	57.10	1.0	86.78	4.34	91.12
421	1.74	6.73	2.87	0.4	10	3.45	10.04	1.0	34.49	1.72	36.21
435	0.81	3.42	6.18	0.4	10	7.41	45.73	1.0	74.14	3.71	77.85
503	1.76	6.76	2.84	0.4	10	3.41	9.69	1.0	34.10	1.71	35.80
504	0.72	2.65	6.95	0.4	10	8.34	54.09	1.0	83.43	4.17	87.61
508	1.21	5.48	4.12	0.4	10	4.94	23.46	1.0	49.40	2.47	51.87
510	1.06	4.88	4.72	0.4	10	5.66	29.97	1.0	56.63	2.83	59.46
511	1.02	4.71	4.89	0.4	10	5.87	31.85	1.0	58.72	2.94	61.65
512	0.67	2.09	7.51	0.4	10	9.01	60.07	1.0	90.08	4.50	94.59
514	2.14	7.26	2.34	0.4	10	2.81	4.27	1.0	28.07	1.40	29.48

⁰ Abbreviations: DTT represents dithiothreitol, IAA represents iodoacetamide, NH₄HCO₃ represents ammonium bicarbonate, ACN represents acetonitrile and TFA represents trifluoroacetic acid.

Appendix 9. Table 2. Child placental protein identification in children with allergic diseases including asthma, eczema and combination of rhinitis in LC-MS/MS (1223 placental proteins). (Highlighted protein name have more than 2 unique peptides (UP).

Protein IDs	Sequence length (bp)	UP	Unique sequence coverage [%]	MW [kDa]
sp Q15149 PLEC_HUMAN	4684	115	32.70	531.78
sp P35579 MYH9_HUMAN	1960	94	47.00	226.53
sp Q09666 AHNK_HUMAN	5890	81	35.60	629.09
sp P24043 LAMA2_HUMAN	3118	74	32.80	343.41
sp P02751 FINC_HUMAN	2355	73	45.70	259.21
sp P21333 FLNA_HUMAN	2647	72	42.80	280.74
sp P01024 CO3_HUMAN	1663	68	52.40	187.15
sp P02768 ALBU_HUMAN	609	66	85.70	69.37
sp Q9Y490 TLN1_HUMAN	2541	58	39.60	269.76
sp Q00610 CLH1_HUMAN	1675	55	42.00	191.61
sp P08670 VIME_HUMAN	466	52	74.70	53.65
sp P01023 A2MG_HUMAN	1474	49	47.40	163.29
sp P11047 LAMC1_HUMAN	1573	46	39.30	174.01
sp P98160 PGBM_HUMAN	4346	46	16.90	464.01
sp Q05707 COEA1_HUMAN;	1796	45	34.90	193.51
sp P02545 LMNA_HUMAN	664	44	64.60	74.14
sp P55268 LAMB2_HUMAN	1798	43	34.30	195.98
sp P14625 ENPL_HUMAN	802	41	53.10	92.34
sp P02675 FIBB_HUMAN	491	39	70.50	55.93
sp P08133 ANXA6_HUMAN	673	39	61.40	75.87
sp P05787 K2C8_HUMAN	483	37	64.00	53.70
sp P02671 FIBA_HUMAN	866	36	39.80	94.97
sp P18206 VINC_HUMAN	1066	35	41.20	116.72
sp P46940 IQGA1_HUMAN	1657	34	32.00	189.28
sp P08729 K2C7_HUMAN	469	33	57.40	51.39
sp P11021 GRP78_HUMAN	654	33	52.00	72.33
sp P02647 APOA1_HUMAN	267	32	80.10	30.78
sp P35580 MYH10_HUMAN	1976	32	22.40	229.00
sp Q13813 SPTN1_HUMAN	2472	32	19.90	284.54
sp Q9P2E9 RRBP1_HUMAN	1410	32	28.70	152.45
sp O75369 FLNB_HUMAN	2602	31	19.90	278.16
sp O15230 LAMA5_HUMAN	3695	30	12.30	399.73
sp P04083 ANXA1_HUMAN	346	30	75.10	38.71
sp P21980 TGM2_HUMAN	687	30	51.50	77.33
sp P07355 ANXA2_HUMAN;sp A6NMY6 AXA2L_HUMAN	339	29	66.10	38.58
sp P11511 CP19A_HUMAN;	503	29	54.10	57.88
sp P30101 PDIA3_HUMAN	480	29	60.20	54.10
sp P35749 MYH11_HUMAN	1938	29	19.10	223.57
sp P02679 FIBG_HUMAN	453	27	63.80	51.51
sp P07942 LAMB1_HUMAN	1786	26	20.50	198.04
sp P55072 TERA_HUMAN	806	26	47.50	89.32
sp P08603 CFAH_HUMAN	1231	25	28.90	138.95
sp P14618 KPYM_HUMAN	531	25	56.90	57.94
sp P00738 HPT_HUMAN;sp P00739 HPTR_HUMAN	406	24	50.50	45.21
sp P10809 CH60_HUMAN	573	24	49.20	61.05
sp P12109 CO6A1_HUMAN	1026	24	33.30	108.34
sp P00450 CERU_HUMAN	1006	23	39.40	115.47
sp P00558 PGK1_HUMAN	417	23	70.00	44.61
sp P05783 K1C18_HUMAN	430	23	55.10	48.03
sp P08758 ANXA5_HUMAN	320	23	77.80	35.94

sp Q13576 QGA2_HUMAN	1575	23	24.10	180.59
sp Q14697 GANAB_HUMAN	944	23	30.50	106.87
sp P06396 GELS_HUMAN	715	22	36.50	78.83
sp P06733 ENOA_HUMAN	434	22	59.90	47.17
sp P13639 EF2_HUMAN	858	22	30.70	95.34
sp P15311 EZRI_HUMAN	586	22	39.10	69.24
sp O43707 ACTN4_HUMAN	911	21	31.10	104.85
sp P04114 APOB_HUMAN	4563	21	7.20	515.55
sp P05187 PPB1_HUMAN;sp P10696 PPBN_HUMAN	535	21	55.10	57.95
sp P07237 PDIA1_HUMAN	508	21	44.70	57.12
sp P20742 PZP_HUMAN	1482	21	21.70	163.87
sp P29401 TKT_HUMAN	576	21	53.80	62.88
sp P04040 CATA_HUMAN	527	20	46.90	59.76
sp P11142 HSP7C_HUMAN	646	20	43.50	70.90
sp P12814 ACTN1_HUMAN	892	20	35.10	103.06
sp P20936 RASA1_HUMAN	880	20	28.60	101.54
sp Q96TA1 NIBL1_HUMAN	733	20	50.80	82.68
sp P02730 B3AT_HUMAN	911	19	31.00	101.81
sp P04075 ALDOA_HUMAN	364	19	67.60	39.42
sp P07900 HS90A_HUMAN	732	19	30.90	84.66
sp P14543 NID1_HUMAN	1247	19	18.90	136.38
sp P17661 DESM_HUMAN	470	19	45.70	53.54
sp P25705 ATPA_HUMAN	553	19	43.80	59.75
sp P49748 ACADV_HUMAN	579	19	45.10	62.58
sp P02549 SPTA1_HUMAN	2419	18	11.50	280.01
sp P04792 HSPB1_HUMAN	205	18	90.20	22.78
sp P05108 CP11A_HUMAN	521	18	39.90	60.10
sp P06576 ATPB_HUMAN	445	18	61.30	48.11
sp P21397 AOFA_HUMAN	527	18	39.80	59.68
sp P22626 ROA2_HUMAN	353	18	53.00	37.43
sp P27824 CALX_HUMAN	592	18	34.50	67.57
sp P30041 PRDX6_HUMAN	224	18	77.20	25.04
sp Q07065 CKAP4_HUMAN	560	18	46.10	62.06
sp Q14204 DYHC1_HUMAN	4646	18	5.80	532.40
sp Q9Y6Q1 CAN6_HUMAN	641	18	38.20	74.59
sp P00734 THRB_HUMAN	622	17	40.70	70.04
sp P13645 K1C10_HUMAN	584	17	38.70	58.83
sp P19338 NUCL_HUMAN	633	17	29.10	68.44
sp P27797 CALR_HUMAN	406	17	56.90	46.92
sp P35527 K1C9_HUMAN	623	17	44.60	62.06
sp P60174 TPIS_HUMAN	249	17	78.70	26.67
sp Q14112 NID2_HUMAN	1373	17	15.20	150.54
sp Q5VTE0 EF1A3_HUMAN;sp P68104 EF1A1_HUMAN;sp Q05639 EF1A2_HUMAN	462	17	50.90	50.18
sp O43852 CALU_HUMAN	315	16	63.20	37.11
sp P02790 HEMO_HUMAN	462	16	48.90	51.68
sp P04406 G3P_HUMAN	335	16	64.80	36.05
sp P13489 RINI_HUMAN	456	16	61.60	49.42
sp P13796 PLSL_HUMAN	627	16	35.60	70.29
sp P14060 3BHS1_HUMAN;sp P26439 3BHS2_HUMAN	373	16	58.40	42.25
sp P14314 GLU2B_HUMAN	527	16	39.70	59.30
sp P23381 SYWC_HUMAN	471	16	47.30	53.17
sp P36871 PGM1_HUMAN	562	16	38.30	61.45
sp Q15084 PDIA6_HUMAN	440	16	49.10	48.12
sp P01042 KNG1_HUMAN	415	15	41.90	46.50
sp P05556 ITB1_HUMAN	798	15	21.90	88.41
sp P08238 HS90B_HUMAN	724	15	29.10	83.26
sp P22314 UBA1_HUMAN	1058	15	21.70	117.85

sp P37802 TAGL2_HUMAN	199	15	79.40	22.39
sp P40926 MDHM_HUMAN	305	15	65.60	31.97
sp P48735 IDHP_HUMAN	452	15	37.20	50.91
sp P50454 SERPH_HUMAN	418	15	51.70	46.44
sp P61978 HNRPK_HUMAN	428	15	40.90	47.56
sp Q01082 SPTB2_HUMAN	2278	15	8.90	264.44
sp O75923 DYSF_HUMAN	2080	14	10.20	237.29
sp P00491 PNPH_HUMAN	289	14	62.30	32.12
sp P00747 PLMN_HUMAN	810	14	25.40	90.54
sp P04843 RPN1_HUMAN	568	14	32.60	64.58
sp P09525 ANXA4_HUMAN	319	14	49.80	35.88
sp P13667 PDIA4_HUMAN	645	14	27.10	72.93
sp P26038 MOES_HUMAN	577	14	28.20	67.82
sp P32119 PRDX2_HUMAN	183	14	53.60	20.11
sp P63104 1433Z_HUMAN	245	14	58.40	27.75
sp Q01518 CAP1_HUMAN	452	14	40.90	49.08
sp Q01995 TAGL_HUMAN	201	14	66.70	22.61
sp Q03252 LMNB2_HUMAN	600	14	26.30	67.69
sp P01011 AACT_HUMAN	423	13	31.20	47.65
sp P04217 A1BG_HUMAN	495	13	41.20	54.25
sp P07585 PGS2_HUMAN	359	13	38.70	39.75
sp P09651 ROA1_HUMAN;sp Q32P51 RA1L2_HUMAN	307	13	55.40	33.16
sp P12956 XRCC6_HUMAN	609	13	32.70	69.84
sp P13010 XRCC5_HUMAN	732	13	24.50	82.70
sp P14061 DHB1_HUMAN	328	13	46.00	34.95
sp P23284 PPIB_HUMAN	208	13	63.50	22.74
sp P23526 SAHH_HUMAN	432	13	35.20	47.72
sp P27487 DPP4_HUMAN	766	13	19.30	88.28
sp P35221 CTNA1_HUMAN	891	13	25.00	98.38
sp P38646 GRP75_HUMAN	665	13	26.60	72.40
sp P40939 ECHA_HUMAN	763	13	27.70	83.00
sp P51991 ROA3_HUMAN	356	13	45.20	37.03
sp P55084 ECHB_HUMAN	474	13	37.60	51.29
sp P62258 1433E_HUMAN	255	13	62.40	29.17
sp P62805 H4_HUMAN	103	13	65.00	11.37
sp Q14103 HNRPD_HUMAN	306	13	42.80	32.83
sp Q16555 DPYL2_HUMAN	572	13	38.10	62.29
sp Q9GZM7 TINAL_HUMAN	467	13	49.00	52.39
sp Q9Y4L1 HYOU1_HUMAN;	848	13	25.70	94.20
sp O00299 CLIC1_HUMAN	241	12	59.80	26.92
sp O75874 IDHC_HUMAN	414	12	35.70	46.66
sp P00338 LDHA_HUMAN	332	12	37.30	36.69
sp P00751 CFAB_HUMAN	764	12	22.40	85.53
sp P07737 PROF1_HUMAN	140	12	77.10	15.05
sp P0DML3 CSH2_HUMAN;sp P0DML2 CSH1_HUMAN	217	12	68.70	24.99
sp P16435 NCPR_HUMAN	677	12	23.30	76.69
sp P21796 VDAC1_HUMAN	283	12	59.00	30.77
sp P21810 PGS1_HUMAN	307	12	54.10	34.88
sp P23396 RS3_HUMAN	243	12	60.50	26.69
sp P63261 ACTG_HUMAN;sp P60709 ACTB_HUMAN	363	12	42.70	40.50
sp P80365 DHI2_HUMAN	405	12	48.10	44.13
sp P98082 DAB2_HUMAN	770	12	19.50	82.47
sp Q06830 PRDX1_HUMAN	199	12	59.80	22.11
sp O60664 PLIN3_HUMAN	434	11	39.40	47.07
sp P00915 CAH1_HUMAN	261	11	57.90	28.87
sp P01008 ANT3_HUMAN	464	11	34.30	52.60
sp P04844 RPN2_HUMAN	631	11	26.50	69.33
sp P07195 LDHB_HUMAN	334	11	32.90	36.64

sp P30153 2AAA_HUMAN	534	11	30.70	59.14
sp P50395 GDIB_HUMAN	445	11	36.00	50.66
sp P51884 LUM_HUMAN	338	11	37.90	38.43
sp P61158 ARP3_HUMAN	418	11	40.00	47.37
sp P67936 TPM4_HUMAN	248	11	32.30	28.52
sp P80723 BASP1_HUMAN	227	11	84.60	22.69
sp Q08211 DHX9_HUMAN	1270	11	11.40	140.96
sp Q16658 FSCN1_HUMAN	472	11	31.60	52.28
sp Q5SSJ5 HP1B3_HUMAN	553	11	19.90	61.21
sp Q96KP4 CNBP2_HUMAN	463	11	35.40	51.50
sp Q9UHQ9 NB5R1_HUMAN	305	11	47.90	34.09
sp Q9Y678 COPG1_HUMAN	874	11	19.80	97.72
sp O15347 HMGB3_HUMAN	188	10	36.70	21.44
sp O75367 H2AY_HUMAN	372	10	36.30	39.62
sp P00367 DHE3_HUMAN;sp P49448 DHE4_HUMAN	490	10	28.80	54.26
sp P00441 SODC_HUMAN	154	10	82.50	15.94
sp P00533 EGFR_HUMAN	1210	10	12.60	134.32
sp P00918 CAH2_HUMAN	260	10	50.80	29.25
sp P01019 ANGT_HUMAN	466	10	28.10	51.06
sp P02749 APOH_HUMAN	326	10	47.50	36.25
sp P08842 STS_HUMAN	583	10	21.10	65.49
sp P10909 CLUS_HUMAN	449	10	27.80	52.49
sp P15121 ALDR_HUMAN	316	10	33.90	35.85
sp P15924 DESP_HUMAN	2871	10	5.60	331.77
sp P21128 ENDOU_HUMAN	410	10	32.00	46.87
sp P27105 STOM_HUMAN	237	10	56.50	25.96
sp P30043 BLVRB_HUMAN	206	10	65.50	22.12
sp P39656 OST48_HUMAN	456	10	28.90	50.70
sp P45880 VDAC2_HUMAN	282	10	51.80	30.35
sp P62937 PPIA_HUMAN	165	10	66.70	18.01
sp P63244 GBLP_HUMAN	317	10	46.40	35.08
sp Q86VP6 CAND1_HUMAN	1230	10	12.10	136.32
sp Q9C075 K1C23_HUMAN	422	10	29.10	48.18
sp Q9H4M9 EHD1_HUMAN	534	10	34.30	60.63
sp Q9NYU2 UGGG1_HUMAN	1531	10	11.40	175.00
sp O75083 WDR1_HUMAN	606	9	24.60	66.19
sp O95782 AP2A1_HUMAN	977	9	12.50	107.54
sp P00488 F13A_HUMAN	732	9	18.90	83.27
sp P04899 GNAI2_HUMAN	355	9	34.90	40.45
sp P07910 HNRPC_HUMAN	262	9	40.50	28.92
sp P08107 HSP71_HUMAN	641	9	22.60	70.05
sp P09211 GSTP1_HUMAN	210	9	43.30	23.36
sp P11166 GTR1_HUMAN	416	9	17.10	45.87
sp P12110 CO6A2_HUMAN	1019	9	10.70	108.58
sp P16152 CBR1_HUMAN	277	9	48.00	30.38
sp P19823 ITIH2_HUMAN	935	9	16.70	105.21
sp P20774 MIME_HUMAN	298	9	27.90	33.92
sp P30044 PRDX5_HUMAN	162	9	73.50	17.03
sp P35606 COPB2_HUMAN	906	9	14.50	102.49
sp P49411 EFTU_HUMAN	452	9	24.10	49.54
sp P51888 PRELP_HUMAN	382	9	31.90	43.78
sp P52272 HNRPM_HUMAN	730	9	19.20	77.57
sp P53621 COPA_HUMAN	1224	9	11.00	138.34
sp P62140 PP1B_HUMAN;sp P36873 PP1G_HUMAN;sp P62136 PP1A_HUMAN	327	9	35.50	37.19
sp P62826 RAN_HUMAN	199	9	41.70	22.54
sp Q00839 HNRPU_HUMAN	722	9	16.30	79.72
sp Q06323 PSME1_HUMAN	249	9	40.20	28.72

sp Q12905 ILF2_HUMAN	352	9	40.10	38.91
sp Q13228 SBP1_HUMAN	472	9	24.20	52.39
sp Q13642 FHL1_HUMAN	260	9	47.70	29.50
sp Q14624 ITIH4_HUMAN	888	9	12.40	98.35
sp Q14974 IMB1_HUMAN	876	9	14.20	97.18
sp Q15019 SEPT2_HUMAN	361	9	39.90	41.49
sp Q15293 RCN1_HUMAN	331	9	36.60	38.89
sp Q15907 RB11B_HUMAN;sp P62491 RB11A_HUMAN	218	9	47.70	24.49
sp Q6NZI2 PTRF_HUMAN	363	9	35.50	40.48
sp Q8NBS9 TXND5_HUMAN	324	9	39.50	36.18
sp Q99798 ACON_HUMAN	780	9	18.20	85.42
sp Q9BS26 ERP44_HUMAN	406	9	29.30	46.97
sp Q9NZM1 MYOF_HUMAN	2061	9	7.20	234.71
sp O95833 CLIC3_HUMAN	236	8	49.20	26.65
sp P02008 HBAZ_HUMAN	142	8	67.60	15.64
sp P04004 VTNC_HUMAN	478	8	23.00	54.31
sp P07738 PMGE_HUMAN	259	8	32.00	30.01
sp P08195 4F2_HUMAN;	511	8	22.10	55.94
sp P08572 CO4A2_HUMAN	1712	8	7.20	167.55
sp P09382 LEG1_HUMAN	135	8	60.00	14.72
sp P18669 PGAM1_HUMAN	254	8	42.10	28.82
sp P20700 LMNB1_HUMAN	586	8	18.90	66.41
sp P21291 CSRP1_HUMAN	187	8	66.30	20.04
sp P23528 COF1_HUMAN	149	8	54.40	16.81
sp P26599 PTBP1_HUMAN	527	8	22.40	56.51
sp P30050 RL12_HUMAN	165	8	64.20	17.82
sp P30086 PEBP1_HUMAN	187	8	69.00	21.06
sp P31946 1433B_HUMAN	246	8	39.80	28.08
sp P36578 RL4_HUMAN	427	8	29.00	47.70
sp P39059 COFA1_HUMAN	1374	8	7.00	140.06
sp P40925 MDHC_HUMAN	210	8	51.00	23.04
sp P48047 ATPO_HUMAN	213	8	49.80	23.28
sp P51149 RAB7A_HUMAN	207	8	51.70	23.49
sp P60660 MYL6_HUMAN	152	8	65.10	17.09
sp P61247 RS3A_HUMAN	223	8	41.70	25.61
sp P61981 1433G_HUMAN	247	8	37.20	28.30
sp P62241 RS8_HUMAN	208	8	44.20	24.21
sp P62701 RS4X_HUMAN	243	8	33.70	27.26
sp P78527 PRKDC_HUMAN	4128	8	2.90	469.08
sp Q02218 ODO1_HUMAN	1023	8	10.00	115.93
sp Q02878 RL6_HUMAN	288	8	35.10	32.74
sp Q06210 GFPT1_HUMAN	699	8	15.90	78.81
sp Q14847 LASP1_HUMAN	261	8	31.40	29.72
sp Q8WZ42 TITIN_HUMAN	33423	8	0.30	3713.70
sp Q96QK1 VPS35_HUMAN	796	8	13.10	91.71
sp Q9BW30 TPPP3_HUMAN	176	8	42.60	18.99
sp A1L0T0 ILVBL_HUMAN	632	7	19.10	67.87
sp O75396 SC22B_HUMAN	215	7	34.40	24.59
sp P02462 CO4A1_HUMAN	229	7	54.60	25.33
sp P02649 APOE_HUMAN	317	7	32.80	36.15
sp P02792 FRIL_HUMAN	175	7	50.30	20.02
sp P05155 IC1_HUMAN	333	7	25.50	37.29
sp P05388 RLA0_HUMAN;sp Q8NHW5 RLA0L_HUMAN	317	7	36.60	34.30
sp P06748 NPM_HUMAN	294	7	32.00	32.58
sp P07339 CATD_HUMAN	412	7	25.50	44.55
sp P08123 CO1A2_HUMAN	1364	7	5.00	129.15
sp P08865 RSSA_HUMAN	264	7	38.30	29.51
sp P10253 LYAG_HUMAN	952	7	10.60	105.34

sp P12111 CO6A3_HUMAN	3177	7	3.60	343.67
sp P14550 AK1A1_HUMAN	325	7	24.30	36.57
sp P15880 RS2_HUMAN	195	7	43.60	21.15
sp P16157 ANK1_HUMAN	1881	7	5.80	206.26
sp P16284 PECA1_HUMAN	733	7	13.60	81.78
sp P16401 H15_HUMAN	226	7	16.80	22.58
sp P16403 H12_HUMAN;sp P10412 H14_HUMAN;sp P16402 H13_HUMAN	213	7	17.80	21.36
sp P17980 PRS6A_HUMAN	397	7	27.70	44.32
sp P18124 RL7_HUMAN	208	7	38.90	24.43
sp P19827 ITI1_HUMAN	645	7	15.00	72.12
sp P26641 EF1G_HUMAN	437	7	19.00	50.12
sp P29966 MARCS_HUMAN	330	7	39.70	31.60
sp P30040 ERP29_HUMAN	261	7	35.60	28.99
sp P31937 3HIDH_HUMAN	336	7	33.00	35.33
sp P31949 S10AB_HUMAN	105	7	56.20	11.74
sp P35555 FBN1_HUMAN	2871	7	3.40	312.24
sp P37059 DHB2_HUMAN	387	7	24.80	42.76
sp P39023 RL3_HUMAN	354	7	25.10	40.14
sp P49368 TCPG_HUMAN	500	7	20.40	55.67
sp P50453 SPB9_HUMAN	376	7	26.60	42.40
sp P52907 CAZA1_HUMAN	286	7	45.50	32.91
sp P54652 HSP72_HUMAN	639	7	16.90	70.02
sp P62158 CALM_HUMAN	149	7	47.00	16.84
sp P62753 RS6_HUMAN	249	7	29.30	28.71
sp P63241 IF5A1_HUMAN;sp Q9GZV4 IF5A2_HUMAN;sp Q6IS14 IF5AL_HUMAN	147	7	58.50	16.12
sp Q00325 MPCP_HUMAN	324	7	32.10	36.16
sp Q01469 FABP5_HUMAN	135	7	64.40	15.16
sp Q02978 M2OM_HUMAN	296	7	25.70	32.18
sp Q13838 DX39B_HUMAN;sp O00148 DX39A_HUMAN	428	7	27.10	48.99
sp Q16181 SEPT7_HUMAN	410	7	23.70	47.88
sp Q53SF7 COBL1_HUMAN	1204	7	8.80	131.79
sp Q96AC1 FERM2_HUMAN	680	7	18.20	77.86
sp Q96AG4 LRC59_HUMAN	307	7	30.90	34.93
sp Q96G03 PGM2_HUMAN	612	7	19.80	68.28
sp Q99497 PARK7_HUMAN	189	7	58.70	19.89
sp Q9BTV4 TMM43_HUMAN	400	7	31.00	44.88
sp Q9HDC9 APMAP_HUMAN	416	7	25.50	46.48
sp Q9NZN4 EHD2_HUMAN	533	7	18.90	60.04
sp Q9Y2B0 CNPY2_HUMAN	182	7	52.70	20.65
sp Q9Y6N6 LAMC3_HUMAN	1575	7	5.70	171.23
sp O00151 PDLI1_HUMAN	329	6	35.30	36.07
sp O14773 TPP1_HUMAN	385	6	27.00	41.68
sp P01860 IGHG3_HUMAN	517	6	22.10	56.89
sp P02511 CRYAB_HUMAN	174	6	44.30	20.03
sp P02765 FETUA_HUMAN	367	6	16.60	39.32
sp P02766 TTHY_HUMAN	147	6	49.00	15.89
sp P05387 RLA2_HUMAN	115	6	73.90	11.67
sp P06702 S10A9_HUMAN	114	6	51.80	13.21
sp P06744 G6PI_HUMAN	531	6	16.80	59.99
sp P06756 ITAV_HUMAN	1048	6	11.50	116.04
sp P07384 CAN1_HUMAN	714	6	11.80	81.89
sp P09429 HMGB1_HUMAN	215	6	30.70	24.89
sp P10599 THIO_HUMAN	105	6	51.40	11.74
sp P12429 ANXA3_HUMAN	323	6	24.10	36.38
sp P13727 PRG2_HUMAN	222	6	36.00	25.21
sp P13804 ETFA_HUMAN	333	6	27.90	35.08

sp P14866 HNRPL_HUMAN	530	6	13.80	58.36
sp P17858 PFKAL_HUMAN	780	6	12.40	85.02
sp P19367 HXK1_HUMAN	915	6	10.20	102.31
sp P23229 ITA6_HUMAN	1130	6	8.80	126.60
sp P23246 SFPQ_HUMAN	634	6	18.60	68.63
sp P25398 RS12_HUMAN	132	6	53.00	14.52
sp P28066 PSA5_HUMAN	241	6	29.50	26.43
sp P28838 AMPL_HUMAN	519	6	17.30	56.05
sp P30048 PRDX3_HUMAN	256	6	36.30	27.69
sp P31930 QCR1_HUMAN	365	6	32.60	40.37
sp P34932 HSP74_HUMAN	782	6	11.80	88.00
sp P35237 SPB6_HUMAN	376	6	22.10	42.62
sp P35754 GLRX1_HUMAN	106	6	55.70	11.78
sp P35908 K22E_HUMAN	639	6	16.60	65.43
sp P36957 ODO2_HUMAN	453	6	15.90	48.82
sp P39019 RS19_HUMAN	145	6	40.70	16.06
sp P40227 TCPZ_HUMAN	488	6	19.30	53.68
sp P43652 AFAM_HUMAN	599	6	14.00	69.07
sp P47756 CAPZB_HUMAN	260	6	28.80	29.30
sp P49419 AL7A1_HUMAN	539	6	16.50	58.49
sp P50502 F10A1_HUMAN;sp Q8IZP2 ST134_HUMAN;sp Q8NFI4 F10A5_HUMAN	310	6	22.60	35.02
sp P50991 TCPD_HUMAN	483	6	22.80	52.33
sp P51858 HDGF_HUMAN	240	6	34.20	26.81
sp P53396 ACLY_HUMAN;t	1091	6	7.50	119.77
sp P61019 RAB2A_HUMAN;sp Q8WUD1 RAB2B_HUMAN	212	6	38.20	23.55
sp P61106 RAB14_HUMAN	215	6	45.60	23.90
sp P61160 ARP2_HUMAN	394	6	17.50	44.76
sp P61224 RAP1B_HUMAN;sp A6NIZ1 RP1BL_HUMAN;sp P62834 RAP1A_HUMAN	184	6	34.20	20.83
sp P62081 RS7_HUMAN	194	6	41.20	22.13
sp P62244 RS15A_HUMAN	130	6	53.80	14.84
sp P62249 RS16_HUMAN	146	6	43.80	16.45
sp P62873 GBB1_HUMAN	340	6	31.80	37.30
sp P62879 GBB2_HUMAN	340	6	32.10	37.33
sp P62979 RS27A_HUMAN;sp P62987 RL40_HUMAN;sp P0CG47 UBB_HUMAN;sp P0CG48 UBC_HUMAN	156	6	50.60	17.91
sp P63010 AP2B1_HUMAN	934	6	11.20	104.22
sp P78371 TCPB_HUMAN	530	6	20.80	56.81
sp P84077 ARF1_HUMAN;sp P61204 ARF3_HUMAN	181	6	35.40	20.70
sp Q00013 EM55_HUMAN	449	6	25.80	50.15
sp Q01105 SET_HUMAN;sp P0DME0 SETLP_HUMAN	277	6	23.80	32.13
sp Q04917 I433F_HUMAN	246	6	31.70	28.22
sp Q13011 ECH1_HUMAN	328	6	20.40	35.82
sp Q13263 TIF1B_HUMAN	835	6	12.80	88.56
sp Q14019 COTL1_HUMAN	142	6	55.60	15.95
sp Q15365 PCBP1_HUMAN	356	6	23.60	37.50
sp Q86VB7 C163A_HUMAN	1109	6	7.90	120.32
sp Q96CX2 KCD12_HUMAN	325	6	29.50	35.73
sp Q9Y2J2 E41L3_HUMAN	756	6	10.70	84.68
sp Q9Y696 CLIC4_HUMAN;	245	6	39.60	27.82
sp Q07955 SRSF1_HUMAN	233	6	27.90	25.62
sp O00425 IF2B3_HUMAN	579	5	13.60	63.70
sp O14980 XPO1_HUMAN	1071	5	8.00	123.38
sp O15143 ARC1B_HUMAN	372	5	22.00	40.95
sp O15144 ARPC2_HUMAN	300	5	21.00	34.33
sp O15145 ARPC3_HUMAN	178	5	33.10	20.56
sp O60829 PAGE4_HUMAN	102	5	60.80	11.15

