Cardiovascular Risk Assessment in Women : Impact of Ageing, Polycystic Ovarian Syndrome and Menopause on Nitric Oxide Signalling

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Table of contents

List of figures **x**

List of tables **xii**

Thesis summary **xiii**

Glossary of abbreviations **xvi**

Declaration **xix**

Acknowledgements **xx**

Publications, presentations and awards **xxi**

Chapter 1 INTRODUCTION 1

CHAPTER 1A

Polycystic ovary syndrome and cardiovascular risk

Section A, Part 1: Polycystic ovary syndrome – clinical features and diagnosis 2

A1.1 Introduction 2

A1.2 PCOS clinical phenotypes and diagnosis 3

A1.3 Natural history of PCOS 6

Section A, Part 2: Evidence of PCOS and cardiovascular disease 7

A2.1 Introduction 7

A2.1.1 What are the "conventional"coronary risk factors associated with PCOS? 7

A2.1.2 PCOS and premature atherogeneis 9

A2.1.3 Studies evaluating cardiovascular event rates associated with PCOS 11

A2.2 Disturbances of circulatory physiology in PCOS: an intrinsic problem? 13

CHAPTER 1B

The Nitric Oxide/Cyclic Guanosine Monophosphate pathway and cardiovascular homeostasis

Section B, Part 1: Nitric oxide and its mechanisms of actions 15

B1.1 Introduction 15

B1.2 Nitric oxide production 16

B.1.3. Nitric oxide bioreactivity 17

B1.4 Physiology of Nitric Oxide 19

B.1.5 Functions of NO in blood vessels 20

B.1.5.1 Regulation of vascular tone and blood flow 20
B1.5.2 Vascular smooth muscle proliferation 21
B1.5.3 Leukocyte-endothelial interactions 21
B1.5.4 Recruitment of endothelial progenitor cells 22

B1.6 Function of NO in platelets 24

B1.6.1 Normal platelet function in hemostasis 24
B1.6.2 NO in platelet function 27

Section B, Part 2: Pathological abnormalities in the NO/cGMP pathway 30

B2.1 Introduction 30

B2.2 Reduction in NO generation 31

B2.2.1 Substrate and cofactor availability 31
B2.2.2 NOS activity and expression : ADMA/DDAH pathway 32

B2.3 Reduced sensitivity to NO 38

B2.3.1 NO resistance: Increased clearance of NO- role of oxidative stress 38
B2.3.2"NO resistance": Alterations at soluble guanylate cyclase level 40

B2.4 Role of thrombospondin-1 in NO/cGMP pathway 42

B2.5 Role of thioredoxin system in NO/cGMP signalling 43

Section B, Part 3: Clinical concepts involving NO/cGMP signalling pathway impairment 46

B3.1 Introduction 46

B 3.2 Endothelial dysfunction in cardiovascular disease 47

B3.3 Nitric oxide resistance in cardiovascular disease 50

B3.3.1 Nitric oxide resistance in blood vessels 50
B3.3.2 Nitric oxide resistance and its relationship to endothelial dysfunction 51
B3.3.3 Nitric oxide resistance at the platelet level 52

Section B, Part 4: Assessment of NO/cGMP system 54

B4.1 Introduction 54

B4.2 Biochemical measures of NO/cGMP system 54

B4.2.1 Plasma ADMA concentrations 54
B4.2.2 Inflammatory markers 55

B4.3 Physiological measures of NO/cGMP system 56

B4.3.1 Endothelial function 56
B4.3.2 Assessment of platelet NO responsiveness 60

CHAPTER 1C

Polycystic ovary syndrome and the NO/cGMP pathway

Section C, Part 1: Polycystic ovary syndrome and the NO/cGMP pathway 63

C1.1. Introduction 63

C1.2 Endothelial dysfunction in PCOS 64

C1.3 Platelet dysfunction in PCOS 65

C1.3.1 Platelet hyperaggregability 65
C1.3.2 Altered physiological inhibition of platelet aggregation 65

C1.4 Potential mechanisms underlying disruption of NO/cGMP pathways in PCOS 67

C1.4.1 Evidence of altered NO production and bioavailability in PCOS 67

C1.5 Limitations of current studies: pathophysiological implications 69

CHAPTER 1D

Cardiovascular ageing: rapproachment between physiology and pathology

Section D, Part 1: Ageing as a cardiovascular risk factor 71

D1.1 Introduction 71

D1.2 Postulated mechanisms leading from physiology to pathology 72

D1.3 Therapeutic implications: can we stop cardiovascular ageing? 77

Section D, Part 2: Ageing of the NO system 80

D2.1 Evidence of ageing of NO system 80

CHAPTER 1E

Cardiovascular ageing in women

Section E , Part 1: Cardiovascular Ageing in Women: Scope of cardiovascular disease in women 81

E1.1 Introduction 81

E1.2 Trends by age and gender 82

Section E, Part 2: Role of menopause in CVD trends 85

E2.1 Introduction 855

E2.2 Effects of oestrogen on endothelial and vascular smooth muscle cells 87

E2.3 Effects of oestrogen on cardiovascular risk factors 89

E2.4 Hormone replacement therapy and cardiovascular risk 90

Section E, Part 3: Gender specific ageing of the NOS/cGMP system 92

CHAPTER 1F

Scope of the present study 93

Chapter 2 STUDY DESIGN, MATERIALS AND METHODS 94

2.1 Study design 95

2.2 Materials 96

2.2.1 Subjects studied 96

2.2.2 Blood sampling 96

2.3 Methods 97

2.3.1 Technique for evaluation of platelet NO responses 97

2.3.2 Techniques for evaluation of endothelial function 99

2.3.3 Establishing flow cytometric analysis of EPC cell counts 103

2.3.4 Other biochemical measures 107

Chapter 3 DETERMINATION OF THE IMPACT OF AGEING ON PLATELET AND ENDOTHELIAL FUNCTION IN HEALTHY WOMEN AND IN POLYCYSTIC OVARIAN SYNDROME 109

3.1 Introduction 110

3.2 Objectives of the study 114

3.3 Methods 116

3.3.1 Study population 116
3.3.2 Study protocol 116
3.3.3 Assessment of platelet response to NO 117
3.3.4 Assessment of endothelial function 118
3.3.5 Definitions 119

3.4 Data analysis 120

3.5 Results 121

3.5.1 Results of platelet and vascular responses, and correlation with biochemical markers in ageing women 121
3.5.2 Results of platelet and vascular function studies, comparing PCOS with normal women 135
3.5.3 Effects of menopause on platelet and vascular responses 152

3.6 Discussion 157

3.7 Implications of study findings 164

3.8 Study limitations 165

3.9 Conclusions 166

Chapter 4 CONCLUSIONS AND FUTURE DIRECTIONS 167

4.1 **Ageing and NOS/cGMP system** 169

4.2 **Ageing in women with polycystic ovary syndrome** 170

4.3 **Menopause and NO/cGMP pathway** 171

Future directions 173

Conclusions 174

References **175**

Appendices **198**

List of Figures

Figure 1.1 Venn diagram representing the three cardinal features of PCOS 4

Figure 1.2 Some putative biochemical mechanisms underlying anomalies of NO/sGC/cGMP signalling: categorical summary 30

Figure 1.3 Schematic overview of biochemical pathways related to ADMA 34

Figure 1.4 The TRX system 44

Figure 1.5 Differences between endothelial dysfunction and NO resistance 46

Figure 1.6 Coronary heart disease prevalence by age and sex, 2007-08 82

Figure 2.1 Tracings from platelet aggregometry 98

Figure 2.2 Tracings from pulse wave analysis 101

Figure 2.3 Typical results from flow cytometry 105

Figure 3.1 Platelet aggregation (A) and NO responses (B) in normal women, by age groups. 124

Figure 3.2 Vascular NO (A) and endothelial-dependent (B) responses in normal women, by age groups. 126

Figure 3.3 ADMA concentrations (μM) in normal women, by age groups. 127

Figure 3.4 SDMA concentrations (μM) in normal women, by age groups. 128

Figure 3.5 L-arginine concentrations (μM) in normal women, by age groups. 128

Figure 3.6 ADMA/SDMA ratio in normal women, by age groups. 129

Figure 3.7 Relationship between platelet NO responses (%) and ADMA concentrations (μM). 130

Figure 3.8 EPC counts in normal women, by age groups. 131

Figure 3.9 hs-CRP (mg/L) in normal women, by age groups. 132

Figure 3.10 Platelet aggregation (A) and NO responses (B) in normal and PCOS women, by age groups. 139

Figure 3.11 Platelet aggregation (A) and NO responses (B) in normal women, according to OC use. 141

Figure 3.12 Endothelial-dependent (A) and vascular NO responses (B) in PCOS (-●-) and normal (-■-) women, by age groups. 144

Figure 3.13 ADMA concentrations (μM) for PCOS (-●-) and controls (-■-), by age groups. 145

Figure 3.14 SDMA concentrations (μM) for PCOS (-●-) and controls (-■-), by age groups. 146

Figure 3.15 Arginine/ADMA ratio with increasing age for PCOS (-●-) and controls (-■-). 147

Figure 3.16 EPC cell counts with increasing age for PCOS (-●-) and controls (-■-). 150

Figure 3.17 hs-CRP concentrations with increasing age for PCOS (-●-) and controls (-■-). 150

Figure 3.18 Correlation of platelet NO responses (A) and vascular responses, salbutamol (-●-) and NTG (-■-) with oestradiol levels. 156

List of Tables

Table 1.1 Diagnostic criteria for PCOS 5

Table 1.2 Summary of studies demonstrating premature atherosclerosis in PCOS 10

Table 1.3 Epidemiological studies on cardiovascular outcomes in PCOS individuals 11

Table 1.4 Impact of intra-group variability on outcome measures in clinical studies 36

Table 1.5 Clinical associations of vascular endothelial function 47

Table 1.6 Effects of ageing on major structural and functional characteristics of the cardiovascular system 72

Table 2.1 Intraassay variability 106

Table 2.2 Interassay variability 106

Table 3.1 Baseline characteristics of all normal women (n=133) 122

Table 3.2 Cardiovascular risk factors and medications (n, %) among normal subjects 123

Table 3.3 Univariate correlates of platelet NO responses in normal women 133

Table 3.4 Significant correlates of platelet NO responsiveness on multivariate analysis 134

Table 3.5 Subject characteristics PCOS women in comparison to normal women, by age groups 136

Table 3.6 Cardiovascular risk factors and medications (n, %), comparing PCOS and normal women 137

Table 3.7A Significant correlates of PCOS status on binary logistic regression in the entire cohort 149

Table 3.7B Significant physiological/biochemical correlates of ADMA concentrations on backward multiple linear regression in the PCOS cohort only 149

Table 3.8 Baseline characteristics of perimenopausal women (n=40) 153

Table 3.9 Results of platelet and vascular responses 154

Thesis Summary

Ageing represents an independent and strong risk factor for cardiovascular disease (Lakatta and Levy 2003), and in women, menopause appears to trigger a substantial increase in cardiovascular disease incidence (Castelli 1984). One potential basis for this observation is impairment of vascular endothelial function (Yasue, Matsuyama et al. 1990; Egashira, Inou et al. 1993). However no stratified comparisons of endothelial function or tissue responsiveness to nitric oxide (NO) with increasing age have previously been reported in either gender.

The objectives of the experiments contained in this thesis were therefore to:-

1) Characterise the putative variability in platelet and vascular responsiveness to NO in women of ages 18 to 60 years.

2) Compare this variability with that in vascular endothelial function and its biochemical determinants.

3) Compare the above-mentioned putative fluctuations to those present in age-matched patients with PCOS, a condition characterised by presence of impaired NO signalling in early adult life.

4) Determine the possible impact of menopause on NO signalling in vessels and platelets.

Methods

In order to examine these objectives, we conducted a case-control study of women aged between 18 and 60 years, which allowed us to firstly, examine

NO signalling and various parameters in normal ageing women and then secondly, to compare these with women with PCOS. A subset of 40 perimenopausal women was studied prospectively to assess the relationship between menopause and platelet and vascular parameters.

PCOS women were selected based on Rotterdam criteria and women who were pregnant or on clopidogrel were excluded from the study. Inhibition of platelet aggregation by nitric oxide was the primary outcome measure. Vascular endothelial function utilizing applanation tonometry, plasma concentrations of N^G,N^G-dimethyl-L-arginine (ADMA) and endothelial progenitor cell count (EPC) were assessed as markers of endothelial dysfunction. High-sensitivity C-reactive protein (hs-CRP) was measured as a marker of inflammation.

Results

The key findings from this thesis are:

(1) With increasing age in normal women, there was progressive attenuation of platelet responses to NO (ANOVA, $P<0.0001$) with no significant changes in vascular NO responses.

(2) There was also evidence of endothelial dysfunction with increasing age ($p<0.0001$) which was accompanied by elevation of ADMA concentrations with increasing age ($p=0.003$).

(3) Irrespective of age, PCOS women exhibited greater impairment of platelet NO responses and endothelial function ($p<0.05$, 2 way ANOVA) compared to normal women. Furthermore, these anomalies were evident in PCOS women from an early age but had a tendency to converge with normal women above the age of 40 years.

(4) The changes in platelet and endothelial function in normal women were not correlated with oestradiol levels.

Conclusions

Normal ageing in women is associated with attenuation of NO-based signalling in platelets and blood vessels. In women with PCOS, these changes are present from early adult life, which may form the pathophysiological basis for premature atherogenesis seen in these individuals. The changes in NO signalling are not totally attributable to the onset of menopause.

Glossary of abbreviations

Abbreviation	Definition
AA	Arachidonic Acid
ACEI	Angiotensin converting enzyme inhibitor
ASK-1	Apoptosis signal-regulating kinase 1
ADMA	Asymmetric N^G,N^G-dimethyl-L-arginine
ADP	Adenosine diphosphate
Aix	Augmentation Index
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ATRA	Angiotensin II receptor antagonist
BH_4	Tetrahydrobiopterin
BMI	Body mass index
C	Celsius
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophsophate
cGMP	Cyclic guanosine monophosphate
CVD	Cardiovascular disease
DAG	Diacylglycerol
DDAH	Dimethylarginine dimethlyaminohydrolase
DM	Diabetes mellitus
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
FBF	Forearm blood flow
FA	Flavin adenine dinucleotide

FMD	Flow-mediated dilatation
FMN	Flavin mononucleotide
GP	Glycoprotein
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HOMA-IR	Homeostasis model of insulin resistance
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy
hs-CRP	High-sensitivity C-reactive protein
IL	Interlukin
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 1,4,5-triphosphate
IR	Insulin resistance
ISDN	Isosorbide dinitrate
LDL	Low-density lipoprotein
L-NMMA	N^G-monomethyl-L-arginine
MBS	Metabolic syndrome
MDA	Malondialdehyde
NADPH	Nicotinamide adenine dinucleotide phosphate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
$NO^{\cdot}$	Nitric oxide free radical
NO^+	Nitrosonium
NO^-	Nitoxyl
NOS	Nitric oxide synthase
NTG	Nitroglycerin
O_2^-	Superoxide

OC	Oral contraceptive
PAI-1	Plasminogen activator-1
PCOS	Polycystic ovary syndrome
PG	Prostaglandin
PIP_2	Phosphatidylinositol 4,5-bisphosphonate
PKC	Protein kinase C
PLA_2	Phospholiapse A_2
PLC	Phospholipase C
PRMT	Protein arginine methyltransferases
PRP	Platelet-rich plasma
PWA	Pulse wave analysis
ROS	Reactive oxygen species
SD	Standard deviation
SDMA	N^G,$N^{G'}$-dimethyl-L-arginine
SEM	Standard error of mean
sGC	Soluble guanylate cyclase
SNP	Sodium Nitroprusside
SOD	Superoxide dismutase
TGFβ	Transforming growth factor-beta
TRX	Thioredoxin
TSP-1	Thrombospondin-1
TxA_2	Thromboxane A
TXNIP	Thioredoxin-interacting protein
vWf	Von Willebrand factor

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma 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 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.

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Publications, presentations and awards

Peer reviewed articles relating to this thesis

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Rajendran S, Willoughby SR, **Chan WP**, Liberts EA, Heresztyn T, Saha M, Marber MS, Norman RJ, Horowitz JD. Polycystic ovary syndrome is associated with severe platelet and endothelial dysfunction in both obese and lean subjects. Atherosclerosis 2009 Jun;204(2):509-14.

Ngo DT, **Chan WP**, Rajendran S, Heresztyn T, Amarasekera A, Sverdlov AL, O'Loughlin PD, Morris HA, Chirkov YY, Norman RJ, Horowitz JD. Determinants of insulin responsiveness in young women: Impact of polycystic ovarian syndrome, nitric oxide, and vitamin D. Nitric Oxide 2011 Oct 30;25(3):326-30

Willoughby SR, Rajendran S, **Chan WP,** Procter N, Leslie S, Liberts EA, Heresztyn T, Chirkov YY, Horowitz JD. Ramipril Sensitizes Platelets to Nitric Oxide: Implications for Therapy in High-Risk Patients. J Am Coll Cardiol 60(10): 887-894

Chan WP Ngo DT, Sverdlov AL, Rajendran S, Heresztyn T, Stafford I, Chirkov YY, Horowitz JD. Premature ageing of cardiovascular/platelet function in polycystic ovarian syndrome. Accepted by American Journal of Medicine 16 Jan 2013, in press.

Accepted presentations at international meetings

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Implications of insulin sensitivity on inflammatory activation and atherogenic risk in normal and PCOS females
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Polycystic ovary syndrome is associated with insulin resistance independent of obesity, vitamin D status and inflammatory activation
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Ngo DT, **Chan WP**, Rajendran S, Heresztyn T, Amaresekera A,Sverdlov AL, Chirkov YY, Horowitz JD.
Insulin resistance in polycystic ovarian syndrome and obesity: correlations with ADMA and inflammatory activation
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Impact of ageing on the platelet and vascular responses in women with polycystic ovary syndrome
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Chan WP, Sverdlov AL, Ngo DT, Chirkov YY, Horowitz JD.
Does oral contraceptive therapy induce platelet nitric oxide resistance?
European Society of Cardiology Congress 2011, Paris

Chan WP, Ngo DT, Sverdlov AL, Chirkov YY, Horowitz JD.
Effects of Ageing on Nitric Oxide Signalling in Women: Comparison with Polycystic Ovarian Syndrome.
American Heart Association Scientific Sessions 2012, Los Angeles
(Nominated for Outstanding Research by an Early Career Investigator by Arteriosclerosis, Thrombosis and Vascular Biology Council, 2012)
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Chapter 1

INTRODUCTION

CHAPTER 1, SECTION A

Polycystic ovary syndrome and cardiovascular risk

Section A, Part 1: Polycystic ovary syndrome – clinical features and diagnosis

A1.1 Introduction

Polycystic ovary syndrome (PCOS) was first described in 1935 by Stein and Levanthal, in a cohort of seven women with features of amenorrhoea, hirsutism, obesity and characteristic polycystic appearance in their ovaries (1935). Since then the syndrome has been recognised to be the most common endocrinopathy in women, affecting up to 10% of premenopausal women (Azziz, Woods et al. 2004) and up to 30% of obese women (Alvarez-Blasco, Botella-Carretero et al. 2006), and is also the leading cause of infertility. It is a lifelong condition, with features at menarche of reproductive and hormonal complications, and on a longer term, evidence of associated metabolic complications including insulin resistance, hypercholesterolemia and hypertension (Talbott, Guzick et al. 1995; Legro, Kunselman et al. 1999). It is the prevalence of these metabolic features, which represent cardiovascular risk factors, which initially raised the possibility of increased cardiovascular morbidity/mortality in PCOS individuals.

A1.2 PCOS clinical phenotypes and diagnosis

PCOS is a very heterogeneous disorder. In its complete or "classic" phenotype, an individual will display all three characteristic features, which include (i) hyperandrogenism, (ii) ovulatory disturbances and (iii) polycystic ovaries on imageing. Hyperandrogenism typically is manifested as hirsutism, acne and/or male pattern balding. Ovulatory disturbances may range from oligomenorrhoea (less than 9 menses per year), amenorrhoea, dysfunctional uterine bleeding to infertility. Infertility may be manifested as either difficulty in conception or spontaneous early abortions. These disturbances are usually present from the time of onset of menarche. Menarche itself may be delayed or less commonly, absent and presenting as primary amenorrhoea. Obesity is also often present, in up to 70% of PCOS individuals (Ehrmann 2005).

Besides the biological/physical effects of PCOS, there have also been increasing reports of associated psychological disturbances including higher risk of depression, anxiety, low self-esteem, negative body image and psychosexual dysfunction in this cohort of women (Jones, Hall et al. 2008; Bishop, Basch et al. 2009).

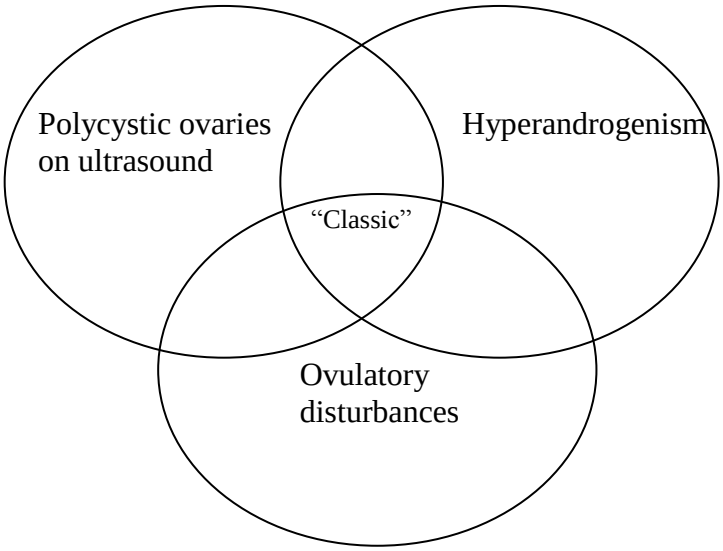

Figure 1.1 Venn diagram representing the three cardinal features of PCOS

Diagnosis of PCOS is often made on the basis of a combination of clinical, biochemical and ultrasound imaging. The typical abnormalities on biochemistry are elevated testosterone and androstenedione levels, with mean concentrations up to 50-150 percent higher than normal women, although this has been reported to be highly variable between individuals (Conway, Honour et al. 1989; Franks 1989). In PCOS, the ovulatory disturbances have been attributed to a lack of preovulatory or mid-luteal increase in oestradiol concentrations, rather than due to an oestradiol-deficient state (Polson, Franks et al. 1987). Therefore plasma oestradiol levels are often normal in the early follicular and mid-follicular phases of the menstrual cycle. The ultrasonographic criteria for diagnosis of polycystic ovaries include bilateral ovarian enlargement (>9 cm in maximal diameter), 10 or more follicles measuring 2 to 10 mm in diameter per ovary, and increased density and area of stroma (Balen, Laven et al. 2003).

Due to the marked clinical heterogeneity, various diagnostic criteria (listed below) have been introduced to aid with diagnosis. These criteria are only applicable once other causes of hyperandrogenism (for example late-onset congenital adrenal hyperplasia) or ovulatory disturbances (e.g hyperprolactinemia) have been excluded.

Table 1.1 Diagnostic criteria for PCOS

Criteria	Oligomenorrhoea	Hyperandrogenism	Polycystic ovaries on ultrasound
NIH 1990[a]	+	+	+/-
Rotterdam 2003[b]	+/-	+/-	+/-
AE-PCOS[c]	+/-	+	+/-

[a] National Institutes for Health (NIH) (1990) requires the presence of both oligomenorrhoea and clinical/biochemical hyperandrogenism
[b] Rotterdam (2003), any two of the above criteria
[c] AE-PCOS (2009), presence of hyperandrogenism and one other criterion

One of the limitations of these criteria is that there is no consensus to date, as to which is the most precise. Studies have frequently reported different frequencies of diagnosis using different criteria. Generally, the NIH criteria are more restrictive compared the latter two. The Rotterdam criteria include the broadest clinical phenotypes, including the normoandrogenic anovulatory and hyperandrogenic ovulatory individuals. This lack of uniformity poses a major problem to PCOS research.

A1.3 Natural history of PCOS

a) PCOS

As previously mentioned, at presentation (around menarche), the clinical picture is dominated by the hormonal changes and reproductive problems. However, the natural history of PCOS is that the above symptoms (predominantly ovulatory disturbances) tend to abate once they reach middle age (late thirties/early forties), with reports of late unassisted pregnancies in their forties. Puurunen et al (2011) recently demonstrated that hyperandrogenism in PCOS women persist till after menopause.

b) Cardiovascular risk factors

By their thirties, PCOS individuals will start to demonstrate evidence of metabolic complications associated with this syndrome (see next section). Insulin resistance is reported in up to 25% of PCOS individuals at age 30, with increasing frequency with increasing age (Ehrmann, Barnes et al. 1999; Legro, Kunselman et al. 1999).

Section A, Part 2 : Evidence of PCOS and cardiovascular disease

A2.1 Introduction

Given that PCOS is frequently associated with insulin resistance, hypertension (Talbott, Guzick et al. 1995) and hyperlipidemia (Wild and Bartholomew 1988), it constitutes a potential basis for increased cardiovascular risk (Dahlgren, Janson et al. 1992). There is indeed evidence that PCOS may be associated with increased atherogenesis and probably with increased atherothrombotic event rates, although it is not certain whether this risk is mediated exclusively via the "conventional" coronary risk factors associated with PCOS, or whether PCOS *per se* is a cardiovascular risk factor.

Therefore it is appropriate first to review the evidence of an association with atherogenesis/increased ischemic events and then to discuss the available data regarding the pathophysiology of this process.

A2.1.1 What are the "conventional" coronary risk factors associated with PCOS?

Higher incidences of cardiovascular risk factors have been found in individuals with PCOS, specifically higher rates of insulin resistance (IR), dyslipidemia and metabolic syndrome (MBS).

It is estimated that the incidence of IR is between 60-80%, which increases to 95% in obese PCOS individuals (Carmina and Lobo 2004; DeUgarte, Bartolucci et al. 2005). IR has been associated with the development of dyslipidemia, MBS (two to three fold increase compared to age-matched controls)(Ehrmann, Liljenquist et al. 2006) and frank Type 2 diabetes mellitus (T2DM) in PCOS individuals (up to a 5-fold increase over 8 years compared to controls (Boudreaux, Talbott et al. 2006)). The underlying cause/mechanisms of impaired insulin sensitivity are unknown.

Epidemiological studies have revealed an increased prevalence of the MBS, which encompasses insulin resistance, dyslipidemia, hypertension, central obesity and microalbuminuria [NCEP-ATP III Guidelines (2002)], in the PCOS population as compared to age-matched normal, healthy women (Ehrmann, Liljenquist et al. 2006). The general opinion is that the development of this syndrome in PCOS individuals depended on the presence of insulin resistance and obesity (Cussons, Watts et al. 2008), (Faloia, Canibus et al. 2004). However, Apridonidze et al (2005) reported up to a two-fold increase of prevalence of MBS in PCOS women which was independent of body mass index, suggesting that presence of PCOS itself (rather than obesity), results in an increased risk of MBS. Toprak et al (2001) also demonstrated that insulin resistance is not confined to obese women with PCOS but that a significant degree of insulin resistance is found in non-obese women with PCOS.

To date, no attempt has been made to insert these variables into predictive risk equations such as the Framingham Risk Equation (Anderson, Odell et al. 1991; Anderson, Wilson et al. 1991) in order to determine whether

actual coronary event rates in PCOS are predicted adequately by these indirect associations.

A2.1.2 PCOS and premature atherogeneis

Evidence of premature atherosclerosis has been demonstrated in several studies in PCOS when compared to age-matched controls, which are summarized in Table 1.2.

However, the limitations of the interpretation of these studies are that there has been several studies which have not demonstrated a difference between PCOS and controls (Ketel, Stehouwer et al.; Costa, dos Santos et al. 2008; Heutling, Schulz et al. 2008; Pamuk, Torun et al.), and there is no conclusive evidence whether PCOS status or its associated risk factors is the major determinant of premature atherogenesis (Ketel, Stehouwer et al.; Talbott, Guzick et al. 2000; Talbott, Zborowski et al. 2004).

Table 1.2 Summary of studies demonstrating premature atherosclerosis in PCOS

Authors	Study size	Mean age ± SD	Results in PCOS p values (compared to controls)
(Talbott, Guzick et al. 2000)	125 PCOS 142 controls	37.5 ± 6.2 39.0 ± 6.2	Increased CIMT in PCOS ≥45 years p=0.005
(Orio, Palomba et al. 2004)	30 PCOS 30 controls	22.2 ± 2.5 22.6 ± 2.3	Increased CIMT p<0.05
(Vural, Caliskan et al. 2005)	43 PCOS 43 controls	21.4 ± 1.8 20.8 ± 2.2	Increased CIMT p<0.001
(Luque-Ramirez, Mendieta-Azcona et al. 2007)	40 PCOS 20 controls	24.5 ± 5.8 27.2 ± 6.8	Increased CIMT p=0.005
(Trakakis, Balanika et al. 2008)	53 PCOS 53 controls	26.1 ± 5.5 25.4 ± 4.7	Increased CIMT p<0.0001
(Christian, Dumesic et al. 2003)	36 PCOS 71 controls	38.5 ± 4.2 39.0 ± 4.1	Increased coronary artery calcification p<0.001
(Talbott, Zborowski et al. 2004)	61 PCOS 85 controls	38.7 ± 4.8 40.4 ± 5.3	Increased prevalence of coronary and aortic calcification

CIMT Carotid intima-media thickness

A2.1.3 Studies evaluating cardiovascular event rates associated with PCOS

Several of the currently available epidemiological studies on cardiovascular disease (CVD) mortality and morbidity (including coronary heart disease, CHD) in PCOS individuals are summarised below.

Table 1.3 Epidemiological studies on cardiovascular outcomes in PCOS individuals

Author	Study design	No of subjects	Results
(Pierpoint, McKeigue et al. 1998)	Prospective long term follow-up (mean 30 years)	786 PCOS National rates	No difference in Standardized mortality ratio (SMR) for CV deaths in PCOS Vs national rates
(Wild, Pierpoint et al. 2000)	Retrospective case control study	319 PCOS 1060 controls	No difference in all cause or CV mortality. Crude odds ratio (OR) CHD 1.5 Crude OR cerebrovascular disease 2.8
(Cibula, Cifkova et al. 2000)	Cross sectional study women age between 45-59 years	28 PCOS 752 controls	Increased prevalence of coronary artery disease in PCOS
(Elting, Korsen et al. 2001)	Cross sectional study	346 PCOS Danish national rates	No difference in cardiac complaints
(Solomon, Hu et al. 2002)	Prospective long term follow up (mean 14 years)	Total 82439 (Criteria: menstrual irregularity)	Relative risk (RR) nonfatal CHD 1.25 RR fatal CHD 1.67 RR all stroke 1.30 RR ischemic stroke 1.40
(Azevedo, Duarte et al. 2006)	Cross sectional case control	Total 414 postmenopausal women (Criteria: menstrual irregularity)	Menstrual irregularity was associated with increased angioplasty rates (OR 6.82)
(Krentz, von Muhlen et al. 2007)	Cross sectional cohort study	713 (9.3%)	Stepwise graded association of PCOS phenotype and CVD (p=0.02) and CHD alone (p=0.03)
(Shaw et al, 2008)	Substudy WISE cross sectional	390 postmenopausal women 104 with PCOS	PCOS women had more angiographic CAD, lower 5 year event-free survival

Overall, these epidemiological studies examining this relationship have been criticised for various reasons including their study design, lack of uniformity of definition of PCOS, small studies and inadequate duration of follow-up.

Nevertheless, from the table above, one can conclude that there is a trend towards an increase in cardiovascular disease burden in PCOS individuals compared to age-matched controls. Data from Shaw et al (2008) is perhaps the most convincing: 390 postmenopausal women enrolled in the Women's Ischemia Syndrome Evaluation (WISE) study were evaluated for PCOS (diagnosed based on history of menstrual irregularity and hyperandrogenemia). Women with clinical features of PCOS (104 women) were more often diabetic ($P < 0.0001$), obese ($P = 0.005$), had the metabolic syndrome ($P < 0.0001$), and had more angiographic coronary artery disease (CAD) ($P = 0.04$) compared to women without clinical features of PCOS (286 women). The cumulative 5-yr CV event-free survival was also lower for women with clinical features of PCOS compared to women without clinical features of PCOS (78.9% vs. 88.7%, $P = 0.006$). Furthermore, using prognostic models including diabetes, waist circumference, hypertension, and angiographic CAD as covariates, PCOS remained a significant predictor ($P < 0.01$).

A2.2 Disturbances of circulatory physiology in PCOS: an intrinsic problem?

In addition to the above morphological and epidemiological studies, there has been evidence of abnormalities in circulatory physiology demonstrated in PCOS women as well, namely increased arterial stiffness and abnormal endothelial function. Such abnormalities have previously been demonstrated to subclinical markers of increased cardiovascular risk with prognostic value as well (discussed in detail Section B3.2). An overview discussion of such studies is provided due to the large number of studies performed to date.

Firstly, these studies have been performed predominantly in young PCOS women under the age of 40 as case-control studies. Whilst the metabolic, hormonal and inflammatory anomalies associated with PCOS have been demonstrated to persist beyond menopause (Puurunen, Piltonen et al.), there has been no long-term follow-up/longitudinal studies performed to evaluate the change in vascular physiology. Therefore it remains to be confirmed if the early detectable anomalies translate into actual increased cardiovascular events.

Secondly, whilst most have shown a difference in endothelial function between age-matched and weight-matched individuals, a relatively smaller number have not shown such differences (Mather, Verma et al. 2000; Bickerton, Clark et al. 2005; Brinkworth, Noakes et al. 2006; Arikan, Akay et al. 2009). The discrepancy may be accounted by the use of different diagnostic criteria and the relatively small size of these studies.

Thirdly, multiple factors have been associated with endothelial dysfunction in PCOS women, including insulin resistance, testosterone levels, total cholesterol and various inflammatory markers (e.g. hsCRP, endothelin-1), and therefore have not demonstrated an independent association with PCOS. We have recently published findings which suggest that PCOS per se may represent an independent risk factor for CVD (Rajendran, Willoughby et al. 2009). In a cohort of young PCOS women without any known cardiovascular risk factors, markers of CVD (endothelial and platelet function) were abnormal in PCOS individuals irrespective of their weight, hormonal status or presence/absence of insulin resistance.

In view of these findings, it is appropriate to review the physiology and pathophysiology of endothelial and platelet NO signalling.

The Nitric Oxide/cyclic Guanosine Monophosphate pathway and cardiovascular homeostasis

Section B, Part 1 : Nitric oxide and its mechanisms of actions

B1.1 Introduction

Nitric oxide (NO) is a free radical gas which plays an important role in the maintenance of cardiovascular homeostasis, specifically by regulating vascular and platelet function. It is a highly reactive molecule, with a half-life of only a few seconds once released by cells. Abnormalities in NO bioavailability or signalling pathways have been demonstrated in multiple cardiovascular disease states and risk factors including atherosclerotic heart disease, heart failure, hypertension and diabetes, and have been the focus for development of therapeutic agents.

B1.2 Nitric oxide production

NO synthesis occurs in a wide range of cell types and tissues in the vasculature. However, it is released predominantly from endothelial cells, and in lower levels from macrophages and activated platelets.

NO is formed from the breakdown of L-arginine into NO and L-citrulline. This action is mediated primarily by a class of enzymes known as nitric oxide synthases (NOS). Furthermore, this reaction requires several cofactors including flavin mononucleotide (FMN), flavin adenine dinucleotide (FA), tetrahydrobiopterin (BH_4), calcium-calmodulin and haem, and several cosubstrates including molecular oxygen and nicotinamide adenine dinucleotide phosphate (NADPH).

There are three isoforms of NOS, which are characterized by their site of synthesis, pattern of expression and calcium-dependency: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). nNOS is found in neurons, iNOS in macrophages, neutrophils, platelets, smooth muscle cells and non-vascular cells and eNOS especially in endothelial cells and probably in platelets. nNOS and eNOS are constitutively expressed NOS isoforms whereas iNOS can be induced by a large variety of cytokines. The activities of both eNOS and nNOS are strongly calcium-dependent which enables rapid and reversible binding of calmodulin. In contrast, the binding of iNOS to calmodulin is largely unaffected by intracellular Ca^{2+} levels.

B.1.3. Nitric oxide bioreactivity

NO has a short half-life of 3 to 5 seconds. It is highly hydrophobic and therefore diffuses across cell membranes easily to exert a predominantly local effect.

NO can accept or donate electrons to form stable adducts and exist in three closely related redox forms that have distinct properties and reactivities: the free radical $NO^{\cdot}$, NO^{+} (nitrosonium, resulting from a one-electron oxidation of $NO^{\cdot}$) and NO^{-} (nitroxyl anion, resulting from one-electron reduction of $NO^{\cdot}$) (Stamler, Singel et al. 1992). These varieties of reactive nitrogen species, and NO itself, allows for both positive and negative biological interactions with multiple substances including molecular oxygen, thiols, reduced hemoproteins and redox metals.

In its target cells, the interaction of NO with the haem iron moiety of soluble guanylate cyclase (sGC) is responsible for its principal action, which is the production of the second messenger cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) (Murad, Mittal et al. 1978). Another important interaction is with sulfhydryl-containing proteins in the presence of molecular oxygen to produce S-nitrosothiols. S-nitrosothiols are relatively stable and may serve as a functional storage for bioavailable NO (Stamler, Simon et al. 1992). Furthermore, this post-translational modification of proteins by S-nitrosylation may alter their physiological properties, and mediates some of the actions of NO.

In contrast to the positive biological reactions described above, other reactions, specifically the interaction with molecular oxygen, reactive oxygen species (ROS) and oxidized low density lipoproteins (LDL), limit NO bioavailability and may have deleterious effects including direct cellular cytotoxicity causing apoptosis and necrosis . NO can be inactivated by ROS such as free radical superoxide (O_2^-) to form peroxynitrite, ($OONO^-$); hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$). Furthermore, NO may undergo oxidation to nitrite and nitrate. Conversely, although once thought to an inactive metabolite, nitrite has now been demonstrated especially under hypoxic conditions, to be able to be reduced in turn to produce NO in a NOS-independent pathway.

B1.4 Physiology of Nitric Oxide

As mentioned above, the principal action of NO occurs via activation of sGC and the production of cGMP. cGMP has a number of effects including the activation of protein kinase G (PKG). Activation of PKG leads to changes in intracellular calcium levels and therefore smooth muscle contractility, and also other effects in cells, for example activation of transcription factors which can lead to changes in gene expression which in turn can alter the response of the cell to a variety of stimuli. Importantly also, cGMP can also be converted back to GTP by proteins known as phosphodiesterases, which therefore effectively blocks further cGMP-dependent NO signalling. The principal effects of NO include the regulation of vascular tone, vascular smooth muscle cell proliferation, endothelial-leukocyte interactions, anti-inflammatory/anti-apoptotic and anti-aggregatory effects, which are discussed in detail below.

B.1.5 Functions of NO in blood vessels

Blood vessels consist of endothelial cells and vascular smooth muscle cells. The endothelium is a monolayer of squamous epithelial cells which lines the inside of all blood vessels. It is a dynamic tissue which performs many active autocrine and paracrine actions to maintain a healthy vasculature, including the secretion and modification of vasoactive substances and the contraction and relaxation of underlying vascular smooth muscle. Additionally it plays a critical role in the mechanics of blood flow, the regulation of coagulation, leucocyte adhesion and blood vessel structure.

B.1.5.1 Regulation of vascular tone and blood flow

Under normal physiological conditions, endothelial cells produce continuous, low levels of NO to maintain basal vascular tone. These levels can be increased in response to both biochemical stimuli (e.g. thrombin, adenosine diphosphate (ADP), serotonin, bradykinin and acetylcholine) and mechanical stimuli (e.g. shear stress and cyclic strain).

NO plays a crucial role in the maintenance of vascular smooth muscle tone primarily by the activation of cGMP via soluble guanylate cyclase, which leads to a decrease in cytosolic calcium concentration. This occurs via 1) direct inhibition of voltage-gated calcium channels and 2) the activation of protein kinases that phosphorylate proteins in sarcoplasmic reticulum and calcium-dependent potassium channels. This reduction in cytosolic Ca^{2+} concentration, in turn, affects phosphorylation of the regulatory myosin light chains which results in smooth muscle relaxation (Horowitz, Menice et al. 1996).

Furthermore, NO is involved in the autoregulation of blood flow, both in large arteries and in the microcirculation, and therefore is a critical determinant of the distribution of flow among vascular beds (White, Vallance et al. 2000).

B1.5.2 Vascular smooth muscle proliferation

Besides regulation of smooth muscle contraction, NO is also important in the normal physiological regulation of smooth muscle migration, growth, and proliferation. Excess smooth muscle proliferation and increase in mitosis is are seen in atherosclerosis, which can be inhibited by NO (Walford and Loscalzo (2003). Gard and Hassig (1989) have demonstrated that exogenous NO donors dose-dependently inhibited vascular smooth muscle DNA synthesis, measured by thymidine incorporation, through c-GMP mechanisms in rat aortic smooth muscle. Others have demonstrated that this process may also be regulated by cGMP –independent mechanisms.

B1.5.3 Leukocyte-endothelial interactions

NO also plays an anti-inflammatory role in the vasculature by modulating leukocyte chemotaxis by altering the expression of adhesion molecules and/or the secretion of chemoattractant proteins (Tsao, Lewis et al. 1995). During inflammation, following exposure to cytokines such TNF-α, endothelial cells are activated to produce chemoattractants (such as monocyte chemotactic protein-1) and endothelial-leukocyte adhesion molecules, which promote leukocyte recruitment and binding. Niebauer et al (1999) demonstrated that

increased NO production from endothelial cells transfected with eNOS was associated with a reduction in monocyte chemotactic protein-1 expression. Conversely, inhibition of NO production either pharmacologically via the use of N(G)-nitro-L-arginine methyl ester (Kurose, Wolf et al. 1995) or genetically in iNOS-deficient mice (Hickey, Granger et al. 2001) resulted in increased leukocyte adhesion to the endothelium.

B1.5.4 Recruitment of endothelial progenitor cells

Disruption of a healthy intact endothelium is an important precursor of atherosclerosis. Endothelial progenitor cells (EPCs) are now increasingly recognized as important biomarkers of vascular function, particularly in "vascular repair". Circulating EPCs, which originate from the bone marrow and has proliferative capacity (Bompais, Chagraoui et al. 2004), play an important role in promoting vascular growth (Asahara, Masuda et al. 1999), repairing ongoing vascular damage (Werner, Junk et al. 2003) and in endothelialization (Peichev, Naiyer et al. 2000), thereby potentially mitigating disease processes such as atherosclerosis. There is now evidence that traditional cardiovascular risk factors are associated with low levels of circulating EPC. Hill et al (2003) found EPC numbers to be inversely correlated with the Framingham risk score and is a better predictor of vascular function than the Framingham risk score. Recent studies have also shown the value of both EPC numbers and function in predicting cardiovascular events (Werner, Kosiol et al. 2005).

NO and eNOS in the bone marrow appear to be play an important role in EPC-mediated angiogenesis, including its production, mobilization and maintenance of their function. Aicher et al (2003) demonstrated that eNOS plays a critical role in bone marrow microenvironment, in the recruitment of stem and progenitor cells. Furthermore, Ii et al (2005) demonstrated that eNOS was important for the recruitment and cardioprotective effect of myocardial ischemic preconditioning. Kazakov et al (2011) recently further demonstrated the key role of bone marrow eNOS in regulation of EPC mobilization and function in a pressure overload mouse model.

B1.6 Function of NO in platelets

Platelets are discoid enucleated cells which play a major role in haemostasis and thrombosis. Under normal conditions, they circulate in blood in a quiescent state and are activated within seconds upon vascular injury, leading to platelet aggregation and formation of a platelet/vascular plug at the site of injury. In disease states, the number or reactivity of platelets may be altered leading to the formation of occlusive thrombi within major arteries, as seen in a stroke or myocardial infarction. NO has been shown to exert effects on both platelet function and production. In order to understand the role of NO, normal platelet function in primary haemostasis is firstly described.

B1.6.1 Normal platelet function in haemostasis

The 3 critical steps involved in primary haemostasis are

(a) platelet adhesion

(b) activation and secretion and

(c) aggregation.

a) Platelet adhesion

Platelet adhesion is the initiating event in response to vascular damage, and is dependent on the adhesion of platelet glycoproteins (Gp) receptors to subendothelial matrix proteins. Within a few seconds, platelets adhere to the collagen fibrils in the subendothelial matrix, via the specific platelet collagen receptor, Gp Ia/IIa. Under intermediate and high shear conditions, this interaction is stabilised by the adhesion of von Willebrand factor (vWf), which acts as an adhesive glycoprotein linking the Gp Ib/IX receptor and subendothelial collagen fibrils. Collagen itself also serves a strong stimulus for platelet activation.

b) Platelet activation

Platelet activation occurs upon binding of a platelet agonist to their cell surface receptors, which enables heterotrimeric G-proteins within cytoplasm to be "switched on". There are various physiological agonists (thrombin, collagen, ADP, epinephrine, vasopressin, serotonin) and non-physiological agonists (divalent cationophores, cyclic endoperoxide analogues). Upon binding of an agonist, 2 major intracellular signalling pathways are involved:

(i) The phosphoinositide hydrolysis pathway

This pathway begins with the activation of phospholipase C (PLC). PLC then cleaves membrane phosphatidylinositol 4,5-biphosphate (PIP_2) to form

inositol 1,4,5-triphosphate (IP_3) and diacylglcerol (DAG), which both act as second messengers leading to the release of granular contents, increase in intracellular calcium levels, and subsequent platelet shape change and aggregation responses.

(ii) The eicosanoid pathway

Phospholipase A_2, released form intraplatelet granules, is able to hydrolyse membrane phospholipids (phosphatidylcholine and phosphatidylethanolamine), to release arachidonic acid (AA). AA is then converted by thromboxane synthase to thomboxane A_2 (TxA_2), which is a potent agonist of platelet activation.

Upon activation, platelets undergo a dramatic conformational change into a spherical shape with long spidery pseudopods. This step improves platelet attachment to other platelets and vessel wall. There is a further shape change upon adhesion to a reactive surface which shifts granules and organelles into the centre of the cell to be secreted into the surface open canalicular system and released into surrounding medium.

c) Platelet aggregation

This step refers to the cross-linking of platelets via the interaction between the integrin Gp IIb/IIIa and fibrinogen. Under the normal state Gp IIb/IIIa are unable to bind to fibrinogen. However, once activated, fibrinogen is able to bind to two Gp IIb/IIIa receptors simultaneously and therefore able to link platelets together. Each platelet has 40000 to 800000 copies of Gp IIb/IIIa receptors on its surface, and therefore large clumps of platelet aggregates can be formed.

B1.6.2 NO in platelet function

NO is the most potent physiological inhibitor of platelet function, which is necessary to prevent platelet activation in normal vasculature and also limit the thrombus growth during haemostasis.

In view of the considerable physiological importance of NO signalling for maintenance of platelet homeostasis, the issue of source of NO is critical. It appears that a substantial component of platelet NO exposure is exogenous whether from contact with endothelial cells in the microvasculature, "unloading" of NO during desaturation of haemoglobin (Jia, Bonaventura et al. 1996) or release from leukocytes (Cha, Chang et al. 2000). In this sense, the platelet NO response functions as a sort of "circulatory litmus test": this provides a physiological rationale for interest in changes in platelet NO signalling efficiency.

On the other hand, platelets may produce endogenous NO: - this is somewhat controversial because the existence of eNOS within platelets is a subject of active debate (Mehta, Chen et al. 1995; Sase and Michel 1995). However, it has been suggested that vWF may also function to generate platelet NO (Riba, Oberprieler et al. 2006). The issue of the extent of "endogenous" NO formation is, however, incidental to the subject matter of this thesis.

NO exerts its effects via cGMP production (Moro, Russel et al. 1996) and a secondary calcium-ATPase-dependent refilling of calcium stores to inhibit platelet activation (Wang, Zhu et al. 1998). cGMP results in the activation of protein kinase G (PKG). One of the actions of PKG is to inhibit type III phosphodiesterase (PDE), which then leads to increases in 3'-5'-cyclic adenosine monophosphate (cAMP) concentration and activation of protein kinase A (PKA). PKA and PKG inhibit platelet activation through a number of shared pathways, including inhibition of phosphoinositide metabolism and extrusion of calcium. Furthermore cGMP and its associated protein kinases also bind to TxA_2 receptors to inhibit platelet activation and also reduce the expression of the surface adhesion molecule, p-selectin through the down-regulation of protein kinase C (from DAG pathway)(Willoughby, Holmes et al. 2002).

NO also affects platelet aggregation. This occurs through cGMP-associated impairment of expression of fibrinogen binding conformation of GpIIb/IIIa and subsequent reduction in fibrinogen binding to GpIIb/IIIa (Gries, Bottiger et al. 1997).

There are several smaller studies which suggest that NO also affects platelet production as well as platelet function. Battinelli et al (2001) demonstrated that the addition of S-nitrososglutathione with and without the addition of thrombopoeitin, to megakaryocytes resulted in increased platelet production, highlighting the importance of NO in megakaryocyte development and thrombopoeisis.

Section B, Part 2 Pathological abnormalities in the NO/cGMP pathway

B2.1 Introduction

As previously mentioned, abnormalities in the NO/cGMP pathway have been shown in association with various cardiovascular disease states and risk factors. These can be categorised broadly as reductions in (1) NO generation or (2) NO biological effect independent of generation i.e. "NO resistance".

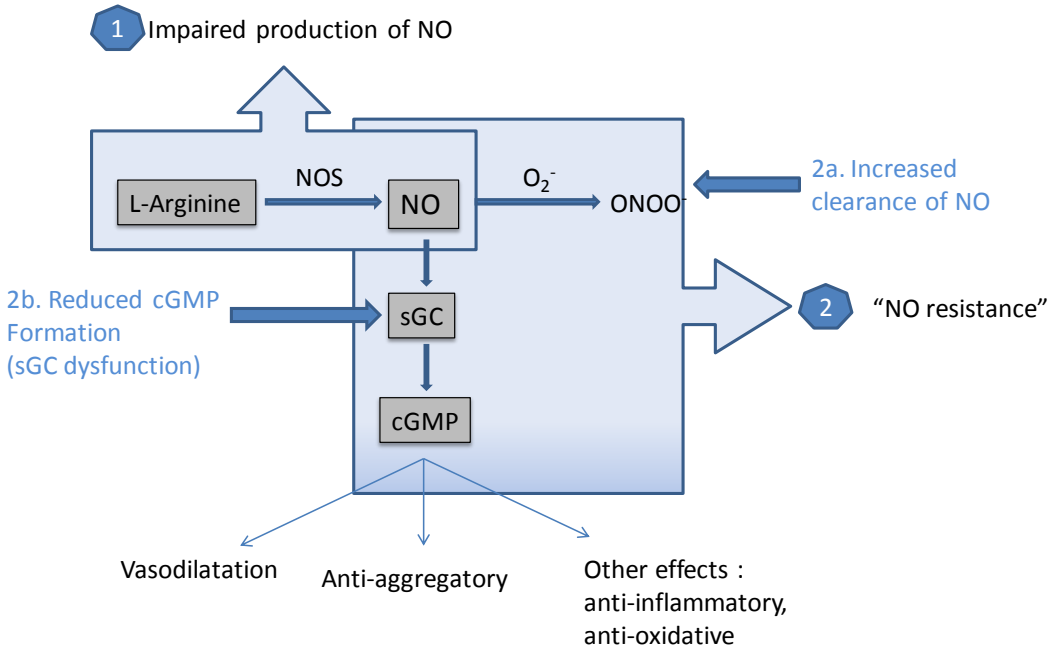

Figure 1.2 Some putative biochemical mechanisms underlying anomalies of NO/sGC/cGMP signalling: categorical summary

B2.2 Reduction in NO generation

B2.2.1 Substrate and cofactor availability

As schematized in Figure 1.2, reduced NO production may result at least in theory, from a lack of substrate, cofactors for NOS or NOS activity/expression itself, as well as from the presence of NOS inhibitors and/or of NOS dysfunction.

Plasma L-arginine is the exclusive substrate for NOS. The "arginine paradox" widely described in the literature, is based on the fact that concentrations of arginine exceed the K_m of endothelial NO synthase by approximately 30-fold (Bredt and Snyder 1990) and are unlikely to be the rate-limiting for enzyme activity. Despite that, multiple in vivo studies of L-arginine supplementation in humans have demonstrated incremental endothelial NO production. These include studies showing both acute intravenous and chronic administration of L-arginine improved the brachial and coronary vasodilator response to acetylcholine in hypercholesterolaemic patients (Creager, Gallagher et al. 1992; Clarkson, Adams et al. 1996). Although the underlying mechanism for this paradox is unknown, it has been proposed that L-arginine in plasma may be exchanged with NOS inhibitors such as asymmetric dimethylarginine via the y^+ transporter on NOS producing cells, to increase NO production (Tsikas, Boger et al. 2000).

Arginases are enzymes which catalyse the hydrolysis of L-arginine to L-ornithine and urea. Recently arginase(s) have been demonstrated in extra-hepatic tissues including the vasculature and shown to compete with NOS for L-arginine and therefore NO production. Several studies have now shown

evidence of increased arginase-mediated impairment of NO production in association with ageing (Berkowitz, White et al. 2003), hypertension (Xu, Kaneko et al. 2004; Johnson, Johnson et al. 2005), diabetes (Bivalacqua, Hellstrom et al. 2001) and animal models of ischemia-reperfusion (Hein, Zhang et al. 2003). Therefore arginases may play a role in reduction of NO bioavailability.

An essential cofactor for the synthesis of NO is tetrahydrobiopterin (BH_4). Under normal conditions, BH_4 acts to stabilize the dimeric structure of NOS, coupling L-arginine oxidation to NADPH consumption and preventing dissociation of the ferrous-dioxygen complex. Decreased levels of BH_4 have been shown to reduce the production of NO (Schmidt, Werner et al. 1992) and also results in eNOS "uncoupling", where NOS reduces molecular oxygen to O_2^- rather than catalyzing electron transfer to L-arginine (Cosentino and Katusic 1995). O_2^- generated preferentially by this reaction can then inactivate NO (Xia, Tsai et al. 1998). In hypercholesterolemic patients, BH_4 was shown to improve endothelial function (Stroes, Kastelein et al. 1997).

B2.2.2 NOS activity and expression : ADMA/DDAH pathway

Asymmetric N^G,N^G-dimethyl-L-arginine (ADMA) is an endogenous inhibitor of NO synthase (NOS), and was first shown by Vallance et al (1992) to be able to antagonize endothelium-dependent vasodilatation.

Kinetics of ADMA formation and clearance are summarized in Figure 1.3. ADMA is derived from the catabolism of proteins containing methylated arginine

residues, by enzymes known as protein arginine methyl transferase types I and II (PRMT I and PRMT II). PRMT I forms ADMA and PRMT 2 forms symmetric dimethyarginine (SDMA), which does not inhibit NOS. ADMA is then degraded by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) or excreted in the urine.