sp O75947 ATP5H_HUMAN	161	5	46.00	18.49
sp O95865 DDAH2_HUMAN	285	5	32.30	29.64
sp P01031 CO5_HUMAN	1106	5	8.70	123.35
sp P02743 SAMP_HUMAN	223	5	26.50	25.39
sp P02753 RET4_HUMAN	199	5	35.70	22.97
sp P02763 A1AG1_HUMAN	201	5	25.40	23.51
sp P02794 FRIH_HUMAN	183	5	37.20	21.23
sp P05455 LA_HUMAN	408	5	17.60	46.87
sp P07305 H10_HUMAN	194	5	27.30	20.86
sp P07437 TBB5_HUMAN	426	5	16.90	47.77
sp P09497 CLCB_HUMAN	211	5	19.90	23.18
sp P0CW22 RS17L_HUMAN;sp P08708 RS17_HUMAN	135	5	51.90	15.55
sp P10109 ADX_HUMAN	184	5	33.20	19.39
sp P10768 ESTD_HUMAN	253	5	36.40	28.23
sp P18085 ARF4_HUMAN	180	5	31.10	20.51
sp P21399 ACOC_HUMAN	889	5	10.30	98.40
sp P25311 ZA2G_HUMAN	298	5	22.10	34.26
sp P25815 S100P_HUMAN	95	5	38.90	10.40
sp P26373 RL13_HUMAN	211	5	25.10	24.27
sp P27816 MAP4_HUMAN	2297	5	3.20	245.44
sp P29692 EF1D_HUMAN	261	5	28.40	28.82
sp P30084 ECHM_HUMAN	290	5	26.90	31.39
sp P31948 STIP1_HUMAN	543	5	13.80	62.64
sp P35232 PHB_HUMAN	272	5	25.70	29.82
sp P37837 TALDO_HUMAN	318	5	17.60	35.33
sp P43243 MATR3_HUMAN	847	5	12.20	94.62
sp P46781 RS9_HUMAN	139	5	24.50	16.63
sp P48643 TCPE_HUMAN	520	5	12.50	57.14
sp P49755 TMEDA_HUMAN	219	5	22.40	24.98
sp P50914 RL14_HUMAN	215	5	25.60	23.43
sp P50990 TCPQ_HUMAN	497	5	13.10	54.11
sp P53618 COPB_HUMAN	953	5	8.50	107.14
sp P54819 KAD2_HUMAN	232	5	29.70	25.63
sp P55060 XPO2_HUMAN	971	5	7.30	110.42
sp P55327 TPD52_HUMAN	224	5	47.80	24.33
sp P55735 SEC13_HUMAN	322	5	23.90	35.54
sp P61313 RL15_HUMAN	204	5	26.50	24.15
sp P61970 NTF2_HUMAN	127	5	56.70	14.48
sp P62263 RS14_HUMAN	151	5	38.40	16.27
sp P62269 RS18_HUMAN	152	5	30.30	17.72
sp P62328 TYB4_HUMAN	44	5	56.80	5.05
sp P62424 RL7A_HUMAN	266	5	16.50	30.00
sp P62906 RL10A_HUMAN	217	5	26.30	24.83
sp P62917 RL8_HUMAN	205	5	31.70	22.39
sp P63000 RAC1_HUMAN;sp P60763 RAC3_HUMAN;sp P15153 RAC2_HUMAN	192	5	27.60	21.45
sp P99999 CYC_HUMAN	101	5	57.40	11.33
sp Q00341 VIGLN_HUMAN	1268	5	5.00	141.38
sp Q00765 REEP5_HUMAN	156	5	30.80	17.76
sp Q07020 RL18_HUMAN	164	5	35.40	18.73
sp Q07021 C1QBP_HUMAN	282	5	31.60	31.38
sp Q07960 RHG01_HUMAN	439	5	17.10	50.44
sp Q12906 ILF3_HUMAN	702	5	10.80	75.96
sp Q13162 PRDX4_HUMAN	271	5	27.70	30.54
sp Q13268 DHRS2_HUMAN	225	5	30.70	23.82
sp Q13492 PICAL_HUMAN	610	5	15.20	66.39
sp Q13724 MOGS_HUMAN	837	5	9.40	91.92
sp Q15063 POSTN_HUMAN	779	5	11.20	87.02

sp Q16851 UGPA_HUMAN;	438	5	18.30	49.22
sp Q16853 AOC3_HUMAN	763	5	6.30	84.62
sp Q53EL6 PDCD4_HUMAN	455	5	16.70	50.23
sp Q8NFW8 NEUA_HUMAN	434	5	17.70	48.33
sp Q92499 DDX1_HUMAN	659	5	11.70	73.92
sp Q92841 DDX17_HUMAN	729	5	8.40	80.27
sp Q92945 FUBP2_HUMAN	711	5	9.10	73.03
sp Q99623 PHB2_HUMAN	267	5	24.30	29.72
sp Q9NZ08 ERAP1_HUMAN	941	5	8.20	107.18
sp Q9NZA1 CLIC5_HUMAN	251	5	35.50	28.18
sp Q9UL46 PSME2_HUMAN	239	5	31.00	27.40
sp Q9UNF0 PACN2_HUMAN	445	5	13.50	51.35
tr B4DUV1 B4DUV1_HUMAN;tr B1AHL2 B1AHL2_HUMAN	641	5	15.60	70.15
sp O14745 NHRF1_HUMAN	358	4	15.40	38.90
sp O15173 PGRC2_HUMAN	223	4	18.80	23.82
sp O43390 HNRPR_HUMAN	595	4	8.60	67.01
sp O60506 HNRPO_HUMAN	453	4	15.20	50.65
sp O75347 TBCA_HUMAN	84	4	60.70	10.08
sp O75608 LYPA1_HUMAN	166	4	35.50	17.98
sp O94973 AP2A2_HUMAN	930	4	8.50	103.06
sp P00387 NB5R3_HUMAN	291	4	22.30	33.24
sp P00403 COX2_HUMAN	217	4	25.80	24.56
sp P02652 APOA2_HUMAN	95	4	43.20	10.65
sp P02748 CO9_HUMAN	559	4	7.90	63.20
sp P02750 A2GL_HUMAN	330	4	15.50	36.49
sp P02760 AMBP_HUMAN	352	4	13.40	39.00
sp P02788 TRFL_HUMAN	666	4	8.60	73.17
sp P05090 APOD_HUMAN	189	4	22.80	21.28
sp P05546 HEP2_HUMAN	499	4	6.80	56.96
sp P06727 APOA4_HUMAN	396	4	13.60	45.40
sp P06737 PYGL_HUMAN	819	4	7.00	94.07
sp P07093 GDN_HUMAN	334	4	16.80	37.16
sp P07858 CATB_HUMAN	245	4	20.00	26.86
sp P07954 FUMH_HUMAN	510	4	14.30	54.64
sp P08559 ODPA_HUMAN	390	4	11.00	43.30
sp P08571 CD14_HUMAN	375	4	24.00	40.05
sp P09622 DLDH_HUMAN	459	4	13.30	48.90
sp P11177 ODPB_HUMAN	359	4	14.20	39.23
sp P11940 PABP1_HUMAN;sp Q4VXU2 PAP1L_HUMAN;sp Q9H361 PABP3_HUMAN;sp Q13310 PABP4_HUMAN	419	4	12.20	47.33
sp P12270 TPR_HUMAN	687	4	9.50	79.39
sp P13693 TCTP_HUMAN	172	4	22.70	19.60
sp P15090 FABP4_HUMAN	132	4	43.20	14.72
sp P17096 HMGA1_HUMAN	107	4	41.10	11.68
sp P17987 TCPA_HUMAN	401	4	13.50	43.89
sp P18621 RL17_HUMAN	129	4	28.70	14.89
sp P19105 ML12A_HUMAN;sp O14950 ML12B_HUMAN	171	4	29.20	19.79
sp P19652 A1AG2_HUMAN	201	4	18.40	23.60
sp P21964 COMT_HUMAN	271	4	19.90	30.04
sp P22061 PIMT_HUMAN	192	4	39.60	20.68
sp P22392 NDKB_HUMAN;sp P15531 NDKA_HUMAN;sp O60361 NDK8_HUMAN	152	4	34.90	17.30
sp P22413 ENPP1_HUMAN	873	4	6.50	99.87
sp P22695 QCR2_HUMAN	453	4	14.80	48.44
sp P24534 EF1B_HUMAN	225	4	34.70	24.76
sp P24539 AT5F1_HUMAN	256	4	21.50	28.84
sp P24844 MYL9_HUMAN	172	4	29.10	19.85
sp P27695 APEX1_HUMAN	318	4	17.90	35.55

sp P31150 GDIA_HUMAN	417	4	13.90	46.91
sp P31939 PUR9_HUMAN	592	4	12.30	64.62
sp P35222 CTNB1_HUMAN	709	4	7.20	77.52
sp P36542 ATPG_HUMAN	291	4	16.50	32.25
sp P36955 PEDF_HUMAN	418	4	16.30	46.31
sp P39060 COIA1_HUMAN	1336	4	5.80	135.51
sp P39687 AN32A_HUMAN	177	4	29.90	20.00
sp P43686 PRS6B_HUMAN	418	4	17.20	47.37
sp P45974 UBP5_HUMAN	858	4	7.80	95.79
sp P46977 STT3A_HUMAN	703	4	5.40	80.43
sp P49257 LMAN1_HUMAN	510	4	14.10	57.52
sp P49591 SYSC_HUMAN	511	4	7.40	58.41
sp P50570 DYN2_HUMAN;sp Q05193 DYN1_HUMAN	870	4	6.70	98.06
sp P50895 BCAM_HUMAN	588	4	8.80	63.69
sp P50995 ANX11_HUMAN	411	4	12.70	45.60
sp P51148 RAB5C_HUMAN	216	4	27.30	23.48
sp P52209 PGD_HUMAN	483	4	13.90	53.14
sp P52565 GDIR1_HUMAN	194	4	23.70	21.61
sp P53999 TCP4_HUMAN	127	4	26.00	14.40
sp P54727 RD23B_HUMAN	403	4	12.40	42.31
sp P59998 ARPC4_HUMAN	168	4	22.60	19.67
sp P60842 IF4A1_HUMAN	406	4	14.80	46.12
sp P60903 S10AA_HUMAN	97	4	50.50	11.19
sp P60953 CDC42_HUMAN	191	4	30.90	21.26
sp P61088 UBE2N_HUMAN;sp Q5JXB2 UE2NL_HUMAN	152	4	34.20	17.14
sp P61604 CH10_HUMAN	71	4	54.90	7.54
sp P62277 RS13_HUMAN	148	4	27.00	16.73
sp P62280 RS11_HUMAN	158	4	31.60	18.43
sp P62888 RL30_HUMAN	114	4	51.80	12.66
sp P67775 PP2AA_HUMAN;sp P62714 PP2AB_HUMAN	309	4	21.00	35.55
sp P78417 GSTO1_HUMAN	200	4	18.50	23.34
sp Q00577 PURA_HUMAN	285	4	30.50	32.02
sp Q02543 RL18A_HUMAN	141	4	31.20	16.71
sp Q03135 CAV1_HUMAN	178	4	29.80	20.47
sp Q04760 LGUL_HUMAN	184	4	30.40	20.78
sp Q07954 LRP1_HUMAN	4544	4	1.20	504.60
sp Q10567 AP1B1_HUMAN	949	4	8.60	104.64
sp Q12907 LMAN2_HUMAN	285	4	23.90	32.58
sp Q13126 MTAP_HUMAN	283	4	24.40	31.25
sp Q13151 ROA0_HUMAN	305	4	19.00	30.84
sp Q13765 NACA_HUMAN;sp E9PAV3 NACAM_HUMAN	136	4	40.40	15.02
sp Q13885 TBB2A_HUMAN;sp Q9BVA1 TBB2B_HUMAN	445	4	19.30	49.91
sp Q14011 CIRBP_HUMAN	172	4	27.30	18.65
sp Q14257 RCN2_HUMAN	317	4	23.00	36.88
sp Q15185 TEBP_HUMAN	160	4	30.60	18.70
sp Q15366 PCBP2_HUMAN;sp P57721 PCBP3_HUMAN	282	4	19.10	29.74
sp Q15582 BGH3_HUMAN	366	4	16.40	39.79
sp Q7Z4H8 KDEL2_HUMAN	402	4	12.40	46.93
sp Q8NF91 SYNE1_HUMAN	8392	4	0.50	964.83
sp Q8WUM4 PDC6I_HUMAN	683	4	7.60	75.93
sp Q92688 AN32B_HUMAN	250	4	21.20	28.67
sp Q96AP7 ESAM_HUMAN	390	4	20.00	41.18
sp Q96CW1 AP2M1_HUMAN	385	4	12.50	43.91
sp Q96FW1 OTUB1_HUMAN	241	4	23.20	28.05
sp Q96HC4 PDLI5_HUMAN	483	4	12.00	52.70
sp Q96JY6 PDLI2_HUMAN	146	4	36.30	16.10
sp Q99536 VAT1_HUMAN	300	4	18.70	32.56
sp Q99729 ROAA_HUMAN	280	4	19.30	30.30

sp Q99832 TCPH_HUMAN	543	4	13.80	59.34
sp Q9BVK6 TMED9_HUMAN	214	4	22.00	25.08
sp Q9NZ01 TECR_HUMAN	308	4	15.30	36.05
sp Q9UBI6 GBG12_HUMAN	72	4	66.70	8.01
sp Q9UIQ6 LCAP_HUMAN	1025	4	4.80	117.34
sp Q9UJU6 DBNL_HUMAN	406	4	17.20	45.75
sp Q9UKS6 PACN3_HUMAN	424	4	11.80	48.49
sp Q9ULV4 COR1C_HUMAN	474	4	10.50	53.28
sp Q9UQ80 PA2G4_HUMAN	367	4	16.60	40.91
tr V9HW25 V9HW25_HUMAN;tr A7XZE4 A7XZE4_HUMAN;tr B4DGC2_HUMAN;tr B4E3P1 B4E3P1_HUMAN	284	4	11.30	32.99
sp O00231 PSD11_HUMAN	422	3	8.50	47.46
sp O00571 DDX3X_HUMAN;sp O15523 DDX3Y_HUMAN	362	3	9.90	40.19
sp O14818 PSA7_HUMAN;sp Q8TAA3 PSA7L_HUMAN	238	3	16.80	26.71
sp O14880 MGST3_HUMAN	152	3	33.60	16.53
sp O14979 HNRDL_HUMAN	271	3	14.80	30.21
sp O60493 SNX3_HUMAN	162	3	21.60	18.76
sp O60716 CTND1_HUMAN	610	3	6.70	68.00
sp O60749 SNX2_HUMAN	519	3	6.70	58.44
sp O75368 SH3L1_HUMAN	114	3	36.00	12.77
sp O75390 CISY_HUMAN	421	3	9.00	47.08
sp O94979 SC31A_HUMAN	969	3	4.60	105.70
sp P00390 GSHR_HUMAN	522	3	8.60	56.26
sp P02042 HBD_HUMAN	147	3	19.00	16.06
sp P04196 HRG_HUMAN	525	3	7.40	59.51
sp P05023 AT1A1_HUMAN;sp P13637 AT1A3_HUMAN;sp Q13733 AT1A4_HUMAN	1020	3	3.00	112.44
sp P05109 S10A8_HUMAN	93	3	32.30	10.83
sp P05141 ADT2_HUMAN	298	3	11.40	32.85
sp P05543 THBG_HUMAN	415	3	11.60	46.32
sp P07108 ACBP_HUMAN	87	3	52.90	10.04
sp P07602 SAP_HUMAN	452	3	7.30	50.65
sp P07996 TSP1_HUMAN	401	3	11.50	43.94
sp P08185 CBG_HUMAN	405	3	9.10	45.09
sp P08246 ELNE_HUMAN	192	3	27.10	20.66
sp P08311 CATG_HUMAN	255	3	12.20	28.84
sp P08697 A2AP_HUMAN	491	3	9.60	54.57
sp P09496 CLCA_HUMAN	248	3	12.10	27.08
sp P10515 ODP2_HUMAN	479	3	9.60	51.20
sp P11279 LAMP1_HUMAN	395	3	8.10	42.59
sp P12532 KCRU_HUMAN	417	3	14.90	47.04
sp P12830 CADH1_HUMAN	647	3	8.30	71.34
sp P13611 CSPG2_HUMAN	354	3	9.30	39.26
sp P13716 HEM2_HUMAN	313	3	14.40	34.52
sp P13798 ACPH_HUMAN	732	3	5.30	81.22
sp P13861 KAP2_HUMAN	404	3	10.40	45.50
sp P13987 CD59_HUMAN	108	3	29.60	11.99
sp P14174 MIF_HUMAN	115	3	35.70	12.48
sp P15374 UCHL3_HUMAN	230	3	17.00	26.18
sp P16144 ITB4_HUMAN	1515	3	2.60	169.15
sp P16671 CD36_HUMAN	396	3	10.90	44.73
sp P17174 AATC_HUMAN	413	3	13.30	46.32
sp P19801 AOC1_HUMAN	710	3	5.80	80.53
sp P19971 TYPH_HUMAN	482	3	10.80	49.96
sp P20674 COX5A_HUMAN	150	3	26.70	16.76
sp P21589 5NTD_HUMAN	264	3	21.20	28.84
sp P23634 AT2B4_HUMAN;sp P20020 AT2B1_HUMAN;sp Q16720 AT2B3_HUMAN	1164	3	3.60	128.78

sp P24666 PPAC_HUMAN	158	3	20.30	18.04
sp P25787 PSA2_HUMAN	234	3	26.10	25.84
sp P25789 PSA4_HUMAN	182	3	26.40	20.08
sp P26885 FKBP2_HUMAN	142	3	38.00	15.65
sp P27348 1433T_HUMAN	245	3	21.20	27.76
sp P28072 PSB6_HUMAN	239	3	13.00	25.36
sp P28331 NDUS1_HUMAN	611	3	8.50	66.92
sp P31942 HNRH3_HUMAN	346	3	11.00	36.93
sp P31943 HNRH1_HUMAN	429	3	10.00	47.09
sp P35613 BASI_HUMAN	189	3	25.40	20.54
sp P35998 PRS7_HUMAN	409	3	9.80	45.83
sp P37198 NUP62_HUMAN	446	3	11.00	45.62
sp P37840 SYUA_HUMAN	98	3	48.00	10.02
sp P40429 RL13A_HUMAN	142	3	19.00	16.73
sp P42765 THIM_HUMAN	397	3	14.40	41.90
sp P42766 RL35_HUMAN	123	3	22.80	14.55
sp P46108 CRK_HUMAN	304	3	15.80	33.83
sp P46776 RL27A_HUMAN	91	3	36.30	10.13
sp P46777 RL5_HUMAN	130	3	34.60	14.11
sp P46778 RL21_HUMAN	160	3	27.50	18.57
sp P46782 RS5_HUMAN	200	3	18.00	22.39
sp P46783 RS10_HUMAN	165	3	24.20	18.90
sp P47755 CAZA2_HUMAN	286	3	17.10	32.97
sp P48307 TFPI2_HUMAN	167	3	31.70	19.37
sp P49189 AL9A1_HUMAN	484	3	8.50	52.77
sp P50213 IDH3A_HUMAN	175	3	25.70	19.25
sp P51571 SSRD_HUMAN	173	3	24.90	19.00
sp P51572 BAP31_HUMAN	246	3	15.40	27.96
sp P52566 GDIR2_HUMAN	201	3	31.80	22.99
sp P52943 CRIP2_HUMAN	192	3	34.90	20.60
sp P53634 CATC_HUMAN	446	3	12.60	50.15
sp P54920 SNAA_HUMAN	256	3	14.80	29.16
sp P55145 MANF_HUMAN	182	3	17.00	20.70
sp P55786 PSA_HUMAN	825	3	4.40	92.87
sp P60033 CD81_HUMAN	165	3	35.80	17.96
sp P60866 RS20_HUMAN	119	3	22.70	13.37
sp P60900 PSA6_HUMAN	107	3	31.80	11.57
sp P61163 ACTZ_HUMAN	302	3	18.20	34.51
sp P61769 B2MG_HUMAN	119	3	35.30	13.71
sp P62330 ARF6_HUMAN	175	3	24.60	20.21
sp P62333 PRS10_HUMAN	262	3	15.60	29.54
sp P62829 RL23_HUMAN	134	3	33.60	14.15
sp P62851 RS25_HUMAN	125	3	16.00	13.74
sp P62899 RL31_HUMAN	108	3	29.60	12.70
sp P62910 RL32_HUMAN	133	3	27.80	15.62
sp P62913 RL11_HUMAN	177	3	16.90	20.09
sp P62942 FKB1A_HUMAN	108	3	41.70	11.95
sp P80303 NUCB2_HUMAN	338	3	14.20	40.37
sp P83731 RL24_HUMAN	121	3	24.80	14.37
sp P84103 SRSF3_HUMAN	95	3	49.50	10.32
sp Q07507 DERM_HUMAN	201	3	21.90	24.01
sp Q08830 FGL1_HUMAN	312	3	9.90	36.38
sp Q13200 PSMD2_HUMAN	900	3	5.80	99.31
sp Q13561 DCTN2_HUMAN	314	3	14.30	34.49
sp Q14152 EIF3A_HUMAN	634	3	6.20	74.67
sp Q14258 TRI25_HUMAN	630	3	6.70	70.97
sp Q14956 GPNMB_HUMAN	455	3	7.50	50.58
sp Q15056 IF4H_HUMAN	248	3	17.30	27.39

sp Q15121 PEA15_HUMAN	130	3	30.00	15.07
sp Q15181 IPYR_HUMAN	289	3	18.30	32.66
sp Q15404 RSU1_HUMAN	277	3	15.90	31.54
sp Q15417 CNN3_HUMAN	329	3	14.90	36.41
sp Q15435 PP1R7_HUMAN	274	3	17.50	31.36
sp Q16629 SRSF7_HUMAN	165	3	23.00	18.82
sp Q16881 TRXR1_HUMAN	482	3	10.00	53.17
sp Q16891 MIC60_HUMAN	659	3	7.90	73.16
sp Q1KMD3 HNRL2_HUMAN	746	3	5.60	84.69
sp Q6B0K9 HBM_HUMAN	141	3	31.90	15.62
sp Q6XQN6 PNCB_HUMAN	490	3	8.00	52.16
sp Q86Y82 STX12_HUMAN	269	3	16.40	30.88
sp Q8WZ42 TITIN_HUMAN	33423	3	0.00	3713.70
sp Q92616 GCN1L_HUMAN	2432	3	2.00	266.87
sp Q969H8 CS010_HUMAN	173	3	20.20	18.80
sp Q96F07 CYFP2_HUMAN	1178	3	3.00	137.02
sp Q96M27 PRRC1_HUMAN	445	3	10.30	46.70
sp Q99733 NP1L4_HUMAN	375	3	13.30	42.82
sp Q9BR76 COR1B_HUMAN	489	3	10.20	54.23
sp Q9BRF8 CPPED_HUMAN	314	3	15.60	35.55
sp Q9BSJ8 ESYT1_HUMAN	1058	3	3.40	117.39
sp Q9BT6 LRRC1_HUMAN	524	3	9.20	59.23
sp Q9BUP0 EFHD1_HUMAN;sp Q96C19 EFHD2_HUMAN	239	3	14.20	26.96
sp Q9BX66 SRBS1_HUMAN	428	3	10.70	49.07
sp Q9NPH2 INO1_HUMAN	356	3	15.40	38.82
sp Q9NVJ2 ARL8B_HUMAN;sp Q96BM9 ARL8A_HUMAN	177	3	23.70	20.60
sp Q9NYL9 TMOD3_HUMAN	352	3	15.30	39.55
sp Q9P0L0 VAPA_HUMAN	242	3	15.70	27.32
sp Q9UKM9 RALY_HUMAN	237	3	13.50	24.67
sp Q9UNM6 PSD13_HUMAN	311	3	14.10	35.35
sp Q9Y224 CN166_HUMAN	244	3	15.60	27.97
sp Q9Y2Q3 GSTK1_HUMAN	173	3	22.50	19.39
sp Q9Y3I0 RTCB_HUMAN	430	3	8.60	47.38
sp Q9Y5Z4 HEBP2_HUMAN	205	3	18.00	22.88
sp B9A064 IGLL5_HUMAN;sp P0CG04 LAC1_HUMAN	234	2	12.40	25.15
sp O00483 NDUA4_HUMAN	81	2	27.20	9.37
sp O00764 PDXK_HUMAN	166	2	22.90	18.97
sp O14558 HSPB6_HUMAN	160	2	14.40	17.14
sp O14617 AP3D1_HUMAN	865	2	3.80	98.54
sp O15260 SURF4_HUMAN	186	2	16.70	21.13
sp O15400 STX7_HUMAN	216	2	11.60	24.55
sp O15511 ARPC5_HUMAN	151	2	16.60	16.32
sp O43143 DHX15_HUMAN	784	2	2.90	89.55
sp O43184 ADA12_HUMAN	906	2	4.30	99.22
sp O43242 PSMD3_HUMAN	498	2	6.00	57.23
sp O43294 TGFI1_HUMAN	444	2	5.60	47.94
sp O43399 TPD54_HUMAN	186	2	18.80	19.90
sp O43765 SGTA_HUMAN	162	2	17.30	17.79
sp O60610 DIAP1_HUMAN	1215	2	2.10	135.35
sp O60763 USO1_HUMAN	962	2	4.50	107.89
sp O60784 TOM1_HUMAN	454	2	5.30	49.58
sp O75131 CPNE3_HUMAN;sp Q96FN4 CPNE2_HUMAN;sp Q8IYJ1 CPNE9_HUMAN;sp Q96A23 CPNE4_HUMAN;sp O95741 CPNE6_HUMAN;sp Q86YQ8 CPNE8_HUMAN;sp Q9HCH3 CPNE5_HUMAN;sp Q9UBL6 CPNE7_HUMAN	532	2	4.30	59.78
sp O75340 PDCD6_HUMAN	104	2	36.50	12.17
sp O75531 BAF_HUMAN	89	2	34.80	10.06
sp O75643 U520_HUMAN	2136	2	1.10	244.50
sp O75964 ATP5L_HUMAN	76	2	31.60	8.45

sp O94905 ERLN2_HUMAN;sp O75477 ERLN1_HUMAN	338	2	8.60	37.73
sp O95336 6PGL_HUMAN	179	2	23.50	18.91
sp O95373 IPO7_HUMAN	1038	2	2.60	119.52
sp O95810 SDPR_HUMAN	425	2	7.80	47.10
sp O95834 EMAL2_HUMAN	181	2	18.20	18.89
sp O95881 TXD12_HUMAN	172	2	13.40	19.21
sp P00167 CYB5_HUMAN	132	2	25.80	14.57
sp P00505 AATM_HUMAN	371	2	8.40	41.33
sp P00748 FA12_HUMAN	300	2	12.00	32.22
sp P01009 A1AT_HUMAN;tr Q13747 Q13747_HUMAN	418	2	5.70	46.74
sp P01598 KV106_HUMAN;sp P01596 KV104_HUMAN	108	2	26.90	11.79
sp P02100 HBE_HUMAN	147	2	24.50	16.20
sp P02452 CO1A1_HUMAN	1069	2	2.70	97.44
sp P02656 APOC3_HUMAN	99	2	27.30	10.85
sp P02724 GLPA_HUMAN	72	2	43.10	7.84
sp P02786 TFR1_HUMAN	760	2	2.00	84.87
sp P04080 CYTB_HUMAN	98	2	33.70	11.14
sp P04179 SODM_HUMAN	213	2	13.10	23.67
sp P04216 THY1_HUMAN	145	2	16.60	15.90
sp P04632 CPNS1_HUMAN	268	2	16.00	28.32
sp P05120 PAI2_HUMAN	415	2	7.20	46.60
sp P05386 RLA1_HUMAN	113	2	52.20	11.40
sp P06454 PTMA_HUMAN	107	2	22.40	11.76
sp P06703 S10A6_HUMAN	85	2	17.60	9.68
sp P07741 APT_HUMAN	153	2	9.80	16.62
sp P08397 HEM3_HUMAN	330	2	11.20	35.76
sp P08493 MGP_HUMAN	103	2	23.30	12.35
sp P08574 CY1_HUMAN	315	2	11.40	34.49
sp P08648 ITA5_HUMAN	1049	2	2.80	114.48
sp P0C0L5 CO4B_HUMAN	1698	2	1.50	187.67
sp P10155 RO60_HUMAN	538	2	5.20	60.67
sp P11465 PSG2_HUMAN	335	2	7.80	37.22
sp P12277 KCRB_HUMAN	381	2	7.60	42.64
sp P13073 COX41_HUMAN	83	2	28.90	9.29
sp P14406 CX7A2_HUMAN	83	2	27.70	9.43
sp P14854 CX6B1_HUMAN	86	2	45.30	10.19
sp P14923 PLAK_HUMAN	745	2	5.10	81.73
sp P16615 AT2A2_HUMAN;sp Q93084 AT2A3_HUMAN	933	2	3.00	102.48
sp P17931 LEG3_HUMAN	121	2	20.70	13.90
sp P18428 LBP_HUMAN	477	2	6.10	52.93
sp P18859 ATP5J_HUMAN	108	2	35.20	12.59
sp P20290 BTF3_HUMAN	206	2	23.30	22.17
sp P20618 PSB1_HUMAN	241	2	14.10	26.49
sp P20962 PTMS_HUMAN	102	2	22.50	11.53
sp P23142 FBLN1_HUMAN	703	2	7.30	77.21
sp P25788 PSA3_HUMAN	255	2	8.60	28.42
sp P26583 HMGB2_HUMAN	208	2	13.00	23.90
sp P27169 PON1_HUMAN	355	2	8.50	39.73
sp P27635 RL10_HUMAN	201	2	20.40	23.07
sp P30046 DOPD_HUMAN;sp A6NHG4 DDTL_HUMAN	118	2	19.50	12.71
sp P30049 ATPD_HUMAN	168	2	13.70	17.49
sp P30533 AMRP_HUMAN	357	2	8.70	41.48
sp P30626 SORCN_HUMAN	155	2	14.80	17.61
sp P30740 ILEU_HUMAN	341	2	6.20	38.69
sp P31040 SDHA_HUMAN	519	2	6.00	56.66
sp P31930 QCR1_HUMAN	480	2	0.00	52.65
sp P32969 RL9_HUMAN	182	2	15.90	20.87
sp P35637 FUS_HUMAN	157	2	21.00	16.11

sp P38606 VATA_HUMAN	617	2	5.80	68.30
sp P42356 PI4KA_HUMAN	1257	2	2.50	140.77
sp P43121 MUC18_HUMAN	531	2	10.40	59.02
sp P43307 SSRA_HUMAN	265	2	9.80	29.57
sp P43487 RANG_HUMAN	163	2	13.50	18.76
sp P46779 RL28_HUMAN	89	2	21.30	9.66
sp P47914 RL29_HUMAN	157	2	14.60	17.55
sp P48147 PPCE_HUMAN	710	2	3.50	80.73
sp P48444 COPD_HUMAN	511	2	4.50	57.21
sp P49327 FAS_HUMAN	2511	2	1.20	273.42
sp P49720 PSB3_HUMAN	145	2	23.40	16.16
sp P50148 GNAQ_HUMAN;sp O95837 GNA14_HUMAN;sp P29992 GNA11_HUMAN	359	2	8.40	42.11
sp P50440 GATM_HUMAN	423	2	8.70	48.46
sp P51692 STA5B_HUMAN	390	2	7.70	44.95
sp P52597 HNRPF_HUMAN	338	2	9.80	37.26
sp P52788 SPSY_HUMAN	275	2	14.20	31.10
sp P53990 IST1_HUMAN	141	2	19.90	16.11
sp P54136 SYRC_HUMAN	660	2	6.10	75.38
sp P55036 PSMD4_HUMAN	203	2	21.20	21.78
sp P55209 NP1L1_HUMAN	198	2	17.70	23.42
sp P56134 ATPK_HUMAN	54	2	44.40	5.91
sp P58546 MTPN_HUMAN	118	2	38.10	12.90
sp P61353 RL27_HUMAN	126	2	13.50	14.26
sp P61513 RL37A_HUMAN	68	2	39.70	7.62
sp P61586 RHOA_HUMAN	193	2	12.40	21.77
sp P61960 UFM1_HUMAN	85	2	58.80	9.12
sp P62070 RRAS2_HUMAN;sp P10301 RRAS_HUMAN	204	2	13.20	23.40
sp P62191 PRS4_HUMAN	440	2	5.20	49.18
sp P62316 SMD2_HUMAN	118	2	16.10	13.53
sp P62318 SMD3_HUMAN	126	2	15.10	13.92
sp P62750 RL23A_HUMAN	156	2	14.70	17.70
sp P62820 RAB1A_HUMAN	205	2	18.50	22.68
sp P62847 RS24_HUMAN	131	2	20.60	15.20
sp P62854 RS26_HUMAN;sp Q5JNZ5 RS26L_HUMAN	115	2	20.90	12.99
sp P63173 RL38_HUMAN	64	2	39.10	7.56
sp P63208 SKP1_HUMAN	142	2	14.80	16.03
sp P63220 RS21_HUMAN	81	2	33.30	8.85
sp P68366 TBA4A_HUMAN	448	2	7.80	49.92
sp P68402 PA1B2_HUMAN	229	2	12.20	25.57
sp P69892 HBG2_HUMAN	147	2	9.50	16.13
sp P78386 KRT85_HUMAN	507	2	2.40	55.80
sp P80362 KV125_HUMAN;sp P01610 KV118_HUMAN;sp P01600 KV108_HUMAN;sp P01609 KV117_HUMAN;sp P01608 KV116_HUMAN;sp P01607 KV115_HUMAN;sp P01599 KV114_HUMAN	96	2	25.00	10.42
sp P84098 RL19_HUMAN	173	2	15.00	20.82
sp Q00887 PSG9_HUMAN;sp Q00889 PSG6_HUMAN	387	2	7.80	44.01
sp Q01130 SRSF2_HUMAN;sp Q9BRL6 SRSF8_HUMAN	130	2	19.20	15.16
sp Q04637 IF4G1_HUMAN	1395	2	2.20	153.20
sp Q07157 ZO1_HUMAN	1267	2	2.40	142.57
sp Q13177 PAK2_HUMAN;sp Q13153 PAK1_HUMAN;sp O75914 PAK3_HUMAN	455	2	5.90	50.88
sp Q13185 CBX3_HUMAN	183	2	14.20	20.81
sp Q13188 STK3_HUMAN	491	2	4.50	56.30
sp Q13219 PAPP1_HUMAN	1627	2	1.70	180.97
sp Q13404 UB2V1_HUMAN;sp Q15819 UB2V2_HUMAN	105	2	15.20	12.02
sp Q13418 ILK_HUMAN	452	2	6.20	51.42
sp Q13510 ASA1_HUMAN	305	2	11.50	34.59

sp Q13641 TPBG_HUMAN	420	2	7.90	46.03
sp Q14126 DSG2_HUMAN	232	2	12.90	25.94
sp Q14683 SMC1A_HUMAN	832	2	2.80	97.95
sp Q14696 MESD_HUMAN	234	2	15.80	26.08
sp Q14980 NUMA1_HUMAN	963	2	3.20	107.30
sp Q15005 SPCS2_HUMAN	87	2	42.50	10.16
sp Q15075 EEA1_HUMAN	807	2	3.60	92.61
sp Q15431 SYCP1_HUMAN	951	2	2.30	111.24
sp Q15436 SC23A_HUMAN	736	2	4.10	82.97
sp Q16527 CSRP2_HUMAN	193	2	13.00	20.95
sp Q16543 CDC37_HUMAN	230	2	15.20	27.44
sp Q3ZCM7 TBB8_HUMAN	444	2	7.40	49.78
sp Q5XX13 FBW10_HUMAN;sp O95170 CDRT1_HUMAN	1052	2	0.00	119.84
sp Q63ZY3 KANK2_HUMAN	221	2	9.00	23.95
sp Q68EM7 RHG17_HUMAN	211	2	15.20	24.01
sp Q6PIU2 NCEH1_HUMAN	408	2	10.50	45.81
sp Q6ZRR7 LRRC9_HUMAN	1453	2	1.40	166.91
sp Q6ZUB1 S31E1_HUMAN	586	2	4.80	64.85
sp Q71DI3 H32_HUMAN;sp Q16695 H31T_HUMAN;sp P84243 H33_HUMAN;sp P68431 H31_HUMAN;sp Q6NXT2 H3C_HUMAN	92	2	12.00	10.33
sp Q71UI9 H2AV_HUMAN;sp P0C0S5 H2AZ_HUMAN	122	2	13.90	13.16
sp Q71UM5 RS27L_HUMAN;sp P42677 RS27_HUMAN	84	2	25.00	9.48
sp Q7KZF4 SND1_HUMAN	885	2	5.10	99.67
sp Q7Z406 MYH14_HUMAN	1995	2	1.00	227.87
sp Q86V81 THOC4_HUMAN	257	2	16.70	26.89
sp Q8IUR7 ARMC8_HUMAN	468	2	5.10	52.74
sp Q8IWA5 CTL2_HUMAN	704	2	2.80	79.91
sp Q8N163 CCAR2_HUMAN	923	2	4.20	102.93
sp Q8NC51 PAIRB_HUMAN	387	2	8.80	42.44
sp Q8NCW5 NNRE_HUMAN	288	2	12.80	31.67
sp Q8NFV4 ABHDB_HUMAN	315	2	11.70	34.69
sp Q8TCJ2 STT3B_HUMAN	826	2	2.90	93.67
sp Q8WXH0 SYNE2_HUMAN	6818	2	0.00	787.71
sp Q92522 H1X_HUMAN	213	2	10.30	22.49
sp Q93077 H2A1C_HUMAN;sp Q7L7L0 H2A3_HUMAN;sp P04908 H2A1B_HUMAN	130	2	13.80	14.14
sp Q969G5 PRDBP_HUMAN	261	2	9.60	27.70
sp Q96E39 RMXL1_HUMAN;sp P38159 RBMX_HUMAN;sp Q75526 RMXL2_HUMAN;sp Q8N7X1 RMXL3_HUMAN	78	2	34.60	8.61
sp Q96GT9 XAGE2_HUMAN	111	2	23.40	12.35
sp Q96HE7 ERO1A_HUMAN	468	2	8.80	54.39
sp Q96KK5 H2A1H_HUMAN;sp Q9BTM1 H2AJ_HUMAN;sp Q16777 H2A2C_HUMAN;sp Q6FI13 H2A2A_HUMAN;sp P20671 H2A1D_HUMAN;sp P0C0S8 H2A1_HUMAN	128	2	14.10	13.91
sp Q99714 HCD2_HUMAN	261	2	15.30	26.92
sp Q99829 CPNE1_HUMAN	517	2	3.50	56.76
sp Q99880 H2B1L_HUMAN;sp Q99879 H2B1M_HUMAN;sp Q99877 H2B1N_HUMAN;sp Q93079 H2B1H_HUMAN;sp Q5QNW6 H2B2F_HUMAN;sp P62807 H2B1C_HUMAN;sp P58876 H2B1D_HUMAN;sp O60814 H2B1K_HUMAN;sp P57053 H2BFS_HUMAN	126	2	7.90	13.83
sp Q9BPY8 HOP_HUMAN	73	2	35.60	8.26
sp Q9BQE3 TBA1C_HUMAN	449	2	9.10	49.82
sp Q9BV40 VAMP8_HUMAN	100	2	24.00	11.44
sp Q9H0U4 RAB1B_HUMAN	201	2	18.90	22.17
sp Q9H0W9 CK054_HUMAN	239	2	12.10	26.33
sp Q9H299 SH3L3_HUMAN	88	2	33.00	9.38
sp Q9H9B4 SFXN1_HUMAN	144	2	19.40	16.56

sp Q9HC38 GLOD4_HUMAN	242	2	9.50	26.73
sp Q9NR31 SAR1A_HUMAN	198	2	16.20	22.37
sp Q9NR46 SHLB2_HUMAN	374	2	11.00	41.67
sp Q9NUB1 ACS2L_HUMAN	99	2	25.30	10.85
sp Q9NVD7 PARVA_HUMAN	209	2	13.40	23.62
sp Q9NZB8 MOCS1_HUMAN	636	2	5.80	70.10
sp Q9NZD4 AHSP_HUMAN	102	2	24.50	11.84
sp Q9P1F3 ABRAL_HUMAN	81	2	27.20	9.06
sp Q9P260 K1468_HUMAN	1018	2	5.00	112.80
sp Q9P289 STK26_HUMAN;sp O00506 STK25_HUMAN;sp Q9Y6E0 STK24_HUMAN	148	2	14.90	16.37
sp Q9UBQ7 GRHPR_HUMAN	328	2	12.50	35.67
sp Q9UHD8 SEPT9_HUMAN	195	2	14.40	22.22
sp Q9UHG3 PCYOX_HUMAN	487	2	6.80	54.55
sp Q9UJ70 NAGK_HUMAN	182	2	19.80	19.39
sp Q9UJS0 CMC2_HUMAN	567	2	5.50	62.29
sp Q9UKN1 MUC12_HUMAN	5478	2	0.00	558.16
sp Q9ULA0 DNPEP_HUMAN	378	2	8.50	41.40
sp Q9ULC4 MCTS1_HUMAN	181	2	19.90	20.56
sp Q9UNH7 SNX6_HUMAN	216	2	13.00	24.65
sp Q9UPN3 MACF1_HUMAN	4434	2	0.60	505.64
sp Q9Y262 EIF3L_HUMAN	188	2	15.40	22.28
sp Q9Y277 VDAC3_HUMAN	283	2	8.50	30.66
sp Q9Y289 SC5A6_HUMAN	426	2	6.10	45.86
sp Q9Y2V2 CHSP1_HUMAN	147	2	18.40	15.89
sp Q9Y3D6 FIS1_HUMAN	152	2	15.80	16.94
sp Q9Y3U8 RL36_HUMAN	105	2	20.00	12.25
sp Q9Y6E2 BZW2_HUMAN	274	2	10.20	31.25
tr A8K6Q8 A8K6Q8_HUMAN	760	2	2.00	84.87
tr B2R7Y0 B2R7Y0_HUMAN	415	2	6.50	46.61
tr B4E1B2 B4E1B2_HUMAN	678	2	1.90	74.83
tr B4E2L8 B4E2L8_HUMAN	626	2	4.20	70.34
tr D3DTX7 D3DTX7_HUMAN	885	2	2.50	84.74
tr D6RF35 D6RF35_HUMAN	476	2	2.70	53.02
tr D9YZU8 D9YZU8_HUMAN	147	2	7.50	16.13
sp Q16719 KYNU_HUMAN	164	1	9.80	18.52
sp Q5EB52 MEST_HUMAN	173	1	10.40	20.05
sp Q9Y3E0 GOT1B_HUMAN	74	1	18.90	8.21
sp Q15029 U5S1_HUMAN	850	1	1.40	95.37
sp Q9Y3A5 SBDS_HUMAN	86	1	25.60	9.77
sp O00159 MYO1C_HUMAN	1039	1	1.40	118.99
sp O00160 MYO1F_HUMAN;sp Q12965 MYO1E_HUMAN	90	1	18.90	9.65
sp O00161 SNP23_HUMAN	97	1	14.40	11.14
sp O00303 EIF3F_HUMAN	100	1	17.00	10.81
sp O00410 IPO5_HUMAN	718	1	2.80	81.23
sp O15069 NACAD_HUMAN	1562	1	0.80	161.10
sp O15269 SPTC1_HUMAN	473	1	3.00	52.74
sp O43169 CYB5B_HUMAN	107	1	31.80	11.87
sp O43708 MAAI_HUMAN	70	1	28.60	7.55
sp O60229 KALRN_HUMAN	1654	1	0.00	191.24
sp O60282 KIF5C_HUMAN;sp P33176 KINH_HUMAN;sp Q12840 KIF5A_HUMAN	265	1	4.90	30.58
sp O60476 MA1A2_HUMAN	641	1	2.70	73.00
sp O60613 SEP15_HUMAN	162	1	9.90	17.79
sp O75915 PRAF3_HUMAN	165	1	11.50	19.21
sp O95198 KLHL2_HUMAN	117	1	20.50	13.36
sp O95299 NDUAA_HUMAN	201	1	10.90	22.37
sp O95433 AHSA1_HUMAN	90	1	24.40	10.03

sp P00846 ATP6_HUMAN	43	1	23.30	5.11
sp P01617 KV204_HUMAN	102	1	23.50	11.04
sp P01621 KV303_HUMAN	100	1	9.00	10.73
sp P01623 KV305_HUMAN;sp P01620 KV302_HUMAN;sp P04206 KV307_HUMAN;sp P01622 KV304_HUMAN;sp P18136 KV313_HUMAN;sp P18135 KV312_HUMAN	109	1	16.50	11.65
sp P01625 KV402_HUMAN	114	1	15.80	12.64
sp P01717 LV403_HUMAN	96	1	19.80	10.35
sp P01765 HV304_HUMAN;sp P01764 HV303_HUMAN;sp P01779 HV318_HUMAN;sp P01776 HV315_HUMAN;sp P01774 HV313_HUMAN;t	238	1	8.00	25.16
sp P01871 IGHM_HUMAN;sp P04220 MUCB_HUMAN	599	1	1.20	65.70
sp P01876 IGHA1_HUMAN	494	1	6.50	53.09
sp P02533 K1C14_HUMAN;sp P08779 K1C16_HUMAN;sp P19012 K1C15_HUMAN	472	1	3.20	51.56
sp P02774 VTDB_HUMAN	474	1	5.50	52.96
sp P04208 LV106_HUMAN;sp P01700 LV102_HUMAN	235	1	5.50	24.82
sp P04921 GLPC_HUMAN	128	1	20.30	13.81
sp P05114 HMG1_HUMAN	44	1	29.50	4.74
sp P05164 PERM_HUMAN	745	1	1.90	83.87
sp P05198 IF2A_HUMAN	252	1	6.00	28.87
sp P05204 HMG2_HUMAN;sp Q15651 HMG3_HUMAN	90	1	10.00	9.39
sp P05976 MYL1_HUMAN;sp P08590 MYL3_HUMAN	140	1	11.40	15.60
sp P07919 QCR6_HUMAN	85	1	21.20	9.95
sp P08134 RHOC_HUMAN	189	1	6.30	21.58
sp P08727 K1C19_HUMAN	400	1	7.50	44.11
sp P09466 PAEP_HUMAN	126	1	11.10	14.80
sp P09543 CN37_HUMAN	120	1	19.20	13.65
sp P09972 ALDOC_HUMAN	325	1	7.10	35.42
sp P0C0L4 CO4A_HUMAN	1744	1	0.70	192.78
sp P10636 TAU_HUMAN	354	1	6.50	36.99
sp P11532 DMD_HUMAN	57	1	21.10	6.52
sp P11766 ADHX_HUMAN	115	1	14.80	12.22
sp P12236 ADT3_HUMAN	298	1	4.40	32.87
sp P16452 EPB42_HUMAN	543	1	2.40	59.83
sp P16949 STMN1_HUMAN	86	1	15.10	9.86
sp P17066 HSP76_HUMAN;sp P48741 HSP77_HUMAN	643	1	1.70	71.00
sp P18564 ITB6_HUMAN	693	1	1.30	75.85
sp P18827 SDC1_HUMAN	161	1	10.60	16.94
sp P19404 NDUV2_HUMAN	231	1	4.30	25.43
sp P20073 ANXA7_HUMAN	336	1	4.50	37.81
sp P20337 RAB3B_HUMAN	219	1	8.20	24.74
sp P20340 RAB6A_HUMAN	208	1	10.60	23.55
sp P20591 MX1_HUMAN	197	1	7.60	20.90
sp P20810 ICAL_HUMAN	642	1	5.80	69.75
sp P21912 SDHB_HUMAN	123	1	11.40	14.14
sp P23434 GCSH_HUMAN	125	1	16.00	13.81
sp P23786 CPT2_HUMAN	658	1	1.80	73.78
sp P24752 THIL_HUMAN	141	1	12.10	15.16
sp P25325 THM_HUMAN	84	1	19.00	9.48
sp P26232 CTNA2_HUMAN	953	1	1.90	105.31
sp P28074 PSB5_HUMAN	118	1	11.00	12.75
sp P28482 MK01_HUMAN;sp P27361 MK03_HUMAN	87	1	17.20	10.18
sp P28906 CD34_HUMAN	193	1	10.40	20.71
sp P30038 AL4A1_HUMAN	214	1	7.50	23.13
sp P30457 1A66_HUMAN;sp P30456 1A43_HUMAN;sp P30450 1A26_HUMAN;sp P18462 1A25_HUMAN;sp Q95604 1C17_HUMAN	7544	1	38.15	337.00
sp P30531 SC6A1_HUMAN	421	1	2.60	47.35

sp P31146 COR1A_HUMAN	461	1	2.20	51.03
sp P31323 KAP3_HUMAN	405	1	4.20	44.87
sp P31947 1433S_HUMAN	248	1	3.20	27.77
sp P35241 RADI_HUMAN	583	1	2.60	68.56
sp P35268 RL22_HUMAN	47	1	27.70	5.08
sp P35858 ALS_HUMAN	605	1	2.60	66.07
sp P36406 TRI23_HUMAN	574	1	1.70	64.03
sp P36543 VATE1_HUMAN	203	1	6.90	23.54
sp P37108 SRP14_HUMAN	115	1	12.20	13.06
sp P42126 ECI1_HUMAN	224	1	5.80	24.70
sp P42167 LAP2B_HUMAN;sp P42166 LAP2A_HUMAN	237	1	9.70	26.87
sp P43003 EAA1_HUMAN	428	1	2.80	46.95
sp P43363 MAGAA_HUMAN	161	1	6.80	17.22
sp P43490 NAMPT_HUMAN	491	1	4.10	55.52
sp P47985 UCRI_HUMAN;sp P0C7P4 UCRIL_HUMAN	274	1	7.70	29.67
sp P49407 ARRB1_HUMAN	258	1	9.30	29.24
sp P49721 PSB2_HUMAN	84	1	13.10	9.56
sp P51452 DUS3_HUMAN	132	1	9.80	14.25
sp P51659 DHB4_HUMAN	500	1	3.00	53.93
sp P53007 TXTP_HUMAN	311	1	3.90	34.01
sp P54578 UBP14_HUMAN	20	1	65.00	2.23
sp P54709 AT1B3_HUMAN	43	1	32.60	4.81
sp P54725 RD23A_HUMAN	362	1	8.30	39.47
sp P55010 IF5_HUMAN	193	1	7.80	22.54
sp P55795 HNRH2_HUMAN	449	1	3.80	49.26
sp P56537 IF6_HUMAN	245	1	9.80	26.60
sp P57088 TMM33_HUMAN	222	1	5.40	25.22
sp P60981 DEST_HUMAN	135	1	8.10	15.40
sp P60983 GMFB_HUMAN	112	1	20.50	12.87
sp P61020 RAB5B_HUMAN	215	1	6.50	23.71
sp P61026 RAB10_HUMAN	200	1	5.50	22.54
sp P61081 UBC12_HUMAN	183	1	8.70	20.90
sp P61225 RAP2B_HUMAN	181	1	6.60	20.29
sp P61626 LYSC_HUMAN	104	1	11.50	11.49
sp P61803 DAD1_HUMAN	65	1	18.50	7.29
sp P61923 COPZ1_HUMAN	89	1	25.80	10.04
sp P62195 PRS8_HUMAN	345	1	4.60	38.76
sp P62266 RS23_HUMAN	143	1	7.70	15.81
sp P62304 RUXE_HUMAN	92	1	12.00	10.80
sp P62736 ACTA_HUMAN;sp P63267 ACTH_HUMAN	377	1	2.90	42.01
sp P62841 RS15_HUMAN	68	1	27.90	8.03
sp P62857 RS28_HUMAN	69	1	17.40	7.84
sp P62995 TRA2B_HUMAN	252	1	6.30	29.20
sp P63162 RSMN_HUMAN;sp P14678 RSMB_HUMAN	169	1	4.70	17.55
sp P67809 YBOX1_HUMAN	266	1	7.10	29.37
sp P68036 UB2L3_HUMAN	154	1	14.30	17.79
sp P68371 TBB4B_HUMAN	445	1	2.70	49.84
sp P84085 ARF5_HUMAN	180	1	10.00	20.53
sp P84090 ERH_HUMAN	71	1	23.90	8.20
sp P84095 RHOG_HUMAN	191	1	9.90	21.31
sp Q00005 2ABB_HUMAN;sp P63151 2ABA_HUMAN;sp Q66LE6 2ABD_HUMAN	135	1	10.40	14.82
sp Q00888 PSG4_HUMAN	419	1	2.40	47.09
sp Q10589 BST2_HUMAN	180	1	8.30	19.77
sp Q13103 SPP24_HUMAN	211	1	5.70	24.34
sp Q13148 TADBP_HUMAN	200	1	6.00	22.84
sp Q13283 G3BP1_HUMAN	284	1	6.00	31.51
sp Q13509 TBB3_HUMAN	406	1	4.20	45.62

sp Q13938 CAYP1_HUMAN	118	1	11.00	12.86
sp Q14213 IL27B_HUMAN	229	1	5.20	25.40
sp Q14728 MFS10_HUMAN;	356	1	3.70	38.25
sp Q15111 PLCL1_HUMAN	1012	1	1.50	114.69
sp Q15437 SC23B_HUMAN	331	1	3.90	37.14
sp Q15714 T22D1_HUMAN	86	1	14.00	9.37
sp Q15836 VAMP3_HUMAN	68	1	35.30	7.78
sp Q15847 ADIRF_HUMAN	76	1	40.80	7.85
sp Q16363 LAMA4_HUMAN	1816	1	0.90	201.82
sp Q16401 PSMD5_HUMAN	504	1	3.80	56.22
sp Q16557 PSG3_HUMAN	428	1	3.70	47.94
sp Q16563 SYPL1_HUMAN	224	1	4.90	24.78
sp Q16778 H2B2E_HUMAN;sp P33778 H2B1B_HUMAN;sp P23527 H2B1O_HUMAN;sp P06899 H2B1J_HUMAN;sp Q8N257 H2B3B_HUMAN;sp Q96A08 H2B1A_HUMAN	126	1	7.90	13.90
sp Q29974 2B1G_HUMAN;sp P01911 2B1F_HUMAN	180	1	15.00	20.94
sp Q32P28 P3H1_HUMAN	207	1	4.80	23.09
sp Q3SYG4 PTHB1_HUMAN	146	1	15.80	16.40
sp Q562R1 ACTBL_HUMAN	376	1	4.80	42.00
sp Q5T8P6 RBM26_HUMAN	1007	1	0.00	113.60
sp Q5VZK9 LR16A_HUMAN	1365	1	0.00	150.91
sp Q5XKP0 QIL1_HUMAN	118	1	16.90	13.09
sp Q6EEV6 SUMO4_HUMAN;sp P61956 SUMO2_HUMAN;sp P55854 SUMO3_HUMAN	95	1	12.60	10.87
sp Q6IBS0 TWF2_HUMAN	254	1	6.70	28.88
sp Q6P474 PDXD2_HUMAN	469	1	0.00	51.81
sp Q71U36 TBA1A_HUMAN;sp Q13748 TBA3C_HUMAN;sp Q6PEY2 TBA3E_HUMAN	451	1	3.10	50.14
sp Q7Z7G0 TARSH_HUMAN	1029	1	0.00	113.43
sp Q86SX6 GLRX5_HUMAN	157	1	8.90	16.63
sp Q86UP2 KTN1_HUMAN	153	1	7.80	17.31
sp Q8IYT4 KATL2_HUMAN	128	1	9.40	14.00
sp Q8IZ83 A16A1_HUMAN	802	1	1.50	85.13
sp Q8N5K1 CISD2_HUMAN	135	1	10.40	15.28
sp Q8N6L1 KTAP2_HUMAN	136	1	12.50	14.68
sp Q8NBJ7 SUMF2_HUMAN	165	1	6.10	18.58
sp Q8NBN7 RDH13_HUMAN	260	1	9.20	28.84
sp Q8NC56 LEMD2_HUMAN	114	1	14.90	12.88
sp Q8NFZ4 NLGN2_HUMAN	194	1	0.00	20.94
sp Q8TD55 PKHO2_HUMAN	490	1	4.70	53.35
sp Q8TD84 DSCL1_HUMAN	1843	1	0.00	201.11
sp Q92504 S39A7_HUMAN	374	1	3.70	39.60
sp Q92804 RBP56_HUMAN	395	1	8.60	40.08
sp Q92882 OSTF1_HUMAN	214	1	5.60	23.71
sp Q92973 TNPO1_HUMAN	898	1	2.70	102.35
sp Q969Q0 RL36L_HUMAN;sp P83881 RL36A_HUMAN	106	1	8.50	12.53
sp Q96EP5 DAZP1_HUMAN	327	1	4.90	35.02
sp Q96IY4 CBPB2_HUMAN	386	1	3.10	44.02
sp Q96K17 BT3L4_HUMAN	145	1	19.30	15.78
sp Q96MP5 ZSWM3_HUMAN	690	1	1.60	78.36
sp Q96P70 IPO9_HUMAN	1041	1	1.70	115.96
sp Q96QR8 PURB_HUMAN	312	1	9.90	33.24
sp Q99439 CNN2_HUMAN	148	1	11.50	16.50
sp Q99471 PFD5_HUMAN	152	1	11.80	17.05
sp Q99538 LGMN_HUMAN	91	1	18.70	9.66
sp Q99805 TM9S2_HUMAN	573	1	2.60	65.43
sp Q9BPX5 ARP5L_HUMAN	153	1	7.80	16.97
sp Q9BRX8 F213A_HUMAN	229	1	6.60	25.76
sp Q9BUF5 TBB6_HUMAN	374	1	4.00	41.95

sp Q9BX68 HINT2_HUMAN	128	1	15.60	13.92
sp Q9H8H3 MET7A_HUMAN	175	1	8.60	20.51
sp Q9H8Y8 GORS2_HUMAN	405	1	5.20	42.04
sp Q9NP97 DLRB1_HUMAN;sp Q8TF09 DLRB2_HUMAN	96	1	12.50	10.92
sp Q9NQC3 RTN4_HUMAN	1192	1	2.80	129.93
sp Q9NRP0 OSTC_HUMAN	83	1	14.50	9.37
sp Q9NTK5 OLA1_HUMAN	116	1	12.90	12.30
sp Q9NVA2 SEP11_HUMAN	126	1	15.90	14.13
sp Q9NVW2 RNF12_HUMAN	624	1	0.00	68.55
sp Q9UBE0 SAE1_HUMAN	172	1	11.60	19.15
sp Q9UBV2 SE1L1_HUMAN	794	1	2.40	88.75
sp Q9UH99 SUN2_HUMAN	442	1	4.30	49.42
sp Q9UIA9 XPO7_HUMAN	237	1	6.80	26.84
sp Q9UIJ7 KAD3_HUMAN	157	1	8.90	18.18
sp Q9UNL2 SSRG_HUMAN	174	1	8.00	20.16
sp Q9UNZ2 NSF1C_HUMAN	274	1	5.50	30.22
sp Q9UQ72 PSG11_HUMAN	219	1	7.30	24.01
sp Q9Y263 PLAP_HUMAN	795	1	2.10	87.16
sp Q9Y281 COF2_HUMAN	166	1	12.00	18.74
sp Q9Y333 LSM2_HUMAN	95	1	20.00	10.83
sp Q9Y371 SHLB1_HUMAN	365	1	4.10	40.80
sp Q9Y376 CAB39_HUMAN	339	1	3.20	39.45
sp Q9Y3I1 FBX7_HUMAN	233	1	5.60	26.62
sp Q9Y3R5 DOP2_HUMAN	2298	1	0.00	258.23
sp Q9Y3Z3 SAMH1_HUMAN	626	1	2.40	72.20
sp Q9Y5L4 TIM13_HUMAN	95	1	14.70	10.50
sp Q9Y6F6 MRVI1_HUMAN	428	1	3.70	46.63
tr A0A024QZP4 A0A024QZP4_HUMAN	113	1	14.20	13.65
tr A0A024R6I7 A0A024R6I7_HUMAN	418	1	3.80	46.71
tr A0A068LKQ0 A0A068LKQ0_HUMAN	120	1	10.00	13.35
tr A0A068LKQ2 A0A068LKQ2_HUMAN	120	1	13.30	13.16
tr A0A087X079 A0A087X079_HUMAN;tr A0A087WYC5 A0A087WYC5_HUMAN	473	1	2.50	51.91
tr A0N5G5 A0N5G5_HUMAN;tr B1N7B8 B1N7B8_HUMAN	118	1	15.30	12.77
tr A6MUU8 A6MUU8_HUMAN	113	1	8.00	12.40
tr B2RMN7 B2RMN7_HUMAN;tr A0A024R674 A0A024R674_HUMAN	2137	1	0.60	246.57
tr B2RWP4 B2RWP4_HUMAN	2826	1	1.00	296.74
tr B4DI57 B4DI57_HUMAN	571	1	4.20	63.44
tr B4DMW1 B4DMW1_HUMAN	428	1	2.60	48.91
tr B4DVL8 B4DVL8_HUMAN;tr Q6PJ76 Q6PJ76_HUMAN;tr Q9BVJ8 Q9BVJ8_HUMAN;tr B4DVA7 B4DVA7_HUMAN	301	1	8.30	34.58
tr B4E337 B4E337_HUMAN	562	1	2.00	64.16
tr B7Z5V6 B7Z5V6_HUMAN	644	1	3.30	71.80
tr B7Z7M2 B7Z7M2_HUMAN	136	1	8.80	14.97
tr B7Z938 B7Z938_HUMAN;tr Q5D044 Q5D044_HUMAN;tr H0UI49 H0UI49_HUMAN	1413	1	1.10	157.47
tr C0KRQ8 C0KRQ8_HUMAN	92	1	38.00	10.21
tr C7DJS2 C7DJS2_HUMAN	151	1	11.90	16.67
tr C8C504 C8C504_HUMAN	147	1	10.90	16.00
tr D3DTH7 D3DTH7_HUMAN	862	1	1.70	98.93
tr E9LUX2 E9LUX2_HUMAN	71	1	28.20	7.73
tr H7BZJ3 H7BZJ3_HUMAN	123	1	11.40	13.52
tr L0R4Y9 L0R4Y9_HUMAN	40	1	27.50	4.59
tr L0R512 L0R512_HUMAN	42	1	21.40	4.73
tr L8E7E2 L8E7E2_HUMAN	193	1	7.80	22.59
tr Q5XTR9 Q5XTR9_HUMAN	34	1	35.30	3.88
tr Q68D26 Q68D26_HUMAN	158	1	9.50	18.17