ADMA, similar to other NOS inhibitors, exerts adverse cardiovascular effects, reflecting the diverse physiological roles of NO. In the vasculature, ADMA leads to impairment of endothelium-dependent vasorelaxation, vasoconstriction, increased cell endothelial cell adhesiveness and elevation of blood pressure. In the heart, ADMA reduces heart rate and cardiac output and may contribute to left ventricular hypertrophy. There have also been studies showing ADMA inhibition of angiogenesis

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Figure 1.3 Schematic overview of biochemical pathways related to ADMA. Methylation of arginine residues within proteins or polypeptides occurs through N-methyltransferases, which utilize S-adenosylmethionine as a methyl group donor. After proteolytic breakdown of proteins, free ADMA is present in the cytoplasm. It can also be detected circulating in human blood plasma. ADMA acts as an inhibitor of NO synthase by competing with the substrate of this enzyme, L-arginine, and causes endothelial dysfunction. ADMA is eliminated from the body in part via urinary excretion and more importantly, via metabolism by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) to citrulline and dimethylamine. *[Adapted from (Boger 2003]).*

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ADMA has been shown to be elevated in association with multiple cardiovascular risk factors (hypercholesterolemia, hypertension, diabetes) and disease states (coronary artery disease, cardiac failure, pre-eclampsia and chronic renal impairment). ADMA has been shown to be associated with endothelial dysfunction and/or reduced NO production these conditions.

Apart from its pathophysiological role in cardiovascular diseases, ADMA levels have been shown to have prognostic implications. In a prospective analysis of non-smoking middle-aged men, risk of acute coronary events was raised on average 27-fold (p=0·04) per 0·1 μmol/L of ADMA as measured by high-performance liquid chromatography (HPLC) in serum, after adjustment for traditional cardiovascular risk factors (Valkonen, Paiva et al. 2001). Schnabel et al (2005) also demonstrated an incremental cardiovascular risk with higher ADMA levels in a cohort of patients with coronary artery disease. The median concentrations of ADMA levels were higher among individuals who subsequently developed the primary end point (death from cardiovascular causes or nonfatal myocardial infarction) than among those who did not (0.70 versus 0.63 micromol/L; P<0.001). In this study, individuals with ADMA levels in the highest tertile at entry had a hazard ratio 2.48 times higher than those in the lowest tertile (95% confidence interval, 1.52 to 4.06; P<0.001).

A recent review of the fluctuation of ADMA levels in plasma with various disease states (Horowitz and Heresztyn 2007) noted the substantial prognostic impact of small changes in levels, as summarized in Table 1.4.

Table 1.4 Impact of intra-group variability on outcome measures in clinical studies

Patient group	*N*	Differential ADMA (%)	Adverse outcome	Authors
Previous coronary event	70	+19	Acute coronary events	(Valkonen, Paiva et al. 2001)
Haemodialysis	225	+34[a]	Mortality	(Zoccali, Bode-Boger et al. 2001)
Stable angina/PCI	153	+22	Acute coronary events	(Lu, Ding et al. 2003)
Organ failure	52	+42[b]	Mortality	(Nijveldt, Teerlink et al. 2003)
Unstable angina	36	+15	Acute coronary events	(Krempl, Maas et al. 2005)
CAD	1872	+11[a]	Mortality/AMI	(Schnabel, Blankenberg et al. 2005)

Differential ADMA is the percent difference in mean ADMA concentration between the subgroup with adverse outcomes and those without adverse outcomes
[1] N, number of patient studied; PCI, percutaneous coronary intervention; CAD, coronary artery disease; AMI, acute myocardial infarction.
[a] ADMA values provided were expressed as median, not mean.
[b] This value is based on the highest quartile vs. mean of the whole group, relative risk=17.2 (mean data for patients with adverse and favourable outcomes not available).
Table adapted from (Horowitz and Heresztyn 2007).

Furthermore there was at one stage substantial criticism of the potential physiological role of ADMA generation, despite these compelling associations, on the grounds that plasma ADMA concentrations were considerably less than the Km for inhibition of NOS. However, this issue has been resolved in practice by the findings of a number of studies of manipulation of ADMA kinetics for example by DDAH over-expression (see below).

DDAH is the primary enzyme involved in the breakdown of ADMA. DDAH exists in two isoforms: DDAH-1, which is found predominantly in the proximal tubules of the kidney and liver, and DDAH-2, in the vasculature. Accordingly,

Dayoub et al (2003) demonstrated that over-expression of DDAH-1 using gene transfer and transgenic approaches in vivo is associated with an approximate two-fold increase in NOS activity and two-fold reduction in plasma ADMA. Ito et al demonstrated that incubation of transformed human umbilical vein endothelial cells with oxidized LDL and TNF-α (markers of oxidative stress and inflammation) resulted in reduction of DDAH activity and elevation of ADMA levels, and that these changes are associated with endothelial dysfunction (Ito, Tsao et al. 1999). There is also emerging evidence from recent DDAH-1 knockout animal studies that DDAH may also mediate NOS inhibition independent of ADMA. Leiper et al (2007) induced a 50% decrease in DDAH which was associated with a 20% rise in ADMA levels but significant alterations in vascular function (up to 40%), raising the possibility of ADMA independent actions of DDAH. Furthermore, the addition of exogenous L-arginine only partially reversed the loss of endothelial relaxation.

The role or significance of PRMT in regulation of ADMA and endothelial dysfunction is still largely unknown. Boger et al (1998) demonstrated that the expression and activity of PRMTs are upregulated in the presence of low-density lipoproteins (LDL), with corresponding rise in ADMA concentration, thus providing a possible partial basis for endothelial dysfunction seen in hypercholesterolemic patients. Furthermore there is some evidence that shear stress affects PRMT gene expression and ADMA release via the NF-κB pathway. Despite these studies, the contribution of PRMT in cardiovascular disease has not been studied thoroughly.

B2.3 Reduced sensitivity to NO

The concept of reduced sensitivity or "NO resistance", refers to the hypo-responsiveness to the antiaggregatory and vasodilatory effects of NO previously demonstrated in both platelets and coronary arteries of patients with stable angina. This phenomenon has been associated with various cardiovascular risk factors and disease states, and also has prognostic implications which will be described in detail below.

This phenomenon may arise from several mechanisms, involving the disruption of the NO/cGMP signalling pathway: (a) increased clearance of NO by O_2^-, resulting in reduced NO bioavailability for the interaction with sGC and (b) alterations/dysfunction at the sGC level. These are described in detail below.

B2.3.1 NO resistance: Increased clearance of NO- role of oxidative stress

As mentioned above, NO can be inactivated by reactions with molecular oxygen, ROS and oxidized LDL: products of increased oxidative stress. Oxidative stress occurs as a result of an imbalance between the production of reactive oxygen species (ROS) and their inactivation by antioxidant systems. ROS (including O_2^-, H_2O_2, lipid peroxides) can be generated by many enzymes, including xanthine oxidase, cytochrome P450 monooxygenase, "uncoupled" NOS and NADPH oxidases within all aerobic cells. ROS promote lipid oxidation, stimulate smooth muscle growth and initiate expression of proinflammatory genes (Griendling and FitzGerald 2003). Furthermore, ROS lead to endothelial

cell apoptosis (Dimmeler and Zeiher 2000), a prothrombotic state in the vascular lumen and activation of matrix metalloproteinases, which may lead to plaque instability and rupture (Rajagopalan, Meng et al. 1996). Endogenous scavengers of ROS include reduced gluthathione (GSH), superoxide dismutase (SOD) and gluthathione peroxidase (GSH-Px).

In regards to its relationship to NO resistance, Chirkov et al (1999) demonstrated a fourfold elevation of O_2^- levels and a reduction in the anti-aggregatory effect to the exogenous NO donor, sodium nitroprusside in patients with stable angina pectoris compared to healthy controls. Upon addition of SOD, a O_2^- scavenger, platelet aggregability was reduced and the anti-aggregatory effect of NO was enhanced, suggesting that O_2^- plays a significant role in NO resistance.

The role of oxidative stress in cardiovascular disease has been widely demonstrated. Increased oxidative stress has been documented in patients with traditional risk factors of cardiovascular disease, such as smoking (Morrow, Frei et al. 1995), diabetes (Davi, Ciabattoni et al. 1999) and hypertension (Minuz, Patrignani et al. 2002). Furthermore, ROS have been shown to play a pathophysiological role in myocardial ischemic injury (Hammond and Hess 1985; McCord 1985) and atherogenesis (Morel, DiCorleto et al. 1984; Gesquiere, Loreau et al. 1999). In animal models, the major underlying mechanism has been shown to be inactivation of NO by O_2^- (Lockette, Otsuka et al. 1986).

Despite clear evidence of the contributory role of ROS/superoxide in pathogenesis of atherosclerosis and cardiovascular disease states, results from

clinical trials on the use of antioxidant vitamin therapy are largely negative. While some studies demonstrate that acute vitamin C therapy resulted in the improvement in endothelial-dependent vasodilatation in subjects with coronary artery disease or risk factors of vascular disease (Heitzer, Just et al. 1996; Ting, Timimi et al. 1997; Taddei, Virdis et al. 1998; Tousoulis, Xenakis et al. 2005), larger population studies with vitamins E and C have failed to demonstrate any benefit in progression of atherosclerosis or cardiovascular outcomes (Yusuf, Dagenais et al. 2000; Sesso, Buring et al. 2008).

Thus in summary, the generation of ROS as a component of oxidative stress results in increased clearance of NO, largely via interaction with O_2^-. This leads to the formation of peroxynitrite ($ONOO^-$), which largely via activation of PARP receptors, may induce DNA damage and/or activation of apoptosis. Furthermore, this increased "scavenging" of NO by O_2^- represents a major source of endothelial/platelet dysfunction by virtue of lack of NO effect, as has been documented especially in diabetes mellitus and coronary atherogenesis.

The role of the thioredoxin system in modulating both oxidative stress and NO bioavailability is discussed later.

B2.3.2 "NO resistance": Alterations at soluble guanylate cyclase level

Alterations at the soluble guanylate cyclase level have also been implicated in the pathophysiology of NO resistance, possibly due to reduced sensitivity to NO or loss of sGC function. Chirkov et al (1999) demonstrated that in the presence of an inhibitor of guanylate cyclase activation, ODQ, the

antiaggregatory effects of SNP and NTG were suppressed in both patients with angina and controls, but to a significantly lesser extent in angina patients. These findings suggest a loss of sGC sensitivity to NO, which may in part also be linked again to O_2^- which has been shown to be able to inhibit human platelet sGC function.

There is also an important issue underlying the observed change in sGC activity in NO resistance. sGC is a haem protein which is substantially inactivated by loss of its haem moiety, to the extent where it becomes unresponsive to NO. This effect can be overcome with the use of sGC stimulators such as BAY41-2667, which stabilizes the nitrosyl-haem complex in low levels of NO to maintain its active configuration, and also sGC activators such as BAY 58-2667, which binds to the sGC haem-pocket to mimic the haem group [for review see (Stasch, Pacher et al. 2011)]. A number of recent studies suggest that resistance to NO may be ameliorated by agents such as perhexiline and angiotensin converting enzyme inhibitors (ACEIs), without necessarily affecting haem/sGC dissociation. The implication is that sGC dysfunction is not necessarily engendered by haem depletion.

B2.4 Role of thrombospondin-1 in NO/cGMP pathway

There has been recent interest in the role of thrombospondin-1(TSP-1) as a negative regulator of NO/cGMP signalling in the vasculature. TSP-1 is a glycoprotein that is expressed in abundance in α-granules of platelets and also in megakaryocytes and sites of tissue injury or remodelling (Chen, Herndon et al. 2000). TSP-1 mediates its actions by binding to different receptors found on platelets, vascular smooth muscle cells and endothelial cells, the most important of these being the CD47 receptor which is necessary for its actions (Gao, Lindberg et al. 1996). Current literature suggests that the primary mechanism of action of TSP-1 is as a soluble guanylate cyclase inhibitor, which leads to inhibition of NO-driven cGMP production in endothelial and vascular smooth muscle cells (Isenberg, Ridnour et al. 2005). Although the exact mechanism(s) are yet to be resolved, it does not appear that TSP-1 induces dissociation of sGC from its haem moiety. Other possible mechanisms of actions include inhibition of eNOS activity and on downstream cGMP targets as well (Isenberg, Jia et al. 2007). Furthermore, TSP-1 inhibits adenylate cyclase (Yao, Roberts et al. 2011) and increases the generation of the proinflammatory mediator TGFβ1 (Murphy-Ullrich and Poczatek 2000).

Whilst the role of TSP-1 has been demonstrated in various animal models of chronic diseases such as hypertension (Bauer, Qin et al. 2010) and diabetes (Stenina, Krukovets et al. 2003), no data are currently available on its pathophysiological role or its contribution to chronic diseases in humans.

B2.5 Role of thioredoxin system in NO/cGMP signalling

The thioredoxin (TRX) system (comprising of TRX, TRX-reductase and NADPH) have also been demonstrated to play a role in the modulation of NO signalling and cardiovascular diseases.

TRX is ubiquitous and comprises of two isoforms, TRX-1 which is primarily found in cytosol and nucleus of cells and TRX-2 in mitochondria. TRX acts essentially as an endogenous antioxidant, via several mechanisms. In its reduced state, TRX reduces oxidized cysteine groups on various proteins to form a disulfide bond, which is then reduced back by the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent TRX-reductase, as depicted in Figure 1.4A. More importantly, TRX induces the scavenging of reactive oxygen species (ROS) by reducing oxidized TRX peroxidases. The actions of TRX can be inhibited by thioredoxin-interacting protein (TXNIP), which acts on the reduced forms of TRX (Figure 1.4B).

TRX levels have been shown to be elevated in models of injury/stress (Shioji, Kishimoto et al. 2002) and reduced in association with chronic diseases such as hypertension (Ebrahimian and Touyz 2008). It has also been shown to play a role in the modulation of endothelial cell and vascular smooth muscle cell function including migratory and proliferative capacity (Dai, He et al. 2009) (Schulze, De Keulenaer et al. 2002; Xu, He et al. 2009). Its cardioprotective effects include the regulation of oxidative stress and the inhibition of pathological cardiac hypertrophy (Yamamoto, Yang et al. 2003; Yang, Ago et al. 2011).

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Figure 1.4 **The TRX system.** A, TRX in the oxidized (TRX-S2) or reduced (TRX-(SH)2) state. In the reduced state, TRX directly reduces disulfides in oxidized substrate proteins (PR−-S2). The resultant oxidation of TRX in this process is reversible and maintained by thioredoxin reductase (TRXR) and the electron donor NADPH. B, TXNIP can form a mixed disulfide with reduced TRX, inhibiting the ability of TRX to reduce disulfides of other protein substrates or undergo reversible oxidation.
Adapted from (Dunn, Buckle et al. 2010)

However, it is its inhibitor, TXNIP, which appears to play a pivotal role in the modulation of various disease states. TXNIP expression has been shown to be induced by hyperglycemia. In microarray studies of human pancreatic β cells (Shalev, Pise-Masison et al. 2002), TXNIP was identified as the gene most strongly induced by glucose, driven by a carbohydrate response element within the TXNIP promoter site. This response has also been demonstrated in a variety of other cell types, including vascular smooth muscle cells (Schulze, Yoshioka et al. 2004), endothelial cells (Li, Rong et al. 2009) and various cancer cells (Turturro, Friday et al. 2007; Yamaguchi, Takata et al. 2008). The hyperglycaemic induction of TxNIP results in increased apoptosis by competitive inhibition of the TRX1/apoptosis signal-

regulating kinase (ASK-1) complex (Chen, Saxena et al. 2008; Chen, Cha-Molstad et al. 2009) in pancreatic β islets and cardiomyocytes; and the suppression of TRX activity, induction of ROS accumulation (Schulze, Yoshioka et al. 2004) and inhibition of vascular network formation (Buckle A 2007) in vascular smooth muscle and endothelial cells.

There is evidence that dysregulation of TRX/TXNIP is involved in the pathophysiological mechanisms in metabolic disorders such as diabetes; various cancers (Goldberg, Miele et al. 2003; Nishinaka, Nishiyama et al. 2004; Dutta, Nishinaka et al. 2005) and inflammation (Schulze, De Keulenaer et al. 2002). Shalev et al (2002) demonstrated that TxNIP is associated with pancreatic β-cell toxicity. Furthermore, there is evidence also that TXNIP regulates both insulin-dependent and insulin-independent pathways of glucose uptake in human skeletal muscle (Parikh, Carlsson et al. 2007). Of particular interest to this thesis are studies suggesting that TRX/TXNIP is also implicated in the regulation of cellular proliferation and the aging process (Yoshida, Nakamura et al. 2005).

To date, there are limited data on the potential interaction between the TRX system and NO signalling. Schulze et al (2006) demonstrated that incubation of pulmonary artery smooth muscle cells with NO resulted in the suppression of TXNIP and increased TRX reductase and thioredoxin activity. Further studies investigating this relationship are required.

Section B, Part 3: Clinical concepts involving NO/cGMP signalling pathway impairment

B3.1 Introduction

2 major clinical concepts have been described related to this pathway, and have been demonstrated in association with various cardiovascular risk factors and disease states: endothelial dysfunction and NO resistance. Although both abnormalities have been described together, they are conceptually different as outlined in figure 1.5.

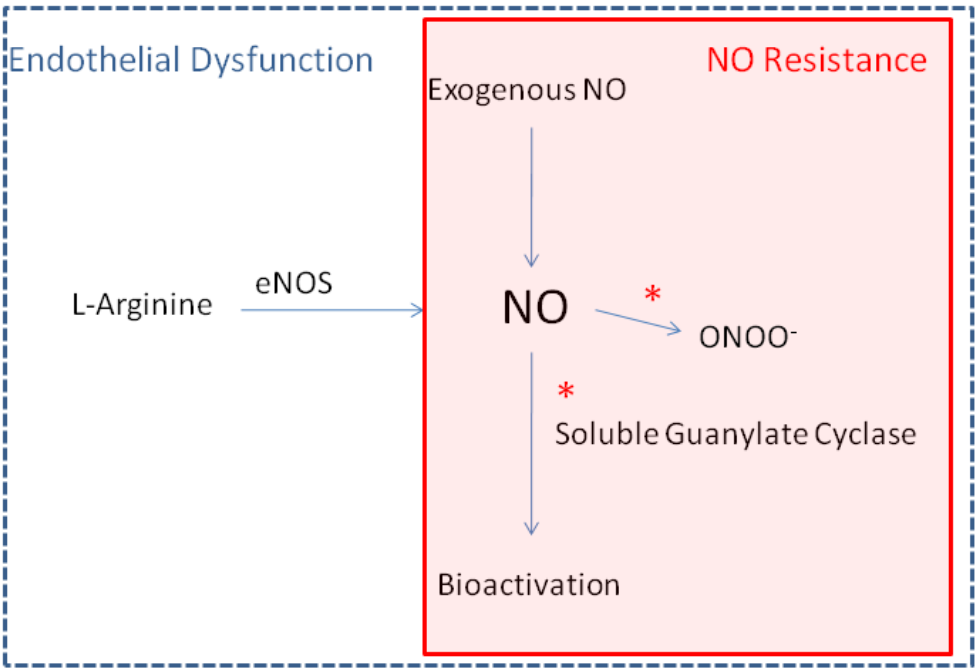

Figure 1.5 **Differences between endothelial dysfunction and NO resistance.** ---- box represents concept of endothelial dysfunction, which encompasses abnormalities from NO production to sGC/cGMP downstream signalling to NO effects. __ box encompasses abnormalities involved in NO resistance, which assumes NO availability.

B 3.2 Endothelial dysfunction in cardiovascular disease

As mentioned previously, the endothelium plays an important role in regulation and maintenance of blood flow by production of various vasoactive agents. Accordingly, endothelial injury and dysfunction are thought to be critical events in the pathogenesis of atherosclerosis. Endothelial dysfunction is characterized by impaired endothelial-mediated vasodilatation and/or paradoxical vasoconstriction responses to endothelial-dependent vasomotor agents. On a cellular level, the endothelial cells lose their ability to produce NO and also demonstrate increased expression of vasoconstrictor, proinflammatory and prothrombotic factors.

Endothelial dysfunction has been reported in many conditions, some of which are listed below (Table 1.5).

Table 1.5. Clinical associations of vascular endothelial function

Cardiovascular Risk Factors	**Cardiovascular Diseases**	**Non-cardiac Diseases**
Hypercholesterolemia High HDL Hypertension High Lp(a) Hyperhomocystinemia Active/passive smoking Insulin resistance Diabetes- type I and II	Atherosclerosis Syndrome X/Variant angina Heart failure Dilated cardiomyopathy Pulmonary hypertension Atrial fibrillation Aortic stenosis	Aging Postmenopuasal Vasculitic conditions Chagas disease Kawasaki's disease Preeclampsia

Endothelial dysfunction has been demonstrated in both coronary and peripheral vasculature. Ludmer et al (1986) demonstrated evidence of acetylcholine-induced coronary spasm at sites of atheroma, presumably as a result of impaired release of NO. Multiple cardiovascular risk factors have been demonstrated to be associated with peripheral endothelial dysfunction. Experimentally, evidence of endothelial dysfunction precedes that of atherosclerosis, and this may well be the case in most clinical circumstances.

Besides the implications in pathogenesis, endothelial dysfunction also has been shown to have prognostic implications. Halcox et al (2002) demonstrated in a cohort of individuals, with and without coronary artery disease, epicardial and microvascular coronary endothelial dysfunction were independent predictors of acute cardiovascular events. Similarly, Schachinger et al (2000) demonstrated the prognostic value of coronary vasoreactivity, after adjustment for cardiovascular risk factors and presence of atherosclerosis, in terms of predicting long-term progression of atherosclerotic disease and cardiovascular event rates. Peripheral endothelial dysfunction has also been shown to be an adverse prognostic marker in those with peripheral arterial disease (Brevetti, Silvestro et al. 2003), congestive cardiac failure (Fischer, Rossa et al. 2005) and hypertension (Perticone, Ceravolo et al. 2001).

Various therapies have now also been shown to improve/attenuate endothelial dysfunction. Leung et al (1993) were the first to demonstrate the beneficial effects of lipid lowering achieved by diet and the use of cholestyramine, on endothelial-dependent vasodilatation. Similar

improvements were then demonstrated with the use of statins (Wassmann, Laufs et al. 2001; Weis, Pehlivanli et al. 2001) and other cholesterol lowering treatment (Keaney, Xu et al. 1995). Studies using vitamin C as an antioxidant have also previously shown improved endothelial function (Heitzer, Just et al. 1996; Ting, Timimi et al. 1997). Angiotensin-converting enzyme inhibitors (ACEIs) have also been shown to improve coronary (Mancini, Henry et al. 1996) and peripheral endothelial function (Taddei, Virdis et al. 1998). It has been speculated that the beneficial effects of ACE inhibition are likely due to the attenuation of vasoconstriction and superoxide-generating effects of angiotensin II and to enhancement of endothelial cell release of NO secondary to diminished breakdown of bradykinin (Hornig, Kohler et al. 1997).

B3.3 Nitric oxide resistance in cardiovascular disease

”NO resistance” as described above, refers to the observed reduced or lack of responsiveness to the vasodilatory and anti-aggregatory effects of NO. This phenomenon has been demonstrated particularly in blood vessels and platelets, and evidence is accumulating of its importance in myocardium (Kelm, Schafer et al. 1997).

B3.3.1 Nitric oxide resistance in blood vessels

As depicted in the above figure, endothelial dysfunction encompasses abnormalities in the production of NO (which involves the endothelial cells) and also in the endothelium-independent pathways (NO/cGMP signalling pathway). It is the abnormalities in the endothelium-independent pathway that manifest as NO resistance in blood vessels. Schachinger et al (1995) demonstrated an impairment of coronary vasodilator function to both nitroglycerin (NTG) and acetylcholine (ACh) (endothelium-independent and endothelium-dependent function respectively) in a cohort of patients with no or minimal coronary artery disease (Schachinger and Zeiher 1995). Katz et al (1992) demonstrated a similar impairment in both ACh and NTG femoral bed vasodilatation in patients with heart failure.

A number of investigations of NO resistance have utilized NTG as an NO donor. In theory, this is problematic because of the issue of nitrate tolerance in patients chronically treated with organic nitrates. Therefore, investigations of NO resistance should avoid utilizing NTG except on a de novo basis.

NO resistance in blood vessels has been demonstrated in association with multiple cardiovascular risk factors and conditions. In a large study of 800 asymptomatic, high-risk individuals by Adams et al (1998), impaired de novo vascular NTG responses were associated with cardiovascular risk factors of hypercholesterolemia, cigarette smoking, diabetes, male gender, advanced age and larger vessel size on univariate analysis. On multivariate analysis, diabetes and larger vessel size were associated with impaired NTG responses. Individuals with essential hypertension and hypertensive stroke patients also demonstrate evidence of impaired NTG responsiveness. Raitakari et al (2001) demonstrated impaired responsiveness to NTG in the brachial arteries of patients with coronary artery disease. As mentioned above, Katz et al (1992) also demonstrated impaired NTG responses in congestive cardiac failure patients (NYHA Class I-IV).

B3.3.2 Nitric oxide resistance and its relationship to endothelial dysfunction

The relationship between NO resistance and endothelial dysfunction was demonstrated in the study by Adams et al (1998), where a significant direct correlation was found between impaired endothelium-dependent vasodilatation and impaired NTG-related vasodilatation. Furthermore, Schachinger and Zeiher et al (1995) demonstrated that the presence of atherosclerosis (utilizing a combination of quantitative angiography and intracoronary ultrasound) was associated with impairment of the vasodilator response to both NTG and acetylcholine in epicardial arteries *in vivo*, with an inverse relation between local atherosclerotic plaque load and NTG-induced changes in vasomotor tone

($r = -0.65$, $P < 0.0001$). Moreover, both impaired endothelial-dependent and endothelium-independent coronary vasodilatation have been shown to be significant, independent adverse predictors of cardiovascular events and of the presence of atherosclerosis itself (Schachinger, Britten et al. 2000). These results suggest that the abnormalities in vasodilatory function arise from a combination of endothelial as well as smooth muscle dysfunction, which may be structural (e.g. due to smooth muscle fibrosis, atrophy) or functional (e.g. impaired cGMP signalling).

B3.3.3 Nitric oxide resistance at the platelet level

At the platelet level, NO resistance manifests as impaired responsiveness to the anti-aggregatory effect to NO, which is measurable (discussed below). Again this phenomenon has been associated with multiple cardiovascular conditions and also has prognostic implications.

Chirkov et al (1999) have previously demonstrated evidence of platelet hyporesponsiveness to exogenous NO in patients with stable angina. Furthermore, the degree of resistance was more marked in patients who presented with an acute coronary syndrome (ACS) in comparison to those with stable angina (Chirkov, Holmes et al. 2001). This phenomenon was also demonstrated in patients with congestive cardiac failure not previously treated with ACEIs (Chirkov, Holmes et al. 2004) and aortic stenosis (Chirkov, Holmes et al. 2002). Cardiovascular risk factors e.g. hypertension (Woods, Edwards et al. 1993) and type II diabetes (Anderson, Ellis et al. 2005) have also been associated with impaired platelet NO responsiveness.

Willoughby et al (2005) demonstrated that impaired platelet NO responsiveness at baseline in a cohort of ACS patients was associated with an increased in cardiovascular readmission and/or death (relative risk 2.7, p=0.04) and all-cause mortality (relative risk 6.3, p=0.03) at 7 years. These data therefore suggests that impaired platelet NO responsiveness is an adverse prognostic marker of cardiovascular events/mortality.

Section B, Part 4 : Assessment of NO/cGMP system

B4.1 Introduction

In practice, we are now able to assess the integrity of NOS/cGMP signalling pathways, utilizing various biochemical or physiological methods. These are discussed below.

B4.2 Biochemical measures of NO/cGMP system

B4.2.1Plasma ADMA concentrations

The role of ADMA, the endogenous NOS inhibitor, in the NOS/cGMP pathway has been discussed above. Plasma concentrations of ADMA can now be measured utilising high performance liquid chromatography (HPLC), various methods of mass spectrometry (MS) or utilizing commercially available ELISA kits.