tr Q6IPN0 Q6IPN0_HUMAN;tr F8W914 F8W914_HUMAN;tr Q53SY1 Q53SY1_HUMAN;tr A6XGP7 A6XGP7_HUMAN;tr H7C106 H7C106_HUMAN;tr D6W5C2 D6W5C2_HUMAN;tr Q3LIF4 Q3LIF4_HUMAN	343	1	4.10	36.92
tr Q6LAM1 Q6LAM1_HUMAN	321	1	4.40	35.88
tr Q6VFQ6 Q6VFQ6_HUMAN	42	1	28.60	4.51
tr Q6ZW64 Q6ZW64_HUMAN;tr Q96DK0 Q96DK0_HUMAN;tr Q6N092 Q6N092_HUMAN	494	1	6.50	53.32
tr Q7Z351 Q7Z351_HUMAN	482	1	2.50	52.85
tr Q86TT1 Q86TT1_HUMAN	375	1	3.50	41.27
tr Q86WI6 Q86WI6_HUMAN	26	1	0.00	3.06
tr Q8IWy7 Q8IWy7_HUMAN	1649	1	0.70	182.46
tr Q9BUM6 Q9BUM6_HUMAN	425	1	6.80	47.45
tr Q9BWU5 Q9BWU5_HUMAN	105	1	16.20	11.50
tr Q9GZL9 Q9GZL9_HUMAN	115	1	8.70	12.53
tr Q9UK54 Q9UK54_HUMAN	128	1	9.40	13.96
tr Q9UL85 Q9UL85_HUMAN;tr Q9UL83 Q9UL83_HUMAN	109	1	16.50	11.76
tr Q9UL88 Q9UL88_HUMAN	131	1	14.50	14.14
tr U3PXP0 U3PXP0_HUMAN	51	1	19.60	5.70
tr U6A3P2 U6A3P2_HUMAN	41	1	31.70	4.37
tr V9H1D9 V9H1D9_HUMAN	128	1	9.40	13.58
sp P01614 KV201_HUMAN;sp P06309 KV205_HUMAN;sp P06310 KV206_HUMAN	219	0	0.00	24.03
sp P01825 HV207_HUMAN;sp P06331 HV209_HUMAN	465	0	0.00	51.08
sp P01834 IGKC_HUMAN	231	0	0.00	25.14
sp P01857 IGHG1_HUMAN	475	0	0.00	52.11
sp P01859 IGHG2_HUMAN	417	0	0.00	46.06
sp P01861 IGHG4_HUMAN	327	0	0.00	35.94
sp P02787 TRFE_HUMAN	698	0	0.00	77.08
sp P04264 K2C1_HUMAN	644	0	0.00	66.11
sp P04350 TBB4A_HUMAN	444	0	0.00	49.59
sp P04433 KV309_HUMAN	233	0	0.00	25.60
sp P0CG06 LAC3_HUMAN;sp P0CG05 LAC2_HUMAN;sp P0CF74 LAC6_HUMAN;tr S6C4R7 S6C4R7_HUMAN	234	0	0.00	24.79
sp P11464 PSG1_HUMAN	291	0	0.00	33.08
sp P48668 K2C6C_HUMAN;sp P04259 K2C6B_HUMAN;sp P02538 K2C6A_HUMAN	520	0	0.00	55.80
sp P68032 ACTC_HUMAN	377	0	0.00	42.02
sp P68363 TBA1B_HUMAN	416	0	0.00	46.34
sp P68871 HBB_HUMAN;	147	0	0.00	16.00
sp P69891 HBG1_HUMAN	147	0	0.00	16.14
sp P69905 HBA_HUMAN	142	0	0.00	15.26
sp Q04826 1B40_HUMAN;sp P30488 1B50_HUMAN;sp P30487 1B49_HUMAN;sp P30485 1B47_HUMAN;sp P30483 1B45_HUMAN;sp P30481 1B44_HUMAN;sp P30461 1B13_HUMAN;sp P03989 1B27_HUMAN;sp P04222 1C03_HUMAN	273	0	0.00	31.81
sp Q05682 CALD1_HUMAN	557	0	0.00	64.17
sp Q13046 PSG7_HUMAN	419	0	0.00	47.24
sp Q5XKE5 K2C79_HUMAN	535	0	0.00	57.84
tr A0A087WWU8 A0A087WWU8_HUMAN;tr D6R904 D6R90	227	0	0.00	26.42
tr B2RDE1 B2RDE1_HUMAN;tr Q5VU59 Q5VU59_HUMAN;tr Q5HYB6 Q5HYB6_HUMAN	248	0	0.00	29.02
tr B4DE59 B4DE59_HUMAN	563	0	0.00	62.62
tr D9YZV5 D9YZV5_HUMAN;tr Q15657 Q15657_HUMAN;tr H0YKX5 H0YKX5_HUMAN	284	0	0.00	32.88
tr E7ENL6 E7ENL6_HUMAN;tr B7ZW00 B7ZW00_HUMAN;tr B4E0A4 B4E0A4_HUMAN	2569	0	0.00	278.11
tr E9PGZ1 E9PGZ1_HUMAN;tr Q9NYG1 Q9NYG1_HUMAN;tr C9J813 C9J813_HUMAN;tr Q6PJM5 Q6PJM5_HUMAN	536	0	0.00	61.71

tr H6VRG2 H6VRG2_HUMAN	644	0	0.00	66.07
tr H7BYY1 H7BYY1_HUMAN;tr Q1ZYL5 Q1ZYL5_HUMAN;tr F5H7S3 F5H7S3_HUMAN;tr B7Z596 B7Z596_HUMAN;tr Q8TCG4 Q8TCG4_HUMAN	248	0	0.00	28.75
tr Q2NKY5 Q2NKY5_HUMAN	447	0	0.00	50.09
tr Q6MZQ6 Q6MZQ6_HUMAN;tr Q6N094 Q6N094_HUMAN;tr Q6N096 Q6N096_HUMAN;tr Q6N097 Q6N097_HUMAN;tr Q6N095 Q6N095_HUMAN	475	0	0.00	52.04
tr Q6MZV7 Q6MZV7_HUMAN;tr Q6PYX1 Q6PYX1_HUMAN	473	0	0.00	52.12
tr Q6P5S8 Q6P5S8_HUMAN	236	0	0.00	25.77
tr Q6ZN40 Q6ZN40_HUMAN	326	0	0.00	37.45
tr Q9NPP6 Q9NPP6_HUMAN	416	0	0.00	44.79
tr Q9UL72 Q9UL72_HUMAN	118	0	0.00	12.87
tr S6B291 S6B291_HUMAN	466	0	0.00	50.87
tr S6BGE0 S6BGE0_HUMAN	300	0	0.00	32.18
tr V9HW34 V9HW34_HUMAN;tr Q6PJF2 Q6PJF2_HUMAN;tr Q6PIL8 Q6PIL8_HUMAN	235	0	0.00	25.65

Appendix 10. Table 3. Child salivary protein identification in children with allergic diseases including asthma, eczema and combination of rhinitis in LC-MS/MS. (177 salivary proteins)
(Highlighted protein name have more than 2 unique peptides (UP)).

Protein IDs	Sequence length (bp)	UP	Unique sequence coverage [%]	MW [kDa]
sp P02768 ALBU_HUMAN	609	78	96.10	69.37
sp P01833 PIGR_HUMAN	764	52	55.00	83.28
sp Q9HC84 MUC5B_HUMAN	5762	47	12.50	596.33
sp P02788 TRFL_HUMAN	711	37	59.50	78.38
sp P06733 ENOA_HUMAN	434	28	61.80	47.17
sp P02787 TRFE_HUMAN	698	27	27.50	77.08
sp P25311 ZA2G_HUMAN	298	24	56.70	34.26
sp Q9UGM3 DMBT1_HUMAN	2413	21	38.30	260.73
sp Q9UBG3 CRNN_HUMAN	495	16	50.30	53.56
sp P01037 CYTN_HUMAN	141	15	70.20	16.39
sp P61626 LYSC_HUMAN	148	15	77.00	16.54
sp P01036 CYTS_HUMAN	141	14	80.10	16.21
sp P01591 IGJ_HUMAN	159	14	68.60	18.10
sp P09228 CYTT_HUMAN	141	14	77.30	16.45
sp P22079 PERL_HUMAN	712	12	27.70	80.29
sp P04080 CYTB_HUMAN	98	11	84.70	11.14
sp P12273 PIP_HUMAN	146	10	70.50	16.57
sp P31947 I433S_HUMAN	248	10	51.60	27.77
sp P31025 LCN1_HUMAN	176	9	43.80	19.25
sp P00738 HPT_HUMAN;sp P00739 HPTR_HUMAN	404	8	19.60	44.93
sp P07237 PDIA1_HUMAN	492	8	22.40	55.38
sp A8K2U0 A2ML1_HUMAN	1454	7	6.10	161.10
sp P01034 CYTC_HUMAN	146	7	52.10	15.80
sp P04406 G3P_HUMAN;	335	7	24.50	36.05
sp P04792 HSPB1_HUMAN	205	7	48.30	22.78
sp P06702 S10A9_HUMAN	114	7	50.00	13.21
sp P60174 TPIS_HUMAN	249	7	52.60	26.67
sp P68871 HBB_HUMAN	147	7	49.70	16.00
sp Q01469 FABP5_HUMAN	135	7	59.30	15.16
sp Q8TAX7 MUC7_HUMAN	377	7	14.60	39.16
sp P02766 TTHY_HUMAN	147	6	41.50	15.89
sp P04217 A1BG_HUMAN	326	6	30.10	35.24
sp P10599 THIO_HUMAN	105	6	65.70	11.74
sp P54108 CRIS3_HUMAN	245	6	23.30	27.63
sp Q6P5S2 CF058_HUMAN	330	6	13.30	37.93
sp Q9H299 SH3L3_HUMAN	88	6	50.00	9.38
sp P01871 IGHM_HUMAN;sp P04220 MUCB_HUMAN	588	6	7.50	64.11
sp Q95274 LYPD3_HUMAN	294	5	29.90	30.50
sp P03973 SLPI_HUMAN	132	5	32.60	14.33
sp P06870 KLK1_HUMAN	262	5	29.00	28.89
sp P07476 INVO_HUMAN	585	5	12.60	68.48
sp P18510 IL1RA_HUMAN	143	5	41.30	16.14
sp P23280 CAH6_HUMAN	308	5	23.70	35.37
sp P61769 B2MG_HUMAN	92	5	60.90	10.40
sp P62937 PPIA_HUMAN	165	5	37.60	18.01
sp Q02487 DSC2_HUMAN	758	5	8.80	83.79
sp P01009 A1AT_HUMAN	197	4	18.30	22.83
sp P01023 A2MG_HUMAN	1474	4	3.90	163.29
sp P04075 ALDOA_HUMAN	364	4	18.70	39.42
sp P15515 HIS1_HUMAN	57	4	43.90	6.96

sp P30086 PEBP1_HUMAN	187	4	42.80	21.06
sp P32926 DSG3_HUMAN	999	4	5.80	107.50
sp Q99877 H2B1N_HUMAN;sp P33778 H2B1B_HUMAN;sp Q93079 H2B1H_HUMAN;sp Q5QNW6 H2B2F_HUMAN;sp Q16778 H2B2E_HUMAN;sp P62807 H2B1C_HUMAN;sp P58876 H2B1D_HUMAN;sp P23527 H2B1O_HUMAN;sp Q96A08 H2B1A_HUMAN;sp Q8N257 H2B3B_HUMAN;sp P57053 H2BFS_HUMAN;sp P06899 H2B1J_HUMAN;sp O60814 H2B1K_HUMAN	126	4	36.50	13.92
sp Q06830 PRDX1_HUMAN	171	4	24.00	18.98
sp Q16610 ECM1_HUMAN	540	4	13.30	60.67
sp Q9BPY8 HOP_HUMAN	73	4	19.20	8.26
sp Q9UBC9 SPRR3_HUMAN	169	4	36.70	18.15
sp Q9Y6R7 FCGBP_HUMAN	4204	4	2.10	445.21
sp P67936 TPM4_HUMAN;sp P09493 TPM1_HUMAN;sp P07951 TPM2_HUMAN	227	4	18.10	26.42
sp P00709 LALBA_HUMAN	142	3	37.30	16.23
sp P01024 CO3_HUMAN	1663	3	2.50	187.15
sp P01040 CYTA_HUMAN	98	3	50.00	11.01
sp P01617 KV204_HUMAN;sp P01614 KV201_HUMAN;sp P06309 KV205_HUMAN	102	3	46.10	11.04
sp P02765 FETUA_HUMAN	367	3	19.10	39.32
sp P02808 STAT_HUMAN	62	3	54.80	7.30
sp P04083 ANXA1_HUMAN	115	3	45.20	12.64
sp P04745 AMY1_HUMAN	511	3	3.50	57.75
sp P05109 S10A8_HUMAN	93	3	29.00	10.83
sp P11142 HSP7C_HUMAN;sp P54652 HSP72_HUMAN	210	3	20.50	23.08
sp P18669 PGAM1_HUMAN;sp P15259 PGAM2_HUMAN	129	3	35.70	14.53
sp P28325 CYTD_HUMAN	142	3	38.70	16.08
sp P30044 PRDX5_HUMAN	162	3	32.10	17.03
sp P47710 CASA1_HUMAN	185	3	14.60	21.67
sp Q6MZM9 PRR27_HUMAN	219	3	23.70	22.72
tr Q6N041 Q6N041_HUMAN	498	3	7.20	54.13
sp O60309 L37A3_HUMAN;sp A6NM11 L37A2_HUMAN;sp A6NMS7 L37A1_HUMAN	1634	2	1.00	180.62
sp O15397 IPO8_HUMAN	1037	2	3.30	119.94
sp P00441 SODC_HUMAN	135	2	23.00	13.91
sp P02750 A2GL_HUMAN	330	2	10.60	36.49
sp P02763 A1AG1_HUMAN;sp P19652 A1AG2_HUMAN	201	2	13.40	23.51
sp P02774 VTDB_HUMAN	474	2	11.40	52.96
sp P02814 SMR3B_HUMAN	79	2	65.80	8.19
sp P05164 PERM_HUMAN	745	2	4.00	83.87
sp P05814 CASB_HUMAN	226	2	11.90	25.38
sp P06396 GELS_HUMAN	485	2	9.50	52.37
sp P07108 ACBP_HUMAN	87	2	18.40	10.04
sp P14174 MIF_HUMAN	115	2	11.30	12.48
sp P14618 KPYM_HUMAN	151	2	27.20	16.43
sp P19971 TYPH_HUMAN	482	2	9.10	49.96
sp P20061 TCO1_HUMAN	433	2	11.30	48.21
sp P27482 CALL3_HUMAN	149	2	31.50	16.89
sp P36952 SPB5_HUMAN	375	2	9.60	42.10
sp P47929 LEG7_HUMAN	136	2	30.90	15.08
sp P63104 1433Z_HUMAN	52	2	21.20	5.86
sp P69905 HBA_HUMAN	100	2	26.00	10.75
sp P80303 NUCB2_HUMAN	338	2	10.10	40.37
sp Q02413 DSG1_HUMAN	1049	2	3.10	113.75
sp Q08380 LG3BP_HUMAN	413	2	14.80	46.42
sp Q08999 RBL2_HUMAN	1139	2	2.20	128.37
;sp Q13085 ACACA_HUMAN	2268	2	1.90	257.24
sp Q16378 PROL4_HUMAN	134	2	15.70	15.10

sp Q16777 H2A2C_HUMAN;sp Q6F113 H2A2A_HUMAN;sp Q99878 H2A1J_HUMAN;sp Q96KK5 H2A1H_HUMAN;sp Q71U19 H2AV_HUMAN;sp P0C0S5 H2AZ_HUMA;sp Q9BTM1 H2AJ_HUMAN;sp Q93077 H2A1C_HUMAN;sp Q8IUE6 H2A2B_HUMAN;sp Q7L7L0 H2A3_HUMAN;sp P20671 H2A1D_HUMAN;sp P0C0S8 H2A1_HUMAN;sp P04908 H2A1B_HUMAN;sp Q96QV6 H2A1A_HUMAN;sp P16104 H2AX_HUMAN	129	2	29.50	13.99
sp Q86W28 NALP8_HUMAN	1048	2	2.20	119.43
sp Q8WZ42 TITIN_HUMAN	33423	2	0.10	3713.70
sp Q9GZZ8 LACRT_HUMAN	138	2	13.80	14.25
sp Q9NQ38 ISK5_HUMAN	1064	2	1.60	120.71
tr A0A075B6N7 A0A075B6N7_HUMAN	340	2	7.10	36.59
sp P01042 KNG1_HUMAN	427	2	8.20	47.85
sp P01876 IGHA1_HUMAN	494	2	9.50	53.09
sp P80362 KV125_HUMAN;sp P01610 KV118_HUMAN;sp P01600 KV108_HUMAN	117	2	42.10	12.85
sp Q5T2W1 NHRF3_HUMAN	330	1	4.80	36.67
sp O00584 RNT2_HUMAN	218	1	9.60	25.31
sp P00558 PGK1_HUMAN	161	1	6.80	17.05
sp P00734 THRB_HUMAN	324	1	5.90	35.93
sp P00751 CFAB_HUMAN	122	1	19.70	13.29
sp P01593 KV101_HUMAN	108	1	11.10	11.99
sp P01602 KV110_HUMAN	117	1	13.70	12.77
sp P01613 KV121_HUMAN	108	1	14.80	11.84
sp P01620 KV302_HUMAN	109	1	20.20	11.78
sp P01621 KV303_HUMAN	100	1	9.00	10.73
sp P01625 KV402_HUMAN	114	1	15.80	12.64
sp P01714 LV301_HUMAN	108	1	16.70	11.39
sp P05386 RLA1_HUMAN	113	1	28.30	11.40
sp P06310 KV206_HUMAN	86	1	30.20	9.76
sp P08493 MGP_HUMAN	103	1	10.70	12.35
sp P09211 GSTP1_HUMAN	52	1	25.00	5.82
sp P11021 GRP78_HUMAN	654	1	2.80	72.33
sp P19878 NCF2_HUMAN;tr Q59F14 Q59F14_HUMAN	143	1	9.10	16.25
sp P19961 AMY2B_HUMAN;sp P04746 AMYP_HUMAN	511	1	4.70	57.71
sp P35326 SPR2A_HUMAN;sp P35325 SPR2B_HUMAN;sp P22532 SPR2D_HUMAN;sp P22531 SPR2E_HUMAN;sp Q9BYE4 SPR2G_HUMAN	72	1	29.20	7.97
sp P22681 CBL_HUMAN	906	1	3.30	99.63
sp P23528 COF1_HUMAN	91	1	13.20	10.18
sp P27797 CALR_HUMAN	163	1	17.80	19.03
sp P29508 SPB3_HUMAN	390	1	3.60	44.65
sp P30740 ILEU_HUMAN	341	1	4.40	38.69
sp P58062 ISK7_HUMAN	59	1	15.30	6.56
sp P60709 ACTB_HUMAN	351	1	4.80	39.23
sp P62158 CALM_HUMAN	65	1	46.20	7.37
sp P63261 ACTG_HUMAN;sp P63267 ACTH_HUMAN;sp P68133 ACTS_HUMAN;sp P68032 ACTC_HUMAN;sp P62736 ACTA_HUMAN	333	1	5.10	37.35
sp Q00765 REEP5_HUMAN	99	1	11.10	10.58
sp Q08188 TGM3_HUMAN	693	1	2.30	76.63
sp Q14515 SPRL1_HUMAN	490	1	3.30	55.52
sp Q562R1 ACTBL_HUMAN	376	1	4.80	42.00
sp Q6UWE0 LRSM1_HUMAN	723	1	1.70	83.59
sp Q7Z6J0 SH3R1_HUMAN	888	1	1.60	93.13
sp Q8N4F0 BPIB2_HUMAN	433	1	3.70	46.38
sp Q8NGH3 OR2D3_HUMAN	330	1	9.40	37.49
sp Q8NHH9 ATLA2_HUMAN	102	1	11.80	11.35
sp Q96MG8 PCMD1_HUMAN	130	1	8.50	14.39

sp Q99575 POP1_HUMAN	1024	1	0.70	114.68
sp Q9BRF8 CPPED_HUMAN	314	1	3.50	35.55
sp Q9Y4I5 MTL5_HUMAN	204	1	12.30	22.77
tr Q5NV69 Q5NV69_HUMAN	100	1	30.00	10.34
sp P01777 HV316_HUMAN	101	1	16.80	11.17
sp P04433 KV309_HUMAN	233	1	9.00	25.60
sp P01765 HV304_HUMAN;sp P01779 HV318_HUMAN;sp P01776 HV315_HUMAN;sp P01774 HV313_HUMAN	238	1	8.00	25.16
sp P01717 LV403_HUMAN	96	1	19.80	10.35
tr B1N7B8 B1N7B8_HUMAN	107	1	22.40	11.80
tr B4DRV8 B4DRV8_HUMAN	204	1	5.90	21.40
tr B4E1S8 B4E1S8_HUMAN	155	1	20.00	17.35
tr L8E9Y3 L8E9Y3_HUMAN	60	1	30.00	7.14
tr Q6ZW64 Q6ZW64_HUMAN	494	1	6.50	53.32
tr Q8N355 Q8N355_HUMAN	234	1	4.70	24.79
tr Q9UL88 Q9UL88_HUMAN	131	1	14.50	14.14
sp P01623 KV305_HUMAN;sp P04206 KV307_HUMAN;sp P01622 KV304_HUMAN	109	0	0.00	11.75
tr Q6N096 Q6N096_HUMAN	466	0	0.00	50.93
sp P01834 IGKC_HUMAN	231	0	0.00	25.14
tr A0A5E4 A0A5E4_HUMAN	235	0	0.00	24.75
tr A0N5G5 A0N5G5_HUMAN	118	0	0.00	12.77
sp P01766 HV305_HUMAN	118	0	0.00	12.84
sp P01781 HV320_HUMAN	479	0	0.00	51.64
tr Q6MZX9 Q6MZX9_HUMAN	483	0	0.00	52.24
tr Q6NS95 Q6NS95_HUMAN	234	0	0.00	25.15
tr Q6P5S8 Q6P5S8_HUMAN	236	0	0.00	25.77
tr V9HW34 V9HW34_HUMAN	235	0	0.00	25.65
sp P04208 LV106_HUMAN;sp P01700 LV102_HUMAN	235	0	0.00	24.82
tr Q9UL78 Q9UL78_HUMAN	109	0	0.00	11.65

References

1. Mullins RJ. Paediatric food allergy trends in a community-based specialist allergy practice, 1995-2006. *The Medical journal of Australia*. 2007 Jun 18;186(12):618-21.
2. Prescott SL. Allergic disease: understanding how in utero events set the scene. *The Proceedings of the Nutrition Society*. 2010 Aug;69(3):366-72. DOI: 10.1017/s0029665110001874.
3. Prescott SL, Clifton V. Asthma and pregnancy: emerging evidence of epigenetic interactions in utero. *Current Opinion in Allergy and Clinical Immunology*. 2009;9(5):417-26. DOI: 10.1097/ACI.0b013e328330634f.
4. Martino D, Prescott S. Epigenetics and prenatal influences on asthma and allergic airways disease. *Chest*. 2011 Mar;139(3):640-7. DOI: 10.1378/chest.10-1800.
5. Dietert RR. Maternal and childhood asthma: Risk factors, interactions, and ramifications. *Reproductive toxicology*. 2011 9//;32(2):198-204. DOI: <http://dx.doi.org/10.1016/j.reprotox.2011.04.007>.
6. Eckl-Dorna J, Niederberger V. What is the source of serum allergen-specific IgE? *Current allergy and asthma reports*. 2013 Jun;13(3):281-7. DOI: 10.1007/s11882-013-0348-x.
7. Barker DJ. In utero programming of chronic disease. *Clin Sci*. 1998 1998/08//;95(2):115-28.
8. Kumar R, Story RE, Pongracic JA, Hong X, Arguelles L, Wang G, et al. Maternal Pre-Pregnancy Obesity and Recurrent Wheezing in Early Childhood. *Pediatr Allergy Immunol Pulmonol*. 2010 Sep;23(3):183-90. DOI: 10.1089/ped.2010.0032.
9. Duijts L. Fetal and infant origins of asthma. *Eur J Epidemiol*. 2012 Jan;27(1):5-14. DOI: 10.1007/s10654-012-9657-y.
10. Sternthal MJ, Coull BA, Chiu YH, Cohen S, Wright RJ. Associations among maternal childhood socioeconomic status, cord blood IgE levels, and repeated wheeze in urban children. *The Journal of allergy and clinical immunology*. 2011 Aug;128(2):337-45 e1. DOI: 10.1016/j.jaci.2011.05.008.
11. Schmidt-Weber CB, Akdis M, Akdis CA. TH17 cells in the big picture of immunology. *The Journal of allergy and clinical immunology*. 2007 Aug;120(2):247-54. DOI: 10.1016/j.jaci.2007.06.039.
12. Scott NM, Hodyl NA, Osei-Kumah A, Stark MJ, Smith R, Clifton VL. The presence of maternal asthma during pregnancy suppresses the placental pro-inflammatory response to an immune challenge in vitro. *Placenta*. 2011 6//;32(6):454-61. DOI: <http://dx.doi.org/10.1016/j.placenta.2011.03.004>.
13. Scott NM, Hodyl NA, Murphy VE, Osei-Kumah A, Wyper H, Hodgson DM, et al. Placental cytokine expression covaries with maternal asthma severity and fetal sex. *J Immunol*. 2009 Feb 1;182(3):1411-20.
14. Gude NM, Roberts CT, Kalionis B, King RG. Growth and function of the normal human placenta. *Thromb Res*. 2004;114(5-6):397-407. DOI: 10.1016/j.thromres.2004.06.038.
15. Prescott SL, Tulic M, Kumah AO, Richman T, Crook M, Martino D, et al. Reduced placental FOXP3 associated with subsequent infant allergic disease. *The Journal of allergy and clinical immunology*. 2011 Oct;128(4):886-7.e5. DOI: 10.1016/j.jaci.2011.05.017.
16. Hernandez E, Barraza-Villarreal A, Escamilla-Nunez MC, Hernandez-Cadena L, Sly PD, Neufeld LM, et al. Prenatal determinants of cord blood total immunoglobulin E levels in Mexican newborns. *Allergy and asthma proceedings : the official journal of regional and state allergy societies*. 2013 Sep-Oct;34(5):e27-34. DOI: 10.2500/aap.2013.34.3688.
17. Martin-Gronert MS, Ozanne SE. Mechanisms underlying the developmental origins of disease. *Reviews in endocrine & metabolic disorders*. 2012 Jun;13(2):85-92. DOI: 10.1007/s11154-012-9210-z.
18. Lucas A. Programming by early nutrition in man. *Ciba Foundation symposium*. 1991;156:38-50; discussion -5.