Although there are uncertainties as to the specificity of some of the earlier assay methods (Horowitz and Heresztyn 2007), recently developed HPLC and MS assays tend to indicate normal plasma concentrations of 0.4-0.5μM (Martens-Lobenhoffer and Bode-Böger 2006). The reproducibility of such assay data (Valtonen, Karppi et al. 2005) has facilitated their utilization as an ideal biochemical marker of potential impairment of the NO system (Cooke 2004).

B4.2.2 Inflammatory markers

Various inflammatory markers have been demonstrated in association with endothelial dysfunction including interleukin-6, tumour necrosis factor-α, soluble P-selectin and soluble intercellular adhesion molecule-1(Blake and Ridker 2001). However high sensitivity C-reactive protein (hs-CRP) is the most widely studied and appears to be the strongest predictor of future cardiovascular events as well (Ridker, Hennekens et al. 2000; Ridker 2001; Ridker, Stampfer et al. 2001).

At a cellular level, CRP has been shown to modulate basal and stimulated endothelial NO release, by mediating the actions of NOS (Verma, Wang et al. 2002). In human aortic endothelial cells, CRP has been shown to decrease eNOS expression and bioactivity (Venugopal, Devaraj et al. 2002). Further studies, have demonstrated that CRP may induce eNOS "uncoupling" (Hein, Singh et al. 2009), with increased superoxide generation, decreased NO production and altered eNOS phosphorylation.

In clinical studies, CRP levels are correlated with endothelium-dependent vasodilatation (Fichtlscherer, Rosenberger et al. 2000). Furthermore CRP levels is associated with increased oxidative stress production in patients with known coronary artery disease, which is ameliorated with the use of Vitamin C (Heitzer, Schlinzig et al. 2001), and appear to be involved in the regulation of systemic NO bioavailability.

B4.3 Physiological measures of NO/cGMP system

These include various assessments of endothelial and platelet function, which are invasive and non-invasive.

B4.3.1 Endothelial function

Both endothelium-dependent and –independent function can be determined by measuring the vasodilatory response to various pharmacological and physiological stimuli, both in the coronary and peripheral circulation [for latest review see (Lekakis, Abraham et al. 2011)].

a) Invasive intra-coronary studies

Ludmer et al. (1986) first demonstrated that endothelial function in human coronary arteries can be assessed in vivo by measuring the vasomotor responses of epicardial arteries by quantitative coronary angiography in response to graded concentrations of acetylcholine and other agonists. They found that in arteries with preserved endothelial function, infusion of acetylcholine resulted in vasodilatation, reflecting the release of NO from the endothelium. However, infusion of acetylcholine into atherosclerotic vessels resulted in paradoxical vasoconstriction of the vessels, reflecting unopposed vasoconstrictor effect of acetylcholine and effective loss of NO in atherosclerotic vessels (Quyyumi, Dakak et al. 1997).

However the invasive nature of such studies limits its widespread applicability, especially in well, asymptomatic individuals. In practice, the only circumstance in which there is general consensus about the clinical utility of intracoronary

acetylcholine administration relates to its use as a diagnostic test for coronary vasospasm (Sueda, Kohno et al. 2004).

b) Venous occlusion plethysmography

Endothelial function can also be assessed by using a semi-invasive technique: venous occlusion plethysmography (in combination with intra-arterial drug administration), which measures forearm volume and therefore indirectly forearm blood flow (FBF). Details of this method and its reproducibility have been described previously (Benjamin, Calver et al. 1995; Petrie, Ueda et al. 1998). In brief, a small needle is inserted into the brachial artery of the non-dominant arm for infusion of endothelium-dependent (e.g. acetylcholine) or endothelium-independent (e.g. NTG, SNP) agonists and recording of blood pressure using a pressure transducer. Venous return is occluded using an upper arm cuff inflated to just above venous pressure (about 40mmHg) and then the drugs are administered. A strain gauge placed around the forearm is connected to a plethysmography device to enable the measurement of changes in forearm volumes in response to the degree of vasodilatation/vasoconstriction, simultaneously in the test and control forearms.

A reduction in blood flow response to Ach in the forearm has been shown to be closely related to both the impairment in coronary endothelial-dependent vasodilatation and the presence of coronary artery disease (Anderson, Uehata et al. 1995).

c) Flow-mediated vasodilatation

Increased blood flow (shear stress) acts as a mechanical stimulus for NO production from endothelial cells. Celermajer et al. (1992) were one of the first groups to describe a non-invasive way of assessing endothelial function based on changing the shear stress in the forearm. Hyperemia is induced by inflating a forearm cuff to suprasystolic pressures to induce forearm ischemia and released to produce reactive hyperaemia (endothelium-mediated vasodilatation). High-resolution ultrasound or magnetic resonance imaging is used to measure the diameter of the brachial artery at rest and during reactive hyperaemia. Imaging can also be performed with the use of sublingual nitroglycerine to assess for endothelium-independent vasodilatation.

Several studies have shown a correlation between flow-mediated vasodilatation (FMD) in the brachial artery and intra-coronary endothelium-dependent vasomotor responses to ACh (Anderson, Uehata et al. 1995), reflecting again that changes in endothelial function are systemic rather than localised to coronary vessels.

The major limitation of this technique is that it is highly operator-dependent.

d) Pulse wave analysis (PWA)

PWA is a simple non-invasive technique used to assess arterial stiffness (O'Rourke and Gallagher 1996; Wilkinson, Cockcroft et al. 1998). It involves

the use of applanation tonometry to obtain peripheral (usually radial) artery waveforms, from which central aortic pressure can be derived. The central aortic pressure is composed of a forward-travelling wave generated by left ventricular ejection and a later-arriving reflected wave from the periphery. Augmentation index (AIx, in percentage) can then be calculated from the ratio of the augmentation pressure (difference of pressure between the first systolic shoulder to the systolic peak) and the pulse pressure. AIx has been validated as a measure of arterial stiffness (Wilkinson, Fuchs et al. 1998) and is an independent risk marker of premature coronary artery disease (Weber, Auer et al. 2004). AIx increases with age (O'Rourke, Pauca et al. 2001) as well as certain cardiovascular risk factors.

The effect of sublingual NTG on AIx provides a measure of endothelium-independent vasodilatation or vascular NO resistance. Furthermore, the concomitant use of salbutamol, a $ß_2$ agonist, enables the assessment of endothelial-dependent vasodilatation as well. Salbutamol has been shown to result in endothelial release of NO in humans (Dawes, Chowienczyk et al. 1997) and in animal studies. The use of this combination of medications with PWA has been illustrated in several studies (Hayward, Kraidly et al. 2002; Wilkinson, Hall et al. 2002). In Wilkinson et al's study, they demonstrated a significant reduction in baseline AIx to salbutamol (400µg) but not to NTG in hypercholesterolaemic subjects when compared with matched controls. The effect of salbutamol but not NTG, was abolished by the introduction of a NOS inhibitor LNMMA, reflecting its endothelium-dependent effect. Furthermore, a significant linear correlation was found between the change in AIx after salbutamol and the change in forearm blood flow in response to acetylcholine,

both endothelium dependent. No relationship was seen with Ach response and AIx following NTG administration.

Again this technique is operator-dependent, and adequate doses of NTG and salbutamol must be administered to elicit adequate responses.

B4.3.2 Assessment of platelet NO responsiveness

Several methods are currently available to evaluate platelet NO responsiveness, which involves the measurement of platelet aggregation and subsequent inhibition of aggregation by exogenous NO donors.

a) Impedance platelet aggregometry

This is the most widely used technique in studies of platelet NO resistance. It involves the use of an aggregometer which measures changes in electrical impedance between two platinum wire electrodes immersed in whole blood. Whole blood is placed in a cuvette and stirred at 37ºC. An exogenous agonist such as ADP is then added to whole blood which causes the platelets to aggregate around the electrode, thus increasing the electrical impedance. SNP, an exogenous NO donor, is then added for 1 minute prior to the addition of ADP. The anti-aggregatory effect can then be calculated (expressed in %).

Although not time-consuming, this technique is limited by the lifespan of platelets (approximately an hour) and therefore has to be performed as soon as blood is collected. Stringent conditions are required as well to reduce the

degree of basal platelet activation including at least 15 minutes of resting of subject and adequate needle bore size with atraumatic collection into a heparinised tube to avoid haemolysis/clotting and platelet activation. Post collection samples should also be rested for these reasons.

It must also be appreciated that platelet aggregometry performed utilizing whole blood is affected by interactions between platelets and other formed elements of blood, such as neutrophils.

b) Optical platelet aggregometry

The similar effect can be performed using platelet-rich plasma (PRP) and optical platelet aggregometry. This technique relies on changes in light transmission of PRP during platelet aggregation (Born 1962). PRP is obtained by centrifugation of venous whole blood sample at 250 g for 10 minutes. Then, the PRP is placed in a cuvette under similar conditions as above, between a light source and a photocell within an aggregometer. Upon addition of an agonist, the aggregated platelets clump together and fall to the bottom of the tube, thus increasing light transmittance. The magnitude of aggregation is detected by a maximum change in light transmission.

Although results obtained from PRP are comparable to those in whole blood(Ingerman-Wojenski, Smith et al. 1983), there are several advantages of whole blood above PRP. The use of whole blood allows platelets to be analysed in their physiological milieu and therefore takes into consideration the contribution of other blood constituents, namely, leukocytes and erythrocytes on

platelet activation. PRP also requires more preparation and therefore more time-consuming and may result in higher sources of variability such as variation in centrifugation speeds and the adjustment of platelet concentration to some standard count.

CHAPTER 1, SECTION C

Polycystic ovary syndrome and the NO/cGMP pathway

C1.1. Introduction

There are currently physiological and biochemical studies available in PCOS individuals to suggest that disruption in NO/cGMP signalling (see below) frequently occurs in association with this syndrome. However it remains debatable from these studies if the disruption cumulates into excess cardiovascular events in this cohort or if PCOS *per se*, independent of its metabolic complications are associated with such abnormalities.

C1.2 Endothelial dysfunction in PCOS

Physiological assessment of endothelial function has been performed in PCOS individuals with majority of the studies utilising FMD. Tarkun et al (2004) demonstrated evidence of impaired FMD and NTG responses in a cohort of PCOS women when compared to control women. Furthermore, FMD was correlated with hsCRP and insulin resistance. Paradisi et al (Paradisi, Steinberg et al. 2001), utilizing leg blood flow responses, found that the endothelial dysfunction in PCOS women was correlated with testosterone levels and body mass index. Kelly et al (Kelly, Speirs et al. 2002) demonstrated increased brachial artery stiffness (utilising pulse wave velocity) and impaired response of microvessels to insulin in PCOS individuals. Furthermore, Orio et al (Orio, Palomba et al. 2004) demonstrated the presence of endothelial dysfunction in a normal-weight, nondyslipidemic and nonhypertensive cohort of young PCOS women. Furthermore endothelial dysfunction can be ameliorated by use of metformin (Orio, Palomba et al. 2005) and thioglitazones (Tarkun, Cetinarslan et al. 2005; Jensterle, Sebestjen et al. 2008).

However, not all studies have demonstrated endothelial dysfunction in PCOS patients. For example, Mather et al (2000) were unable to detect any difference in brachial artery flow mediated dilatation (FMD) between young obese PCOS patients and age-matched controls.

C1.3 Platelet dysfunction in PCOS

C1.3.1 Platelet hyperaggregability

Platelet hyperaggregability was shown to be present in lean women with PCOS and insulin resistance. Dereli et al (2003) demonstrated that lean women (BMI <25kg/m^2) have significantly greater platelet aggregation (by optical aggregometry) induced by ADP, collagen and epinephrine, compared to age and weight-matched controls. Furthermore platelet aggregation responses were similar when compared to women with non-classical congenital adrenal hyperplasia (NC-CAH), who had similar free testosterone levels as PCOS women. This suggests that platelet hyperaggregability associated with PCOS is not due to elevated androgen levels but instead a positive correlation was found between platelet hyperaggregability and insulin resistance in the PCOS group.

C1.3.2 Altered physiological inhibition of platelet aggregation

We have recently published evidence of impaired platelet NO responsiveness in a cohort of young PCOS women (Rajendran, Willoughby et al. 2009). 24 young women (31±2 years: 12 lean and 12 obese) with PCOS and 12 lean controls were recruited after stringent screening to ensure that they did not have any pre-existing cardiovascular risk factors including smoking and known insulin resistance/type II diabetes. The observed impairment of platelet NO responsiveness in the PCOS cohort was

significant regardless of their BMI or insulin resistance state, and the extent was similar to that previously observed in patient groups with symptomatic myocardial ischaemia (Chirkov, Holmes et al. 1999; Chirkov, Holmes et al. 2001).

C1.4 Potential mechanisms underlying disruption of NO/cGMP pathways in PCOS

It can be assumed that similar biochemical abnormalities in the NO/cGMP signalling pathway are responsible for the endothelial and platelet dysfunction demonstrated in PCOS individuals. Indeed, studies of biomarkers of cardiovascular disease have demonstrated a proinflammatory and proatherogenic profile in PCOS individuals, which may contribute at different levels to the disruption of NO/cGMP pathway (as discussed in Section B).

C1.4.1 Evidence of altered NO production and bioavailability in PCOS

There is evidence in PCOS individuals that NO production may be impaired. Moran et al (2009) demonstrated evidence of elevated ADMA (eNOS inhibitor) concentrations in association with a trend towards impaired FMD in overweight and obese PCOS individuals when compared to BMI-matched individuals. Heutling et al (2008) demonstrated that elevated ADMA concentrations in PCOS individuals were correlated with BMI, insulin sensitivity, androgen levels and carotid intima media thickness.

As mentioned previously, oxidative stress is often associated with reduced NO bioavailability. There are several studies suggesting elevated oxidative stress levels in PCOS individuals. In our study (Rajendran, Willoughby et al. 2009), we found significantly higher levels of malondialdehyde (MDA), a marker of oxidative stress, in PCOS individuals when compared to controls

(0.25±0.02μmol/L vs. 0.17±0.02μmol/L respectively, $p<0.01$). However, there was only a non-significant trend in the relationship between high MDA levels and impaired platelet responsiveness. Furthermore, antioxidant status, as determined by measures of erythrocyte reduced glutathione concentration, glutathione peroxidase and superoxide dismutase concentrations, was also altered in PCOS subjects (Sabuncu, Vural et al. 2001), leading to an increased oxidative state.

Furthermore, inflammatory activation is also frequently associated with impairment of NO signalling (Ito, Tsao et al. 1999). Kelly et al (2001) was the first to demonstrate evidence of low grade inflammation in PCOS individuals compared to controls, which persisted even after correction for age and BMI. We found that elevated hs-CRP levels were found predominantly in the obese PCOS cohort (Rajendran, Willoughby et al. 2009). Furthermore, Tarkun et al (2004) demonstrated the relationship between elevated hs-CRP in PCOS individuals and endothelial-dependent vasodilatation and markers of insulin sensitivity.

C1.5 Limitations of current studies: pathophysiological implications

It is important to highlight that most of these "subclinical" studies have been carried out in young PCOS individuals: for example, in studies of endothelial function - Tarkun et al (2004), mean age 23.5±4.3 years; Paradisi et al (2001), 29.1±1.8 years; Kelly et al (2002), 26±5.8 years, Orio et al (2004) 22.2±2.5 years, suggesting that endothelial function is present/detectable from an early age. On the other hand, studies of atherogenesis in PCOS e.g. measurement of carotid intima media thickness (CIMT) and coronary artery calcification on the other hand focuses on middle aged women : for example, Guzick et al (Guzick, Talbott et al. 1996) studied PCOS women above the age of 40 years and Talbott et al (Talbott, Guzick et al. 2000), mean age 38.7±4.8 years. The obvious question is whether the physiological anomalies detected in younger PCOS subjects provide the nexus to atherogenesis in these individuals. For example, in Orio et al's study, there was evidence of impaired FMD and increased CIMT in the young PCOS women compared to control but the relationship between changes in vascular function and vascular structure was not analysed. To date, there are no specific long-term studies to address this question.

Furthermore, derangements in NO/cGMP signalling pathway appear to be predominantly linked to the metabolic or hormonal complications of this syndrome rather than PCOS *per se.* Talbott et al (2000) demonstrated that PCOS status was a correlate of CIMT in women age >45 years when corrected for age, BMI, LDL but not for other risk factors including insulin

sensitivity. Our study (Rajendran, Willoughby et al. 2009) was the first to demonstrate that both platelet and endothelial dysfunction demonstrated in our cohort of PCOS individuals were independent of BMI and insulin resistant state. Therefore it remains unclear if the presence of PCOS alone confers an increased cardiovascular risk, independent of its associated metabolic and hormonal derangements.

Cardiovascular ageing: rapprochement between physiology and pathology

Section D, Part 1 Ageing as a cardiovascular risk factor

D1.1 Introduction

It is well known that the incidence and prevalence of cardiovascular disease increases with age. Data from the Australian Bureau of Statistics (2004-05) showed that the prevalence rose from 13% for those aged between 35 to 44 years to 23% for those aged 45 to 54 years and to 63% for those aged 75 years and above in a general population. Furthermore, age appears to a strong and independent predictor of cardiovascular disease, when used alone or in multiple risk factor algorithms for predicting cardiovascular disease such as the Framingham risk estimation.

D1.2 Postulated mechanisms leading from physiology to pathology

Ageing has been associated with various age-related changes in the cardiovascular system. A summary of these age-related changes are shown below (Table 1.6).

Table 1.6 Effects of ageing on major structural and functional characteristics of the cardiovascular system

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↓, diminished; ↑, augmented; =, unchanged; VSMC, vascular smooth muscle cells; SOD, superoxide dismutase; MMP, matrix metallo-proteinases. (*Adapted from (Ferrari, Radaelli et al. 2003)*

In the vasculature, alterations in structure and function have been linked to increased risk of atherosclerotic disease. One important functional change is the demonstration of age-related impairment of endothelium-dependent vasodilatation in both animal and human models (Egashira, Inou et al. 1993; Taddei, Virdis et al. 1995). Endothelium-independent vasodilatation does not appear to be affected by age (Celermajer, Sorensen et al. 1994). This anomaly has been shown to be a result of reduced expression and/or function of eNOS, upregulation of iNOS and increased formation of reactive oxygen species in ageing animals (Challah, Nadaud et al. 1997) and humans (Lyons, Roy et al. 1997; Hamilton, Brosnan et al. 2001; Berkowitz, White et al. 2003). Furthermore endothelial cells show increased rates of apoptosis with age, with both increased senescence (Hoffmann, Haendeler et al. 2001) and reduction in rejuvenating capacity (Scheubel, Zorn et al. 2003). The role of endothelial dysfunction as a marker of cardiovascular risk has been discussed.

Structural changes including age-dependent intimal medial thickening also confers an increased cardiovascular risk. The carotid intima media thickness increases 2 to 3 fold between 20 and 90 years of age. This remodelling has been shown to occur in association with age-dependent endothelial dysfunction (Nagai, Metter et al. 1998).

Changes in cardiac structure and function as outlined above is thought to predispose individuals to increased risk of left ventricular hypertrophy (Levy 1988), heart failure (Ho, Pinsky et al. 1993) and the development of atrial fibrillation (Wolf, Abbott et al. 1991). Alterations in cardiomyocyte function and structure, coupled with changes in calcium handling, increased plasma

levels of norepinephrine and epinephrine (Esler, Turner et al. 1995) and impaired responses to β-adrenergic receptor stimulation (Schulman, Lakatta et al. 1992; Fleg, Schulman et al. 1994) are thought to play a role in the resultant increased in myocardial hypertrophy and stiffening. This then leads to increased diastolic filling pressure and left atrial dilatation, leading to diastolic heart failure and atrial fibrillation.

It is however important to highlight that the changes above are heterogeneous and do not always lead to the development of clinically significant atherosclerosis or cardiovascular disease. The factors governing the transition from physiology to pathology remain largely elusive, which appears to be a complex process involving multiple genetic, environmental and biochemical factors.

The general view is that atherosclerosis results from the acceleration of normal vascular ageing by the presence of other traditional cardiovascular risk factors. Animal studies have suggested that the presence of vascular ageing may provide a substrate for worse atherosclerotic disease. Rabbits when fed with an atherogenic (high fat) diet to achieve same plasma levels of cholesterol, demonstrated more severe atherosclerotic lesions in older versus younger rabbits.

The presence of endothelial dysfunction as highlighted above is important as a precursor to atherosclerosis. Ageing has been also associated with increase in oxidative stress. Superoxide generation in 9-12 month old rats were significantly higher than 3-4 month old rats ($p>0.0001$) which can be inhibited by the use of apocynin, an NADPH oxidase activity inhibitor

(Hamilton, Brosnan et al. 2001). In this study, NO bioavailability was also reduced in aged rats, suggesting that the possible role of superoxide in endothelial dysfunction.

Vascular ageing has also been associated with increased endothelial cell senescence, as reflected by telomere shortening. In human abdominal aorta samples, Aviv et al (2001) found a significantly increased frequency of aneuploidy with age and an inverse relationship between telomere length and age. Bekaert et al (2007) also found an age-dependent telomere attrition in a cohort of heathy volunteers, which appeared to be occurring at a significantly faster rate in males than females. The authors also found an association between telomere length and various markers of inflammation and oxidative stress and an unhealthy lifestyle especially in men. In a cohort of women, higher levels of psychological stress were associated with reduction in telomere length on average of the equivalent of at least one decade of additional aging compared to low stress levels, suggesting the role of stress on premature cellular ageing (Epel, Blackburn et al. 2004). Furthermore, the activity of telomerase reverse transcriptase (TERT), an enzyme which can counteract telomere shortening, may be reduced in the presence of age-related increases in oxidative stress (Haendeler, Hoffmann et al. 2003), contributing to increased endothelial cell senescence.

The capacity for endothelial regeneration and vascular angiogenesis is also attenuated with ageing. This may be in part due to impaired secretion of and a reduction in endothelial responsiveness to growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (Swift, Kleinman et al. 1999). Endothelial progenitor cell counts are also

reduced with increasing age, and maybe related to impaired mobilization of these cells from the bone marrow (Scheubel, Zorn et al. 2003) and therefore results in impaired vascular regenerative capacity.

D1.3 Therapeutic implications: can we stop cardiovascular ageing?

If the above hypothesis on "vascular ageing-vascular disease interaction" holds true, then the therapeutic target in the prevention and treatment of cardiovascular disease should be on the modulation of cardiovascular ageing.

Lifestyle interventions including diet and exercise may provide some beneficial effects on cardiovascular ageing. Endurance trained older individuals (age between 61-83 years) had better endothelial vasoreactivity compared to their sedentary counterparts (Rywik, Blackman et al. 1999). In another recent study, middle-aged individuals demonstrated greater magnitude and delayed recovery from endothelial ischemia-reperfusion injury (induced by 20 minutes of forearm venous occlusion) when compared to younger individuals. Habitual exercise in these middle-aged men reduced the magnitude of injury (DeVan, Umpierre et al. 2011). Endurance-aerobic training has also been shown to improve arterial stiffness in middle-aged and older men (Tanaka, Dinenno et al. 2000). Furthermore, exercise training in ageing mice has been shown to reduce age-related vascular inflammation (Lesniewski, Durrant et al. 2011).

Dietary changes have also been shown to provide some benefits in cardiovascular ageing. Consumption of "Mediterranean-type" diet for 6 weeks was associated with an improvement in vascular and smooth muscle function in individuals aged between 57-80 years (Singh, Graves et al.

2002). Low salt diet has also shown to reduce ageing-dependent increases in arterial stiffening (Avolio, Clyde et al. 1986).

Other lifestyle changes such as cessation of smoking (Tell, Polak et al. 1994) and weight reduction (Varady, Bhutani et al. 2011) may influence vascular health and therefore vascular ageing as well.

Several pharmacotherapeutic options have been considered to prevent cardiovascular ageing. In rats inhibition of cyclooxygenase-2 (COX-2) by indomethacin resulted in restoration of age-related endothelium-dependent vascular dysfunction in aged rats, suggesting the role of COX-2 derived vasoconstricting factors in age-related endothelial dysfunction (Mukai, Shimokawa et al. 2002). Clinically, the use of aspirin for COX inhibition, rather than COX-2 inhibitors, has been trialled for primary prevention. Various meta-analyses of such trials have shown an overall benefit use of aspirin on reduction of cardiovascular events and mortality, but only in selected individuals who have a 10 year cardiovascular risk of ≥10-15% (Sanmuganathan, Ghahramani et al. 2001; Greving, Buskens et al. 2008). Furthermore in an Australian study in individuals above the age of 70, the net benefits of aspirin are offset by the risk of haemorrhage (Nelson, Liew et al. 2005). There are no studies to date assessing effects of aspirin on vascular ageing in humans, and therefore remains largely unknown.

Angiotensin converting enzyme inhibitors (ACEIs) have been shown in animal models to improve age-related arterial and cardiac stiffness and remodelling (Michel, Heudes et al. 1994). This improvement in age-related endothelial function in rats may be related to amelioration of COX-2 derived

vasoconstriction and superoxide anions by ACEIs (Mukai, Shimokawa et al. 2002). In humans, ACEIs have been shown to have a cardioprotective effect in high-risk individuals, but whether this benefit is related to improvement in age-related vascular changes is unknown.

Statin therapy has also been shown in ageing mice to ameliorate the age-related impairment in angiogenesis and vasculogenesis (Shimada, Takeshita et al. 2004) and have anti-oxidant effects. However in Mukai et al's study, cerivastatin was not associated with any improvement in age-related endothelial dependent vasorelaxation (Mukai, Shimokawa et al. 2002). In humans, statin therapy has been shown to be effective in primary prevention studies but whether this is due to alterations on vascular ageing is still unclear.

Resveratrol, a phytoalexin (compounds that are produced in plants in response to environmental stress) found in abundance in grapevine and grape skin (therefore red wine) has been shown to exert anti-inflammatory and anti-oxidative effects in ageing vasculature [see (Labinskyy, Csiszar et al. 2006)] and therefore may offer protection against "vascular ageing". Epidemiological studies have observed a cardioprotective effect of light red wine drinking, e.g. Rimm et al. (1999) observed that middle-aged men who reported drinking, on average, on 3–4 days per week had a relative risk of 0.66 for CVD, compared with men who drank less than 1 day a week. However, the exact impact of red wine on vascular ageing is unclear.

Section D, Part 2: Ageing of the NO system

D2.1 Evidence of ageing of NO system

Many of the cardiovascular age changes e.g. endothelial dysfunction are influenced by NO. On a molecular level, multiple studies have shown defects in NOS/cGMP pathway in ageing animal models. In ageing rats, NO production has been shown to decline with age, due to the reduction of eNOS activity with ageing (Cernadas, Sanchez de Miguel et al. 1998; Chou, Yen et al. 1998). This may be in part related to upregulation of arginase with ageing, which modulates NOS activity by regulating the availability of its substrate, L-arginine (Berkowitz, White et al. 2003). This is further supported by the evidence of reduction in L-arginine by 30% and 50% with increasing age from 3 to 25 months in rat (Reckelhoff, Kellum et al. 1994). Oxidative stress also may play a part in NO/cGMP signalling alterations with increasing age either by increasing the breakdown of NO or eNOS uncoupling. The reduced bone marrow mobilization of EPCS is also partly mediated by NO, and therefore provides further evidence of decline in NO with age.

Physiologically, age-related endothelial dysfunction provides indirect evidence of NO ageing. However, endothelium-independent vasodilatation, which reflects smooth muscle (tissue) responsiveness to NO, has yielded mixed results. There are no studies to date assessing platelet NO responsiveness with increasing age.

Cardiovascular Ageing in Women

Section E, Part 1: Scope of cardiovascular disease in women

E1.1 Introduction

Cardiovascular disease (CVD), specifically coronary heart disease (CHD), is the leading cause of death in women in Australia. It is estimated to account for up to 60% of female deaths, and more importantly, a majority of these deaths were premature (ABS 2008).