19. Gluckman PD, Hanson MA. Developmental origins of disease paradigm: a mechanistic and evolutionary perspective. *Pediatric research*. 2004 Sep;56(3):311-7. DOI: 10.1203/01.pdr.0000135998.08025.fb.
20. Turner S. Perinatal programming of childhood asthma: early fetal size, growth trajectory during infancy, and childhood asthma outcomes. *Clinical & developmental immunology*. 2012;2012:962923. DOI: 10.1155/2012/962923.
21. Villamor E, Iliadou A, Cnattingius S. Is the association between low birth weight and asthma independent of genetic and shared environmental factors? *American journal of epidemiology*. 2009 Jun 1;169(11):1337-43. DOI: 10.1093/aje/kwp054.
22. Muraro A, Dreborg S, Halken S, Host A, Niggemann B, Aalberse R, et al. Dietary prevention of allergic diseases in infants and small children. Part II. Evaluation of methods in allergy prevention studies and sensitization markers. Definitions and diagnostic criteria of allergic diseases. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2004 Jun;15(3):196-205. DOI: 10.1111/j.1399-3038.2004.00128.x.
23. Dictionary M. Medical Dictionary. 2013 [17/10/13]. Available from: <http://medical-dictionary.thefreedictionary.com/hypersensitivity>
24. Mackenzie KJ, Anderton SM, Schwarze J. Peptide immunotherapy for childhood allergy - addressing translational challenges. *Clinical and translational allergy*. 2011;1(1):13. DOI: 10.1186/2045-7022-1-13.
25. Chini L, Monteferrario E, Graziani S, Moschese V. Novel treatments of asthma and allergic diseases. *Paediatric respiratory reviews*. 2013 Nov 5. DOI: 10.1016/j.prrv.2013.10.007.
26. Henderson AJ, Warner JO. Fetal origins of asthma. *Seminars in fetal & neonatal medicine*. 2012 Apr;17(2):82-91. DOI: 10.1016/j.siny.2012.01.006.
27. Mantzouranis E, Papadopoulou E, Michailidi E. Childhood asthma: recent developments and update. *Current opinion in pulmonary medicine*. 2014 Jan;20(1):8-16. DOI: 10.1097/mcp.0000000000000014.
28. Galli SJ, Tsai M. IgE and mast cells in allergic disease. *Nature medicine*. 2012 May;18(5):693-704. DOI: 10.1038/nm.2755.
29. Saunes M, Oien T, Dotterud CK, Romundstad PR, Storro O, Holmen TL, et al. Early eczema and the risk of childhood asthma: a prospective, population-based study. *BMC pediatrics*. 2012;12:168. DOI: 10.1186/1471-2431-12-168.
30. Izquierdo-Dominguez A, Valero AL, Mullol J. Comparative analysis of allergic rhinitis in children and adults. *Current allergy and asthma reports*. 2013 Apr;13(2):142-51. DOI: 10.1007/s11882-012-0331-y.
31. Simons FE. Anaphylaxis. *The Journal of allergy and clinical immunology*. 2010 Feb;125(2 Suppl 2):S161-81. DOI: 10.1016/j.jaci.2009.12.981.
32. Simons FE, Arduso LR, Dimov V, Ebisawa M, El-Gamal YM, Lockey RF, et al. World Allergy Organization Anaphylaxis Guidelines: 2013 update of the evidence base. *International archives of allergy and immunology*. 2013;162(3):193-204. DOI: 10.1159/000354543.
33. David Male JB, David B Zroth and Ivan M Roitt. *Immunology*. Eight ed: Elsevier Saunders; 2013.
34. Romagnani S. Immunologic influences on allergy and the TH1/TH2 balance. *The Journal of allergy and clinical immunology*. 2004 Mar;113(3):395-400. DOI: 10.1016/j.jaci.2003.11.025.
35. McFadden JP, Thyssen JP, Basketter DA, Puangpet P, Kimber I. T helper cell 2 immune skewing in pregnancy/early life: chemical exposure and the development of atopic disease and allergy. *Br J Dermatol*. 2015 Mar;172(3):584-91. DOI: 10.1111/bjd.13497.
36. Grieger JA, Clifton VL, Tuck AR, Wooldridge AL, Robertson SA, Gatford KL. In utero Programming of Allergic Susceptibility. *International archives of allergy and immunology*. 2016;169(2):80-92. DOI: 10.1159/000443961.
37. Kramer A, Bekeschus S, Broker BM, Schleibinger H, Razavi B, Assadian O. Maintaining health by balancing microbial exposure and prevention of infection: the hygiene hypothesis versus

- the hypothesis of early immune challenge. *The Journal of hospital infection*. 2013 Feb;83 Suppl 1:S29-34. DOI: 10.1016/s0195-6701(13)60007-9.
38. Tsilochristou OA, Douladiris N, Makris M, Papadopoulos NG. Pediatric allergic rhinitis and asthma: can the march be halted? *Paediatric drugs*. 2013 Dec;15(6):431-40. DOI: 10.1007/s40272-013-0043-3.
 39. Cetinkaya F, Atalay OO. Effects of wheezing in early childhood in the development of allergic rhinitis in later years. *Asia Pacific allergy*. 2014 Jan;4(1):37-41. DOI: 10.5415/apallergy.2014.4.1.37.
 40. Murray CS, Woodcock A, Langley SJ, Morris J, Custovic A. Secondary prevention of asthma by the use of Inhaled Fluticasone propionate in Wheezy INfants (IFWIN): double-blind, randomised, controlled study. *Lancet*. 2006 Aug 26;368(9537):754-62. DOI: 10.1016/s0140-6736(06)69285-4.
 41. Georgy MS, Saltoun CA. Chapter 3: Allergen immunotherapy: definition, indication, and reactions. *Allergy and asthma proceedings : the official journal of regional and state allergy societies*. 2012 May-Jun;33 Suppl 1:S9-11. DOI: 10.2500/aap.2012.33.3533.
 42. Gupta K, Kumar S, Das M, Dwivedi PD. Peptide based immunotherapy: A pivotal tool for allergy treatment. *International immunopharmacology*. 2014 Feb 12. DOI: 10.1016/j.intimp.2014.01.030.
 43. Hargreave FE, Nair P. The definition and diagnosis of asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2009 Nov;39(11):1652-8. DOI: 10.1111/j.1365-2222.2009.03321.x.
 44. Yayan J, Rasche K. Asthma and COPD: Similarities and Differences in the Pathophysiology, Diagnosis and Therapy. *Advances in experimental medicine and biology*. 2016;910:31-8. DOI: 10.1007/5584_2015_206.
 45. Wright RJ. Prenatal maternal stress and early caregiving experiences: implications for childhood asthma risk. *Paediatric and perinatal epidemiology*. 2007 Nov;21 Suppl 3:8-14. DOI: 10.1111/j.1365-3016.2007.00879.x.
 46. Postma DS, Kerstjens HA. Characteristics of airway hyperresponsiveness in asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 1998 Nov;158(5 Pt 3):S187-92. DOI: 10.1164/ajrccm.158.supplement_2.13tac170.
 47. Aoshiba K, Nagai A. Differences in airway remodeling between asthma and chronic obstructive pulmonary disease. *Clin Rev Allergy Immunol*. 2004 Aug;27(1):35-43. DOI: 10.1385/crai:27:1:035.
 48. Gorska K, Krenke R, Kosciuch J, Korczynski P, Zukowska M, Domagala-Kulawik J, et al. Relationship between airway inflammation and remodeling in patients with asthma and chronic obstructive pulmonary disease. *Eur J Med Res*. 2009 Dec 7;14 Suppl 4:90-6.
 49. Holgate ST. Pathogenesis of asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2008 Jun;38(6):872-97. DOI: 10.1111/j.1365-2222.2008.02971.x.
 50. Arshad SH, Bateman B, Matthews SM. Primary prevention of asthma and atopy during childhood by allergen avoidance in infancy: a randomised controlled study. *Thorax*. 2003 Jun;58(6):489-93.
 51. van Schayck OC, Maas T, Kaper J, Knottnerus AJ, Sheikh A. Is there any role for allergen avoidance in the primary prevention of childhood asthma? *The Journal of allergy and clinical immunology*. 2007 Jun;119(6):1323-8. DOI: 10.1016/j.jaci.2007.02.024.
 52. Romano C. Omalizumab therapy for children and adolescents with severe allergic asthma. *Expert Rev Clin Immunol*. 2015;11(12):1309-19. DOI: 10.1586/1744666X.2015.1083860.
 53. Bieber T. Atopic dermatitis. *The New England journal of medicine*. 2008 Apr 3;358(14):1483-94. DOI: 10.1056/NEJMr074081.
 54. Weidinger S, Novak N. Atopic dermatitis. *The Lancet*. 2016;387(10023):1109-22. DOI: 10.1016/s0140-6736(15)00149-x.
 55. de Bruin Weller MS, Knulst AC, Meijer Y, Bruijnzeel-Koomen CA, Pasmans SG. Evaluation of the child with atopic dermatitis. *Clinical and experimental allergy : journal of the British Society*

- for Allergy and Clinical Immunology. 2012 Mar;42(3):352-62. DOI: 10.1111/j.1365-2222.2011.03899.x.
56. Holm T, Rutishauser D, Kai-Larsen Y, Lyutvinskiy Y, Stenius F, Zubarev RA, et al. Protein biomarkers in vernix with potential to predict the development of atopic eczema in early childhood. *Allergy*. 2013 Nov 11. DOI: 10.1111/all.12308.
57. Miyake Y, Ohya Y, Tanaka K, Yokoyama T, Sasaki S, Fukushima W, et al. Home environment and suspected atopic eczema in Japanese infants: the Osaka Maternal and Child Health Study. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2007 Aug;18(5):425-32. DOI: 10.1111/j.1399-3038.2007.00545.x.
58. Flohr C, Mann J. New approaches to the prevention of childhood atopic dermatitis. *Allergy*. 2013 Dec 26. DOI: 10.1111/all.12343.
59. Biagini Myers JM, Wang N, LeMasters GK, Bernstein DI, Epstein TG, Lindsey MA, et al. Genetic and environmental risk factors for childhood eczema development and allergic sensitization in the CCAAPS cohort. *The Journal of investigative dermatology*. 2010 Feb;130(2):430-7. DOI: 10.1038/jid.2009.300.
60. Baek JO, Hong S, Son DK, Lee JR, Roh JY, Kwon HJ. Analysis of the prevalence of and risk factors for atopic dermatitis using an ISAAC questionnaire in 8,750 Korean children. *International archives of allergy and immunology*. 2013;162(1):79-85. DOI: 10.1159/000351403.
61. Flohr C, Mann J. New insights into the epidemiology of childhood atopic dermatitis. *Allergy*. 2013 Nov 21. DOI: 10.1111/all.12270.
62. Sugiura H, Ebise H, Tazawa T, Tanaka K, Sugiura Y, Uehara M, et al. Large-scale DNA microarray analysis of atopic skin lesions shows overexpression of an epidermal differentiation gene cluster in the alternative pathway and lack of protective gene expression in the cornified envelope. *Br J Dermatol*. 2005 Jan;152(1):146-9. DOI: 10.1111/j.1365-2133.2005.06352.x.
63. De Benedetto A, Agnihothri R, McGirt LY, Bankova LG, Beck LA. Atopic dermatitis: a disease caused by innate immune defects? *The Journal of investigative dermatology*. 2009 Jan;129(1):14-30. DOI: 10.1038/jid.2008.259.
64. De Benedetto A, Kubo A, Beck LA. Skin barrier disruption: a requirement for allergen sensitization? *The Journal of investigative dermatology*. 2012 Mar;132(3 Pt 2):949-63. DOI: 10.1038/jid.2011.435.
65. McAleer MA, Irvine AD. The multifunctional role of filaggrin in allergic skin disease. *The Journal of allergy and clinical immunology*. 2013 Feb;131(2):280-91. DOI: 10.1016/j.jaci.2012.12.668.
66. Jarzab J, Filipowska B, Zebracka J, Kowalska M, Istrok, gorzata, et al. Locus 1q21 Gene Expression Changes in Atopic Dermatitis Skin Lesions: Dereglulation of Small Proline-Rich Region 1A. *International archives of allergy and immunology*. 2010;151(1):28-37. DOI: 10.1159/000232568.
67. Howell MD, Kim BE, Gao P, Grant AV, Boguniewicz M, DeBenedetto A, et al. Cytokine modulation of atopic dermatitis filaggrin skin expression. *The Journal of allergy and clinical immunology*. 2009 Sep;124(3 Suppl 2):R7-R12. DOI: 10.1016/j.jaci.2009.07.012.
68. Stein SL, Cifu AS. Management of Atopic Dermatitis. *JAMA*. 2016 Apr 12;315(14):1510-1. DOI: 10.1001/jama.2016.1459.
69. Seidman MD, Gurgel RK, Lin SY, Schwartz SR, Baroody FM, Bonner JR, et al. Clinical practice guideline: Allergic rhinitis. *Otolaryngol Head Neck Surg*. 2015 Feb;152(1 Suppl):S1-43. DOI: 10.1177/0194599814561600.
70. Tomazic PV, Birner-Gruenberger R, Leitner A, Obrist B, Spoerk S, Lang-Loidolt D. Nasal mucus proteomic changes reflect altered immune responses and epithelial permeability in patients with allergic rhinitis. *The Journal of allergy and clinical immunology*. 2014 Mar;133(3):741-50. DOI: 10.1016/j.jaci.2013.09.040.
71. Rimmer J, Santos C, Yli-Panula E, Noronha V, Viander M. Clinical and laboratory studies of the fate of intranasal allergen. *PloS one*. 2015;10(5):e0127477. DOI: 10.1371/journal.pone.0127477.

72. Nurmatov U, van Schayck CP, Hurwitz B, Sheikh A. House dust mite avoidance measures for perennial allergic rhinitis: an updated Cochrane systematic review. *Allergy*. 2012 Feb;67(2):158-65. DOI: 10.1111/j.1398-9995.2011.02752.x.
73. Roberts G, Xatzipsalti M, Borrego LM, Custovic A, Halken S, Hellings PW, et al. Paediatric rhinitis: position paper of the European Academy of Allergy and Clinical Immunology. *Allergy*. 2013 Sep;68(9):1102-16. DOI: 10.1111/all.12235.
74. Calamelli E, Ricci G, Pession A. Recent advances in diagnosis and therapy of allergic rhinitis and asthma in childhood. *European annals of allergy and clinical immunology*. 2012 Dec;44(6):215-24.
75. Okubo K, Kurono Y, Fujieda S, Ogino S, Uchio E, Odajima H, et al. Japanese guideline for allergic rhinitis. *Allergology international : official journal of the Japanese Society of Allergology*. 2011 Mar;60(2):171-89. DOI: 10.2332/allergolint.11-RAI-0334.
76. Dyer AA, Gupta R. Epidemiology of childhood food allergy. *Pediatric annals*. 2013 Jun 1;42(6):91-5. DOI: 10.3928/00904481-20130522-08.
77. Santos AF, Lack G. Food allergy and anaphylaxis in pediatrics: update 2010-2012. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2012 Dec;23(8):698-706. DOI: 10.1111/pai.12025.
78. Prescott S, Allen KJ. Food allergy: riding the second wave of the allergy epidemic. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2011 Mar;22(2):155-60. DOI: 10.1111/j.1399-3038.2011.01145.x.
79. Kandyil RM, Davis CM. Shellfish allergy in children. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2009 Aug;20(5):408-14; quiz 14. DOI: 10.1111/j.1399-3038.2009.00925.x.
80. Maslova E, Granstrom C, Hansen S, Petersen SB, Strom M, Willett WC, et al. Peanut and tree nut consumption during pregnancy and allergic disease in children-should mothers decrease their intake? Longitudinal evidence from the Danish National Birth Cohort. *The Journal of allergy and clinical immunology*. 2012 Sep;130(3):724-32. DOI: 10.1016/j.jaci.2012.05.014.
81. Nicolaou N, Tsabouri S, Priftis KN. Reintroduction of Cow's Milk in Milk-Allergic Children. *Endocrine, metabolic & immune disorders drug targets*. 2014 Jan 21.
82. Jarvinen KM, Konstantinou GN, Pilapil M, Arrieta MC, Noone S, Sampson HA, et al. Intestinal permeability in children with food allergy on specific elimination diets. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2013 Sep;24(6):589-95. DOI: 10.1111/pai.12106.
83. Perrier C, Corthesy B. Gut permeability and food allergies. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2011 Jan;41(1):20-8. DOI: 10.1111/j.1365-2222.2010.03639.x.
84. Kalach N, Rocchiccioli F, de Boissieu D, Benhamou PH, Dupont C. Intestinal permeability in children: variation with age and reliability in the diagnosis of cow's milk allergy. *Acta paediatrica (Oslo, Norway : 1992)*. 2001 May;90(5):499-504.
85. Du Toit G, Lack G. Can food allergy be prevented? The current evidence. *Pediatric clinics of North America*. 2011 Apr;58(2):481-509, xii. DOI: 10.1016/j.pcl.2011.02.002.
86. Kattan J. The Prevalence and Natural History of Food Allergy. *Current allergy and asthma reports*. 2016 Jul;16(7):47. DOI: 10.1007/s11882-016-0627-4.
87. Sampson HA, Munoz-Furlong A, Campbell RL, Adkinson NF, Jr., Bock SA, Branum A, et al. Second symposium on the definition and management of anaphylaxis: summary report--Second National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network symposium. *The Journal of allergy and clinical immunology*. 2006 Feb;117(2):391-7. DOI: 10.1016/j.jaci.2005.12.1303.
88. Wolbing F, Fischer J, Koberle M, Kaesler S, Biedermann T. About the role and underlying mechanisms of cofactors in anaphylaxis. *Allergy*. 2013 Sep;68(9):1085-92. DOI: 10.1111/all.12193.
89. Ring J, Behrendt H, de Weck A. History and classification of anaphylaxis. *Chemical immunology and allergy*. 2010;95:1-11. DOI: 10.1159/000315934.

90. De Bisschop MB, Bellou A. Anaphylaxis. *Current opinion in critical care*. 2012 Aug;18(4):308-17. DOI: 10.1097/MCC.0b013e3283557a63.
91. Aysenur K, Akan A, Mustafa E, Muge T, Kocabas CN. Anaphylaxis induced by lentil inhalation. *Asian Pacific journal of allergy and immunology / launched by the Allergy and Immunology Society of Thailand*. 2012 Jun;30(2):167-9.
92. de Silva IL, Mehr SS, Tey D, Tang ML. Paediatric anaphylaxis: a 5 year retrospective review. *Allergy*. 2008 Aug;63(8):1071-6. DOI: 10.1111/j.1398-9995.2008.01719.x.
93. Simons FE. Anaphylaxis pathogenesis and treatment. *Allergy*. 2011 Jul;66 Suppl 95:31-4. DOI: 10.1111/j.1398-9995.2011.02629.x.
94. Lee JK, Vadas P. Anaphylaxis: mechanisms and management. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2011 Jul;41(7):923-38. DOI: 10.1111/j.1365-2222.2011.03779.x.
95. Osborn DA, Sinn JK. Probiotics in infants for prevention of allergic disease and food hypersensitivity. *Cochrane Database Syst Rev*. 2007 Oct 17(4):CD006475. DOI: 10.1002/14651858.CD006475.pub2.
96. Cuello-Garcia CA, Brozek JL, Fiocchi A, Pawankar R, Yepes-Nunez JJ, Terracciano L, et al. Probiotics for the prevention of allergy: A systematic review and meta-analysis of randomized controlled trials. *The Journal of allergy and clinical immunology*. 2015 Oct;136(4):952-61. DOI: 10.1016/j.jaci.2015.04.031.
97. Kuitunen M, Kukkonen K, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, et al. Probiotics prevent IgE-associated allergy until age 5 years in cesarean-delivered children but not in the total cohort. *The Journal of allergy and clinical immunology*. 2009 Feb;123(2):335-41. DOI: 10.1016/j.jaci.2008.11.019.
98. Fleischer DM, Sicherer S, Greenhawt M, Campbell D, Chan E, Muraro A, et al. Consensus communication on early peanut introduction and the prevention of peanut allergy in high-risk infants. *The Journal of allergy and clinical immunology*. 2015 Aug;136(2):258-61. DOI: 10.1016/j.jaci.2015.06.001.
99. Du Toit G, Roberts G, Sayre PH, Bahnson HT, Radulovic S, Santos AF, et al. Randomized trial of peanut consumption in infants at risk for peanut allergy. *The New England journal of medicine*. 2015 Feb 26;372(9):803-13. DOI: 10.1056/NEJMoa1414850.
100. Allen KJ, Koplin JJ. Does LEAP change the screening paradigm for food allergy in infants with eczema? *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2016 Jan;46(1):42-7. DOI: 10.1111/cea.12685.
101. Natsume O, Kabashima S, Nakazato J, Yamamoto-Hanada K, Narita M, Kondo M, et al. Two-step egg introduction for prevention of egg allergy in high-risk infants with eczema (PETIT): a randomised, double-blind, placebo-controlled trial. *The Lancet*. 2017;389(10066):276-86. DOI: 10.1016/s0140-6736(16)31418-0.
102. Waterland RA, Michels KB. Epigenetic epidemiology of the developmental origins hypothesis. *Annu Rev Nutr*. 2007 2007;27:363-88. DOI: 10.1146/annurev.nutr.27.061406.093705.
103. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet*. 1986 May 10;1(8489):1077-81.
104. Ozanne SE, Constancia M. Mechanisms of disease: the developmental origins of disease and the role of the epigenotype. *Nature clinical practice Endocrinology & metabolism*. 2007 Jul;3(7):539-46. DOI: 10.1038/ncpendmet0531.
105. Rinaudo P, Wang E. Fetal programming and metabolic syndrome. *Annual review of physiology*. 2012;74:107-30. DOI: 10.1146/annurev-physiol-020911-153245.
106. Krauss-Etschmann S, Bush A, Bellusci S, Brusselle GG, Dahlen SE, Dehmel S, et al. Of flies, mice and men: a systematic approach to understanding the early life origins of chronic lung disease. *Thorax*. 2013 Apr;68(4):380-4. DOI: 10.1136/thoraxjnl-2012-201902.
107. Tain YL, Hsu CN. Developmental Origins of Chronic Kidney Disease: Should We Focus on Early Life? *Int J Mol Sci*. 2017 Feb 11;18(2). DOI: 10.3390/ijms18020381.

108. Hershkovitz D, Burbea Z, Skorecki K, Brenner BM. Fetal programming of adult kidney disease: cellular and molecular mechanisms. *Clin J Am Soc Nephrol*. 2007 Mar;2(2):334-42. DOI: 10.2215/CJN.03291006.
109. Illi S, Weber J, Zutavern A, Genuneit J, Schierl R, Strunz-Lehner C, et al. Perinatal influences on the development of asthma and atopy in childhood. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology*. 2014 Feb;112(2):132-9.e1. DOI: 10.1016/j.anai.2013.11.019.
110. Joss-Moore LA, Albertine KH, Lane RH. Epigenetics and the developmental origins of lung disease. *Mol Genet Metab*. 2011 Sep-Oct;104(1-2):61-6. DOI: 10.1016/j.ymgme.2011.07.018.
111. Tezza G, Mazzei F, Boner A. Epigenetics of allergy. *Early Human Development*. 2013;89:S20-S1. DOI: 10.1016/s0378-3782(13)70007-0.
112. Chetty A, Andersson S, Lassus P, Nielsen HC. Insulin-like growth factor-1 (IGF-1) and IGF-1 receptor (IGF-1R) expression in human lung in RDS and BPD. *Pediatric pulmonology*. 2004 Feb;37(2):128-36. DOI: 10.1002/ppul.10415.
113. Prescott S, Saffery R. The role of epigenetic dysregulation in the epidemic of allergic disease. *Clinical epigenetics*. 2011 Aug;2(2):223-32. DOI: 10.1007/s13148-011-0028-4.
114. Miller RL, Ho S-M. Environmental epigenetics and asthma: current concepts and call for studies. *Am J Respir Crit Care Med*. 2008 2008/03/15;177(6):567-73. DOI: 10.1164/rccm.200710-1511PP.
115. Joss-Moore LA, Lane RH. Epigenetics and the developmental origins of disease: the key to unlocking the door of personalized medicine. *Epigenomics*. 2012 Oct;4(5):471-3. DOI: 10.2217/epi.12.53.
116. Pastor-Vargas C, Maroto AS, Diaz-Perales A, Villalba M, Esteban V, Ruiz-Ramos M, et al. Detection of major food allergens in amniotic fluid: initial allergenic encounter during pregnancy. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2016 Jun 24. DOI: 10.1111/pai.12608.
117. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proceedings of the National Academy of Sciences of the United States of America*. 2008 Nov 4;105(44):17046-9. DOI: 10.1073/pnas.0806560105.
118. Curtin JA, Simpson A, Belgrave D, Semic-Jusufagic A, Custovic A, Martinez FD. Methylation of IL-2 promoter at birth alters the risk of asthma exacerbations during childhood. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2013 Mar;43(3):304-11. DOI: 10.1111/cea.12046.
119. Saunders. *Dorland's Medical Dictionary for Health Consumers*. Elsevier; 2007. Available from: <http://medical-dictionary.thefreedictionary.com/hemochorial+placentation>
120. Aplin JD. Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence in vivo and in vitro. *Journal of cell science*. 1991 Aug;99 (Pt 4):681-92.
121. Hoffmann P, Saoudi Y, Benharouga M, Graham CH, Schaal JP, Mazouni C, et al. Role of EG-VEGF in human placentation: Physiological and pathological implications. *Journal of cellular and molecular medicine*. 2009 Aug;13(8B):2224-35. DOI: 10.1111/j.1582-4934.2008.00554.x.
122. Merriam-Webster IS. *Merriam-Webster's Medical Dictionary* Merriam-Webster, Incorporated; 2006.
123. Hill MA. Placental membranes.jpg. 2013 [cited 2013 November 7]. Available from: http://embryology.med.unsw.edu.au/embryology/index.php?title=Placental_membranes.jpg
124. Longtine MS, Nelson DM. Placental dysfunction and fetal programming: the importance of placental size, shape, histopathology, and molecular composition. *Semin Reprod Med*. 2011 May;29(3):187-96. DOI: 10.1055/s-0031-1275515.
125. Reynolds LP, Borowicz PP, Caton JS, Vonnahme KA, Luther JS, Buchanan DS, et al. Uteroplacental vascular development and placental function: an update. *The International journal of developmental biology*. 2010;54(2-3):355-66. DOI: 10.1387/ijdb.082799lr.

126. Syme MR, Paxton JW, Keelan JA. Drug transfer and metabolism by the human placenta. *Clin Pharmacokinet*. 2004;43(8):487-514. DOI: 10.2165/00003088-200443080-00001.
127. Joubert BR, Haberg SE, Nilsen RM, Wang X, Vollset SE, Murphy SK, et al. 450K epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. *Environmental health perspectives*. 2012 Oct;120(10):1425-31. DOI: 10.1289/ehp.1205412.
128. Wang M, Ramirez J, Han J, Jia Y, Domenico J, Seibold MA, et al. The steroidogenic enzyme Cyp11a1 is essential for development of peanut-induced intestinal anaphylaxis. *The Journal of allergy and clinical immunology*. 2013 Nov;132(5):1174-83 e8. DOI: 10.1016/j.jaci.2013.05.027.
129. Lee SL, Lam TH, Leung TH, Wong WH, Schooling M, Leung GM, et al. Foetal exposure to maternal passive smoking is associated with childhood asthma, allergic rhinitis, and eczema. *TheScientificWorldJournal*. 2012;2012:542983. DOI: 10.1100/2012/542983.
130. Neuman A, Hohmann C, Orsini N, Pershagen G, Eller E, Kjaer HF, et al. Maternal smoking in pregnancy and asthma in preschool children: a pooled analysis of eight birth cohorts. *Am J Respir Crit Care Med*. 2012 Nov 15;186(10):1037-43. DOI: 10.1164/rccm.201203-0501OC.
131. Guleria I, Sayegh MH. Maternal Acceptance of the Fetus: True Human Tolerance. *J Immunol*. 2007;178(6):3345-51. DOI: 10.4049/jimmunol.178.6.3345.
132. Tuck A, Grzeskowiak L, Osei-Kumah A, Saif Z, Edwards S, Tai A, et al. Distinct Sex-Specific Gene Expression Changes in the Placenta in Association with Childhood Allergy. *International Journal of Respiratory and Pulmonary Medicine*. [Open Access]. 2015(2:4).
133. Aiken CE, Ozanne SE. Sex differences in developmental programming models. *Reproduction (Cambridge, England)*. 2013 Jan;145(1):R1-13. DOI: 10.1530/rep-11-0489.
134. Hill MA. Embryology Embryonic Development. 2016 [cited 2016 23/09//2016]. Available from: https://embryology.med.unsw.edu.au/embryology/index.php/Embryonic_Development
135. Warner JA, Warner JO. Early life events in allergic sensitisation. *British medical bulletin*. 2000;56(4):883-93.
136. Fu B, Li X, Sun R, Tong X, Ling B, Tian Z, et al. Natural killer cells promote immune tolerance by regulating inflammatory TH17 cells at the human maternal-fetal interface. *Proceedings of the National Academy of Sciences of the United States of America*. 2013 Jan 15;110(3):E231-40. DOI: 10.1073/pnas.1206322110.
137. Song D, Shi Y. Immune system modifications and feto-maternal immune tolerance. *Chin Med J (Engl)*. 2014;127(17):3171-80.
138. Ait-Azzouzene D, Caucheteux S, Tchang F, Wantyghem J, Moutier R, Langkopf A, et al. Transgenic major histocompatibility complex class I antigen expressed in mouse trophoblast affects maternal immature B cells. *Biology of reproduction*. 2001 Aug;65(2):337-44.
139. Clark DA, Arck PC, Jalali R, Merali FS, Manuel J, Chaouat G, et al. Psycho-neuro-cytokine/endocrine pathways in immunoregulation during pregnancy. *American journal of reproductive immunology (New York, NY : 1989)*. 1996 Apr;35(4):330-7.
140. Hunt JS, Andrews GK, Wood GW. Normal trophoblasts resist induction of class I HLA. *J Immunol*. 1987 Apr 15;138(8):2481-7.
141. Kapasi K, Albert SE, Yie S, Zavazava N, Librach CL. HLA-G has a concentration-dependent effect on the generation of an allo-CTL response. *Immunology*. 2000 Oct;101(2):191-200.
142. Jiang SP, Vacchio MS. Multiple mechanisms of peripheral T cell tolerance to the fetal "allograft". *J Immunol*. 1998 Apr 1;160(7):3086-90.
143. Qiu Q, Yang M, Tsang BK, Gruslin A. Fas ligand expression by maternal decidual cells is negatively correlated with the abundance of leukocytes present at the maternal-fetal interface. *Journal of reproductive immunology*. 2005 Apr;65(2):121-32. DOI: 10.1016/j.jri.2005.01.007.
144. Smith M, Tourigny MR, Noakes P, Thornton CA, Tulic MK, Prescott SL. Children with egg allergy have evidence of reduced neonatal CD4(+)CD25(+)CD127(lo/-) regulatory T cell function. *The Journal of allergy and clinical immunology*. 2008 2008/06//;121(6):1460-6, 6.e1-7. DOI: 10.1016/j.jaci.2008.03.025.