E1.2 Trends by age and gender

The latest Australian data on the prevalence of CHD according to age and gender are shown below, based on the National Health Survey 2007-08 (AIHW 2011). Due to the method of collection (excludes individuals who are institutionalised or hospitalised at time of survey), the prevalence of CVD is under-estimated.

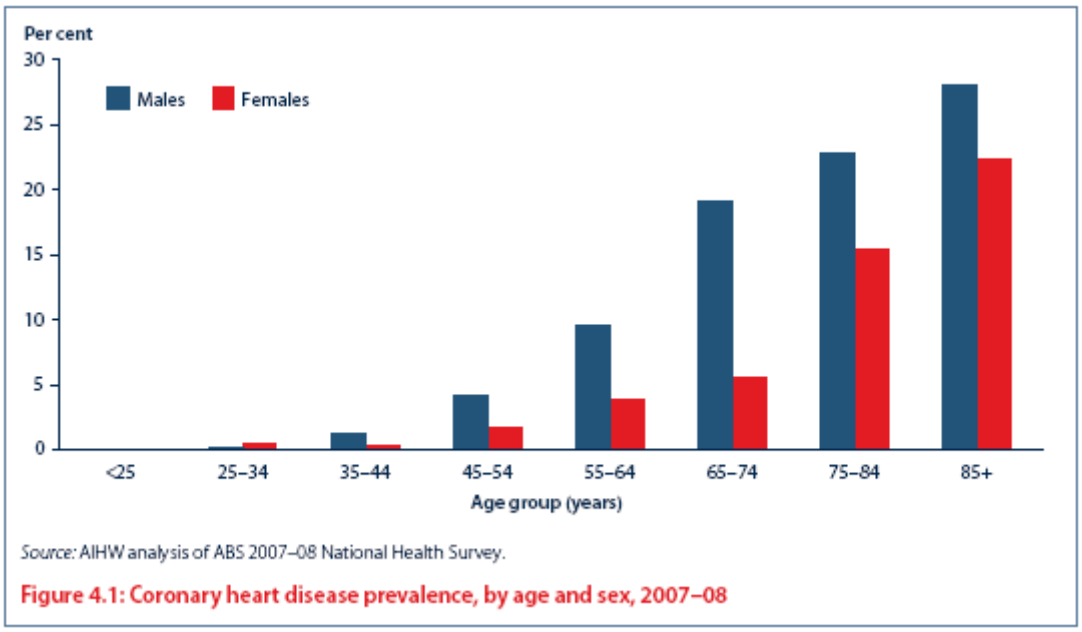

Figure 1.6 Coronary heart disease prevalence by age and sex, 2007-08
[Adapted from (AIHW 2011)]

Overall the prevalence of CVD increased significantly with age from 7% in ages between 55-64 to 24% in those aged 85 years and above. Males had higher prevalence of CHD than females in all age groups above the age of 35. However, there appears to be a differential rise in this prevalence with age between genders: in males the increase with age seems to be more progressive, rising from 19% to 23% from ages 65-74 to 75-84. Females

however had a steeper rise from 6% to 15% (approximately 2.5 fold increase) for the same age groups.

These findings are in concordance to those published in the Framingham study (Castelli 1984), where the incidence of CVD increases with age and is higher in men than in women. For CHD, women appeared to lag behind men in incidence by 10 years with the gap closing with advancing age.

It is also important to highlight that a significant proportion of women who report symptoms of angina are found to have "normal" or non-obstructive coronary artery disease. In the Coronary Artery Surgery Study (CASS) registry, the frequency of non-obstructive coronary disease in approximately 25,000 patients undergoing coronary angiography for angina, was 39% for women compared with 11% of men (Davis, Chaitman et al. 1995). The prevalence was even higher in the Women's Ischemic Syndrome Evaluation (WISE) study, where 62% of women referred for angiography were found to have non-obstructive coronary artery disease (Shaw, Bairey Merz et al. 2006). This pattern persists even in the setting of acute coronary syndromes, where women were still more likely to have non-obstructive CAD compared with men, with prevalence rates of approximately 20% and 10%, respectively (Shaw, Shaw et al. 2008). More importantly, despite the absence of obstructive coronary disease, these women appear to have higher cardiovascular event rates on follow-up. When compared to a healthy cohort of women (n=1000), WISE participants with angina and normal coronary arteries (n=318) or nonobstructive CAD (n=222) had a more than three-fold increase in composite cardiovascular events (2.4% vs. 7.9%, adjusted p=0.002) over five years, including higher rates of stroke

and heart failure hospitalizations. However, there was no statistical difference in rates of myocardial infarction (0.7% vs. 0.9%) or cardiac death (0.6% vs. 1.5%), but WISE women with nonobstructive CAD had significantly higher all cause mortality rates than the control group (2.1 vs. 3.0%, p=0.04) (Gulati, Cooper-DeHoff et al. 2009). The higher cardiovascular morbidity rates in this subgroup of women have also been associated with increased healthcare costs (Shaw, Merz et al. 2006).

Section E, Part 2: Role of menopause in CVD trends

E2.1 Introduction

In the Framingham Study (Castelli 1984), it was noted that in women who have undergone menopause, the risk of cardiovascular events promptly increased by threefold over that of women of the same age who have not undergone menopause. Menopause is a natural state that occurs in women usually in their fifties, where there is a permanent cessation of ovarian function. This time is marked by the absence of menstruation for a period of 12 months. Biochemically there is an associated rise in follicle stimulating hormone (FSH) and decline in oestradiol levels. The relative cardioprotection demonstrated in the epidemiological studies (Barrett-Connor and Bush 1991; Stampfer, Colditz et al. 1991) have been attributed to the abrupt hormonal dysregulation, primarily oestrogen deficiency, and the concomitant worsening of cardiovascular risk factors that occur during the menopausal transition.

Indeed, experimental studies have demonstrated the beneficial effects of oestrogen on cardiovascular health (Paganini-Hill, Ross et al. 1988; Trial 1995). There are three major naturally occurring oestrogens in women: oestrone (E1), oestradiol (E2), and oestriol (E3). Oestriol is the most abundant of the three oestrogens (60-80% of circulating estrogens) but is also the weakest. Oestradiol (10-30%) on the other hand is the strongest, with a potency of approximately 80x that of oestriol, and is therefore is the most important oestrogen from menarche to menopause. Oestradiol has been found to interact directly with oestrogen receptors (ERα and ERβ)

which can be found in myocardial, vascular smooth muscle cells and endothelial cells in humans and animals. There is heterogeneity of ER distribution between male and female animals and also between atherosclerotic and normal vascular beds.

E2.2 Effects of oestrogen on endothelial and vascular smooth muscle cells

One of the major effects of oestradiol on vascular endothelial cells is the induction of NO release. This is mediated by a genomic (via alterations of NOS expression and levels) and a non-genomic pathway (via post-translational activation of NOS enzyme). Acute administration of oestradiol to physiological levels have been shown to potentiate endothelial-dependent vasodilatation in the peripheral (Gilligan, Badar et al. 1994) and coronary vasculatures (Gilligan, Quyyumi et al. 1994) of postmenopausal women. Basal NO release is also enhanced by the administration of oestradiol in perimenopausal women (Sudhir, Jennings et al. 1996).This improvement has been attributed to enhanced calcium-dependent eNOS production and activity, as evident in cultured endothelial cells exposed to oestradiol (Hishikawa, Nakaki et al. 1995). As mentioned previously, NO plays an important anti-atherogenic role including inhibition of smooth muscle proliferation and platelet aggregation. Furthermore, oestradiol has been shown to have a role in endothelial cell regeneration and angiogenesis (Morales, McGowan et al. 1995).

In the vascular smooth muscle cells, oestradiol have been shown to attenuate both myocardial and vascular contractile responses by inhibition of calcium (Zhang, Ram et al. 1994) and activation of potassium channels (White, Darkow et al. 1995). Furthermore oestradiol has been shown to attenuate neointimal formation and media proliferation after balloon injury in rat carotid artery (Sullivan, Karas et al. 1995; Chen, Li et al. 1996).

Most of the above studies demonstrate the effects on the cardiovascular system by restoring/supplementing oestrogen but the effects of oestrogen withdrawal have also been demonstrated. In a group of postmenopausal women on hormone replacement therapy (HRT), acute withdrawal of HRT resulted in evidence of reduced systemic arterial compliance with an increase in peripheral pulse wave velocity. These changes were restored with the reintroduction of hormone replacement therapy (Waddell, Rajkumar et al. 1999). Furthermore NO plays a significant role in these changes seen with acute oestrogen withdrawal (Collins, Shay et al. 1994).

E2.3 Effects of oestrogen on cardiovascular risk factors

Oestrogen has also been shown to exert positive effects on lipid metabolism, indirectly contributing to its cardioprotective role. Ovariectomized rats showed evidence of increased fat and energy gains, with increased hepatic triglyceride content, lipoprotein lipase activity and also increased plasma insulin levels (Deshaies, Dagnault et al. 1997). In postmenopausal women and oophorectomised women, there was a correlation between plasma lipoprotein plasma levels and oestrogen levels, leading to raised low density lipoprotein levels. Withdrawal from HRT also resulted in similar elevations in lipoproteins (Jensen, Riis et al. 1989). Furthermore there is increased in esterification of high density lipoproteins without any change in the levels (Wakatsuki and Sagara 1995). Furthermore postmenopausal women also had higher abdominal and total body fat and less lean tissue mass when compared to premenopausal women, independent of age, indicating an alteration in fat accumulation mechanisms which are oestrogen-dependent.

E2.4 Hormone replacement therapy and cardiovascular risk

Despite evidence from *in vitro* and *in vivo* studies demonstrating the cardioprotective effects of oestrogen, data from the randomised hormone replacement therapy (HRT) trials have shown otherwise. Initial epidemiological studies did suggest a reduction of cardiovascular mortality and morbidity in post-menopausal women (Stampfer, Colditz et al. 1991), at the expense of increased incidence of endometrial cancer, breast cancer and venous thromboembolism. However results from the secondary prevention trial, Heart and Estrogen/Progestin Replacement Study (HERS) (Hulley, Grady et al. 1998), where 2763 postmenopausal women with known coronary disease and who had not had a previous hysterectomy, were randomized to either placebo or HRT for 4 years, and HERS II (Grady, Herrington et al. 2002), an open=labelled follow-up of women in HERS, were disappointing. After 4.1 and 6.8 years, there were no beneficial effects of HRT on coronary events with a statistically significant time trend towards an increased risk of CHD in the first year of HRT and trend towards a benefit between 3 and 5 years of HRT. This was at the expense of increased rates of thromboembolism and gallbladder disease. In the Estrogen Replacement and Atherosclerosis (ERA) study (n=309), HRT use did not influence the angiographic progression of coronary atherosclerosis (Herrington, Reboussin et al. 2000).

Randomized controlled primary prevention trials such as Women's Health Initiative study (WHI) were similarly discouraging. In the WHI study (Rossouw, Anderson et al. 2002), 27347 postmenopausal women aged between 50 to 79 years were intended to be followed up for eight to nine

years. However the first arm of the study involving postmenopausal women with an intact uterus was interrupted at 5.2 years of follow-up due to the excess of coronary events (a 29% increase), breast cancer (26%), stroke (41%) and thromboembolism (50%). In women with prior hysterectomy, HRT was also associated with an increased risk of stroke but not coronary events at 6.8 years of follow-up. Various theories have been put forward to explain these findings including the timing of initiation of HRT, the mode of delivery and the doses used. Various clinical trials to address some of these issues are in progress including Kronos Early Estrogen prevention Study (KEEPS) (Harman, Brinton et al. 2005) and Early vs Late Interventional trial with Estrogen (ELITE) (ClinicalTrials.gov, Identifier no:NCT00114517).

Section E, Part 3: Gender specific ageing the NOS/cGMP system

Celermajer et al (1994) demonstrated that both ageing and gender were important determinants of flow-mediated dilatation in a healthy population as measured in the brachial artery, but not endothelium-independent vasodilatation. In men, FMD was preserved until the age of 40 and declined thereafter at a rate of 0.21%/year. In women however, FMD was preserved for another decade but then declined at a more rapid rate of 0.49%/year ($p=0.002$ when compared to men). The authors attributed the changes in females which coincided with onset of menopause to oestrogen deficiency. Sarabi et al (1999) found that the FMD response was greater in women compared to men before menopause but similar to men after menopause. Furthermore the gradient of change with age was steeper in postmenopausal compared to premenopausal women.

Scope of the present study

The evidence thus far supports differential cardiovascular ageing between genders, with relatively preserved function in women. However, the impact of ageing and hormonal alterations on NO/cGMP remains to be clearly delineated in females.

Therefore this study was designed to assess comprehensively the impact of ageing on the NO/cGMP system in females and potential variability of this process in the presence of PCOS. Four specific aspects were addressed:

1) The normal process of physiological ageing of the NO system, in platelets and vasculature

2) Comparison of these changes with those in vascular endothelial function and its biochemical determinants

3) The impact of PCOS status on cardiovascular ageing

4) The possible role of menopause on the acceleration of cardiovascular ageing

Chapter 2

STUDY DESIGN, MATERIALS AND METHODS

CHAPTER 2

Study Design, Materials and methods

2.1 Study design

A case-control study was conducted involving healthy and PCOS women, between ages of 18 to 64 years. A minimum of 20 women were recruited per age decade per group, in order to facilitate analysis of the impact of ageing on healthy females and to allow comparison between PCOS and healthy females. A subset of healthy women in their premenopausal years was also studied prospectively to assess the contribution of menopausal status on the changes in the NO/cGMP system.

2.2 Materials

2.2.1 Subjects studied

Subjects were recruited from the general population through local media. Screening via telephone/email was performed prior to enrolment into the study. Methods of recruitment were approved by the Ethics of Research Committee of the Queen Elizabeth Hospital and informed consent was obtained prior to subject entry in all cases.

2.2.2 Blood sampling

Blood samples were obtained from venesection via an antecubital vein after 30 minutes of supine rest, up to 60mls per subject. Venous blood was withdrawn with minimal suction into a plastic syringe and aliquoted into various tubes including a plastic tube containing 1:10 volume of acid citrate anticoagulant (2 parts of 0.1mol/L citric oxide to 3 parts of 0.1 mol/L trisodium citrate) for platelet studies, a 10ml EDTA tube for EPC quantification and heparinised lithium tubes for various biochemical assays.

2.3 Methods

2.3.1 Technique for evaluation of platelet NO responses

Given that the principal aim of the current study was to assess the impact of ageing on NO signalling, the primary end-point of the study was the change in platelet NO responsiveness with ageing. As described previously, "NO resistance" has been attributed largely to either increased breakdown of NO by superoxide or dysfunction of sGC. To measure NO responsiveness of platelets, we performed whole blood platelet aggregometry.

Instrumentation. A Lumi-Aggregometer (Model 560CA, Chrono-Log, Harvertown, PA, USA) was used to measure the degree of platelet aggregation. Data were collected via a Chrono-log Model 810CA Aggro/Link computer interface connected to an IBM compatible computer. Continuous measurements of the aggregation response allowed the automatic calculation of the maximal amplitude and slope of the aggregation curve.

Whole blood impedance aggregometry. This technique is based upon the measurement of electrical impedance between two electrodes immersed in whole blood (Cardinal and Flower 1980). A small electric current is passed between these electrodes immersed in a blood sample. Upon initial contact with the electrodes, platelets form a monolayer on their surfaces. When an agonist is added, additional platelets will aggregate on the monolayer, thereby increasing the impedance between the electrodes. The change is recorded over time and is directly proportional to the mass of the platelet aggregate.

In each experiment, samples of whole blood were initially rested on the bench for 15 minutes to reduce platelet activation. For each test, whole blood

(450μL) was diluted with normal saline (500μL) and prewarmed for 5 minutes at 37ºC. Aggregation studies were performed at this temperature with a stirring speed of 900 rpm. Aggregation was induced by adenosine diphosphate (ADP) at a concentration of 2.5μM. Both the extent of aggregation (Ohms) and rate of aggregation (Ohms/min) were recorded for a total duration of 7 minutes. To assess the inhibitory effect of NO on platelet aggregation, we added an exogenous NO donor, sodium nitroprusside (SNP, 10μM), into the diluted sample one minute before the induction of aggregation with the agonist. The extent of inhibition of platelet aggregation, expressed as percentage, was calculated from differences in maximal aggregation in the presence and absence of SNP (Chirkov, Holmes et al. 1999). A typical response to ADP and SNP are depicted in the Figure below.

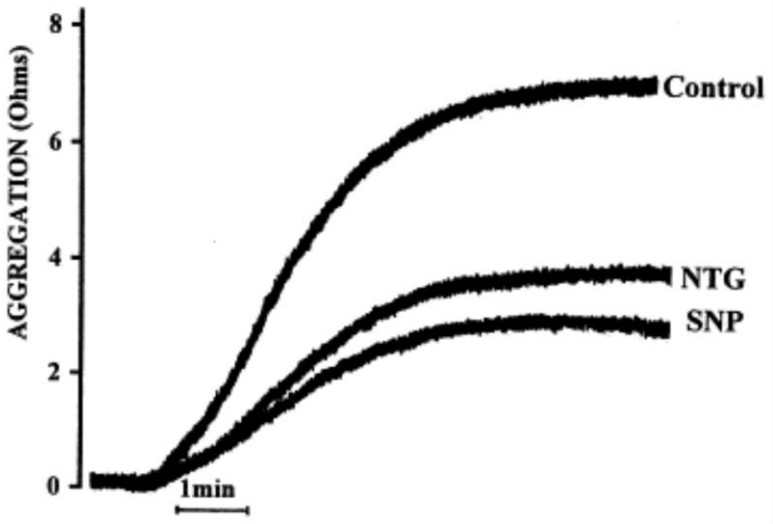

Figure 2.1 **Tracings from platelet aggregometry.** Representative tracings for inhibition of ADP (1 μmol/liter)-induced aggregation by nitroglycerin (NTG) (100 μmol/liter) and sodium nitroprusside (SNP) (10 μmol/liter) in a whole blood sample obtained from a normal male subject.

[Adapted from (Chirkov, Holmes et al. 2001)]

2.3.2Techniques for evaluation of endothelial function

As mentioned previously, the biochemical abnormalities involved in endothelial dysfunction potentially include a wide range of abnormalities in the NO signalling pathway, from the production of NO via eNOS to increased clearance and/or impaired downstream signalling. To further assess endothelial function, we employed several techniques in this thesis including pulse wave analysis (PWA), measurement of asymmetric N^G,N^G-dimethyl-L-arginine (ADMA), which are well-established techniques in our laboratory, and for the first time in our laboratory, quantification of endothelial progenitor cells (EPCs), given that EPC cell counts also reflect bone marrow NO/eNOS signalling (discussed in Section B1.5.4)

(i) Pulse wave analysis (PWA). PWA is a simple non-invasive technique used which measures arterial stiffness (O'Rourke and Gallagher 1996; Wilkinson, Cockcroft et al. 1998). A small ultrasound probe is placed on the radial artery to obtain peripheral arterial waveform, which enables the derivation of the central aortic pressure. The central aortic pressure is composed of a forward-travelling wave generated by left ventricular ejection and a later-arriving reflected wave from the periphery. The augmentation index (AIx) is calculated from the ratio of the augmentation pressure (difference of pressure between the first systolic shoulder to the systolic peak) and the pulse pressure, expressed as a percentage. AIx has been shown to increase with age (O'Rourke, Pauca et al. 2001), , the presence of other cardiovascular risk factors (Patvardhan, Heffernan et al. 2011) and also to represent an independent risk marker of premature coronary artery disease

(Weber, Auer et al. 2004). A recent meta-analysis showed that a 10% increase in AIx was associated with increased cardiovascular events (relative risk (RR) 1.318, 95% CI 1.093-1.588) and all-cause mortality (RR 1.384, 95% CI 1.192-1.606) (Vlachopoulos, Aznaouridis et al. 2010).

However, in the context of the current study, the more important assessment utilising PWA is derived from the change in AIx in response to nitroglycerin (NTG) and the β_2-adrenoreceptor agonist salbutamol, in order to provide a non-invasive measure of global endothelial function. The response to NTG allows measurement of endothelial-independent vasodilatation (that is, a vascular analogue of platelet NO response) and to salbutamol, via its ability to cause the release of NO from endothelial cells, endothelial-dependent vasodilatation. This method has been extensively validated by other investigators, as described in the previous chapter (Hayward, Kraidly et al. 2002; Wilkinson, Hall et al. 2002). A typical response in a normal individual to salbutamol and NTG are shown below.

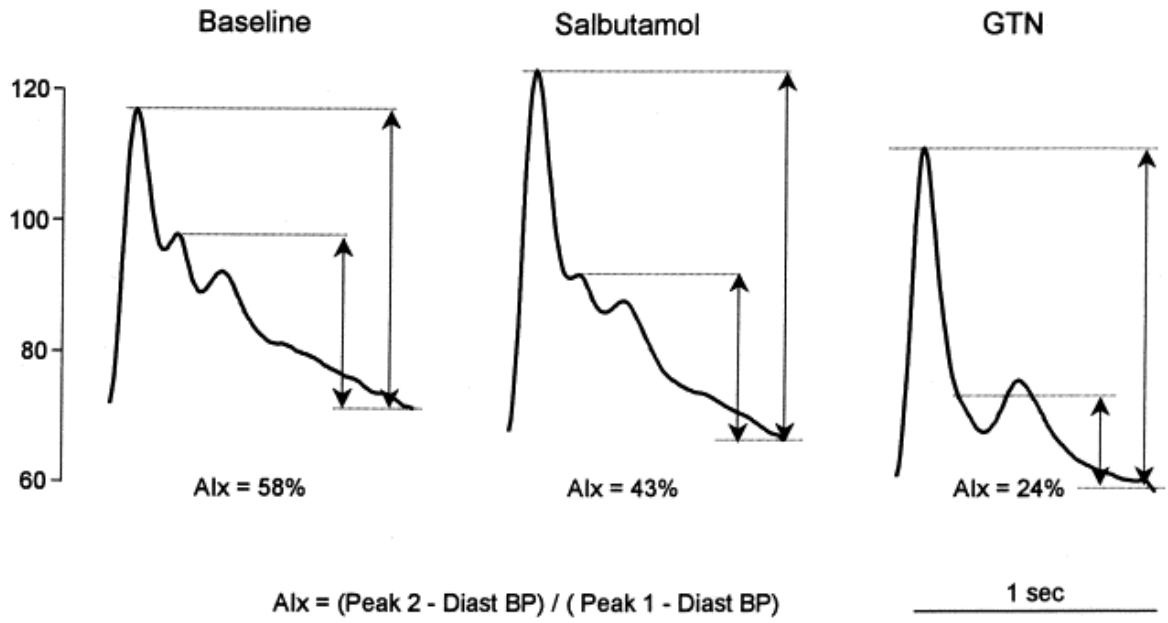

Figure 2.2 Tracings from pulse wave analysis.
An example of the effect of Salbutamol and glyceryl trinitrate (NTG) on the radial waveform in a single individual. It can be seen that the second systolic peak, obvious at baseline, is diminished by Salbutamol and almost completely abolished following NTG. The changes in the wave-shape are quantified using the augmentation index (AIx), calculated as the ratio of the pulse pressure at the second systolic peak to that at the first systolic peak. BP = blood pressure. *[Adapted from (Hayward, Kraidly et al. 2002)]*

(ii) Asymmetric N^G,N^G-dimethyl-L-arginine (ADMA) assay. Plasma concentrations of ADMA, an endogenous eNOS inhibitor and inactivator, has been correlated with extent of endothelial dysfunction by several investigators [for review see (Cooke 2004)]. In our laboratory, we utilise high performance liquid chromatography (HPLC) for the quantification of ADMA, its inactive optical isomer SDMA ($N^G,N^{G'}$-dimethyl-L-arginine) and L-arginine (Horowitz and Heresztyn 2007). In brief, peripheral venous blood was centrifuged at 2700g and 4ºC for 20 minutes. Heparinised plasma was

stored at -80ºC until analysis via a Gilson ASPEC GX-274 automated solid phase extraction system as previously described (Horowitz and Heresztyn 2007). The extracts were derivatised and analysed for ADMA, SDMA and L-arginine on a system consisting of a Waters 717plus autosampler, Agilent 1100 series quaternary pump, and a 1200 series fluorescent detector controlled by Chemstation data acquisition software. Plasma from healthy subjects was pooled and analysed in each sample set as a quality control. The intraassay precision was <4% for ADMA, SDMA and arginine (n=10). Interassay precision for all three analytes over 10 analytical sets was <10%.

Plasma arginase activity was also determined in a selected group of individuals, using Quantichrom arginase assay kit (DARG-200, BioAssay Systems, California, USA) as per manufacturers' instructions and previous publications (Pulichino, Wang et al. 2008; Ndolo, Forrest et al.). In this assay, plasma arginase activity is determined by the measurement of urea formation from the breakdown of arginine by arginase. Plasma samples were firstly, pre-treated to remove urea using Amicon membrane filters (10kDa, Milipore Australia). Samples were then incubated with the provided substrate buffer in a 96-well reaction plate for 2 hours (arginase reaction) and subsequently with the urea reagent (colouring reagent) for 20 minutes, prior to analysis with an optical plate reader (optical density at 430 nm). The arginase activity was then calculated and expressed in U/L, where 1 unit of arginase converts 1 µmole of L-arginine to ornithine and urea per minute at pH 9.5 and 37°C.

(iii) Endothelial progenitor cell counts. EPC counts in peripheral blood reflect the need for endothelial repair in relation to endothelial damage or dysfunction and the release from bone marrow, which is in part NO-mediated (Aicher, Heeschen et al. 2003; Goldstein, Gallagher et al. 2006). Peripheral EPC counts can be performed via flow cytometry which uses "specific" cell surface markers to identify EPCs. The issue of which of the various available markers identify "true" EPCs remains an area of ongoing debate (Timmermans, Plum et al. 2009). Dual (or triple) positive staining of the hematopoietic progenitor cell marker CD34, together with either the immature hematopoietic progenitor cell marker CD133 and/or the endothelial cell receptor VEGFR2 have been widely utilized to identify EPCs (Vasa, Fichtlscherer et al. 2001; Lambiase, Edwards et al. 2004). The methodology is described in detail in the next section.

2.3.3 Establishing flow cytometric analysis of EPC cell counts

Preliminary training in this technique was undertaken in the laboratory of Professor Michael Marber at The Rayne Institute, St Thomas' Hospital, London, after which methodology identical to that utilized in his laboratory (previous publications (Lambiase, Edwards et al. 2004; Murphy, Kanaganayagam et al. 2007), was introduced into The Basil Hetzel Institute, The Queen Elizabeth Hospital laboratory for the purpose of this study.

Methodology. 10ms of venous blood was collected into an EDTA tube, and transferred into a 50ml tube where 10mls of phosphate buffered saline (PBS)

was added and underlaid with 15 mls of Lymphoprep. The sample was centrifuged for 30 minutes at room temperature and the buffy coat layer was collected. The cells were then washed and centrifuged with PBS for 10 minutes to remove any excess plasma. 2 blocking steps with 5% mouse serum and FCR blocker was performed to reduce non-specific binding and to improve sensitivity of collecting rare cell events. The cells were resuspended in 5% mouse serum and distributed equally (150μL) into 6 tubes, 3 "test" and 3 "control" tubes. The cells were then incubated for 10 minutes with phycoerythrin (PE)-conjugated mouse antibody (mAb) against human CD133 (Miltenyi Biotec) and with fluorescein isothiocyanate (FITC)-conjugated mAb against human CD34 (Miltenyi Biotec) in "test" tubes at 4ºC. Isotype IgG1 antibodies were used in "control" tubes (Becton Dickinson, Miltenyi Biotec). The sample tubes were stored at 4°C in dark until measured.