145. Cobbold SP, Adams E, Graca L, Daley S, Yates S, Paterson A, et al. Immune privilege induced by regulatory T cells in transplantation tolerance. *Immunol Rev.* 2006 Oct;213:239-55. DOI: 10.1111/j.1600-065X.2006.00428.x.
146. Sasaki Y, Sakai M, Miyazaki S, Higuma S, Shiozaki A, Saito S. Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. *Mol Hum Reprod.* 2004 May;10(5):347-53. DOI: 10.1093/molehr/gah044.
147. Somerset DA, Zheng Y, Kilby MD, Sansom DM, Drayson MT. Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset. *Immunology.* 2004 May;112(1):38-43. DOI: 10.1111/j.1365-2567.2004.01869.x.
148. Mellor AL, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol.* 2004 Oct;4(10):762-74. DOI: 10.1038/nri1457.
149. Sharpe AH, Abbas AK. T-cell costimulation--biology, therapeutic potential, and challenges. *The New England journal of medicine.* 2006 Sep 7;355(10):973-5. DOI: 10.1056/NEJMp068087.
150. Khoury SJ, Sayegh MH. The roles of the new negative T cell costimulatory pathways in regulating autoimmunity. *Immunity.* 2004 May;20(5):529-38.
151. Jin LP, Zhou YH, Wang MY, Zhu XY, Li DJ. Blockade of CD80 and CD86 at the time of implantation inhibits maternal rejection to the allogeneic fetus in abortion-prone matings. *Journal of reproductive immunology.* 2005 Apr;65(2):133-46. DOI: 10.1016/j.jri.2004.08.009.
152. Holmes CH, Simpson KL. Complement and pregnancy: new insights into the immunobiology of the fetomaternal relationship. *Baillieres Clin Obstet Gynaecol.* 1992 Sep;6(3):439-60.
153. Friedberg SH, Weissman IL. Lymphoid tissue architecture. II. Ontogeny of peripheral T and B cells in mice: evidence against Peyer's patches as the site of generation of B cells. *J Immunol.* 1974 Nov;113(5):1477-92.
154. Haynes BF, Martin ME, Kay HH, Kurtzberg J. Early events in human T cell ontogeny. Phenotypic characterization and immunohistologic localization of T cell precursors in early human fetal tissues. *J Exp Med.* 1988 Sep 1;168(3):1061-80.
155. Hayward AR, Ezer G. Development of lymphocyte populations in the human foetal thymus and spleen. *Clinical and experimental immunology.* 1974 May;17(1):169-78.
156. Kay HE, Doe J, Hockley A. Response of human foetal thymocytes to phytohaemagglutinin (PHA). *Immunology.* 1970 Mar;18(3):393-6.
157. Mold JE, McCune JM. Immunological tolerance during fetal development: from mouse to man. *Adv Immunol.* 2012;115:73-111. DOI: 10.1016/B978-0-12-394299-9.00003-5.
158. Rechavi E, Lev A, Lee YN, Simon AJ, Yinon Y, Lipitz S, et al. Timely and spatially regulated maturation of B and T cell repertoire during human fetal development. *Sci Transl Med.* 2015 Feb 25;7(276):276ra25. DOI: 10.1126/scitranslmed.aaa0072.
159. Brittos T, de Souza WB, Anschau F, Pellanda L. Lipids and leukocytes in newborn umbilical vein blood, birth weight and maternal body mass index. *J Dev Orig Health Dis.* 2016 Aug 30:1-6. DOI: 10.1017/S2040174416000362.
160. Matson AP, Cloutier MM, Dhongade A, Puddington L, Rafti E. Maternal allergy is associated with surface-bound IgE on cord blood basophils. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology.* 2013 Sep;24(6):614-21. DOI: 10.1111/pai.12113.
161. Simister NE, Story CM, Chen HL, Hunt JS. An IgG-transporting Fc receptor expressed in the syncytiotrophoblast of human placenta. *European journal of immunology.* 1996 Jul;26(7):1527-31. DOI: 10.1002/eji.1830260718.
162. Simister NE. Placental transport of immunoglobulin G. *Vaccine.* 2003 Jul 28;21(24):3365-9.
163. Nabavi M, Ghorbani R, Asadi AM, Faranoush M. Factors associated with cord blood IgE levels. *Asian Pacific journal of allergy and immunology / launched by the Allergy and Immunology Society of Thailand.* 2013 Jun;31(2):157-62. DOI: 10.12932/ap0234.31.2.2013.
164. Matson AP, Thrall RS, Rafti E, Lingenheld EG, Puddington L. IgG transmitted from allergic mothers decreases allergic sensitization in breastfed offspring. *Clinical and molecular allergy : CMA.* 2010;8:9. DOI: 10.1186/1476-7961-8-9.

165. de Marco R, Pesce G, Girardi P, Marchetti P, Rava M, Ricci P, et al. Foetal exposure to maternal stressful events increases the risk of having asthma and atopic diseases in childhood. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2012 Dec;23(8):724-9. DOI: 10.1111/j.1399-3038.2012.01346.x.
166. Holloway JA, Warner JO, Vance GH, Diaper ND, Warner JA, Jones CA. Detection of house-dust-mite allergen in amniotic fluid and umbilical-cord blood. *Lancet*. 2000 Dec 2;356(9245):1900-2. DOI: 10.1016/s0140-6736(00)03265-7.
167. Jones AC, Miles EA, Warner JO, Colwell BM, Bryant TN, Warner JA. Fetal peripheral blood mononuclear cell proliferative responses to mitogenic and allergenic stimuli during gestation. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 1996 Aug;7(3):109-16.
168. Warner JA, Miles EA, Jones AC, Quint DJ, Colwell BM, Warner JO. Is deficiency of interferon gamma production by allergen triggered cord blood cells a predictor of atopic eczema? *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 1994 May;24(5):423-30.
169. Martino DJ, Saffery R, Allen KJ, Prescott SL. Epigenetic modifications: mechanisms of disease and biomarkers of food allergy. *Curr Opin Immunol*. 2016 May 21;42:9-15. DOI: 10.1016/j.coi.2016.05.005.
170. Harding R, Maritz G. Maternal and fetal origins of lung disease in adulthood. *Seminars in fetal & neonatal medicine*. 2012 Apr;17(2):67-72. DOI: 10.1016/j.siny.2012.01.005.
171. Prescott SL, Macaubas C, Holt BJ, Smallacombe TB, Loh R, Sly PD, et al. Transplacental Priming of the Human Immune System to Environmental Allergens: Universal Skewing of Initial T Cell Responses Toward the Th2 Cytokine Profile. *J Immunol*. 1998 1998/05/15;160(10):4730-7.
172. Awasthi S, Tripathi P, Ganesh S, Husain N. Association of ADAM33 gene polymorphisms with asthma in Indian children. *J Hum Genet*. 2011 Mar;56(3):188-95. DOI: 10.1038/jhg.2010.157.
173. Kerkhof M, Postma DS, Brunekreef B, Reijmerink NE, Wijga AH, de Jongste JC, et al. Toll-like receptor 2 and 4 genes influence susceptibility to adverse effects of traffic-related air pollution on childhood asthma. *Thorax*. 2010 Aug;65(8):690-7. DOI: 10.1136/thx.2009.119636.
174. Sadeghnejad A, Karmaus W, Arshad SH, Kurukulaaratchy R, Huebner M, Ewart S. IL13 gene polymorphisms modify the effect of exposure to tobacco smoke on persistent wheeze and asthma in childhood, a longitudinal study. *Respiratory research*. 2008;9:2. DOI: 10.1186/1465-9921-9-2.
175. Shaheen SO, Newson RB, Ring SM, Rose-Zerilli MJ, Holloway JW, Henderson AJ. Prenatal and infant acetaminophen exposure, antioxidant gene polymorphisms, and childhood asthma. *The Journal of allergy and clinical immunology*. 2010 Dec;126(6):1141-8 e7. DOI: 10.1016/j.jaci.2010.08.047.
176. Tsai CH, Tung KY, Chen CH, Lee YL. Tumour necrosis factor G-308A polymorphism modifies the effect of home dampness on childhood asthma. *Occup Environ Med*. 2011 Oct;68(10):771-6. DOI: 10.1136/oem.2010.060970.
177. Melen E, Bruce S, Doekes G, Kabesch M, Laitinen T, Lauener R, et al. Haplotypes of G protein-coupled receptor 154 are associated with childhood allergy and asthma. *Am J Respir Crit Care Med*. 2005 May 15;171(10):1089-95. DOI: 10.1164/rccm.200410-1317OC.
178. P K, M K, K K, T L, M K. Interaction of NPSR1 genotypes and probiotics in the manifestation of atopic eczema in early childhood. *Allergologia et immunopathologia*. 2014 Jan 14. DOI: 10.1016/j.aller.2013.10.001.
179. Prescott SL, Clifton V. Asthma and pregnancy: emerging evidence of epigenetic interactions in utero. *Curr Opin Allergy Clin Immunol*. 2009 Oct;9(5):417-26. DOI: 10.1097/ACI.0b013e328330634f.
180. Amarasekera M, Noakes P, Strickland D, Saffery R, Martino DJ, Prescott SL. Epigenome-wide analysis of neonatal CD4(+) T-cell DNA methylation sites potentially affected by maternal fish oil supplementation. *Epigenetics*. 2014 Dec;9(12):1570-6. DOI: 10.4161/15592294.2014.983366.

181. Lockett GA, Huoman J, Holloway JW. Does allergy begin in utero? *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2015 Aug;26(5):394-402. DOI: 10.1111/pai.12408.
182. Cao D, Bromberg PA, Samet JM. COX-2 expression induced by diesel particles involves chromatin modification and degradation of HDAC1. *American journal of respiratory cell and molecular biology*. 2007 Aug;37(2):232-9. DOI: 10.1165/rcmb.2006-0449OC.
183. Perera F, Tang WY, Herbstman J, Tang D, Levin L, Miller R, et al. Relation of DNA methylation of 5'-CpG island of ACSL3 to transplacental exposure to airborne polycyclic aromatic hydrocarbons and childhood asthma. *PloS one*. 2009;4(2):e4488. DOI: 10.1371/journal.pone.0004488.
184. Wang IJ, Karmaus WJ, Chen SL, Holloway JW, Ewart S. Effects of phthalate exposure on asthma may be mediated through alterations in DNA methylation. *Clinical epigenetics*. 2015;7:27. DOI: 10.1186/s13148-015-0060-x.
185. DeVries A, Wlasiuk G, Miller SJ, Bosco A, Stern DA, Lohman IC, et al. Epigenome-wide analysis links SMAD3 methylation at birth to asthma in children of asthmatic mothers. *The Journal of allergy and clinical immunology*. 2016 Dec 21. DOI: 10.1016/j.jaci.2016.10.041.
186. Hong X, Wang X. Early life precursors, epigenetics, and the development of food allergy. *Semin Immunopathol*. 2012 Sep;34(5):655-69. DOI: 10.1007/s00281-012-0323-y.
187. Martino D, Joo JE, Sexton-Oates A, Dang T, Allen K, Saffery R, et al. Epigenome-wide association study reveals longitudinally stable DNA methylation differences in CD4+ T cells from children with IgE-mediated food allergy. *Epigenetics*. 2014 Jul;9(7):998-1006. DOI: 10.4161/epi.28945.
188. Prescott SL. Early origins of allergic disease: a review of processes and influences during early immune development. *Curr Opin Allergy Clin Immunol*. 2003 Apr;3(2):125-32. DOI: 10.1097/01.all.0000064776.57552.32.
189. Herberth G, Hinz D, Roder S, Schlink U, Sack U, Diez U, et al. Maternal immune status in pregnancy is related to offspring's immune responses and atopy risk. *Allergy*. 2011 Aug;66(8):1065-74. DOI: 10.1111/j.1398-9995.2011.02587.x.
190. Ali Z, Ulrik CS. Incidence and risk factors for exacerbations of asthma during pregnancy. *Journal of asthma and allergy*. 2013;6:53-60. DOI: 10.2147/jaa.s43183.
191. Azad MB, Becker AB, Kozyrskyj AL. Association of maternal diabetes and child asthma. *Pediatric pulmonology*. 2013 Jun;48(6):545-52. DOI: 10.1002/ppul.22668.
192. Hodyl NA, Stark MJ, Scheil W, Grzeskowiak LE, Clifton VL. Perinatal outcomes following maternal asthma and cigarette smoking during pregnancy. *European Respiratory Journal*. 2013 July 30, 2013. DOI: 10.1183/09031936.00054913.
193. Wang QP, Wu KM, Li ZQ, Xue F, Chen W, Ji H, et al. Association between maternal allergic rhinitis and asthma on the prevalence of atopic disease in offspring. *International archives of allergy and immunology*. 2012;157(4):379-86. DOI: 10.1159/000328789.
194. Liu J, Radler D, Illi S, Klucker E, Turan E, von Mutius E, et al. TLR2 polymorphisms influence neonatal regulatory T cells depending on maternal atopy. *Allergy*. 2011 Aug;66(8):1020-9. DOI: 10.1111/j.1398-9995.2011.02573.x.
195. Fu Y, Lou H, Wang C, Lou W, Wang Y, Zheng T, et al. T cell subsets in cord blood are influenced by maternal allergy and associated with atopic dermatitis. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2013 Mar;24(2):178-86. DOI: 10.1111/pai.12050.
196. Kumar R, Ouyang F, Story RE, Pongracic JA, Hong X, Wang G, et al. Gestational diabetes, atopic dermatitis, and allergen sensitization in early childhood. *The Journal of allergy and clinical immunology*. 2009 Nov;124(5):1031-8.e1-4. DOI: 10.1016/j.jaci.2009.06.052.
197. Husemoen LL, Glumer C, Lau C, Pisinger C, Mørch LS, Linneberg A. Association of obesity and insulin resistance with asthma and aeroallergen sensitization. *Allergy*. 2008 May;63(5):575-82. DOI: 10.1111/j.1398-9995.2007.01613.x.
198. Patel SP, Rodriguez A, Little MP, Elliott P, Pekkanen J, Hartikainen AL, et al. Associations between pre-pregnancy obesity and asthma symptoms in adolescents. *Journal of*

- epidemiology and community health. 2012 Sep;66(9):809-14. DOI: 10.1136/jech.2011.133777.
199. Scholtens S, Wijga AH, Brunekreef B, Kerkhof M, Postma DS, Oldenwening M, et al. Maternal overweight before pregnancy and asthma in offspring followed for 8 years. *International journal of obesity* (2005). 2010 Apr;34(4):606-13. DOI: 10.1038/ijo.2009.194.
 200. Haberg SE, Stigum H, London SJ, Nystad W, Nafstad P. Maternal obesity in pregnancy and respiratory health in early childhood. *Paediatric and perinatal epidemiology*. 2009 Jul;23(4):352-62. DOI: 10.1111/j.1365-3016.2009.01034.x.
 201. Rao D, Phipatanakul W. Impact of environmental controls on childhood asthma. *Current allergy and asthma reports*. 2011 Oct;11(5):414-20. DOI: 10.1007/s11882-011-0206-7.
 202. Carson C, Sacker A, Kelly Y, Redshaw M, Kurinczuk JJ, Quigley MA. Asthma in children born after infertility treatment: findings from the UK Millennium Cohort Study. *Human reproduction* (Oxford, England). 2013 Feb;28(2):471-9. DOI: 10.1093/humrep/des398.
 203. Prabhu N, Smith N, Campbell D, Craig LC, Seaton A, Helms PJ, et al. First trimester maternal tobacco smoking habits and fetal growth. *Thorax*. 2010 Mar;65(3):235-40. DOI: 10.1136/thx.2009.123232.
 204. Gold DR. Environmental tobacco smoke, indoor allergens, and childhood asthma. *Environmental health perspectives*. 2000 Aug;108 Suppl 4:643-51.
 205. Hong X, Wang X. Epigenetics and development of food allergy (FA) in early childhood. *Current allergy and asthma reports*. 2014 Sep;14(9):460. DOI: 10.1007/s11882-014-0460-6.
 206. Junge KM, Bauer T, Geissler S, Hirche F, Thurmann L, Bauer M, et al. Increased vitamin D levels at birth and in early infancy increase offspring allergy risk-evidence for involvement of epigenetic mechanisms. *The Journal of allergy and clinical immunology*. 2016 Feb;137(2):610-3. DOI: 10.1016/j.jaci.2015.06.040.
 207. Rehan VK, Liu J, Naeem E, Tian J, Sakurai R, Kwong K, et al. Perinatal nicotine exposure induces asthma in second generation offspring. *BMC Med*. 2012 Oct 30;10:129. DOI: 10.1186/1741-7015-10-129.
 208. Wang C, Salam MT, Islam T, Wenten M, Gauderman WJ, Gilliland FD. Effects of in utero and childhood tobacco smoke exposure and beta2-adrenergic receptor genotype on childhood asthma and wheezing. *Pediatrics*. 2008 Jul;122(1):e107-14. DOI: 10.1542/peds.2007-3370.
 209. Leermakers ET, Sonnenschein-van der Voort AM, Heppe DH, de Jongste JC, Moll HA, Franco OH, et al. Maternal fish consumption during pregnancy and risks of wheezing and eczema in childhood: the Generation R Study. *European journal of clinical nutrition*. 2013 Apr;67(4):353-9. DOI: 10.1038/ejcn.2013.36.
 210. Chatzi L, Garcia R, Roumeliotaki T, Basterrechea M, Begiristain H, Iniguez C, et al. Mediterranean diet adherence during pregnancy and risk of wheeze and eczema in the first year of life: INMA (Spain) and RHEA (Greece) mother-child cohort studies. *The British journal of nutrition*. 2013 Dec 14;110(11):2058-68. DOI: 10.1017/s0007114513001426.
 211. Miyake Y, Tanaka K, Okubo H, Sasaki S, Arakawa M. Maternal fat intake during pregnancy and wheeze and eczema in Japanese infants: the Kyushu Okinawa Maternal and Child Health Study. *Annals of epidemiology*. 2013 Nov;23(11):674-80. DOI: 10.1016/j.annepidem.2013.08.004.
 212. Gunaratne AW, Makrides M, Collins CT. Maternal prenatal and/or postnatal n-3 long chain polyunsaturated fatty acids (LCPUFA) supplementation for preventing allergies in early childhood. *Cochrane Database Syst Rev*. 2015 Jul 22(7):CD010085. DOI: 10.1002/14651858.CD010085.pub2.
 213. Lee HS, Barraza-Villarreal A, Hernandez-Vargas H, Sly PD, Biessy C, Ramakrishnan U, et al. Modulation of DNA methylation states and infant immune system by dietary supplementation with omega-3 PUFA during pregnancy in an intervention study. *The American journal of clinical nutrition*. 2013 Aug;98(2):480-7. DOI: 10.3945/ajcn.112.052241.
 214. Kemp AS, Ponsonby AL, Dwyer T, Cochrane JA, Pezic A, Jones G. Maternal antenatal peanut consumption and peanut and rye sensitization in the offspring at adolescence. *Clinical and*

- experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2011 Feb;41(2):224-31. DOI: 10.1111/j.1365-2222.2010.03668.x.
215. Hsu JT, Missmer SA, Young MC, Correia KF, Twarog FJ, Coughlin IB, et al. Prenatal food allergen exposures and odds of childhood peanut, tree nut, or sesame seed sensitization. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology*. 2013 Nov;111(5):391-6. DOI: 10.1016/j.anai.2013.07.014.
 216. Yong SB, Wu CC, Wang L, Yang KD. Influence and mechanisms of maternal and infant diets on the development of childhood asthma. *Pediatrics and neonatology*. 2013 Feb;54(1):5-11. DOI: 10.1016/j.pedneo.2012.12.009.
 217. Stocks J, Sonnappa S. Early life influences on the development of chronic obstructive pulmonary disease. *Ther Adv Respir Dis*. 2013 Jun;7(3):161-73. DOI: 10.1177/1753465813479428.
 218. Baiz N, Dargent-Molina P, Wark JD, Souberbielle JC, Annesi-Maesano I. Cord serum 25-hydroxyvitamin D and risk of early childhood transient wheezing and atopic dermatitis. *The Journal of allergy and clinical immunology*. 2014 Jan;133(1):147-53. DOI: 10.1016/j.jaci.2013.05.017.
 219. Wang SS, Hon KL, Kong AP, Pong HN, Wong GW, Leung TF. Vitamin D deficiency is associated with diagnosis and severity of childhood atopic dermatitis. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2014 Jan 3. DOI: 10.1111/pai.12167.
 220. Prescott SL, Noakes P, Chow BWY, Breckler L, Thornton CA, Hollams EM, et al. Presymptomatic differences in Toll-like receptor function in infants who have allergy. *The Journal of allergy and clinical immunology*. 2008 2008/08//;122(2):391-9, 9.e1-5. DOI: 10.1016/j.jaci.2008.04.042.
 221. Roduit C, Wohlgensinger J, Frei R, Bitter S, Bieli C, Loeliger S, et al. Prenatal animal contact and gene expression of innate immunity receptors at birth are associated with atopic dermatitis. *The Journal of allergy and clinical immunology*. 2011 Jan;127(1):179-85, 85.e1. DOI: 10.1016/j.jaci.2010.10.010.
 222. Lluís A, Depner M, Gaugler B, Saas P, Casaca VI, Raedler D, et al. Increased regulatory T-cell numbers are associated with farm milk exposure and lower atopic sensitization and asthma in childhood. *The Journal of allergy and clinical immunology*. 2014 Feb;133(2):551-9. DOI: 10.1016/j.jaci.2013.06.034.
 223. Levy O. Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat Rev Immunol*. 2007 May;7(5):379-90. DOI: 10.1038/nri2075.
 224. Melville JM, Moss TJ. The immune consequences of preterm birth. *Front Neurosci*. 2013;7:79. DOI: 10.3389/fnins.2013.00079.
 225. Hannet I, Erkeller-Yuksel F, Lydyard P, Deneys V, DeBruyere M. Developmental and maturational changes in human blood lymphocyte subpopulations. *Immunol Today*. 1992 Jun;13(6):215, 8. DOI: 10.1016/0167-5699(92)90157-3.
 226. Walker JC, Smolders MA, Gemen EF, Antonius TA, Leuvenink J, de Vries E. Development of lymphocyte subpopulations in preterm infants. *Scandinavian journal of immunology*. 2011 Jan;73(1):53-8. DOI: 10.1111/j.1365-3083.2010.02473.x.
 227. Carraro S, Scheltema N, Bont L, Baraldi E. Early-life origins of chronic respiratory diseases: understanding and promoting healthy ageing. *Eur Respir J*. 2014 Dec;44(6):1682-96. DOI: 10.1183/09031936.00084114.
 228. Lisciandro JG, Prescott SL, Nadal-Sims MG, Devitt CJ, Richmond PC, Pomat W, et al. Neonatal antigen-presenting cells are functionally more quiescent in children born under traditional compared with modern environmental conditions. *The Journal of allergy and clinical immunology*. 2012 Nov;130(5):1167-74 e10. DOI: 10.1016/j.jaci.2012.06.005.
 229. Prescott SL, Macaubas C, Smallacombe T, Holt BJ, Sly PD, Loh R, et al. Reciprocal age-related patterns of allergen-specific T-cell immunity in normal vs. atopic infants. *Clin Exp Allergy*. 1998 1998/11//;28 Suppl 5:39-44; discussion 50-1.

230. Turturice BA, Ranjan R, Nguyen B, Hughes LM, Andropolis KE, Gold DR, et al. Perinatal Bacterial Exposure Contributes to IL-13 Aeroallergen Response. *American journal of respiratory cell and molecular biology*. 2017 Apr 26. DOI: 10.1165/rcmb.2017-0027OC.
231. Prescott SL, Macaubas C, Smallacombe T, Holt BJ, Sly PD, Holt PG. Development of allergen-specific T-cell memory in atopic and normal children. *The Lancet*. 1999 1999/01/16/;353(9148):196-200. DOI: 10.1016/s0140-6736(98)05104-6.
232. Pesce G, Marcon A, Marchetti P, Girardi P, de Marco R. Febrile and gynecological infections during pregnancy are associated with a greater risk of childhood eczema. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2013 Dec 1. DOI: 10.1111/pai.12160.
233. Sonnenschein-van der Voort AM, Jaddoe VW, Moll HA, Hofman A, van der Valk RJ, de Jongste JC, et al. Influence of maternal and cord blood C-reactive protein on childhood respiratory symptoms and eczema. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2013 Aug;24(5):469-75. DOI: 10.1111/pai.12094.
234. McKeever TM, Lewis SA, Smith C, Hubbard R. The importance of prenatal exposures on the development of allergic disease: a birth cohort study using the West Midlands General Practice Database. *Am J Respir Crit Care Med*. 2002 Sep 15;166(6):827-32. DOI: 10.1164/rccm.200202-158OC.
235. Shaheen SO, Newson RB, Henderson AJ, Headley JE, Stratton FD, Jones RW, et al. Prenatal paracetamol exposure and risk of asthma and elevated immunoglobulin E in childhood. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2005 Jan;35(1):18-25. DOI: 10.1111/j.1365-2222.2005.02151.x.
236. Toro Monjaraz EM, Ramírez Mayans JA, Cervantes Bustamante R, Gómez Morales E, Molina Rosales A, Montijo Barrios E, et al. Perinatal factors associated with the development of cow's milk protein allergy. *Revista de Gastroenterología de México (English Edition)*. 2015;80(1):27-31. DOI: 10.1016/j.rgmex.2015.02.006.
237. Yang IV, Pedersen BS, Liu A, O'Connor GT, Teach SJ, Kattan M, et al. DNA methylation and childhood asthma in the inner city. *The Journal of allergy and clinical immunology*. 2015 Jul;136(1):69-80. DOI: 10.1016/j.jaci.2015.01.025.
238. Michel S, Busato F, Genuneit J, Pekkanen J, Dalphin JC, Riedler J, et al. Farm exposure and time trends in early childhood may influence DNA methylation in genes related to asthma and allergy. *Allergy*. 2013 Mar;68(3):355-64. DOI: 10.1111/all.12097.
239. Martino D, Kesper DA, Amarasekera M, Harb H, Renz H, Prescott S. Epigenetics in immune development and in allergic and autoimmune diseases. *Journal of reproductive immunology*. 2014 Oct;104-105:43-8. DOI: 10.1016/j.jri.2014.05.003.
240. Thygarajan A, Burks AW. American Academy of Pediatrics recommendations on the effects of early nutritional interventions on the development of atopic disease. *Current opinion in pediatrics*. 2008 Dec;20(6):698-702. DOI: 10.1097/MOP.0b013e3283154f88.
241. Greer FR, Sicherer SH, Burks AW. Effects of early nutritional interventions on the development of atopic disease in infants and children: the role of maternal dietary restriction, breastfeeding, timing of introduction of complementary foods, and hydrolyzed formulas. *Pediatrics*. 2008 Jan;121(1):183-91. DOI: 10.1542/peds.2007-3022.
242. Elliott L, Henderson J, Northstone K, Chiu GY, Dunson D, London SJ. Prospective study of breast-feeding in relation to wheeze, atopy, and bronchial hyperresponsiveness in the Avon Longitudinal Study of Parents and Children (ALSPAC). *The Journal of allergy and clinical immunology*. 2008 Jul;122(1):49-54, e1-3. DOI: 10.1016/j.jaci.2008.04.001.
243. Heine RG. Preventing atopy and allergic disease. *Nestle Nutrition Institute workshop series*. 2014;78:141-53. DOI: 10.1159/000354954.
244. Scholtens S, Wijga AH, Brunekreef B, Kerkhof M, Hoekstra MO, Gerritsen J, et al. Breast feeding, parental allergy and asthma in children followed for 8 years. *The PIAMA birth cohort study. Thorax*. 2009 Jul;64(7):604-9. DOI: 10.1136/thx.2007.094938.
245. Sears MR, Greene JM, Willan AR, Taylor DR, Flannery EM, Cowan JO, et al. Long-term relation between breastfeeding and development of atopy and asthma in children and young

- adults: a longitudinal study. *Lancet*. 2002 Sep 21;360(9337):901-7. DOI: 10.1016/s0140-6736(02)11025-7.
246. Duchon K, Casas R, Fageras-Bottcher M, Yu G, Bjorksten B. Human milk polyunsaturated long-chain fatty acids and secretory immunoglobulin A antibodies and early childhood allergy. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2000 Feb;11(1):29-39.
 247. Sanchez-Garcia S, Cipriani F, Ricci G. Food Allergy in childhood: phenotypes, prevention and treatment. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2015 Dec;26(8):711-20. DOI: 10.1111/pai.12514.
 248. Nwaru BI, Takkinen HM, Kaila M, Erkkola M, Ahonen S, Pekkanen J, et al. Food diversity in infancy and the risk of childhood asthma and allergies. *The Journal of allergy and clinical immunology*. 2014 Jan 25. DOI: 10.1016/j.jaci.2013.12.1069.
 249. Jenerowicz D, Silny W, Danczak-Pazdrowska A, Polanska A, Osmola-Mankowska A, Olek-Hrab K. Environmental factors and allergic diseases. *Annals of agricultural and environmental medicine : AAEM*. 2012;19(3):475-81.
 250. Wang J, Zhou L, Chen Y, Luo R, Tao J. [Analysis of inhaled allergen spectrum of children with allergic rhinitis in Guangzhou]. *Lin chuang er bi yan hou tou jing wai ke za zhi = Journal of clinical otorhinolaryngology, head, and neck surgery*. 2012 Nov;26(22):1026-9.
 251. Chaumont A, Voisin C, Sardella A, Bernard A. Interactions between domestic water hardness, infant swimming and atopy in the development of childhood eczema. *Environmental research*. 2012 Jul;116:52-7. DOI: 10.1016/j.envres.2012.04.013.
 252. Silverberg JI, Hanifin J, Simpson EL. Climatic factors are associated with childhood eczema prevalence in the United States. *The Journal of investigative dermatology*. 2013 Jul;133(7):1752-9. DOI: 10.1038/jid.2013.19.
 253. Caffarelli C, Dondi A, Povesi Dascola C, Ricci G. Skin prick test to foods in childhood atopic eczema: pros and cons. *Italian journal of pediatrics*. 2013;39:48. DOI: 10.1186/1824-7288-39-48.
 254. Pesonen M, Kallio MJ, Siimes MA, Elg P, Bjorksten F, Ranki A. Cord serum immunoglobulin E as a risk factor for allergic symptoms and sensitization in children and young adults. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2009 Feb;20(1):12-8. DOI: 10.1111/j.1399-3038.2008.00736.x.
 255. Klimek L, Hoffmann HJ, Renz H, Demoly P, Werfel T, Matricardi PM, et al. Diagnostic test allergens used for in vivo diagnosis of allergic diseases are at risk: a European Perspective. *Allergy*. 2015 Oct;70(10):1329-31. DOI: 10.1111/all.12676.
 256. D'Urbano LE, Pellegrino K, Artesani MC, Donnanno S, Luciano R, Riccardi C, et al. Performance of a component-based allergen-microarray in the diagnosis of cow's milk and hen's egg allergy. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2010 Oct;40(10):1561-70. DOI: 10.1111/j.1365-2222.2010.03568.x.
 257. Castagnola M, Picciotti PM, Messana I, Fanali C, Fiorita A, Cabras T, et al. Potential applications of human saliva as diagnostic fluid. *Acta otorhinolaryngologica Italica : organo ufficiale della Societa italiana di otorinolaringologia e chirurgia cervico-facciale*. 2011 Dec;31(6):347-57.
 258. Lima DP, Diniz DG, Moimaz SA, Sumida DH, Okamoto AC. Saliva: reflection of the body. *Int J Infect Dis*. 2010 Mar;14(3):e184-8. DOI: 10.1016/j.ijid.2009.04.022.
 259. Schulz BL, Cooper-White J, Punyadeera CK. Saliva proteome research: current status and future outlook. *Critical reviews in biotechnology*. 2013 Sep;33(3):246-59. DOI: 10.3109/07388551.2012.687361.
 260. Javaid MA, Ahmed AS, Durand R, Tran SD. Saliva as a diagnostic tool for oral and systemic diseases. *J Oral Biol Craniofac Res*. 2016 Jan-Apr;6(1):66-75. DOI: 10.1016/j.jobcr.2015.08.006.
 261. Wu Z-Z. Diagnostic model of saliva protein finger print analysis of patients with gastric cancer. *World Journal of Gastroenterology*. 2009;15(7):865. DOI: 10.3748/wjg.15.865.