Flow cytometry: Instrument setup and sample acquisition. The sample tubes were acquired and analysed on a FACSCanto II flow cytometer (two-laser, six-color configuration) with FACSDiva 6.1.2 software (Becton Dickinson Biosciences, California, USA). Amplifier settings for forward scatter (FSC) and side scatter (SSC) were used in linear mode and those for fluorescence channels were used in logarithmic mode. The thresholds for detection were set manually. A FSC/SSC scatter gate was set up around the mononuclear cell population (comprising of monocytes and lymphocytes) to collect 400000 gated events per tube. EPCs were identified as dual labelled cells (CD34+/CD133+) and were expressed as counts/100000 events.

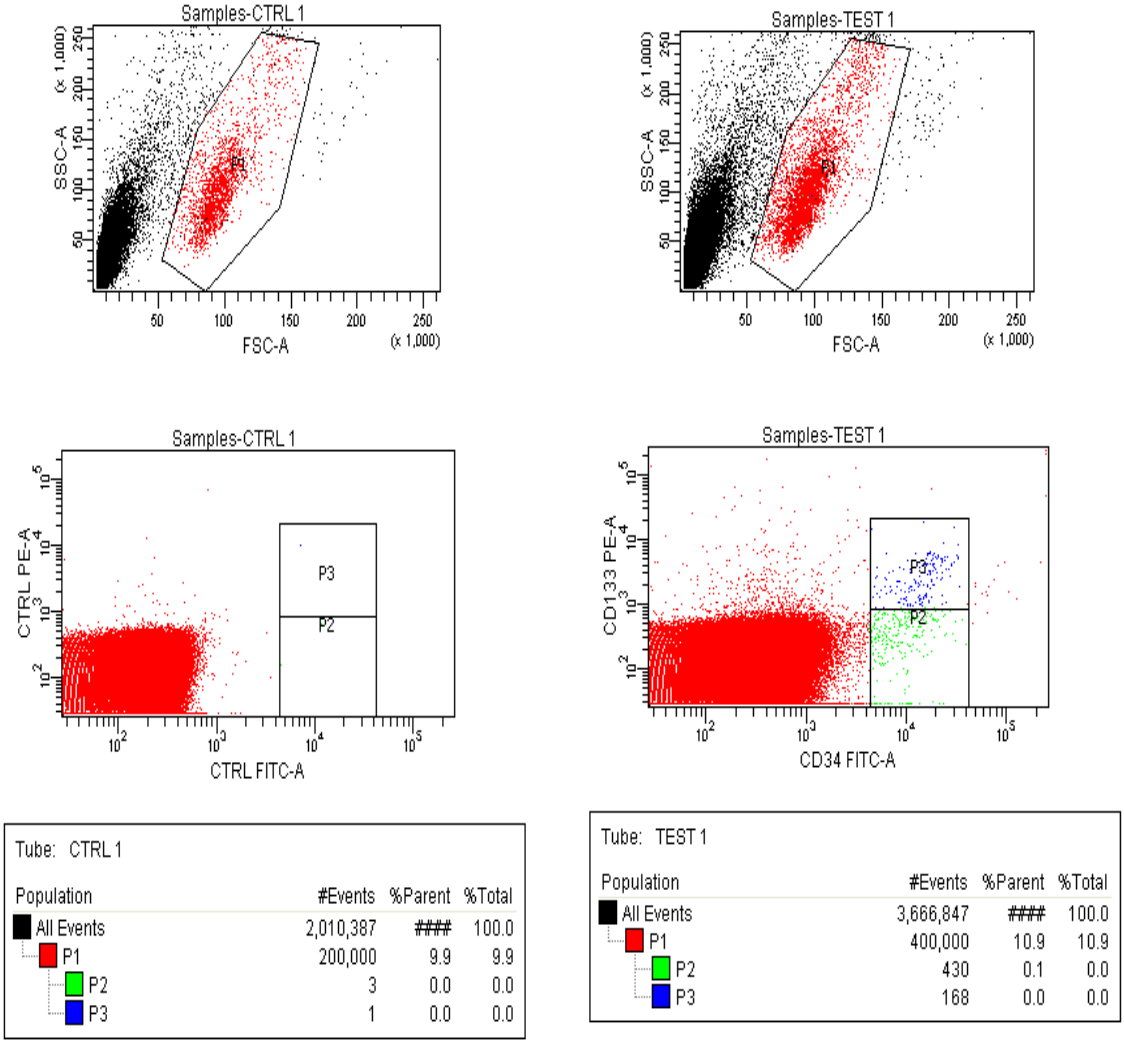

Figure 2.3 **Typical results from flow cytometry.**
CTRL represents negative control tubes, whereas TEST represents CD34 and CD 133 labelled tubes. P1 zone delineates all mononuclear cells (lymphocytes and monocytes). P2 zone represents all CD34-positive cells and P3 zone, dual positive (CD34+/CD133+) cells

Intraassay and interassay variability. With each individual blood sample 3 test and 3 control tubes were analysed. Blood samples were collected from a young healthy individual under the same conditions and time of day on 4 specified days. These allowed the calculation of intraassay variability for each run and interassay variability. Results are shown below.

Table 2.1 Intraassay variability

Sample date	**Sample**	**Coefficient of variation for CD34/133+ cell counts (%): data from multiple estimates on different dates (obtained from 3 readings each sample)**
5th June 2007	1	4.9
6th June 2007	2	13.7
12th June 2007	3	10.9
13th June 2007	4	8.9
	Mean	**9.6**
	SD	**3.7**
	SEM	**1.9**

Table 2.2 Interassay variability

Sample	**Mean CD34/133+ cell counts (expressed as cells/100000 events)**
1	74
2	63
3	47
4	58
Mean	**60.5**
SD	**11.2**
SEM	**5.6**
CV	**18.5%**

The mean coefficient of variation (CV) for the intraassay variability is similar to other laboratories (<10%) (Goon, Lip et al. 2009). In comparison to results from the St Thomas' Hospital laboratory where the interassay CVs varied between 11.5 to 18.6% (unpublished data), our result was also comparable. Previous studies have accepted EPC values with intraassay variability <15% and interassay variability of <20% (Goon and Lip 2007).

2.3.4 Other biochemical measures

Plasma thrombospondin-1 levels (TSP-1). Thrombospondin-1 (TSP-1) has been recently shown to be a physiological regulator of NO signalling, in cell culture and animal models [see Chapter 1, Section B2.4, reviews by (Isenberg, Frazier et al. 2008; Isenberg, Martin-Manso et al. 2009)]. Limited data is known between plasma levels and its correlation to measures of vascular function in humans to date. Therefore, we also determined plasma TSP-1 levels in normal women utilizing a solid phase enzyme-linked immune-sorbent assay (ELISA) kit (Quantikine Human Thrombospondin-1 Immunoassay, R&D Systems, Minneapolis, USA) per manufacturer's instructions and previous publications (Kirsch, Woywodt et al.; Stepanian, Cohen-Moatti et al.). In brief, standards and samples (platelet poor plasma) were added into the provided microplate which has been coated with mouse monoclonal antibody specific for TSP-1, for 2 hours at room temperature. The sample was then washed and an enzyme-linked polyclonal antibody specific to TSP-1 was added into the wells. A further wash was performed and the provided substrate solution was then added. This resulted in varying colour intensity depending on the amount of TSP-1 bound initially which was measured using an optical plate reader.

Thrombospondin-1 levels were expressed as ng/mL. The intraassay and interassay precision for this kit were reported to be less than 10%.

High-sensitive C-reactive protein (hsCRP). The assay for hsCRP was performed using immunonephelometric methodology as previously described elsewhere (Rifai, Tracy et al. 1999) and using a commercial kit manufactured by Dade Behring (Liederbach, Germany). The assay range is between 0.175-11mg/L. The limit of detection is 0.02 mg/L and limit of quantification is 0.15 mg/L.

Other biochemical parameters measured in this thesis including hormonal profile (reproductive hormones and androgens), fasting lipids, insulin, glucose, high sensitive C-reactive protein (hsCRP) were obtained through standard commercially available techniques through SA Pathology Services.

Homeostasis model of insulin resistance (HOMA-IR) calculated by multiplying fasting plasma insulin (mU/L) by fasting plasma glucose (mmol/L), then divided by 22.5 (Matthews, Hosker et al. 1985), was determined as a surrogate index of insulin resistance.

Chapter 3

DETERMINATION OF THE IMPACT OF AGEING ON PLATELET AND ENDOTHELIAL FUNCTION IN HEALTHY WOMEN AND IN POLYCYSTIC OVARIAN SYNDROME

CHAPTER 3

Determination of the impact of ageing on platelet and endothelial function in healthy women and in polycystic ovarian syndrome

3.1 Introduction

Ageing represents the strongest independent risk factor for cardiovascular disease. However, the exact mechanisms underlying this risk are largely unknown. Various potential contributing factors range from physical changes observed in chromosomes including telomere shortening (Fitzpatrick, Kronmal et al. 2007) to physiological abnormalities such as endothelial dysfunction (Yasue, Matsuyama et al. 1990; Egashira, Inou et al. 1993) and biochemical abnormalities including accumulation of reactive oxygen species (Taddei, Virdis et al. 2000) and inflammation (Chung, Lee et al. 2011).

One of the early initiating events for atherosclerosis has been thought to be endothelial dysfunction. Certainly, ageing has been associated with progressive endothelial dysfunction, an independent predictor of cardiovascular events (Lerman and Zeiher 2005). The biochemical abnormalities which underlie endothelial dysfunction involve both impairment of nitric oxide (NO) production

(Oemar, Tschudi et al. 1998) and also increased NO breakdown (Guzik, West et al. 2000), leading to impaired NO signalling.

Impaired tissue responsiveness to NO, which reflects purely impairment in NO signalling (as distinct from NO production), is an emerging independent cardiovascular risk marker (Willoughby, Stewart et al. 2005), which conceptually differs from endothelial dysfunction. Tissue responsiveness to NO assumes NO bioavailability, and is impaired as a result of either increased NO clearance or impaired soluble guanylate cyclase activity (Chirkov, Holmes et al. 1999) resulting in reduction in downstream effect of NO on tissues. This can be demonstrated in platelets and blood vessels, and potentially in other tissues, such as myocardium. Data examining the relationship between ageing and tissue responsiveness to NO are currently limited (Celermajer, Sorensen et al. 1994; Taddei, Virdis et al. 1995; Goubareva, Gkaliagkousi et al. 2007).

Furthermore, oestrogen has been shown to modulate NO signalling (Gilligan, Quyyumi et al. 1994; Hishikawa, Nakaki et al. 1995; Sudhir, Jennings et al. 1996), theoretically conferring a cardioprotective role. As discussed in Section 1.3.1 oestrogen has an important role in mediating NO release and endothelial function. However, its relation to platelet and tissue NO responsiveness is not known.

The relationship between polycystic ovary syndrome (PCOS) and long-term cardiovascular event risk remains unclear. PCOS is the most common endocrinopathy, affecting up to 10% of premenopausal women. (Wang, Davies et al. 2001; Ehrmann 2005). This syndrome is diagnosed on the presence of variable combinations of hyperandrogenism, ovulatory

disturbances and polycystic ovaries, and is well-recognized for its reproductive implications including infertility. However, the metabolic complications associated with this condition [insulin resistance, dyslipidemia, obesity and metabolic syndrome (Dunaif, Hoffman et al. 1985; Norman, Masters et al. 1996; Legro, Kunselman et al. 1999; Norman 2001; Apridonidze, Essah et al. 2005)], which represent cardiovascular risk factors, raise the possibility of an increase in cardiovascular morbidity/mortality in PCOS individuals.

Beyond these factors, there is strong evidence that PCOS in young women is associated with vascular endothelial dysfunction (Paradisi, Steinberg et al. 2001; Orio, Palomba et al. 2004; Tarkun, Arslan et al. 2004) and platelet hyperaggregability (Dereli, Ozgen et al. 2003). Furthermore, we have recently demonstrated that, irrespective of the presence/absence of obesity, vascular endothelial dysfunction in PCOS subjects is accompanied by severe impairment of platelet responsiveness to nitric oxide (NO) (Rajendran, Willoughby et al. 2009), an additional marker of coronary event risk (Willoughby, Stewart et al. 2005).

The majority of physiological studies on PCOS reported to date have been carried out in women in their reproductive years. In contrast, the few available studies of PCOS-associated coronary event risk and atherogenesis (Wild, Grubb et al. 1990; Azevedo, Duarte et al. 2006; Krentz, von Muhlen et al. 2007) have focused on older individuals, sometimes with incompletely defined clinical evidence of PCOS (Azevedo, Duarte et al. 2006; Krentz, von Muhlen et al. 2007).

As only limited data are currently available in relation to the impact of normal ageing in women on NO signalling in platelets and blood vessels, the current study includes characterization of potential changes in these parameters with age in normal women, PCOS subjects being compared with the normal female population. This comparative study of control with PCOS women was performed over an age range of 18 to 60 years to evaluate these processes.

3.2 Objectives of the study

This study was designed to: (1) evaluate changes in NO signalling with increasing age in normal women and (2) to compare these changes with those occurring in women with PCOS. The objectives are outlined in further detail below.

3.2.1. Objective 1. To characterise the putative variability in platelet and vascular responsiveness to NO in normal ageing women.

The *null hypothesis* to be tested was that *ageing in normal women is associated with impairment in NO signalling in platelets or blood vessels*.

3.2.2 Objective 2. To compare this variability with that in vascular endothelial function and its biochemical determinants.

The *null hypothesis* to be tested was *there are no changes in vascular endothelial function or its biochemical determinants with increasing age.*

3.2.3 Objective 3. To compare the above-mentioned putative fluctuations to those present in age-matched patients with PCOS, a condition characterised by presence of impaired NO signalling in early adult life.

The *null hypothesis* was that *women with PCOS do not exhibit abnormalities in platelet and vascular NO signalling compared to healthy normal women, at any age.*

3.2.4 Objective 4. To determine the possible impact of menopause on NO signalling in vessels and platelets.

The *null hypothesis* to be tested was *there is no effect of menopause on NO signalling in platelets and vessels.*

3.3 Methods

3.3.1Study population

We studied age-matched healthy control and PCOS women, aged between 18 to 60 years. Healthy control women and those who had been diagnosed previously with PCOS were invited to participate through advertisement in the local media. Initial screening (via telephone) and assessment (in person) were performed, including confirmation of their diagnosis of PCOS.

We included subjects irrespective of current pharmacotherapy or cardiovascular risk factor profile. The only exclusion criteria were current clopidogrel therapy (which affects platelet aggregation) and pregnancy. Healthy control and ovulatory women were studied in the follicular phase of their menstrual cycle, to avoid variability associated with hormonal changes during the menstrual cycle.

The study was approved by the Ethics of Research Committee of The Queen Elizabeth Hospital and written informed consent was obtained before study entry.

3.3.2Study protocol

A questionnaire was first completed, addressing full medical history, symptomatic status associated with PCOS, cardiovascular risk factors (including previous history of hypertension, hypercholesterolemia, insulin resistance, diabetes, smoking, family history) and pharmacotherapy.

Physical examination included blood pressure measurements using a sphygmomanometer and cardiovascular examination. Height and weight measurements were performed to calculate the body mass index (see below).

Subjects who attended were instructed to have a 12-hour fast and a period of rest for 30 minutes prior to venous blood collection. Samples were obtained for the determination of platelet aggregation (see below) and risk factor evaluation including fasting lipid profile, high-sensitivity C-reactive protein (hs-CRP), glucose and insulin levels. Levels of testosterone and reproductive hormones were also measured.

Endothelial function assessment was then performed (as discussed in Chapter 2) via pulse wave analysis and correlated with endothelial progenitor cell counts [as a NO-sensitive marker of marrow stimulation (Goldstein, Gallagher et al. 2006; de Resende, Huw et al. 2007)] and with plasma concentrations of the endogenous nitric oxide (NO) synthase inhibitor N^G,N^G-dimethyl-L-arginine (ADMA), a biochemical marker/mediator of endothelial dysfunction (Boger, Bode-Boger et al. 1998).

3.3.3 Assessment of platelet response to NO

Determination of ADP and SNP (NO surrogate) responses was performed in whole blood as outlined in Chapter 2.

3.3.4 Assessment of endothelial function

Following blood collection, pulse wave analysis was performed utilizing a Sphygmocor (AtCor Medical, NSW, Australia) device to derive the rate-corrected augmentation index (AIx: a measure of arterial stiffness, defined as the ratio (in %) of the augmentation pressure and the pulse pressure (Wilkinson, Hall et al. 2002). Endothelial-dependent vasodilator responsiveness was determined by determining the change in AIx in response to inhaled salbutamol (400ug). Response to sublingual nitroglycerine (NTG; 50ug) was utilized to quantitate endothelium-independent vascular function, which conceptually reflects vascular NO responsiveness. Three sets of recordings were made at baseline and then consecutively at 1, 2, and then at every 2 minute interval after for a total duration of 20 minutes after salbutamol and NTG administration. Both responses were expressed as peak change in AIx.

Plasma concentrations of ADMA, SDMA and L-arginine were determined via high performance liquid chromatography (HPLC) as described (Heresztyn, Worthley et al. 2004). In a selected group of individuals (see later Section 3.5.2 (v)), we also measured plasma arginase activity in plasma using the Quantichrom arginase assay kit (DARG-200, BioAssay Systems, California, USA) as described in Chapter 2, Section 3.2.

Peripheral blood endothelial progenitor cells (EPCs) were analysed for the expression of dual positive staining cells CD34+/CD133+ (Becton Dickinson, Miltenyi Biotec) via flow cytometric analysis as described in Chapter 2, Section 3.3.

Plasma thrombospondin-1(TSP-1) levels were also measured in a small cohort of women in this study, given its role in NO modulation, utilizing the Quantikine Human Tmrobospondin-1 Immunoassay kit (see Chapter 2, Section 3.4).

3.3.5Definitions

PCOS was defined by the according to the Rotterdam criteria (2004), which specify the presence of any two of the following three components: (i) oligo-/anovulation; (ii) clinical or biochemical evidence of hyperandrogenaemia and/or (iii) polycystic ovaries. Other causes of hyperandrogenism or ovulatory disturbances were excluded.

Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters. Homeostasis Model of Assessment - Insulin Resistance (HOMA-IR) was measured by multiplying fasting plasma insulin (mU/L) and fasting plasma glucose (mmol/L) divided by 22.5 (Matthews, Hosker et al. 1985).

3.4 Data analysis

In accordance with the objectives of the study, initial analyses performed related to impact of ageing and differential effects of PCOS, while we also evaluated potential differences between groups regarding changes with ageing.

The primary parameter examined was platelet responsiveness to nitric oxide, which is diminished in the presence of most cardiovascular risk factors (Anfossi, Mularoni et al. 1998; Stepien, Prideaux et al. 2003) and overt myocardial ischemia (Chirkov, Holmes et al. 2001).

All comparisons between age groups within each cohort were performed utilizing one-way analysis of variance (ANOVA), whilst comparisons between PCOS and normal subjects were performed utilizing two-way ANOVA.

The study had >95% power to detect differences between PCOS and control subjects of the order observed in our initial evaluation (at $p<0.05$) and approximately 80% power to detect a 25% difference in the platelet SNP response:age relationship between groups. Characteristics of participant groups (normal versus PCOS) were compared utilizing non-paired t-test and Chi-square test as appropriate. Backward stepwise multiple regression was used to identify correlates of platelet NO responsiveness. The limit of statistical significance was set at $P<0.05$. All data were expressed as mean ± standard error of mean (SEM), unless otherwise stated. Statistics were analysed using PRISM v5.0 (GraphPad Software, Inc) and SPSS software version 11 (IBM).

3.5 Results

3.5.1 Results of platelet and vascular responses, and correlation with biochemical markers in normal ageing women

(i) Subject characteristics

A total of 133 normal (non-PCOS) subjects participated in this study. Their baseline characteristics are presented below (Table 1), according to age groups. All parameters except for HDL, fasting insulin, HOMA-IR and oestradiol levels increased significantly with increasing age. Plasma oestradiol declined significantly with increasing age, with the biggest change between age groups 40-49 and 50-60 years consistent with changes during menopause. Reported cardiovascular risk factors and subject medications are summarized in Table 2. A total of 38 (28.6%) subjects in the control group were postmenopausal on the basis of absence of menstruation >12 months and FSH levels >20U/L, with only 1 person on hormone replacement therapy. 14 women in the control group were receiving oral contraceptives (OCs).

Table 3.1 Baseline characteristics of all normal women (n=133).
(Data presented are mean, SEM)

Age (years)	**18-29**	**30-39**	**40-49**	**50-60**
N	25	27	33	48
BMI, kg/m^2*	22.2,0.6	24.5,1.0	27.7,0.9	27.8, 0.8
Systolic BP, mmHg *	112.9, 2.5	118.2, 2.6	122.1, 2.8	130.0, 2.6
Diastolic BP, mmHg *	73.2,1.9	74.8,2.1	79.4, 1.8	83.2, 1.6
Total cholesterol, mmol/L*	4.6, 0.2	4.8, 0.2	5.1, 0.1	5.5, 0.1
HDL, mmol/L	1.6, 0.1	1.6, 0.1	1.7, 0.1	1.6, 0.05
Triglycerides, mmol/L †	1.1, 0.1	1.0, 0.2	1.0, 0.1	1.5, 0.2
Fasting glucose, mmol/L †	4.4, 0.1	4.6, 0.1	4.7, 0.1	4.7, 0.1
Insulin, mU/L	6.5, 0.5	6.2, 0.6	6.1, 0.5	10.0, 1.4
HOMA-IR	1.3, 0.1	1.3, 0.1	1.2, 0.1	2.1, 0.3
Testosterone, nmol/L †	1.2, 0.2	1.1, 0.1	0.9, 0.1	0.7, 0.05
Oestradiol, pmol/L †	241.7, 53.2	253.3, 39.8	313.7, 51.1	180.1, 37.2

* $p<0.001$ by 1 way ANOVA
† $p<0.05$ by 1 way ANOVA

Table 3.2. Cardiovascular risk factors and medications (n, %) among normal subjects

Current smoker	**13, 9.8**
Insulin resistance	**4, 3.0**
Type 2 diabetes	**0**
Hypertension	**11, 8.3**
Hypercholesterolemia	**27, 20.3**
Family history	**11, 8.3**
Current OC use	**14, 10.5**
ACEI, ATRA	**6, 4.5**
Statins/HMG-CoA redcutase inhibitors	**6, 4.5**
HRT	**1, 0.8**

(Data presented are mean, SEM)
OC Oral contraceptives
ACEI Angiotensin-converting enzyme inhibitors
ATRA Angiotensin 2 receptor antagonists
HRT Hormone replacement therapy

(ii) Platelet aggregation and NO responses

There was an increase in platelet aggregation responses with increasing age ($p=0.004$, 1-way ANOVA)(Figure 3.1A). Platelet NO responsiveness declined significantly with increasing age ($p<0.0001$, 1-way ANOVA) (Figure 3.1B).

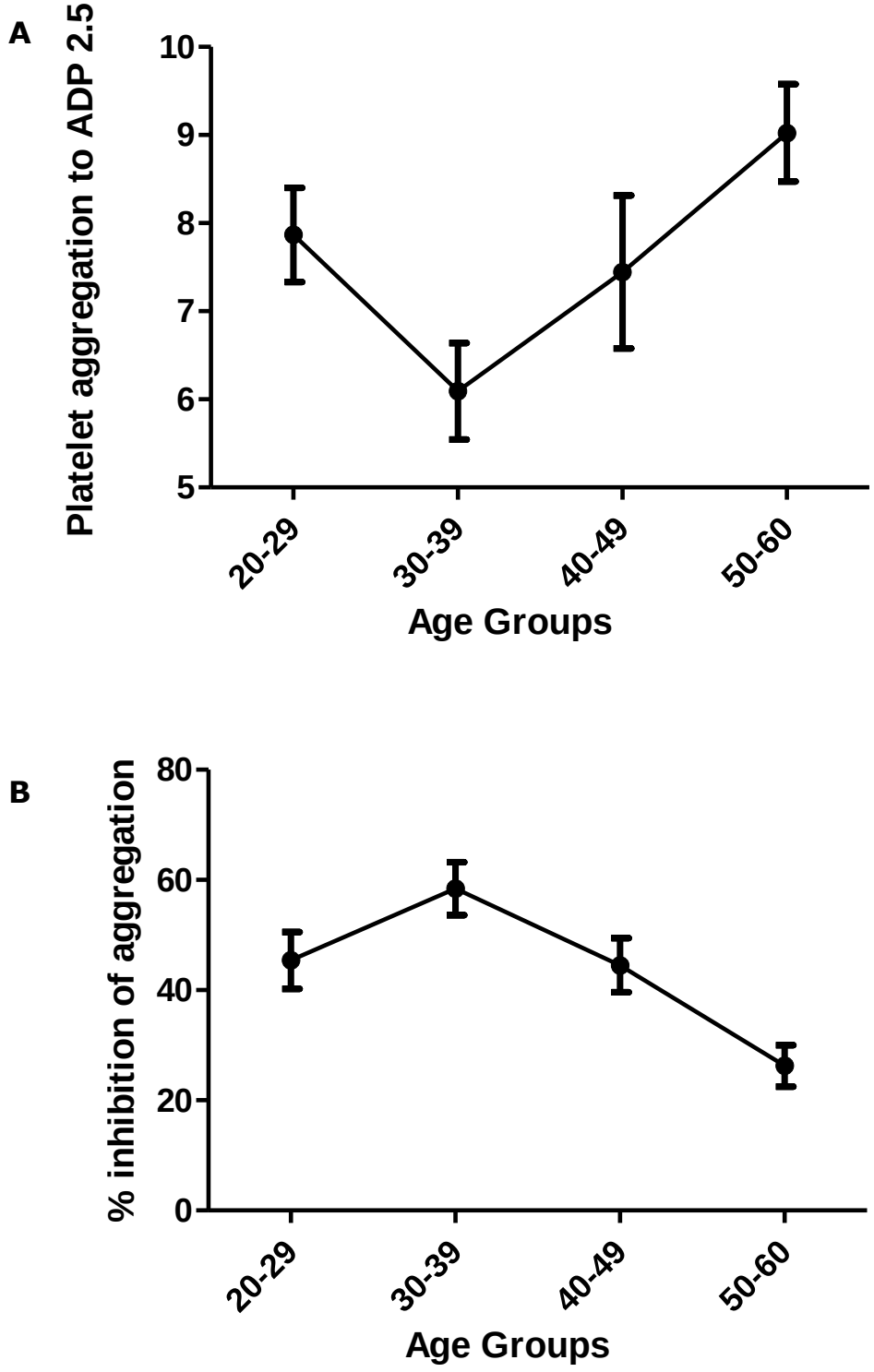

Figure 3.1 Platelet aggregation (A) and NO responses (B) in normal women, by age groups. Platelet aggregation increased whilst NO responsiveness declined with increasing age ($p<0.05$ for both parameters).

(iii) Vascular NO and endothelial-dependent responses

Results for vascular function as assessed by pulse wave analysis are shown in Figure 3.2. Whilst there was no significant change with increasing age in NTG responses ($p=0.5$, 1-way ANOVA), salbutamol responses declined significantly ($p<0.0001$, 1-way ANOVA).