262. Deming FP, Al-Hashimi I, Haghighat N, Hallmon WW, Kerns DG, Abraham C, et al. Comparison of salivary calmodulin binding proteins in Sjogren's syndrome and healthy individuals. *J Oral Pathol Med.* 2007 Mar;36(3):132-5. DOI: 10.1111/j.1600-0714.2006.00494.x.
263. Rahim MA, Rahim ZH, Ahmad WA, Hashim OH. Can Saliva Proteins Be Used to Predict the Onset of Acute Myocardial Infarction among High-Risk Patients? *International journal of medical sciences.* [Review]. 2015;12(4):329-35. DOI: 10.7150/ijms.11280.
264. Rao PV, Reddy AP, Lu X, Dasari S, Krishnaprasad A, Biggs E, et al. Proteomic identification of salivary biomarkers of type-2 diabetes. *Journal of proteome research.* 2009 Jan;8(1):239-45. DOI: 10.1021/pr8003776.
265. Chaparro A, Gaedechens D, Ramirez V, Zuniga E, Kusanovic JP, Inostroza C, et al. Placental Biomarkers and Angiogenic Factors in Oral Fluids of Patients with Preeclampsia. *Prenat Diagn.* 2016 Mar 14. DOI: 10.1002/pd.4811.
266. Messana I, Cabras T, Iavarone F, Manconi B, Huang L, Martelli C, et al. Chrono-proteomics of human saliva: variations of the salivary proteome during human development. *Journal of proteome research.* [Research Support, Non-U.S. Gov't]. 2015 Apr 3;14(4):1666-77. DOI: 10.1021/pr501270x.
267. Prodan A, Brand HS, Ligtenberg AJ, Imangaliyev S, Tsvitsivadze E, van der Weijden F, et al. Interindividual variation, correlations, and sex-related differences in the salivary biochemistry of young healthy adults. *European journal of oral sciences.* 2015 Jun;123(3):149-57. DOI: 10.1111/eos.12182.
268. Pfafe T, Cooper-White J, Beyerlein P, Kostner K, Punyadeera C. Diagnostic potential of saliva: current state and future applications. *Clinical chemistry.* 2011 May;57(5):675-87. DOI: 10.1373/clinchem.2010.153767.
269. Taylor JJ, Preshaw PM. Gingival crevicular fluid and saliva. *Periodontology* 2000. 2016 Feb;70(1):7-10. DOI: 10.1111/prd.12118.
270. Ghallab NA. Diagnostic potential and future directions of biomarkers in gingival crevicular fluid and saliva of periodontal diseases: Review of the current evidence. *Arch Oral Biol.* 2017 Dec 23;87:115-24. DOI: 10.1016/j.archoralbio.2017.12.022.
271. Chiappin S, Antonelli G, Gatti R, De Palo EF. Saliva specimen: a new laboratory tool for diagnostic and basic investigation. *Clin Chim Acta.* 2007 Aug;383(1-2):30-40. DOI: 10.1016/j.cca.2007.04.011.
272. Wang Q, Yu Q, Lin Q, Duan Y. Emerging salivary biomarkers by mass spectrometry. *Clin Chim Acta.* 2015 Jan 1;438:214-21. DOI: 10.1016/j.cca.2014.08.037.
273. Liu J, Duan Y. Saliva: a potential media for disease diagnostics and monitoring. *Oral Oncol.* 2012 Jul;48(7):569-77. DOI: 10.1016/j.oraloncology.2012.01.021.
274. Prasad S, Tyagi AK, Aggarwal BB. Detection of inflammatory biomarkers in saliva and urine: Potential in diagnosis, prevention, and treatment for chronic diseases. *Exp Biol Med* (Maywood). 2016 Mar 24. DOI: 10.1177/1535370216638770.
275. Grzeskowiak LE, Smith B, Roy A, Dekker GA, Clifton VL. An observational study of the impact of an antenatal asthma management service on asthma control during pregnancy. *European journal of obstetrics, gynecology, and reproductive biology.* 2016 Feb;197:48-53. DOI: 10.1016/j.ejogrb.2015.11.038.
276. Grzeskowiak LE, Smith B, Roy A, Dekker GA, Clifton VL. Patterns, predictors and outcomes of asthma control and exacerbations during pregnancy: a prospective cohort study. *ERJ Open Res.* 2016 Jan;2(1). DOI: 10.1183/23120541.00054-2015.
277. Grzeskowiak LE, Smith B, Roy A, Schubert KO, Baune BT, Dekker GA, et al. Impact of a History of Maternal Depression and Anxiety on Asthma Control During Pregnancy. *The Journal of asthma : official journal of the Association for the Care of Asthma.* 2017 Jan 11:0. DOI: 10.1080/02770903.2016.1258080.
278. Ilyin SE, Belkowski SM, Plata-Salaman CR. Biomarker discovery and validation: technologies and integrative approaches. *Trends Biotechnol.* 2004 Aug;22(8):411-6. DOI: 10.1016/j.tibtech.2004.06.005.

279. Silberring J, Ciborowski P. Biomarker discovery and clinical proteomics. *Trends Analyt Chem.* 2010 Feb 1;29(2):128. DOI: 10.1016/j.trac.2009.11.007.
280. Griffiths HR, Moller L, Bartosz G, Bast A, Bertoni-Freddari C, Collins A, et al. Biomarkers. *Mol Aspects Med.* 2002 Feb-Jun;23(1-3):101-208.
281. Ekström J, Khosravani N, Castagnola M, Messina I. Saliva and the Control of Its Secretion. 2011:19-47. DOI: 10.1007/174_2011_481.
282. Topkas E, Keith P, Dimeski G, Cooper-White J, Punyadeera C. Evaluation of saliva collection devices for the analysis of proteins. *Clin Chim Acta.* 2012 Jul 11;413(13-14):1066-70. DOI: 10.1016/j.cca.2012.02.020.
283. Murphy VE, Zakar T, Smith R, Giles WB, Gibson PG, Clifton VL. Reduced 11beta-hydroxysteroid dehydrogenase type 2 activity is associated with decreased birth weight centile in pregnancies complicated by asthma. *The Journal of clinical endocrinology and metabolism.* 2002 Apr;87(4):1660-8. DOI: 10.1210/jcem.87.4.8377.
284. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nature methods.* [Research Support, Non-U.S. Gov't]. 2009 May;6(5):359-62. DOI: 10.1038/nmeth.1322.
285. Ho CS, Lam CW, Chan MH, Cheung RC, Law LK, Lit LC, et al. Electrospray ionisation mass spectrometry: principles and clinical applications. *Clin Biochem Rev.* 2003;24(1):3-12.
286. Westermeier R, Naven T, Höpker H-R. Mass Spectrometry. *Proteomics in Practice: Wiley-VCH Verlag GmbH & Co. KGaA*; 2008. p. 215-72.
287. Woods AG, Sokolowska I, Ngounou Wetie AG, Wormwood K, Aslebagh R, Patel S, et al. Mass spectrometry for proteomics-based investigation. *Advances in experimental medicine and biology.* 2014;806:1-32. DOI: 10.1007/978-3-319-06068-2_1.
288. Westermeier R, Naven T, Höpker H-R. Liquid Chromatography Techniques. *Proteomics in Practice: Wiley-VCH Verlag GmbH & Co. KGaA*; 2008. p. 151-213.
289. Handbook GH. *Hydrophobic Interaction and Reversed Phase Chromatography Principles and Methods.* 2006(11-0012-69).
290. Bahrudin NA. Detection of asthma associated markers in pregnant women using label-free quantification of salivary proteins: The University of Adelaide, Australia; October 2013.
291. Bantscheff M, Lemeer S, Savitski MM, Kuster B. Quantitative mass spectrometry in proteomics: critical review update from 2007 to the present. *Analytical and bioanalytical chemistry.* 2012 Sep;404(4):939-65. DOI: 10.1007/s00216-012-6203-4.
292. Wong JW, Cagney G. An overview of label-free quantitation methods in proteomics by mass spectrometry. *Methods in molecular biology (Clifton, NJ).* 2010;604:273-83. DOI: 10.1007/978-1-60761-444-9_18.
293. Mann M. Comparative analysis to guide quality improvements in proteomics. *Nature methods.* 2009 Oct;6(10):717-19.
294. Patel VJ, Thalassinou K, Slade SE, Connolly JB, Crombie A, Murrell JC, et al. A comparison of labeling and label-free mass spectrometry-based proteomics approaches. *Journal of proteome research.* 2009 Jul;8(7):3752-9. DOI: 10.1021/pr900080y.
295. Arike L, Valgepea K, Peil L, Nahku R, Adamberg K, Vilu R. Comparison and applications of label-free absolute proteome quantification methods on *Escherichia coli*. *Journal of proteomics.* 2012 Sep 18;75(17):5437-48. DOI: 10.1016/j.jprot.2012.06.020.
296. Megger DA, Bracht T, Meyer HE, Sitek B. Label-free quantification in clinical proteomics. *Biochimica et biophysica acta.* 2013 Aug;1834(8):1581-90. DOI: 10.1016/j.bbapap.2013.04.001.
297. Hillenkamp F, Karas M. Mass spectrometry of peptides and proteins by matrix-assisted ultraviolet laser desorption/ionization. *Methods Enzymol.* 1990;193:280-95.
298. Susnea I, Bernevic B, Wicke M, Ma L, Liu S, Schellander K, et al. Application of MALDI-TOF-mass spectrometry to proteome analysis using stain-free gel electrophoresis. *Top Curr Chem.* 2013;331:37-54. DOI: 10.1007/128_2012_321.
299. Pitt JJ. Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. *Clin Biochem Rev.* 2009 Feb;30(1):19-34.

300. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature biotechnology*. [Evaluation Studies Research Support, Non-U.S. Gov't]. 2008 Dec;26(12):1367-72. DOI: 10.1038/nbt.1511.
301. Cox J, Hein MY, Lubner CA, Paron I, Nagaraj N, Mann M. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Molecular & cellular proteomics : MCP*. 2014 Sep;13(9):2513-26. DOI: 10.1074/mcp.M113.031591.
302. Taylor SC, Berkelman T, Yadav G, Hammond M. A defined methodology for reliable quantification of Western blot data. *Molecular biotechnology*. 2013 Nov;55(3):217-26. DOI: 10.1007/s12033-013-9672-6.
303. Mahmood T, Yang PC. Western blot: technique, theory, and trouble shooting. *North American journal of medical sciences*. 2012 Sep;4(9):429-34. DOI: 10.4103/1947-2714.100998.
304. Danfeng Yao JW, Theresa Harper, Weilei Ma, Bettina Heidecker, Chris Clifford, Huddee Ho, Angel Angeles, Bruce Ikin, Lian-She Zhao SH, Wen Tian, Ling Zhang, Tim Barnabei, Sriram Kumaraswamy, Robert Wicke and Krista Witte. Label-free Analysis from Microliter Amounts of Sample2013 [cited 7/4/2016]. Available from: <http://www.fortebio.com/literature.html>
305. ForteBio. Blitz User Guide [Internet]. Forte Bio; 2013. Available from: <http://www.blitzmenow.com/literature.html>
306. ForteBio. Dip and Read™ High Precision Streptavidin (SAX) Biosensors2013. Available from: <http://www.blitzmenow.com/literature.html>
307. Rossotti MA, Gonzalez-Techera A, Guarnaschelli J, Yim L, Camacho X, Fernandez M, et al. Increasing the potency of neutralizing single-domain antibodies by functionalization with a CD11b/CD18 binding domain. *MAbs*. 2015;7(5):820-8. DOI: 10.1080/19420862.2015.1068491.
308. Wadzinski TL, Geromini K, McKinley Brewer J, Bansal R, Abdelouahab N, Langlois MF, et al. Endocrine disruption in human placenta: expression of the dioxin-inducible enzyme, CYP1A1, is correlated with that of thyroid hormone-regulated genes. *The Journal of clinical endocrinology and metabolism*. 2014 Dec;99(12):E2735-43. DOI: 10.1210/jc.2014-2629.
309. Thomadaki K, Helmerhorst EJ, Tian N, Sun X, Siqueira WL, Walt DR, et al. Whole-saliva proteolysis and its impact on salivary diagnostics. *J Dent Res*. 2011 Nov;90(11):1325-30. DOI: 10.1177/0022034511420721.
310. Chevalier F, Hirtz C, Chay S, Cuisinier F, Sommerer N, Rossignol M, et al. Proteomic Studies of Saliva: A Proposal for a Standardized Handling of Clinical Samples. *Clinical Proteomics*. 2007;3(1-4):13-21. DOI: 10.1007/s12014-007-9000-x.
311. Yang Z, Zhang L, Zhang Y, Zhang T, Feng Y, Lu X, et al. Highly efficient production of soluble proteins from insoluble inclusion bodies by a two-step-denaturing and refolding method. *PloS one*. 2011;6(7):e22981. DOI: 10.1371/journal.pone.0022981.
312. Huang CF, Wang CC, Wu TC, Chu CH, Peng HJ. Effect of sublingual administration with a native or denatured protein allergen and adjuvant CpG oligodeoxynucleotides or cholera toxin on systemic T(H)2 immune responses and mucosal immunity in mice. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology*. 2007 Nov;99(5):443-52. DOI: 10.1016/s1081-1206(10)60570-4.
313. Fink AL. Protein aggregation: folding aggregates, inclusion bodies and amyloid. *Fold Des*. 1998;3(1):R9-23. DOI: 10.1016/s1359-0278(98)00002-9.
314. Corp. PF. Biotinylation of Protein for Immobilization onto Streptavidin Biosensors. Pall ForteBio Corp.; 2016 [cited 2016].
315. Freitag S, Le Trong I, Klumb L, Stayton PS, Stenkamp RE. Structural studies of the streptavidin binding loop. *Protein Sci*. 1997 Jun;6(6):1157-66. DOI: 10.1002/pro.5560060604.
316. Corp. PF. Application Note 14 Biomolecular Binding Kinetics on Octet. Pall ForteBio Corp.; 2016 [cited 2016].
317. Takkar. R. Quantitation Best Practices. Pall ForteBio Corp.; 2016 [cited 2016].

318. Corp. PF. Regeneration Strategies for Streptavidin Biosensors on the Octet Platform. Pall ForteBio Corp.; 2016 [cited 2016].
319. Zhao Y, Lin YH. Whole-cell protein identification using the concept of unique peptides. *Genomics Proteomics Bioinformatics*. 2010 Mar;8(1):33-41. DOI: 10.1016/s1672-0229(10)60004-6.
320. Sumara I, Quadroni M, Frei C, Olma MH, Sumara G, Ricci R, et al. A Cul3-based E3 ligase removes Aurora B from mitotic chromosomes, regulating mitotic progression and completion of cytokinesis in human cells. *Dev Cell*. 2007 Jun;12(6):887-900. DOI: 10.1016/j.devcel.2007.03.019.
321. Liu H, Yin W, Liu B, Liu Y, Guo B, Wei Z. Screening of candidate genes in fibroblasts derived from patients with Dupuytren's contracture using bioinformatics analysis. *Rheumatol Int*. 2015 Aug;35(8):1343-50. DOI: 10.1007/s00296-015-3276-3.
322. Raaby L, Otkjaer K, Salvskov-Iversen ML, Johansen C, Iversen L. A Characterization of the expression of 14-3-3 isoforms in psoriasis, basal cell carcinoma, atopic dermatitis and contact dermatitis. *Dermatol Reports*. 2010 Aug 31;2(2):e14. DOI: 10.4081/dr.2010.e14.
323. Asdaghi N, Kilani RT, Hosseini-Tabatabaei A, Odemuyiwa SO, Hackett TL, Knight DA, et al. Extracellular 14-3-3 from human lung epithelial cells enhances MMP-1 expression. *Molecular and cellular biochemistry*. 2012 Jan;360(1-2):261-70. DOI: 10.1007/s11010-011-1065-1.
324. Liu YC, Elly C, Yoshida H, Bonnefoy-Berard N, Altman A. Activation-modulated association of 14-3-3 proteins with Cbl in T cells. *The Journal of biological chemistry*. 1996 Jun 14;271(24):14591-5.
325. Meller N, Liu YC, Collins TL, Bonnefoy-Berard N, Baier G, Isakov N, et al. Direct interaction between protein kinase C theta (PKC theta) and 14-3-3 tau in T cells: 14-3-3 overexpression results in inhibition of PKC theta translocation and function. *Molecular and cellular biology*. 1996 Oct;16(10):5782-91.
326. Liu H, Tang Y, Liu X, Zhou Q, Xiao X, Lan F, et al. 14-3-3 tau (YWHAQ) gene promoter hypermethylation in human placenta of preeclampsia. *Placenta*. 2014 Dec;35(12):981-8. DOI: 10.1016/j.placenta.2014.09.016.
327. Liu B, Xu Y, Voss C, Qiu FH, Zhao MZ, Liu YD, et al. Altered protein expression in gestational diabetes mellitus placentas provides insight into insulin resistance and coagulation/fibrinolysis pathways. *PloS one*. 2012;7(9):e44701. DOI: 10.1371/journal.pone.0044701.
328. Perdomo G, Henry Dong H. Apolipoprotein D in lipid metabolism and its functional implication in atherosclerosis and aging. *Aging (Albany NY)*. 2009 Jan;1(1):17-27. DOI: 10.18632/aging.100004.
329. Barochia AV, Kaler M, Cuento RA, Gordon EM, Weir NA, Sampson M, et al. Serum apolipoprotein A-I and large high-density lipoprotein particles are positively correlated with FEV1 in atopic asthma. *Am J Respir Crit Care Med*. 2015 May 01;191(9):990-1000. DOI: 10.1164/rccm.201411-1990OC.
330. Nagel G, Weiland SK, Rapp K, Link B, Zoellner I, Koenig W. Association of apolipoproteins with symptoms of asthma and atopy among schoolchildren. *International archives of allergy and immunology*. 2009;149(3):259-66. DOI: 10.1159/000199722.
331. Tomazic PV, Birner-Gruenberger R, Leitner A, Darnhofer B, Spoerk S, Lang-Loidolt D. Apolipoproteins have a potential role in nasal mucus of allergic rhinitis patients: a proteomic study. *The Laryngoscope*. 2015 Mar;125(3):E91-6. DOI: 10.1002/lary.25003.
332. Yao X, Gordon EM, Barochia AV, Remaley AT, Levine SJ. The A's Have It: Developing Apolipoprotein A-I Mimetic Peptides Into a Novel Treatment for Asthma. *Chest*. 2016 Aug;150(2):283-8. DOI: 10.1016/j.chest.2016.05.035.
333. Sakai H, Nishizawa Y, Nishimura A, Chiba Y, Goto K, Hanazaki M, et al. Angiotensin II induces hyperresponsiveness of bronchial smooth muscle via an activation of p42/44 ERK in rats. *Pflugers Arch*. 2010 Aug;460(3):645-55. DOI: 10.1007/s00424-010-0844-y.
334. Karnik SS, Singh KD, Tirupula K, Unal H. Significance of angiotensin 1-7 coupling with MAS1 receptor and other GPCRs to the renin-angiotensin system: IUPHAR Review 22. *Br J Pharmacol*. 2017 May;174(9):737-53. DOI: 10.1111/bph.13742.

335. Crowley SD, Rudemiller NP. Immunologic Effects of the Renin-Angiotensin System. *J Am Soc Nephrol*. 2017 Feb 01. DOI: 10.1681/asn.2016101066.
336. Niedoszytko M, Ratajska M, Chelminska M, Makowiecki M, Malek E, Sieminska A, et al. The angiotensinogen AGT p.M235T gene polymorphism may be responsible for the development of severe anaphylactic reactions to insect venom allergens. *International archives of allergy and immunology*. 2010;153(2):166-72. DOI: 10.1159/000312634.
337. Reid AC, Silver RB, Levi R. Renin: at the heart of the mast cell. *Immunol Rev*. 2007 Jun;217:123-40. DOI: 10.1111/j.1600-065X.2007.00514.x.
338. Aldi S, Robador PA, Tomita K, Di Lorenzo A, Levi R. IgE receptor-mediated mast-cell renin release. *Am J Pathol*. 2014 Feb;184(2):376-81. DOI: 10.1016/j.ajpath.2013.10.016.
339. Strushkevich N, MacKenzie F, Cherkesova T, Grabovec I, Usanov S, Park HW. Structural basis for pregnenolone biosynthesis by the mitochondrial monooxygenase system. *Proceedings of the National Academy of Sciences of the United States of America*. 2011 Jun 21;108(25):10139-43. DOI: 10.1073/pnas.1019441108.
340. Gaurav R, Bewtra AK, Agrawal DK. Novel CLC3 transcript variants in blood eosinophils and increased CLC3 expression in nasal lavage and blood eosinophils of asthmatics. *Immun Inflamm Dis*. 2014 Dec;2(4):205-13. DOI: 10.1002/iid3.36.
341. Suginta W, Karoulias N, Aitken A, Ashley RH. Chloride intracellular channel protein CLIC4 (p64H1) binds directly to brain dynamin I in a complex containing actin, tubulin and 14-3-3 isoforms. *The Biochemical journal*. 2001 Oct 01;359(Pt 1):55-64.
342. Qian Z, Okuhara D, Abe MK, Rosner MR. Molecular cloning and characterization of a mitogen-activated protein kinase-associated intracellular chloride channel. *The Journal of biological chemistry*. 1999 Jan 15;274(3):1621-7.
343. Feske S, Wulff H, Skolnik EY. Ion channels in innate and adaptive immunity. *Annu Rev Immunol*. 2015;33:291-353. DOI: 10.1146/annurev-immunol-032414-112212.
344. Money TT, King RG, Wong MH, Stevenson JL, Kalionis B, Erwich JJ, et al. Expression and cellular localisation of chloride intracellular channel 3 in human placenta and fetal membranes. *Placenta*. 2007 May-Jun;28(5-6):429-36. DOI: 10.1016/j.placenta.2006.08.002.
345. Delanghe JR, Speeckaert R, Speeckaert MM. Complement C3 and its polymorphism: biological and clinical consequences. *Pathology*. 2014 Jan;46(1):1-10. DOI: 10.1097/pat.0000000000000042.
346. Hasegawa K, Tamari M, Shao C, Shimizu M, Takahashi N, Mao XQ, et al. Variations in the C3, C3a receptor, and C5 genes affect susceptibility to bronchial asthma. *Human genetics*. 2004 Sep;115(4):295-301. DOI: 10.1007/s00439-004-1157-z.
347. Sunyer JO, Boshra H, Lorenzo G, Parra D, Freedman B, Bosch N. Evolution of complement as an effector system in innate and adaptive immunity. *Immunol Res*. 2003;27(2-3):549-64. DOI: 10.1385/ir.27:2-3:549.
348. Drouin SM, Corry DB, Kildsgaard J, Wetsel RA. Cutting Edge: The Absence of C3 Demonstrates a Role for Complement in Th2 Effector Functions in a Murine Model of Pulmonary Allergy. *J Immunol*. 2001;167(8):4141-5. DOI: 10.4049/jimmunol.167.8.4141.
349. Ghannam A, Fauquert JL, Thomas C, Kemper C, Drouet C. Human complement C3 deficiency: Th1 induction requires T cell-derived complement C3a and CD46 activation. *Mol Immunol*. 2014 Mar;58(1):98-107. DOI: 10.1016/j.molimm.2013.11.010.
350. Mosca T, Menezes MC, Dionigi PC, Stirbulov R, Forte WC. C3 and C4 complement system components as biomarkers in the intermittent atopic asthma diagnosis. *Jornal de pediatria*. 2011 Nov-Dec;87(6):512-6. DOI: doi:10.2223/JPED.2135.
351. Abdel Fattah M, El Baz M, Sherif A, Adel A. Complement components (C3, C4) as inflammatory markers in asthma. *Indian journal of pediatrics*. 2010 Jul;77(7):771-3. DOI: 10.1007/s12098-010-0117-y.
352. Khan MA, Nicolls MR, Surguladze B, Saadoun I. Complement components as potential therapeutic targets for asthma treatment. *Respir Med*. 2014 Apr;108(4):543-9. DOI: 10.1016/j.rmed.2014.01.005.

353. Fukuoka Y, Xia H-Z, Sanchez-Muñoz LB, Dellinger AL, Escribano L, Schwartz LB. Generation of Anaphylatoxins by Human β -Tryptase from C3, C4, and C5. *J Immunol*. 2008;180(9):6307-16. DOI: 10.4049/jimmunol.180.9.6307.
354. Bradding P, Walls AF, Holgate ST. The role of the mast cell in the pathophysiology of asthma. *The Journal of allergy and clinical immunology*. 2006 Jun;117(6):1277-84. DOI: 10.1016/j.jaci.2006.02.039.
355. Wust SK, Blumenthal MN, Corazalla EO, Benson BA, Dalmasso AP. Complement in asthma: sensitivity to activation and generation of C3a and C5a via the different complement pathways. *Transl Res*. 2006 Oct;148(4):157-63. DOI: 10.1016/j.trsl.2006.05.004.
356. Huang P, Ren X, Huang Z, Yang X, Hong W, Zhang Y, et al. Serum proteomic analysis reveals potential serum biomarkers for occupational medicamentosa-like dermatitis caused by trichloroethylene. *Toxicology letters*. 2014 Aug 17;229(1):101-10. DOI: 10.1016/j.toxlet.2014.05.024.
357. Piessens MF, Marien G, Stevens E. Decreased haptoglobin levels in respiratory allergy. *Clin Allergy*. 1984 May;14(3):287-93.
358. Kessel I, Leib M, Levy A, Miller-Lotan R, Waisman D, Jacobson E, et al. Does Haptoglobin Phenotype Influence Postnatal Morbidity in Preterm Neonates? *Am J Perinatol*. 2016 Jan;33(2):130-5. DOI: 10.1055/s-0035-1560042.
359. Stark MJ, Clifton VL, Hodyl NA. Differential effects of docosahexaenoic acid on preterm and term placental pro-oxidant/antioxidant balance. *Reproduction (Cambridge, England)*. 2013 Sep;146(3):243-51. DOI: 10.1530/REP-13-0239.
360. Stark MJ, Hodyl NA, Wright IM, Clifton VL. Influence of sex and glucocorticoid exposure on preterm placental pro-oxidant-antioxidant balance. *Placenta*. 2011 Nov;32(11):865-70. DOI: 10.1016/j.placenta.2011.08.010.
361. Stark MJ, Clifton VL, Wright IM. Sex-specific differences in peripheral microvascular blood flow in preterm infants. *Pediatric research*. 2008 Apr;63(4):415-9. DOI: 10.1203/01.pdr.0000304937.38669.63.
362. Stark MJ, Wright IM, Clifton VL. Sex-specific alterations in placental 11 β -hydroxysteroid dehydrogenase 2 activity and early postnatal clinical course following antenatal betamethasone. *American journal of physiology Regulatory, integrative and comparative physiology*. 2009 Aug;297(2):R510-4. DOI: 10.1152/ajpregu.00175.2009.
363. Kwon HS, Bae YJ, Moon KA, Lee YS, Lee T, Lee KY, et al. Hyperoxidized peroxiredoxins in peripheral blood mononuclear cells of asthma patients is associated with asthma severity. *Life Sci*. 2012 Apr 09;90(13-14):502-8. DOI: 10.1016/j.lfs.2012.01.003.
364. Wood ZA, Schroder E, Robin Harris J, Poole LB. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci*. 2003 Jan;28(1):32-40.
365. Inoue K, Takano H, Koike E, Warabi E, Yanagawa T, Yanagisawa R, et al. Peroxiredoxin I is a negative regulator of Th2-dominant allergic asthma. *International immunopharmacology*. 2009 Oct;9(11):1281-8. DOI: 10.1016/j.intimp.2009.07.010.
366. Ow SH, Chua PJ, Bay BH. Epigenetic regulation of peroxiredoxins: Implications in the pathogenesis of cancer. *Exp Biol Med (Maywood)*. 2017 Jan;242(2):140-7. DOI: 10.1177/1535370216669834.
367. Kim SY, Jo HY, Kim MH, Cha YY, Choi SW, Shim JH, et al. H₂O₂-dependent hyperoxidation of peroxiredoxin 6 (Prdx6) plays a role in cellular toxicity via up-regulation of iPLA2 activity. *The Journal of biological chemistry*. 2008 Nov 28;283(48):33563-8. DOI: 10.1074/jbc.M806578200.
368. Phalen TJ, Weirather K, Deming PB, Anathy V, Howe AK, van der Vliet A, et al. Oxidation state governs structural transitions in peroxiredoxin II that correlate with cell cycle arrest and recovery. *The Journal of Cell Biology*. 2006;175(5):779-89. DOI: 10.1083/jcb.200606005.
369. Gilroy DW. A novel role for phospholipase A2 isoforms in the checkpoint control of acute inflammation. *The FASEB Journal*. 2004;18(3):489-98. DOI: 10.1096/fj.03-0837com.

370. Hallstrand TS, Henderson WR. An update on the role of leukotrienes in asthma. *Current Opinion in Allergy and Clinical Immunology*. 2010;10(1):60-6. DOI: 10.1097/ACI.0b013e32833489c3.
371. Roszer T. Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. *Mediators Inflamm*. 2015;2015:816460. DOI: 10.1155/2015/816460.
372. Robinson MW, Hutchinson AT, Dalton JP, Donnelly S. Peroxiredoxin: a central player in immune modulation. *Parasite Immunol*. 2010 May;32(5):305-13. DOI: 10.1111/j.1365-3024.2010.01201.x.
373. Moon E-Y, Kang JS, Han SH, Yang K-H, Pyo S, Lee M-Y, et al. Differential role of peroxiredoxin II (PrxII) on the expression of toll-like receptor 4 (TLR4) and B-cell activating factor (BAFF) in ovalbumin (OVA)-induced mouse asthma. *International immunopharmacology*. 2008;8(6):935-44. DOI: 10.1016/j.intimp.2008.01.022.
374. Kwon T, Rho JK, Lee JC, Park YH, Shin HJ, Cho S, et al. An important role for peroxiredoxin II in survival of A549 lung cancer cells resistant to gefitinib. *Exp Mol Med*. 2015 May 29;47:e165. DOI: 10.1038/emmm.2015.24.
375. Dittmann LM, Danner A, Gronych J, Wolter M, Stuhler K, Grzendowski M, et al. Downregulation of PRDX1 by promoter hypermethylation is frequent in 1p/19q-deleted oligodendroglial tumours and increases radio- and chemosensitivity of Hs683 glioma cells in vitro. *Oncogene*. 2012 Jul 19;31(29):3409-18. DOI: 10.1038/onc.2011.513.
376. Agrawal-Singh S, Isken F, Agelopoulos K, Klein HU, Thoennissen NH, Koehler G, et al. Genome-wide analysis of histone H3 acetylation patterns in AML identifies PRDX2 as an epigenetically silenced tumor suppressor gene. *Blood*. 2012 Mar 08;119(10):2346-57. DOI: 10.1182/blood-2011-06-358705.
377. Siqueira WL, Dawes C. The salivary proteome: challenges and perspectives. *Proteomics Clin Appl*. 2011 Dec;5(11-12):575-9. DOI: 10.1002/prca.201100046.
378. Holschneider CH, Berek JS. Ovarian cancer: epidemiology, biology, and prognostic factors. *Semin Surg Oncol*. 2000 Jul-Aug;19(1):3-10.
379. Blakemore AI, Cox A, Gonzalez AM, Maskil JK, Hughes ME, Wilson RM, et al. Interleukin-1 receptor antagonist allele (IL1RN*2) associated with nephropathy in diabetes mellitus. *Human genetics*. 1996 Mar;97(3):369-74.
380. Hernandez ML, Mills K, Almond M, Todoric K, Aleman MM, Zhang H, et al. IL-1 receptor antagonist reduces endotoxin-induced airway inflammation in healthy volunteers. *The Journal of allergy and clinical immunology*. 2015 Feb;135(2):379-85. DOI: 10.1016/j.jaci.2014.07.039.
381. Machura E, Szczepanska M, Mazur B, Barc-Czarnecka M, Kasperska-Zajac A. Interleukin 1-beta, interleukin-1 receptor antagonist, and interleukin 18 in children with acute spontaneous urticaria. *BioMed research international*. [Research Support, Non-U.S. Gov't]. 2013;2013:605262. DOI: 10.1155/2013/605262.
382. Mattii M, Ayala F, Balato N, Filotico R, Lembo S, Schiattarella M, et al. The balance between pro- and anti-inflammatory cytokines is crucial in human allergic contact dermatitis pathogenesis: the role of IL-1 family members. *Experimental dermatology*. [Research Support, Non-U.S. Gov't]. 2013 Dec;22(12):813-9. DOI: 10.1111/exd.12272.
383. Cawthorne C, Prenant C, Smigova A, Julyan P, Maroy R, Herholz K, et al. Biodistribution, pharmacokinetics and metabolism of interleukin-1 receptor antagonist (IL-1RA) using [¹⁸F]-IL1RA and PET imaging in rats. *British Journal of Pharmacology*. 2011;162(3):659-72. DOI: 10.1111/j.1476-5381.2010.01068.x.
384. Barksby HE, Lea SR, Preshaw PM, Taylor JJ. The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. *Clinical & Experimental Immunology*. 2007;149(2):217-25. DOI: 10.1111/j.1365-2249.2007.03441.x.
385. Donovan M, Ambach A, Thomas-Collignon A, Prado C, Bernard D, Jammayrac O, et al. Calmodulin-like skin protein level increases in the differentiated epidermal layers in atopic dermatitis. *Experimental dermatology*. 2013 Dec;22(12):836-7. DOI: 10.1111/exd.12274.
386. Yaswen P, Smoll A, Peehl DM, Trask DK, Sager R, Stampfer MR. Down-regulation of a calmodulin-related gene during transformation of human mammary epithelial cells.

- Proceedings of the National Academy of Sciences of the United States of America. 1990 Oct;87(19):7360-4.
387. Bennett RD, Strehler EE. Calmodulin-like protein enhances myosin-10 translation. *Biochemical and biophysical research communications*. 2008;369(2):654-9. DOI: 10.1016/j.bbrc.2008.02.056.
 388. Nwaru BI, Hayes H, Gambling L, Craig LC, Allan K, Prabhu N, et al. An exploratory study of the associations between maternal iron status in pregnancy and childhood wheeze and atopy. *The British journal of nutrition*. [Research Support, Non-U.S. Gov't]. 2014 Dec;112(12):2018-27. DOI: 10.1017/S0007114514003122.
 389. Lok CN, Loh TT. Regulation of transferrin function and expression: review and update. *Biol Signals Recept*. 1998 May-Jun;7(3):157-78.
 390. Shirtcliff EA, Granger DA, Likos A. Gender differences in the validity of testosterone measured in saliva by immunoassay. *Hormones and behavior*. 2002 Aug;42(1):62-9.
 391. Schwartz EB, Granger DA. Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva. *Clinical chemistry*. 2004 Mar;50(3):654-6. DOI: 10.1373/clinchem.2003.028266.
 392. Castellheim A, Brekke OL, Espevik T, Harboe M, Mollnes TE. Innate immune responses to danger signals in systemic inflammatory response syndrome and sepsis. *Scandinavian journal of immunology*. [Review]. 2009 Jun;69(6):479-91. DOI: 10.1111/j.1365-3083.2009.02255.x.
 393. Johnson EE, Wessling-Resnick M. Iron metabolism and the innate immune response to infection. *Microbes and infection / Institut Pasteur*. 2012 Mar;14(3):207-16. DOI: 10.1016/j.micinf.2011.10.001.
 394. Bakkeheim E, Mowinckel P, Carlsen KH, Burney P, Carlsen KC. Altered oxidative state in schoolchildren with asthma and allergic rhinitis. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. [Research Support, Non-U.S. Gov't]. 2011 Mar;22(2):178-85. DOI: 10.1111/j.1399-3038.2010.01092.x.
 395. Has C, Jakob T, He Y, Kiritsi D, Hausser I, Bruckner-Tuderman L. Loss of desmoglein 1 associated with palmoplantar keratoderma, dermatitis and multiple allergies. *British Journal of Dermatology*. 2015;172(1):257-61. DOI: 10.1111/bjd.13247.
 396. Hammers CM, Stanley JR. Desmoglein-1, differentiation, and disease. *J Clin Invest*. 2013 Apr;123(4):1419-22. DOI: 10.1172/jci69071.
 397. Barber AG, Castillo-Martin M, Bonal DM, Jia AJ, Rybicki BA, Christiano AM, et al. PI3K/AKT pathway regulates E-cadherin and Desmoglein 2 in aggressive prostate cancer. *Cancer Medicine*. 2015;4(8):1258-71. DOI: 10.1002/cam4.463.
 398. Cai F, Zhu Q, Miao Y, Shen S, Su X, Shi Y. Desmoglein-2 is overexpressed in non-small cell lung cancer tissues and its knockdown suppresses NSCLC growth by regulation of p27 and CDK2. *Journal of Cancer Research and Clinical Oncology*. 2016;143(1):59-69. DOI: 10.1007/s00432-016-2250-0.
 399. Sherrill JD, Kc K, Wu D, Djukic Z, Caldwell JM, Stucke EM, et al. Desmoglein-1 regulates esophageal epithelial barrier function and immune responses in eosinophilic esophagitis. *Mucosal Immunology*. 2013;7(3):718-29. DOI: 10.1038/mi.2013.90.
 400. Spindler V, Meir M, Vigh B, Flemming S, Hütz K, Germer C-T, et al. Loss of Desmoglein 2 Contributes to the Pathogenesis of Crohn's Disease. *Inflammatory Bowel Diseases*. 2015;1. DOI: 10.1097/mib.0000000000000486.
 401. van Hemert MJ, Steensma HY, van Heusden GP. 14-3-3 proteins: key regulators of cell division, signalling and apoptosis. *Bioessays*. 2001 Oct;23(10):936-46. DOI: 10.1002/bies.1134.
 402. Han X, Han Y, Jiao H, Jie Y. 14-3-3zeta regulates immune response through Stat3 signaling in oral squamous cell carcinoma. *Mol Cells*. 2015;38(2):112-21. DOI: 10.14348/molcells.2015.2101.
 403. Meek SE, Lane WS, Piwnica-Worms H. Comprehensive proteomic analysis of interphase and mitotic 14-3-3-binding proteins. *The Journal of biological chemistry*. 2004 Jul 30;279(31):32046-54. DOI: 10.1074/jbc.M403044200.

404. Wu Y, Shu R, Luo LJ, Ge LH, Xie YF. Initial comparison of proteomic profiles of whole unstimulated saliva obtained from generalized aggressive periodontitis patients and healthy control subjects. *J Periodontal Res.* 2009 Oct;44(5):636-44. DOI: 10.1111/j.1600-0765.2008.01172.x.
405. Giusti L, Baldini C, Ciregia F, Giannaccini G, Giacomelli C, De Feo F, et al. Is GRP78/BiP a potential salivary biomarker in patients with rheumatoid arthritis? *Proteomics Clin Appl.* 2010 Mar;4(3):315-24. DOI: 10.1002/prca.200900082.
406. Lai A, Hosseini-Tabatabaei A, Hartwell R, Rahmani-Neishaboor E, Kilani RT, Ghahary A. Topical application of aminopeptidase N-neutralizing antibody accelerates wound closure. *Molecular and cellular biochemistry.* 2013 Jan;372(1-2):95-100. DOI: 10.1007/s11010-012-1449-x.
407. Lan YJ, Chen H, Chen JQ, Lei QH, Zheng M, Shao ZR. Immunolocalization of vimentin, keratin 17, Ki-67, involucrin, beta-catenin and E-cadherin in cutaneous squamous cell carcinoma. *Pathol Oncol Res.* 2014 Apr;20(2):263-6. DOI: 10.1007/s12253-013-9690-5.
408. Bao L, Alexander JB, Zhang H, Shen K, Chan LS. Interleukin-4 Downregulation of Involucrin Expression in Human Epidermal Keratinocytes Involves Stat6 Sequestration of the Coactivator CREB-Binding Protein. *J Interferon Cytokine Res.* 2016 Jun;36(6):374-81. DOI: 10.1089/jir.2015.0056.
409. Sevilla LM, Nachat R, Groot KR, Klement JF, Uitto J, Djian P, et al. Mice deficient in involucrin, envoplakin, and periplakin have a defective epidermal barrier. *J Cell Biol.* 2007 Dec 31;179(7):1599-612. DOI: 10.1083/jcb.200706187.
410. Trzeciak M, Sakowicz-Burkiewicz M, Wesseling M, Gleń J, Dobaczewska D, Bandurski T, et al. Altered Expression of Genes Encoding Cornulin and Repetin in Atopic Dermatitis. *International archives of allergy and immunology.* 2017;172(1):11-9. DOI: 10.1159/000453452.
411. Palmer CNA, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nature genetics.* 2006;38(4):441-6. DOI: 10.1038/ng1767.
412. Zhang ZK, Yang Y, Bai SR, Zhang GZ, Liu TH, Zhou Z, et al. Screening for key genes associated with atopic dermatitis with DNA microarrays. *Mol Med Rep.* 2014 Mar;9(3):1049-55. DOI: 10.3892/mmr.2014.1908.
413. Gnanaraj P, Dayalan H, Elango T, Malligarjunan H, Raghavan V, Rao R. Downregulation of involucrin in psoriatic lesions following therapy with propylthiouracil, an anti-thyroid thioureyline: immunohistochemistry and gene expression analysis. *Int J Dermatol.* 2015 Mar;54(3):302-6. DOI: 10.1111/ijd.12565.
414. Jensen JM, Folster-Holst R, Baranowsky A, Schunck M, Winoto-Morbach S, Neumann C, et al. Impaired sphingomyelinase activity and epidermal differentiation in atopic dermatitis. *The Journal of investigative dermatology.* 2004 Jun;122(6):1423-31. DOI: 10.1111/j.0022-202X.2004.22621.x.
415. Kim BE, Leung DY, Boguniewicz M, Howell MD. Loricrin and involucrin expression is down-regulated by Th2 cytokines through STAT-6. *Clinical immunology (Orlando, Fla).* 2008 Mar;126(3):332-7. DOI: 10.1016/j.clim.2007.11.006.
416. Hamid Q, Boguniewicz M, Leung DY. Differential in situ cytokine gene expression in acute versus chronic atopic dermatitis. *J Clin Invest.* 1994 Aug;94(2):870-6. DOI: 10.1172/jci117408.
417. Takeda K, Kamanaka M, Tanaka T, Kishimoto T, Akira S. Impaired IL-13-mediated functions of macrophages in STAT6-deficient mice. *J Immunol.* 1996 Oct 15;157(8):3220-2.
418. Takeda K, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura S, et al. Essential role of Stat6 in IL-4 signalling. *Nature.* 1996 Apr 18;380(6575):627-30. DOI: 10.1038/380627a0.
419. Elder JT, Zhao X. Evidence for local control of gene expression in the epidermal differentiation complex. *Experimental dermatology.* 2002 Oct;11(5):406-12.
420. Saha K, Adhikary G, Eckert RL. MEP50/PRMT5 Reduces Gene Expression by Histone Arginine Methylation and this Is Reversed by PKCdelta/p38delta Signaling. *The Journal of investigative dermatology.* 2016 Jan;136(1):214-24. DOI: 10.1038/JID.2015.400.

421. Lieden A, Ekelund E, Kuo IC, Kockum I, Huang CH, Mallbris L, et al. Cornulin, a marker of late epidermal differentiation, is down-regulated in eczema. *Allergy*. 2009 Feb;64(2):304-11. DOI: 10.1111/j.1398-9995.2008.01856.x.
422. Chen K, Li Y, Dai Y, Li J, Qin Y, Zhu Y, et al. Characterization of tumor suppressive function of cornulin in esophageal squamous cell carcinoma. *PloS one*. 2013;8(7):e68838. DOI: 10.1371/journal.pone.0068838.
423. Kligman D, Hilt DC. The S100 protein family. *Trends Biochem Sci*. 1988 Nov;13(11):437-43. DOI: 10.1016/0968-0004(88)90218-6.
424. Contzler R, Favre B, Huber M, Hohl D. Cornulin, a New Member of the "Fused Gene" Family, Is Expressed During Epidermal Differentiation. *Journal of Investigative Dermatology*. 2005;124(5):990-7. DOI: 10.1111/j.0022-202X.2005.23694.x.
425. Thyssen JP, Kezic S. Causes of epidermal filaggrin reduction and their role in the pathogenesis of atopic dermatitis. *Journal of Allergy and Clinical Immunology*. 2014;134(4):792-9. DOI: 10.1016/j.jaci.2014.06.014.
426. Pawar H, Maharudraiah J, Kashyap MK, Sharma J, Srikanth SM, Choudhary R, et al. Downregulation of cornulin in esophageal squamous cell carcinoma. *Acta Histochem*. 2013 Mar;115(2):89-99. DOI: 10.1016/j.acthis.2012.04.003.
427. Salahshourifar I, Vincent-Chong VK, Chang HY, Ser HL, Ramanathan A, Kallarakkal TG, et al. Downregulation of CRNN gene and genomic instability at 1q21.3 in oral squamous cell carcinoma. *Clin Oral Investig*. 2015 Dec;19(9):2273-83. DOI: 10.1007/s00784-015-1467-7.
428. Schaaïj-Visser TB, Bremmer JF, Braakhuis BJ, Heck AJ, Slijper M, van der Waal I, et al. Evaluation of cornulin, keratin 4, keratin 13 expression and grade of dysplasia for predicting malignant progression of oral leukoplakia. *Oral Oncol*. 2010 Feb;46(2):123-7. DOI: 10.1016/j.oraloncology.2009.11.012.
429. Tyszkiewicz T, Jarzab M, Szymczyk C, Kowal M, Krajewska J, Jaworska M, et al. Epidermal differentiation complex (locus 1q21) gene expression in head and neck cancer and normal mucosa. *Folia histochemica et cytobiologica / Polish Academy of Sciences, Polish Histochemical and Cytochemical Society*. 2014;52(2):79-89. DOI: 10.5603/fhc.2014.0018.
430. Akobeng AK. Understanding type I and type II errors, statistical power and sample size. *Acta paediatrica (Oslo, Norway : 1992)*. 2016 Jun;105(6):605-9. DOI: 10.1111/apa.13384.
431. Heidel RE. Causality in Statistical Power: Isomorphic Properties of Measurement, Research Design, Effect Size, and Sample Size. *Scientifica (Cairo)*. 2016;2016:8920418. DOI: 10.1155/2016/8920418.
432. Trapp BDaRG. Basic and Clinical Biostatistics. 4th edition, editor. New York, NY, USA,: McGraw-Hill; 2004.
433. Dill KA, Shortle D. Denatured states of proteins. *Annu Rev Biochem*. 1991;60:795-825. DOI: 10.1146/annurev.bi.60.070191.004051.
434. Restani P, Ballabio C, Cattaneo A, Isoardi P, Terracciano L, Fiocchi A. Characterization of bovine serum albumin epitopes and their role in allergic reactions. *Allergy*. 2004 Aug;59 Suppl 78:21-4. DOI: 10.1111/j.1398-9995.2004.00568.x.
435. Vila L, Beyer K, Jarvinen KM, Chatchatee P, Bardina L, Sampson HA. Role of conformational and linear epitopes in the achievement of tolerance in cow's milk allergy. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2001 Oct;31(10):1599-606.
436. Westritschnig K, Focke M, Verdino P, Goessler W, Keller W, Twardosz A, et al. Generation of an allergy vaccine by disruption of the three-dimensional structure of the cross-reactive calcium-binding allergen, Phl p 7. *J Immunol*. 2004 May 01;172(9):5684-92.
437. Gieni RS, Yang X, Kelso A, Hayglass KT. Limiting dilution analysis of CD4 T-cell cytokine production in mice administered native versus polymerized ovalbumin: directed induction of T-helper type-1-like activation. *Immunology*. 1996 Jan;87(1):119-26.
438. Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. *Lancet*. 1989 Sep 09;2(8663):577-80.

439. Treiber M, Gorenjak M, Pecovnik Balon B. Serum cystatin-C as a marker of acute kidney injury in the newborn after perinatal hypoxia/asphyxia. *Ther Apher Dial.* 2014 Feb;18(1):57-67. DOI: 10.1111/1744-9987.12054.
440. Sweet DG, Turner MA, Stranak Z, Plavka R, Clarke P, Stenson BJ, et al. A first-in-human clinical study of a new SP-B and SP-C enriched synthetic surfactant (CHF5633) in preterm babies with respiratory distress syndrome. *Arch Dis Child Fetal Neonatal Ed.* 2017 May 02. DOI: 10.1136/archdischild-2017-312722.
441. Ochs M, Nenadic I, Fehrenbach A, Albes JM, Wahlers T, Richter J, et al. Ultrastructural alterations in intraalveolar surfactant subtypes after experimental ischemia and reperfusion. *Am J Respir Crit Care Med.* 1999 Aug;160(2):718-24. DOI: 10.1164/ajrccm.160.2.9809060.
442. Gargiulo P, Banfi C, Ghilardi S, Magri D, Giovannardi M, Bonomi A, et al. Surfactant-derived proteins as markers of alveolar membrane damage in heart failure. *PloS one.* 2014;9(12):e115030. DOI: 10.1371/journal.pone.0115030.
443. Cataldi L, Mussap M, Bertelli L, Ruzzante N, Fanos V, Plebani M. Cystatin C in healthy women at term pregnancy and in their infant newborns: relationship between maternal and neonatal serum levels and reference values. *Am J Perinatol.* 1999;16(6):287-95. DOI: 10.1055/s-2007-993874.
444. Bokenkamp A, Dieterich C, Dressler F, Muhlhaus K, Gembruch U, Bald R, et al. Fetal serum concentrations of cystatin C and beta2-microglobulin as predictors of postnatal kidney function. *American journal of obstetrics and gynecology.* 2001 Aug;185(2):468-75. DOI: 10.1067/mob.2001.115283.
445. Murty MS, Sharma UK, Pandey VB, Kankare SB. Serum cystatin C as a marker of renal function in detection of early acute kidney injury. *Indian J Nephrol.* 2013 May;23(3):180-3. DOI: 10.4103/0971-4065.111840.
446. Roche S, D'Ippolito G, Gomez LA, Bouckennooghe T, Lehmann S, Montero-Menei CN, et al. Comparative analysis of protein expression of three stem cell populations: models of cytokine delivery system in vivo. *Int J Pharm.* 2013 Jan 02;440(1):72-82. DOI: 10.1016/j.ijpharm.2011.12.041.
447. Coscia A, Orru S, Di Nicola P, Giuliani F, Varalda A, Peila C, et al. Detection of cow's milk proteins and minor components in human milk using proteomics techniques. *The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet.* 2012 Oct;25 Suppl 4:54-6. DOI: 10.3109/14767058.2012.715015.
448. Kacerovsky M, Lenco J, Musilova I, Tambor V, Lamont R, Torloni MR, et al. Proteomic biomarkers for spontaneous preterm birth: a systematic review of the literature. *Reprod Sci.* 2014 Mar;21(3):283-95. DOI: 10.1177/1933719113503415.
449. Aitken A. 14-3-3 proteins: a historic overview. *Semin Cancer Biol.* 2006 Jun;16(3):162-72. DOI: 10.1016/j.semcancer.2006.03.005.
450. Lodygin D, Yazdi AS, Sander CA, Herzinger T, Hermeking H. Analysis of 14-3-3sigma expression in hyperproliferative skin diseases reveals selective loss associated with CpG-methylation in basal cell carcinoma. *Oncogene.* 2003 Aug 21;22(35):5519-24. DOI: 10.1038/sj.onc.1206854.
451. Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, et al. 14-3-3sigma is a p53-regulated inhibitor of G2/M progression. *Molecular cell.* 1997 Dec;1(1):3-11.
452. Faisal A, Saurin A, Gregory B, Foxwell B, Parker PJ. The scaffold MyD88 acts to couple protein kinase Cepsilon to Toll-like receptors. *The Journal of biological chemistry.* 2008 Jul 04;283(27):18591-600. DOI: 10.1074/jbc.M710330200.
453. Butt AQ, Ahmed S, Maratha A, Miggin SM. 14-3-3epsilon and 14-3-3sigma inhibit Toll-like receptor (TLR)-mediated proinflammatory cytokine induction. *The Journal of biological chemistry.* 2012 Nov 09;287(46):38665-79. DOI: 10.1074/jbc.M112.367490.
454. Wang T, Niu G, Kortylewski M, Burdelya L, Shain K, Zhang S, et al. Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nature medicine.* 2004 Jan;10(1):48-54. DOI: 10.1038/nm976.

455. Yang F, Brune JL, Baldwin WD, Barnett DR, Bowman BH. Identification and characterization of human haptoglobin cDNA. *Proceedings of the National Academy of Sciences of the United States of America*. 1983 Oct;80(19):5875-9.
456. Cid MC, Grant DS, Hoffman GS, Auerbach R, Fauci AS, Kleinman HK. Identification of haptoglobin as an angiogenic factor in sera from patients with systemic vasculitis. *J Clin Invest*. 1993 Mar;91(3):977-85. DOI: 10.1172/jci116319.
457. Dobryszcka W. Biological functions of haptoglobin--new pieces to an old puzzle. *Eur J Clin Chem Clin Biochem*. 1997 Sep;35(9):647-54.
458. Verrills NM, Irwin JA, Yan He X, Wood LG, Powell H, Simpson JL, et al. Identification of Novel Diagnostic Biomarkers for Asthma and Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med*. 2011;183(12):1633-43. DOI: 10.1164/rccm.201010-1623OC.
459. Castell JV, Gomez-Lechon MJ, David M, Andus T, Geiger T, Trullenque R, et al. Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes. *FEBS letters*. 1989 Jan 02;242(2):237-9.
460. Kuhajda FP, Katumuluwa AI, Pasternack GR. Expression of haptoglobin-related protein and its potential role as a tumor antigen. *Proceedings of the National Academy of Sciences of the United States of America*. 1989 Feb;86(4):1188-92.
461. Kauffmann F, Frette C, Annesi I, Oryszczyn MP, Dore MF, Neukirch F. Relationships of haptoglobin level to FEV1, wheezing, bronchial hyper-responsiveness and allergy. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 1991 Nov;21(6):669-74.
462. Koh YY, Kim YW, Park JD, Oh JW. A comparison of serum haptoglobin levels between acute exacerbation and clinical remission in asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 1996 Oct;26(10):1202-9.
463. Lee S-H, Kim K-H, Kim J-M, Yoon S-H, Kim TH, Park S-W, et al. Relationship between Group-Specific Component Protein and the Development of Asthma. *Am J Respir Crit Care Med*. 2011;184(5):528-36. DOI: 10.1164/rccm.201006-0951OC.
464. Yang F, Ghio AJ, Herbert DC, Weaker FJ, Walter CA, Coalson JJ. Pulmonary expression of the human haptoglobin gene. *American journal of respiratory cell and molecular biology*. 2000 Sep;23(3):277-82. DOI: 10.1165/ajrcmb.23.3.4069.
465. Gutteridge JM. The antioxidant activity of haptoglobin towards haemoglobin-stimulated lipid peroxidation. *Biochimica et biophysica acta*. 1987 Feb 14;917(2):219-23.
466. Larsen K, Macleod D, Nihlberg K, Gurcan E, Bjerner L, Marko-Varga G, et al. Specific haptoglobin expression in bronchoalveolar lavage during differentiation of circulating fibroblast progenitor cells in mild asthma. *Journal of proteome research*. 2006 Jun;5(6):1479-83. DOI: 10.1021/pr050462h.
467. Zhang L, Wang M, Kang X, Boontheung P, Li N, Nel AE, et al. Oxidative stress and asthma: proteome analysis of chitinase-like proteins and FIZZ1 in lung tissue and bronchoalveolar lavage fluid. *Journal of proteome research*. 2009 Apr;8(4):1631-8.
468. Chang G, Zhang K, Xu T, Jin D, Guo J, Zhuang S, et al. Epigenetic mechanisms contribute to the expression of immune related genes in the livers of dairy cows fed a high concentrate diet. *PloS one*. 2015;10(4):e0123942. DOI: 10.1371/journal.pone.0123942.
469. Bicho MC, Pereira da Silva A, Matos A, Silva RM, Bicho MD. Sex steroid hormones influence the risk for cervical cancer: modulation by haptoglobin genetic polymorphism. *Cancer Genet Cytogenet*. 2009 Jun;191(2):85-9. DOI: 10.1016/j.cancergencyto.2009.02.005.
470. Ding J, Berryman DE, Jara A, Kopchick JJ. Age- and sex-associated plasma proteomic changes in growth hormone receptor gene-disrupted mice. *J Gerontol A Biol Sci Med Sci*. 2012 Aug;67(8):830-40. DOI: 10.1093/gerona/glr212.
471. Samara A, Herbeth B, Aubert R, Berrahmoune H, Fumeron F, Siest G, et al. Sex-dependent associations of leptin with metabolic syndrome-related variables: the Stanislas study. *Obesity*. 2010 Jan;18(1):196-201. DOI: 10.1038/oby.2009.156.

472. Murphy VE, Gibson PG, Giles WB, Zakar T, Smith R, Bisits AM, et al. Maternal asthma is associated with reduced female fetal growth. *Am J Respir Crit Care Med*. 2003 Dec 1;168(11):1317-23. DOI: 10.1164/rccm.200303-374OC.
473. Song Y, Liu C, Hui Y, Srivastava K, Zhou Z, Chen J, et al. Maternal allergy increases susceptibility to offspring allergy in association with TH2-biased epigenetic alterations in a mouse model of peanut allergy. *The Journal of allergy and clinical immunology*. 2014 Dec;134(6):1339-45 e7. DOI: 10.1016/j.jaci.2014.08.034.
474. Hinz D, Bauer M, Roder S, Olek S, Huehn J, Sack U, et al. Cord blood Tregs with stable FOXP3 expression are influenced by prenatal environment and associated with atopic dermatitis at the age of one year. *Allergy*. 2012 Mar;67(3):380-9. DOI: 10.1111/j.1398-9995.2011.02767.x.
475. Abelius MS, Janefjord C, Ernerudh J, Berg G, Matthiesen L, Duchon K, et al. The placental immune milieu is characterized by a Th2- and anti-inflammatory transcription profile, regardless of maternal allergy, and associates with neonatal immunity. *American journal of reproductive immunology (New York, NY : 1989)*. 2015 May;73(5):445-59. DOI: 10.1111/aji.12350.
476. Duthie SJ, Horgan G, de Roos B, Rucklidge G, Reid M, Duncan G, et al. Blood folate status and expression of proteins involved in immune function, inflammation, and coagulation: biochemical and proteomic changes in the plasma of humans in response to long-term synthetic folic acid supplementation. *Journal of proteome research*. 2010 Apr 05;9(4):1941-50. DOI: 10.1021/pr901103n.
477. McStay CL, Prescott SL, Bower C, Palmer DJ. Maternal Folic Acid Supplementation during Pregnancy and Childhood Allergic Disease Outcomes: A Question of Timing? *Nutrients*. 2017 Feb 09;9(2). DOI: 10.3390/nu9020123.
478. Hollingsworth JW, Maruoka S, Boon K, Garantzotis S, Li Z, Tomfohr J, et al. In utero supplementation with methyl donors enhances allergic airway disease in mice. *Journal of Clinical Investigation*. 2008 2008/09/18/. DOI: 10.1172/jci34378.
479. Steegers-Theunissen RP, Obermann-Borst SA, Kremer D, Lindemans J, Siebel C, Steegers EA, et al. Periconceptional maternal folic acid use of 400 microg per day is related to increased methylation of the IGF2 gene in the very young child. *PloS one*. 2009 Nov 16;4(11):e7845. DOI: 10.1371/journal.pone.0007845.
480. Brower V. Biomarkers: Portents of malignancy. *Nature*. 2011 Mar 24;471(7339):S19-21. DOI: 10.1038/471S19a.
481. Cappellini MD, Motta I. Anemia in Clinical Practice-Definition and Classification: Does Hemoglobin Change With Aging? *Semin Hematol*. 2015 Oct;52(4):261-9. DOI: 10.1053/j.seminhematol.2015.07.006.