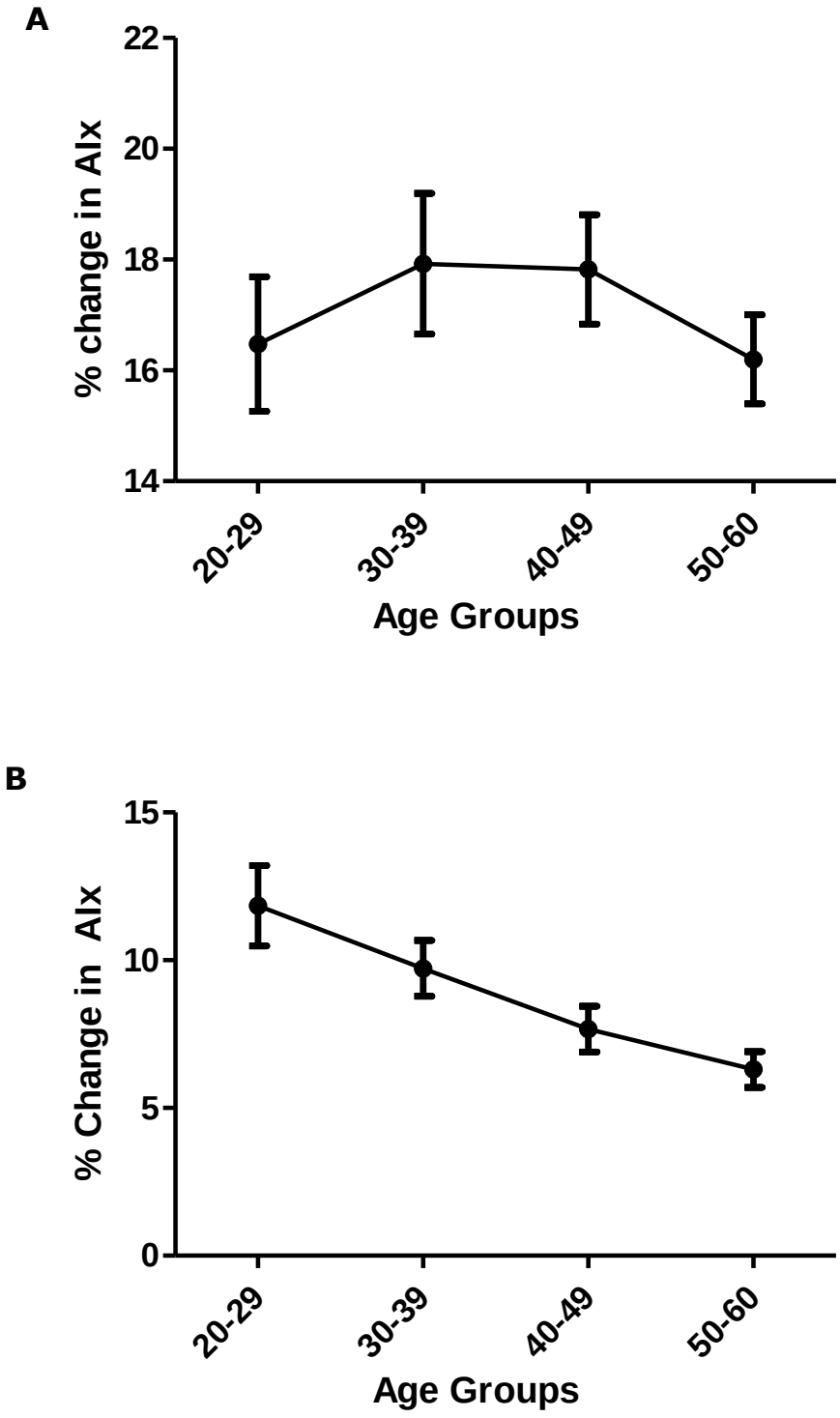

Figure 3.2 Vascular NO (A) and endothelial-dependent (B) responses in normal women, by age groups. NTG responses did not change significantly with age (p=0.5), whilst there was a significant decline in salbutamol responses (p<0.0001).

(iv) Other determinants of endothelial function

(a) ADMA, SDMA and L-arginine concentrations

There was a significant increase in ADMA concentrations with increasing age (F=6.4, p=0.0005, 1-way ANOVA) (Figure 3.3). Concentrations of SDMA, the steoreoisomer of ADMA, also rose significantly with increasing age (p=0.003) (Figure 3.4). However, concentrations of L-arginine did not change with increasing age (F=1.0, p=0.4) (Figure 3.5).

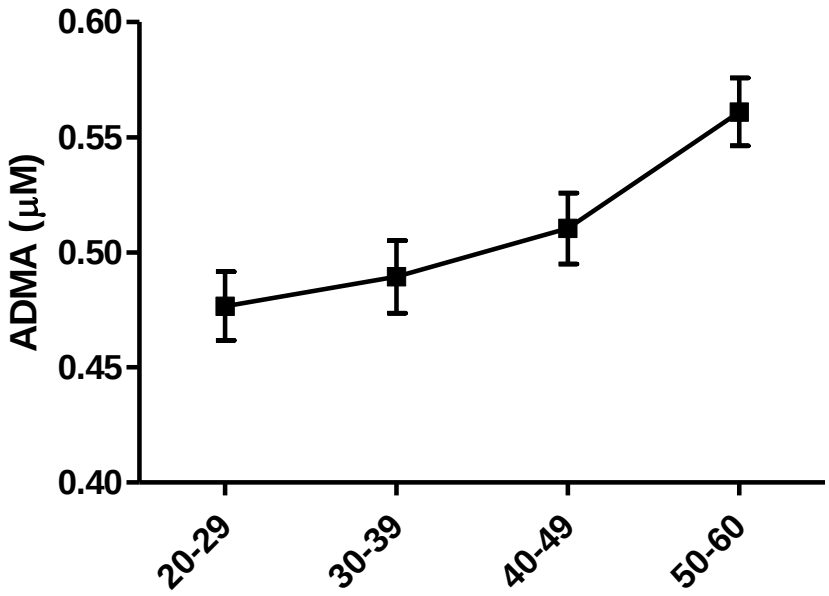

Figure 3.3 ADMA concentrations (μM) in normal women, by age groups. ADMA concentrations rose significantly with age (p

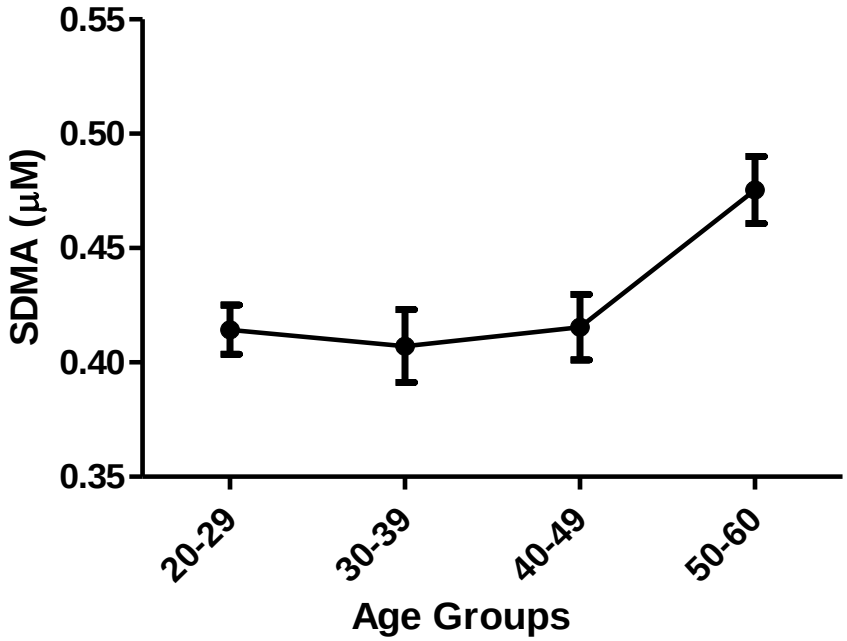

Figure 3.4 SDMA concentrations (μM) in normal women, by age groups. SDMA concentrations increased significantly with age (p=0.003).

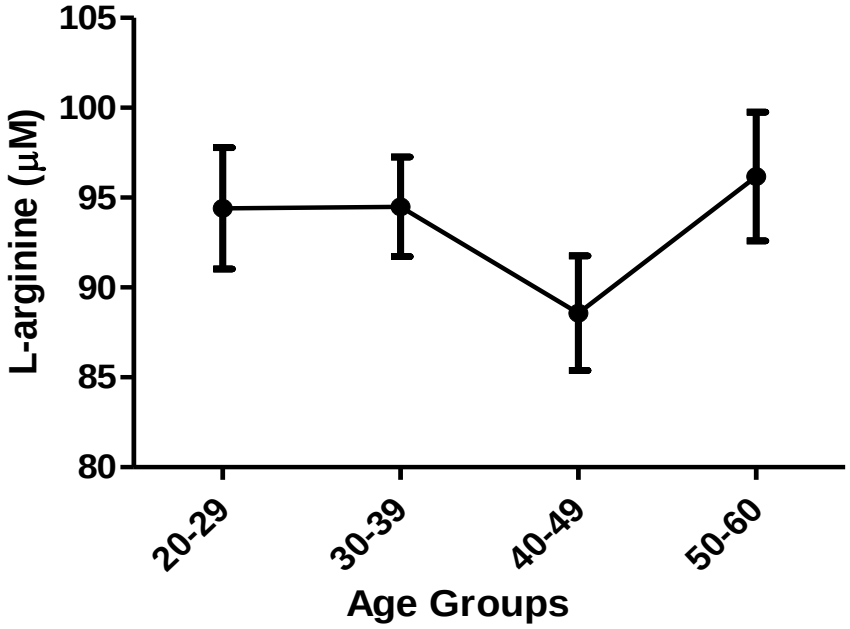

Figure 3.5 L-arginine concentrations (μM) in normal women, by age groups. L-arginine concentrations did not change with increasing age (p=not significant, NS).

ADMA/SDMA ratio, which in part reflects the activity of the ADMA-degrading enzyme DDAH (Bode-Boger, Scalera et al. 2006), also did not change with increasing age (p=0.2) (Figure 3.6).

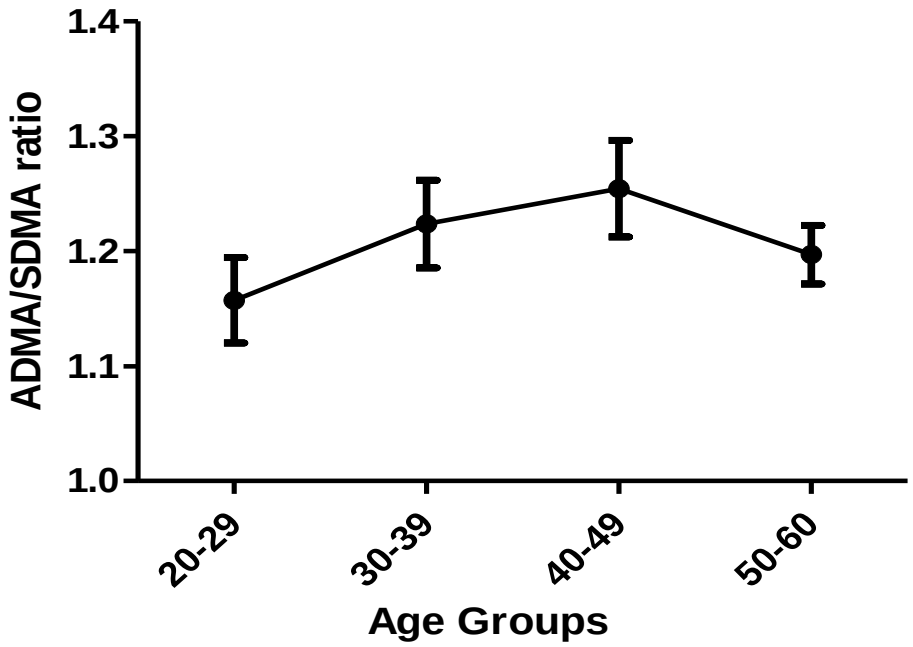

Figure 3.6 ADMA/SDMA ratio in normal women, by age groups. There was no change in the ADMA/SDMA ratio, which in part reflects DDAH activity, with increasing age (p=NS).

On linear regression, platelet NO responsiveness was inversely correlated with ADMA concentrations (r=-0.2, p=0.02) (Figure 3.7).

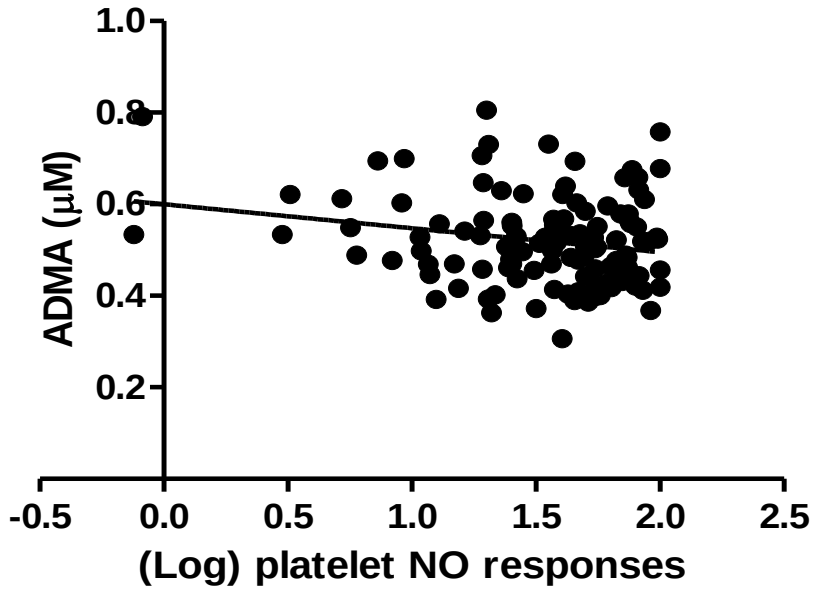

Figure 3.7 Relationship between platelet NO responses (%) and ADMA concentrations (μM). Platelet NO responses were inversely correlated with ADMA concentrations (r=-0.2, p<0.05).

(b) EPC counts

EPC counts varied significantly with increasing age (p=0.02, Kruskal-Wallis test), with an increase to age 50 years followed by a decline.

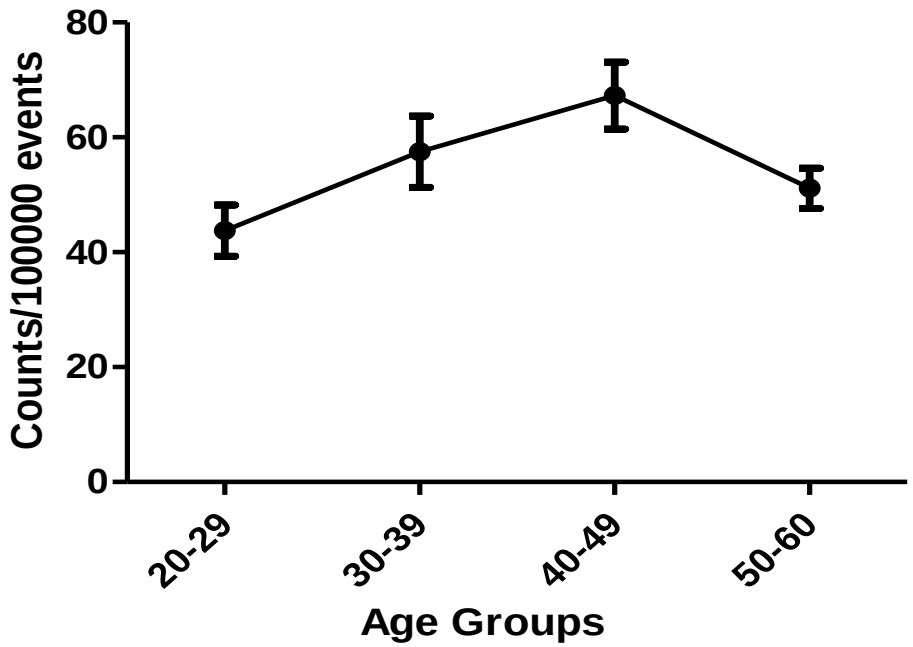

Figure 3.8 EPC counts in normal women, by age groups. There was a significant progressive increase in EPC counts with age, which however declined after age 50 years ($p<0.05$).

(v) hs-CRP concentrations

hs-CRP concentrations also rose significantly with increasing age (p=0.003, Kruskal-Wallis test) in normal women.

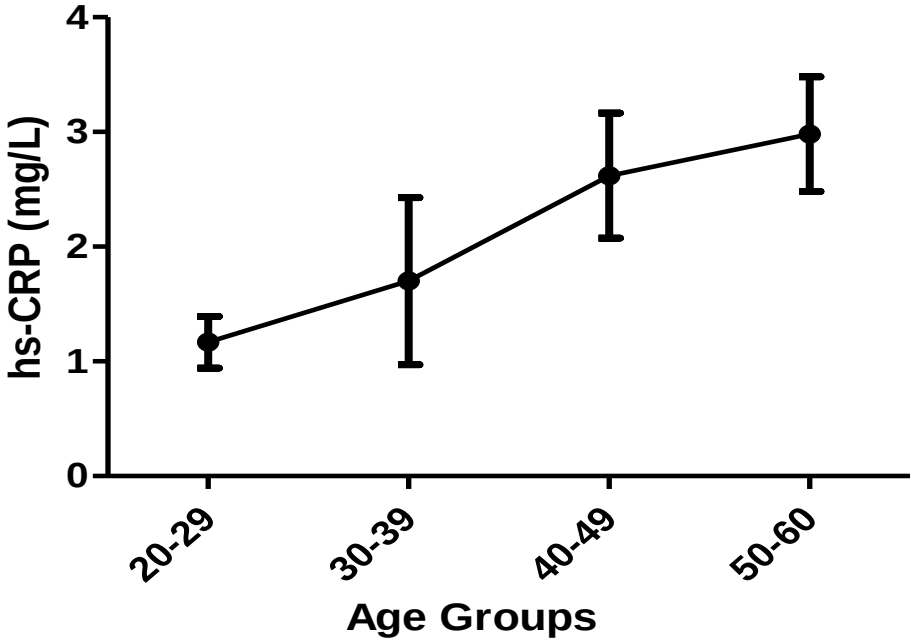

Figure 3.9 hs-CRP (mg/L) in normal women, by age groups. Hs-CRP concentrations rose significantly with age ($p<0.05$).

(vi) Correlates of platelet NO responses

(a) Univariate correlates of platelet NO responses

Univariate correlates of platelet NO responses in normal women are listed in Table 3.3 below.

Table 3.3. Univariate correlates of platelet NO responses in normal women

Parameter	r value	p value
Age, years	-0.4	<0.0001
BMI, kg/m^2	-0.3	0.0004
Platelet aggregation, %	-0.6	<0.0001
hs-CRP, mg/L	-0.2	0.01
Glucose, mmol/L	-0.2	0.02
Insulin, mU/L	-0.2	0.01
HOMA-IR	-0.2	0.02
Total cholesterol, mmol/L	-0.2	0.03
Triglycerides, mmol/L	-0.3	0.007

(b) Multivariate analyses of platelet NO responses

The above parameters were then subjected to backward stepwise regression. Significant correlates of platelet NO responses in normal women were limited to age and extent of ADP-induced platelet aggregation, as summarized below.

Table 3.4 Significant correlates of platelet NO responsiveness on multivariate analysis

Parameter	Beta coefficient	P value
Age, years	-0.32	<0.0001
Platelet aggregation, %	-0.48	<0.0001

3.5.2 Results of platelet and vascular function studies, comparing PCOS with normal women

(i) Subject characteristics

A total of 242 women participated in this study, including 133 normal (non-PCOS) subjects and 109 with a previous diagnosis of PCOS. Their baseline characteristics are compared in Tables 5 and 6.

In the PCOS cohort, there were no indigenous women depite higher rates of PCOS (up to 15%) reported amongst indigenous women in Australia (Boyle, Cunningham et al. 2012). Furthermore, only 11 women fulfilled the NCEP-ATP III criteria for metabolic syndrome (2002).

The normal and PCOS subjects differed significantly in several aspects: PCOS individuals had significantly higher body mass index, testosterone, insulin and HOMA-IR levels, consistent with the metabolic abnormalities associated with PCOS. With increasing age, there were significant increases in BMI, systolic and diastolic blood pressures, total cholesterol, fasting glucose, insulin and HOMA-IR, with decreases in testosterone and oestradiol levels. Prevalence of cardiovascular risk factors was similar between groups except for insulin resistance and diabetes, which were more common in the PCOS group (Table 6).

A total of 38 (28.6%) subjects in the control group and 12 (11.0%) in the PCOS group were postmenopausal on the basis of absence of menstruation >12 months and FSH levels >20U/L. 16 women in the PCOS group and 14 in the control group were receiving oral contraceptives (OCs). Metformin therapy (n=14) was confined to the PCOS group.

Table 3.5 Subject characteristics PCOS women in comparison to normal women, by age groups
(Data presented are mean, SEM)

Age groups	**20-29**		**30-39**		**40-49**		**50-60**	
	PCOS	Control	PCOS	Control	PCOS	Control	PCOS	Control
N	32	25	36	27	21	33	20	48
BMI, kg/m^2*†	29.5, 1.4	22.2,0.6	29.8,1.1	24.5,1.0	32.1,1.5	27.7,0.9	35.0, 1.7	27.8, 0.8
Systolic BP, mmHg *	111.8, 2.5	112.9, 2.5	118.7, 4.3	118.2, 2.6	123.7, 2.4	122.1, 2.8	130.6, 3.5	130.0, 2.6
Diastolic BP, mmHg *	74.0, 1.7	73.2,1.9	76.1,1.5	74.8,2.1	81.4,1.8	79.4, 1.8	80.1, 2.5	83.2, 1.6
Total cholesterol, mmol/L *	5.1, 0.2	4.6, 0.2	4.7, 0.2	4.8, 0.2	5.1, 0.2	5.1, 0.1	5.4, 0.2	5.5, 0.1
HDL, mmol/L	1.6, 0.1	1.6, 0.1	1.5, 0.1	1.6, 0.1	1.5, 0.1	1.7, 0.1	1.6, 0.1	1.6, 0.05
Triglycerides, mmol/L	1.3, 0.2	1.1, 0.1	1.0, 0.1	1.0, 0.2	1.3, 0.1	1.0, 0.1	1.7, 0.2	1.5, 0.2
Fasting glucose, mmol/L *†	4.4, 0.1	4.4, 0.1	4.5, 0.1	4.6, 0.1	4.9, 0.1	4.7, 0.1	5.4, 0.2	4.7, 0.1
Insulin, mU/L *†	14.0, 2.0	6.5, 0.5	9.2, 1.3	6.2, 0.6	15.0,3.0	6.1, 0.5	15.5, 2.6	10.0, 1.4
HOMA-IR *†	2.8, 0.4	1.3, 0.1	1.8, 0.3	1.3, 0.1	3.4, 0.7	1.2, 0.1	3.7, 0.8	2.1, 0.3
Testosterone, nmol/L *†	2.1, 0.3	1.2, 0.2	1.6, 0.2	1.1, 0.1	1.3, 0.1	0.9, 0.1	1, 0.2	0.7, 0.05
Oestradiol, pmol/L*	188.2, 24.2	241.7, 53.2	293.4, 47.1	253.3, 39.8	350.3, 66.9	313.7, 51.1	229.4, 77.8	180.1, 37.2

***** $p<0.05$ by 2 way ANOVA for Age, † $p<0.05$ by 2 way ANOVA for PCOS status
The interaction parameter for all variables was not significant.

TABLE 3.6 Cardiovascular risk factors and medications (n,%), comparing PCOS and normal women
(Data presented are mean, SEM)

	PCOS (109)	Normal (133)
Current smoker	14, 12.8	13, 9.8
Insulin resistance	23, 21.1 *	4, 3.0
Type 2 diabetes	4, 3.7 *	0
Hypertension	9, 8.3	11, 8.3
Hypercholesterolemia	22, 20.2	27, 20.3
Family history	10, 9.2	11, 8.3
Current OC use	16, 14.7	14, 10.5
Current metformin use	14, 12.8 *	0
ACEI, ATRA	7, 6.4	6, 4.5
Statins	4, 3.7	6, 4.5
HRT	2, 1.8	1, 0.8

***** $p<0.05$ by Pearson chi-square analysis

OC Oral contraceptives
ACEI Angiotensin-converting enzyme inhibitors
ATRA Angiotensin 2 receptor antagonists
HRT Hormone replacement therapy

(ii) Platelet aggregation and NO responses

Platelet aggregation in response to ADP (2.5 μmol/L) in blood samples obtained from normal and PCOS subjects according to age decade is depicted in Figure 3.10A. There was a significant increase in platelet aggregation with age ($F=3.30$, $p=0.02$) with a trend towards greater aggregation in PCOS compared to non-PCOS individuals ($F=3.46$, $p=0.06$). The interaction parameter was not significant.

Inhibition of platelet aggregation by the NO donor SNP, the principal outcome measure of the study, is shown in Figure 1B. Platelet NO responses declined overall with age ($F=8.19$, $p<0.0001$). Furthermore, responsiveness to NO was selectively impaired ($F=4.20$, $p=0.04$) in PCOS subjects. However, there was no significant variation in the PCOS:normal relationship with increasing age ($F=2.13$, $p=0.4$).

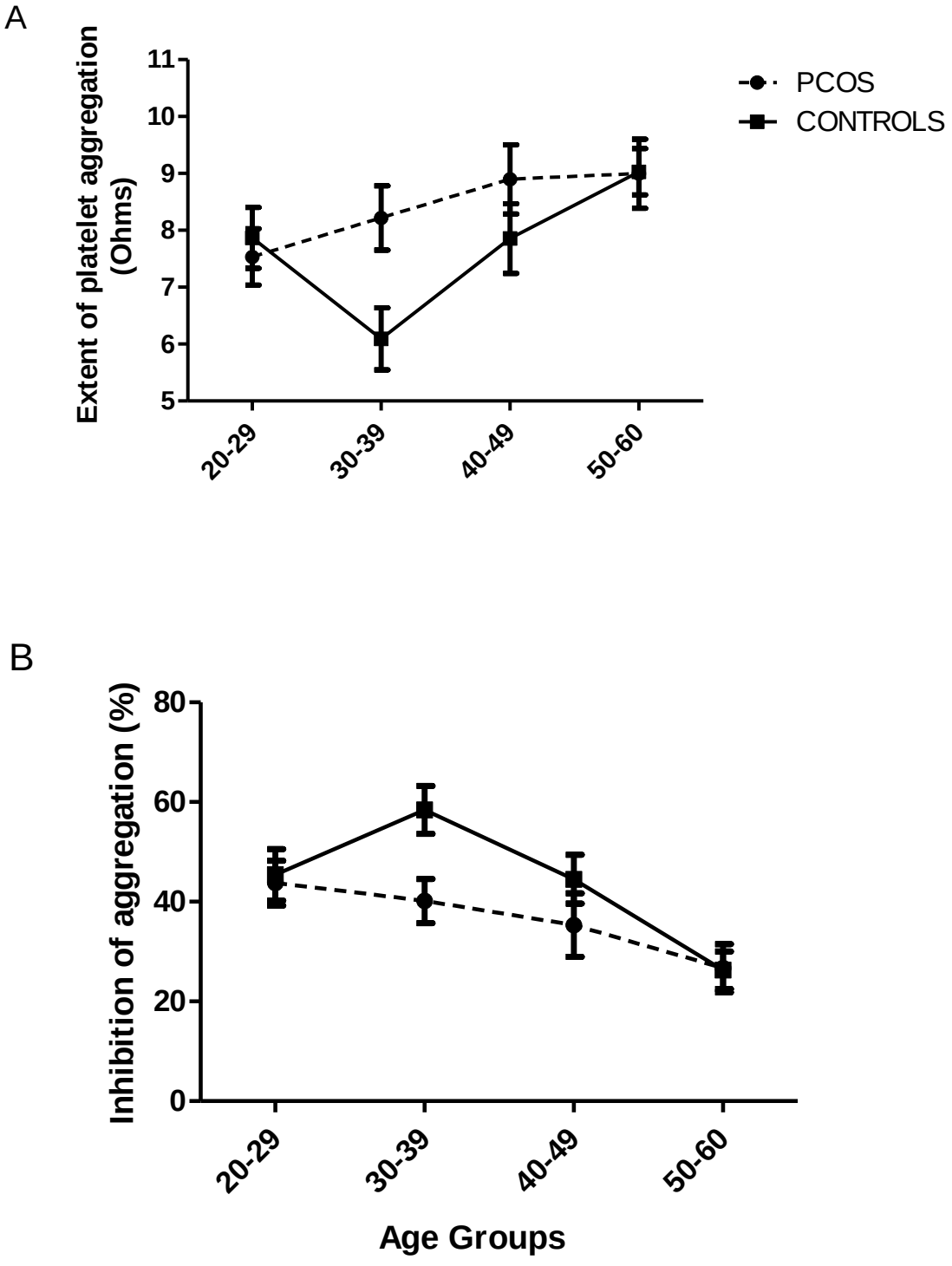

Figure 3.10 **Platelet aggregation (A) and NO responses (B) in normal and PCOS women, by age groups**. Platelet aggregation increased with age (F=3.3, p=0.02) whilst NO responses declined with age (F=8.19, p<0.0001). PCOS women demonstrated a trend towards platelet hyperaggregability (F=3.46, p=0.06) and had significantly impaired NO responses (F=4.2, p=0.04) compared to normal women.

In our previous study, subjects receiving pharmacotherapy with either metformin or OCs were excluded (Rajendran, Willoughby et al. 2009). In order to evaluate whether inclusion of these individuals might have affected the results of the PCOS:control comparison, we compared platelet aggregation and responsiveness to NO in the absence/presence of OC therapy (in both control and PCOS women, limited to age 45 years and younger) and of metformin therapy (in PCOS women only). While there was no heterogeneity according to metformin or OC therapy in PCOS women, it was found that ADP responses were greater ($p=0.047$, unpaired t-test) and platelet NO responses ($p=0.02$) were substantially lower in control subjects taking OCs (Figure 3.11).

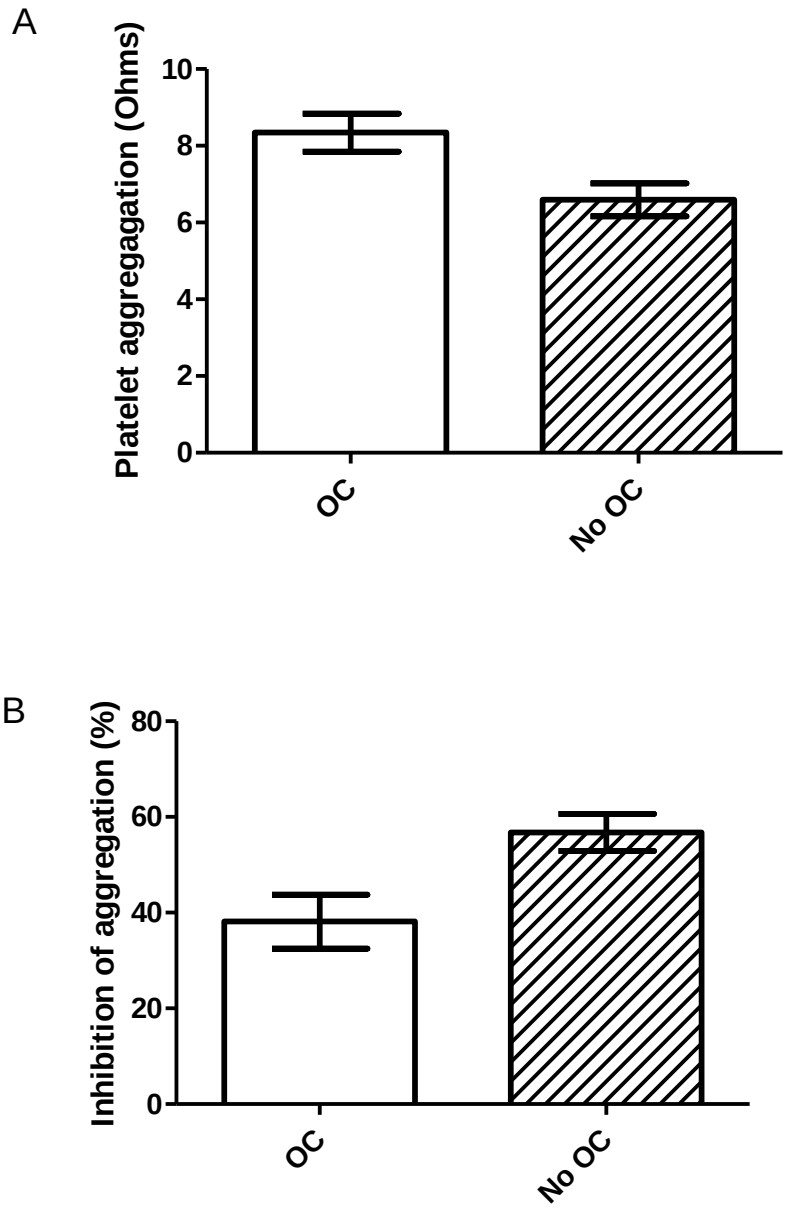

Figure 3.11 **Platelet aggregation (A) and NO responses (B) in normal women, according to OC use.** OC users demonstrated platelet hyperaggregability (p=0.047) and lower NO responses (p=0.02), compared to non-OC users.

(iii) Multivariate analysis of determinants of platelet NO responses in the entire cohort

Backward stepwise regression was performed to identify determinants of the primary end-point in the entire population. The following parameters were included:- age, BMI, baseline augmentation index, salbutamol response, NTG response, hs-CRP, HOMA-IR, testosterone, oestrogen levels, OC use and presence of PCOS. Significant negative correlates of platelet NO responsiveness were age and PCOS status ($p<0.001$ and 0.02 respectively).

(iv) Endothelium-dependent and –independent vasodilatation

Resting AIx increased significantly with age ($F=47.8$, $p<0.0001$) but did not vary significantly between groups.

Figure 3.12A shows the effect of salbutamol on AIx for both groups. There was a significant decline in endothelium-dependent vasodilatation with age ($F=5.14$, $p=0.002$). PCOS status was again a determinant of the responses ($F=5.64$, $p=0.02$), with the most prominent differences seen in those age <40 years. Although the difference between PCOS and normals tended to decrease with age, the interaction parameter did not reach statistical significance ($F=0.98$, $p=0.4$).

Responses to the endothelium-independent vasodilator NTG (Figure 3.12B) exhibited a decline with age of borderline significance ($F=2.59$, $p=0.054$), and there was no significant differential impairment in PCOS subjects

(F=2.52, p=0.1). The interaction parameter was again not significant (F=0.74, p=0.5).

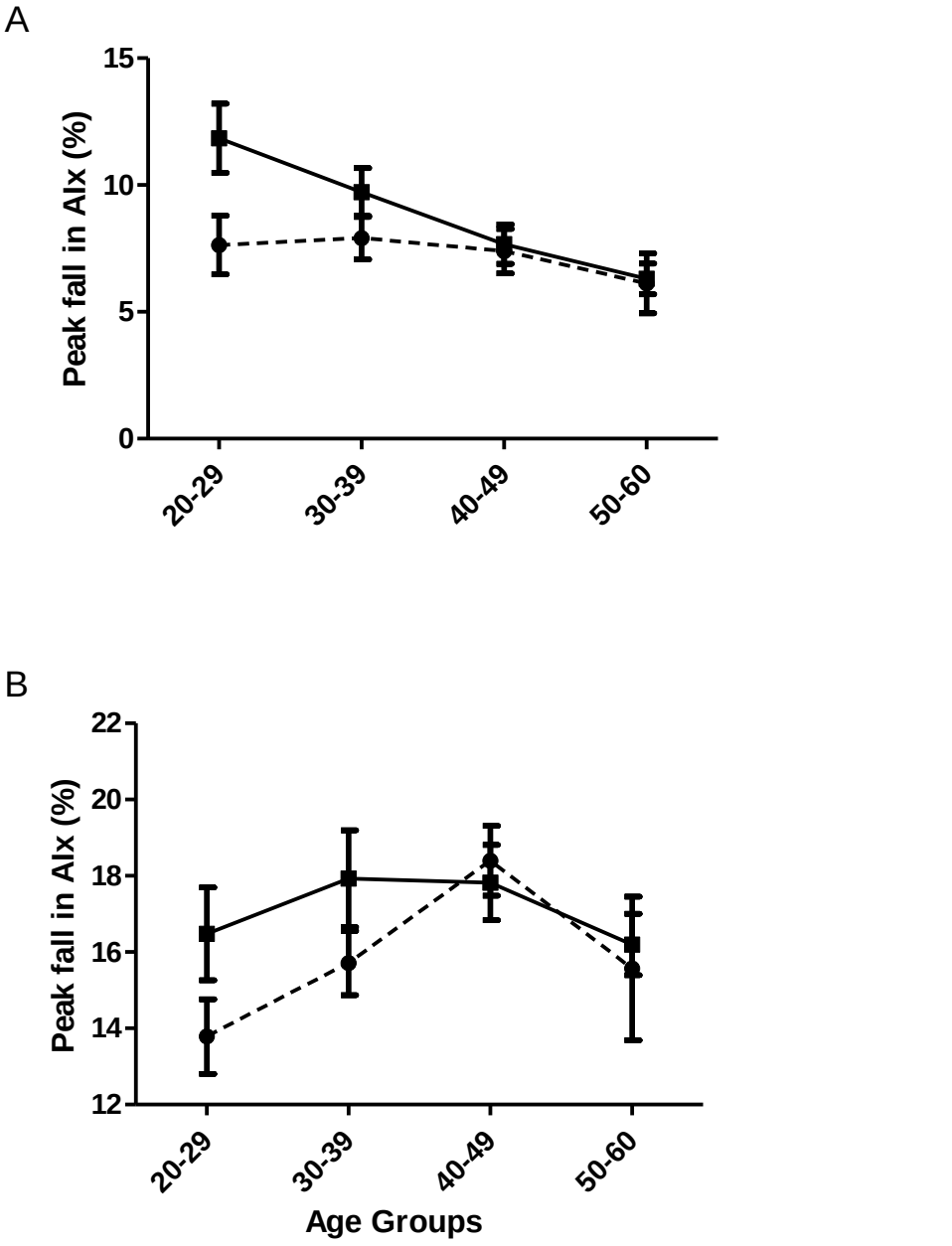

Figure 3.12 **Endothelial-dependent (A) and vascular NO responses (B) in PCOS (-●-) and normal (-■-) women, by age groups.** Both parameters declined with age (Salbutamol: F=5.12, p=0.002, NTG: F=2.59, p=0.05). PCOS women demonstrated impaired endothelial-dependent vasodilatation but not vascular NO responses compared to normal women (F=2.52, p=0.1). The impairment was evident early in young PCOS individuals but had a tendency to converge with normal women above the age of 40 years.

(v) Other determinants of endothelial function

(a) ADMA, SDMA and L-arginine concentrations

Although ADMA levels rose with age (F=3.9, p=0.01), these were significantly greater in the PCOS cohort throughout (F=38.6, p<0.0001). In contrast with NO response in platelets, differences did not tend to diminish with age. The interaction parameter was not significant (F=0.40, p=0.7).

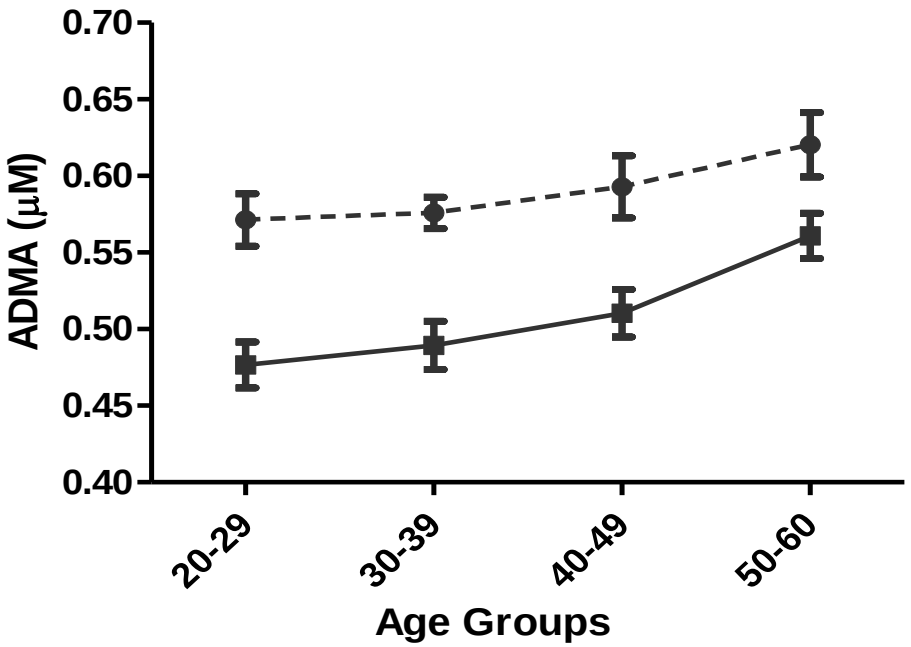

Figure 3.13 **ADMA concentrations (μM) for PCOS (-●-) and controls (-■-), by age groups.** ADMA concentrations increased with age (F=5.7, p=0.002) with higher concentrations in PCOS compared to controls (F=3.7, p=0.002). There was no interaction between both groups (F=1.8, p=0.2).

SDMA concentrations did not vary significantly between populations or with age (Figure 3.14). Plasma arginine concentrations were significantly lower in PCOS patients compared to controls throughout (ANOVA: F=5.7, p= 0.02). Thus the arginine:ADMA ratio was markedly lower for PCOS than control subjects (Figure 3.15). A low arginine:ADMA ratio has been demonstrated in some studies to be correlated with endothelial dysfunction (Boger, Bode-Boger et al. 1998, Sydow, 2003) Furthermore ADMA:SDMA ratios were significantly higher (F=14.1, p=0.0002) in PCOS subjects.

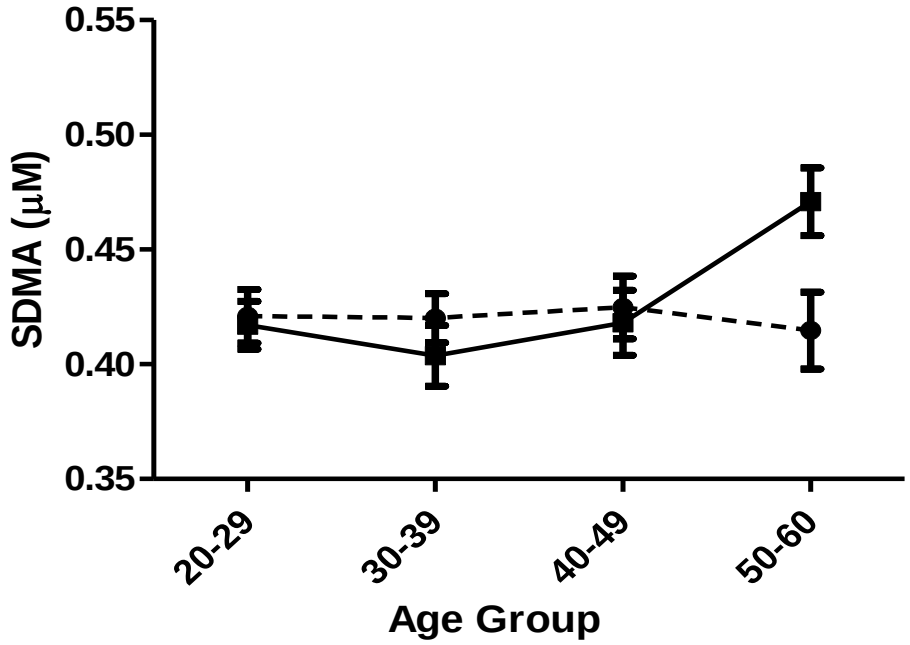

Figure 3.14 **SDMA concentrations (µM) for PCOS (-●-) and controls (-■-), by age groups.** SDMA concentrations did not vary significantly with age (2 way ANOVA, F=2.0, p=0.2) nor PCOS status (F=0.2, p=0.5).

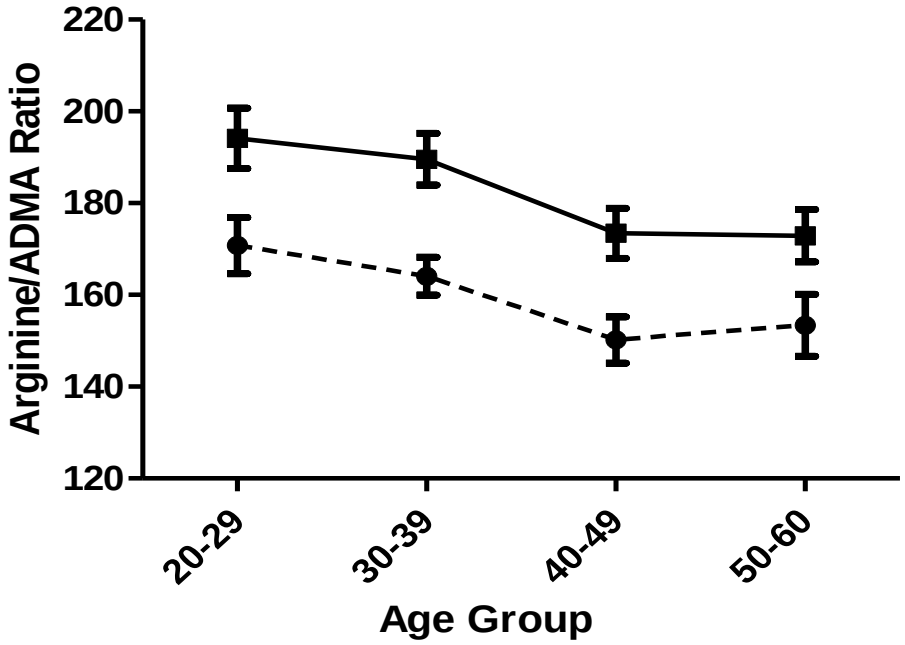

Figure 3.15 **Arginine/ADMA ratio with increasing age for PCOS (-●-) and controls (-■-).**There was a significant decline in the ratio with increasing age (2 way ANOVA, F=6.2, p=0.001) with lower values in the PCOS cohort (F=10.6, p<0.0001). The group:age interaction was not significant.

In view of the finding that plasma arginine concentrations were markedly lower in PCOS patients, plasma arginase assays were performed in a subset of patients above 50 years of age, in whom the differences tended to be most pronounced (Figure 3.15). There was no significant difference between PCOS and control subjects (mean 5.0±1.6 and 6.0±2.2 U/L respectively).

Correlates of ADMA concentrations were also determined. On binary logistic regression analysis, PCOS was directly associated with ADMA elevation (Table 3.7A), and with BMI, and inversely with plasma arginine concentrations, vascular salbutamol and platelet SNP responses.

As summarised in Table 7B, within the PCOS population, ADMA elevation was significantly and directly correlated with free androgen index and with BMI. Furthermore, ADMA and SDMA concentrations were strongly correlated with each other. Within the control population, correlates of ADMA elevation were age (β=0.2, p=0.02), BMI (β=0.3, p<0.0001) and baseline AIx (β=0.0, p-0.02).

Table 3.7A Significant correlates of PCOS status on binary logistic regression in the entire cohort

Parameter	β coefficient	P value
BMI, kg/m^2	12.6	<0.0001
ADMA concentrations, μM	7.0	0.04
Arginine concentrations, μM	-8.5	<0.0001
Salbutamol responses, %	-0.1	<0.0001
SNP responses, %	-0.2	0.02

Variables entered on Step 1: Age, BMI, HOMA-IR, ADMA, SDMA, Arginine, Baseline AIx, Salbutamol and NTG responses in vasculature, SNP response in platelets, CRP, total cholesterol levels

Table 3.7B Significant physiological/biochemical correlates of ADMA concentrations on backward multiple linear regression in the PCOS cohort only

Parameter	β coefficient	P value
BMI, kg/m^2	0.3	0.001
FAI	0.3	0.002
Salbutamol responses, %	-0.2	0.01
SDMA concentrations, μM	0.5	<0.0001
Arginine concentrations, μM	0.2	0.04

Dependent variable: ADMA concentrations
Independent variables: Age, BMI, Salbutamol responses, NTG responses, SNP responses, HOMA-IR and FAI

(b) EPC counts

EPC counts also varied with age (F=5.7, p=0.01), increasing until the age of 50 and declining thereafter. However, there was no difference between controls and PCOS subjects (F=0.5, p=0.5) and the interaction parameter was not significant (F=0.72, p=0.5).

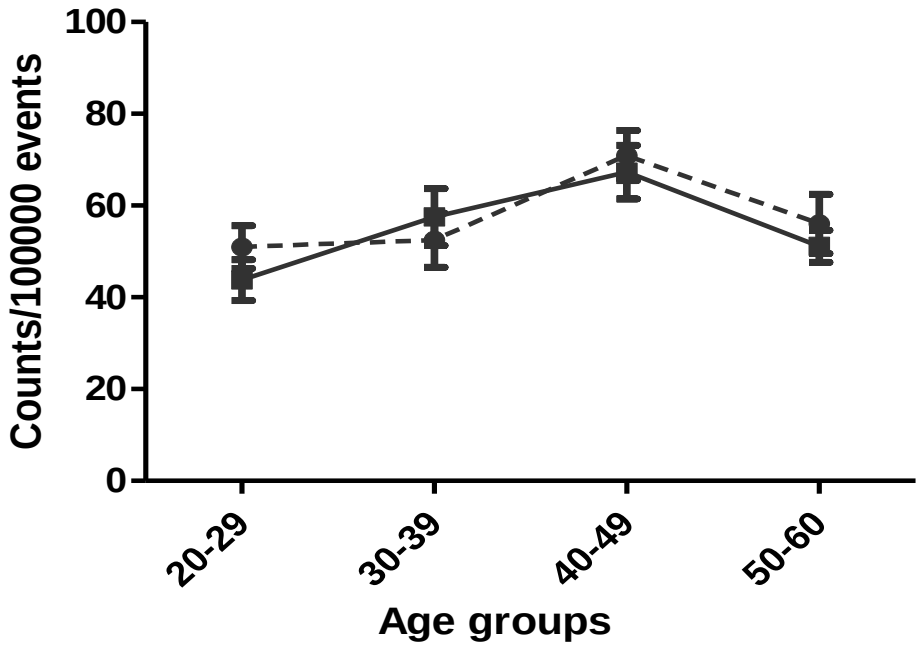

Figure 3.16 **EPC cell counts with increasing age for PCOS (-●-) and controls (-■-).** EPCs increased with age up to 50 years and subsequently declined (2 way ANOVA, F=5.7, p=0.01), with no differences between the two cohorts (F=0.5, p=NS).

(vi) hs-CRP concentrations

In the entire cohort, as distinct from the controls alone, hs-CRP concentrations did not vary significantly with age (F=0.8, p=0.5). In the current cohort, as previously observed (Diamanti-Kandarakis, Alexandraki et al. 2006), hs-CRP concentrations were significantly greater in the PCOS subjects than controls (F=10.1, p=0.002). There was no significant interaction between both groups (F=1.61, p=0.2).

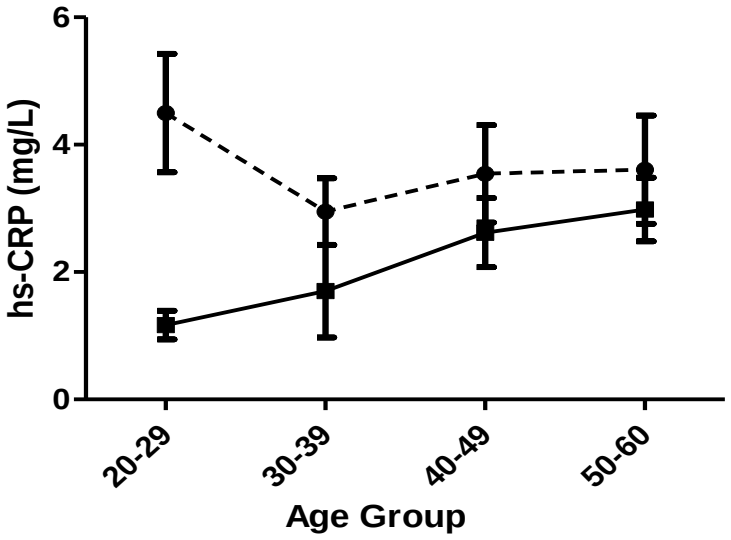

Figure 3.17 **hs-CRP concentrations with increasing age for PCOS (-●-) and controls (-■-).** hs-CRP did not change significantly with age (F=0.8, p=0.5), with significantly higher concentrations in PCOS women compared to normal women (F=10.1, p=0.002).

3.5.3 Effects of menopause on platelet and vascular responses

(i) Subject characteristics

A total of 40 perimenopausal women aged between 40 and 60 years were studied prospectively to determine the relationship between hormonal changes associated with menopause and platelet and vascular responses to NO. Post-menopausal status was defined as the absence of menstruation together with levels of follicle stimulating hormone (FSH) of more than 20U/L. 19 of the women studied were deemed premenopausal and 21 postmenopausal based on these criteria. Subject characteristics are shown below (Table 8). In accordance with these criteria, premenopausal women were younger, had higher oestradiol levels and lower FSH, LH levels than post-menopausal women. Although testosterone levels were higher in premenopausal women, there was no difference between groups in the free androgen index (FAI). Fasting insulin and therefore HOMA-IR was also significantly higher in postmenopausal women (none reported diabetes).

Table 3.8 Baseline characteristics of perimenopausal women (n=40)

(Data presented are mean, SEM)

	Premenopausal n=19	**Postmenopausal n =21**
Age, years *	48.5, 1.0	54.0, 0.8
BMI, kg/m^2	29.2, 1.5	27.4, 0.8
Systolic BP, mmHg	131.4, 3.4	134.4, 4.3
Diastolic BP, mmHg	85.3, 2.3	87.0, 2.2
Total cholesterol, mmol/L	5.2, 0.2	5.6, 0.2
HDL, mmol/L	1.7, 0.1	1.7, 0.1
Triglycerides, mmol/L	1.5, 0.4	1.3, 0.1
Glucose, mmol/L	4.5, 0.1	4.8, 0.1
Insulin, mU/L *	6.8, 1.0	9.6, 1.1
HOMA-IR*	1.4, 0.2	2.1, 0.2
Oestradiol, pmol/L *	484.1, 86.8	65.9, 14.0
FSH, IU/L*	10.4, 1.8	75, 8.7
LH, IU/L*	11.4, 2.4	30.5, 2.9
Testosterone pmol/L *	0.8, 0.1	0.6, 0.1
SHBG, nmol/L*	64.5,5.3	49.1, 5.4
Free androgen index, %	1.4, 0.2	1.4, 0.3

* $p<0.05$

SHBG Sex hormone binding globulin

(ii) Results of platelet and vascular responses

There were no significant differences between groups in both platelet and vascular function parameters, as shown below. In particular, the platelet response to SNP and the vascular response to salbutamol were both marginally (not statistically) lower in post-menopausal women, with mean differences of -13% and -5% respectively (see Table 9). These specific differences approximated to those associated with the commensurate age differences between the 2 groups of women (see Figures 3.1B and 3.2B).

Table 3.9 Results of platelet and vascular responses
(Data presented are mean, SEM)

	Premenopausal	**Postmenopausal**
Platelet aggregation to ADP, Ohms	8.7, 0.5	9.0, 0.8
Inhibition of platelet aggregation, %	33.7, 5.8	29.3, 6.6
Vascular salbutamol response, %	6.0, 1.2	5.7, 0.7
Vascular NTG response, %	17.0, 1.5	17.1, 1.1
ADMA concentrations, µM	0.57, 0.02	0.58, 0.03
EPC counts, per 100000 events	67.0, 6.8	52.0, 5.2
TSP-1, ng/ml	156.3, 19.4	134.4, 23.8

TSP-1 Plasma thrombospondin-1concentrations
There were no significant differences between groups for all parameters.

(iii) Correlation of hormonal levels and platelet NO and vascular responses

In order to evaluate the possibility that the marked falls in oestradiol levels associated with menopause (see Table 8) might interact with platelet NO responses, data were analysed continuously, as shown in Figures 3.18A and 3.18B. Neither platelet NO response (Figure 3.18A) nor vascular salbutamol response (Figure 3.18B) decreased as oestradiol levels fell.

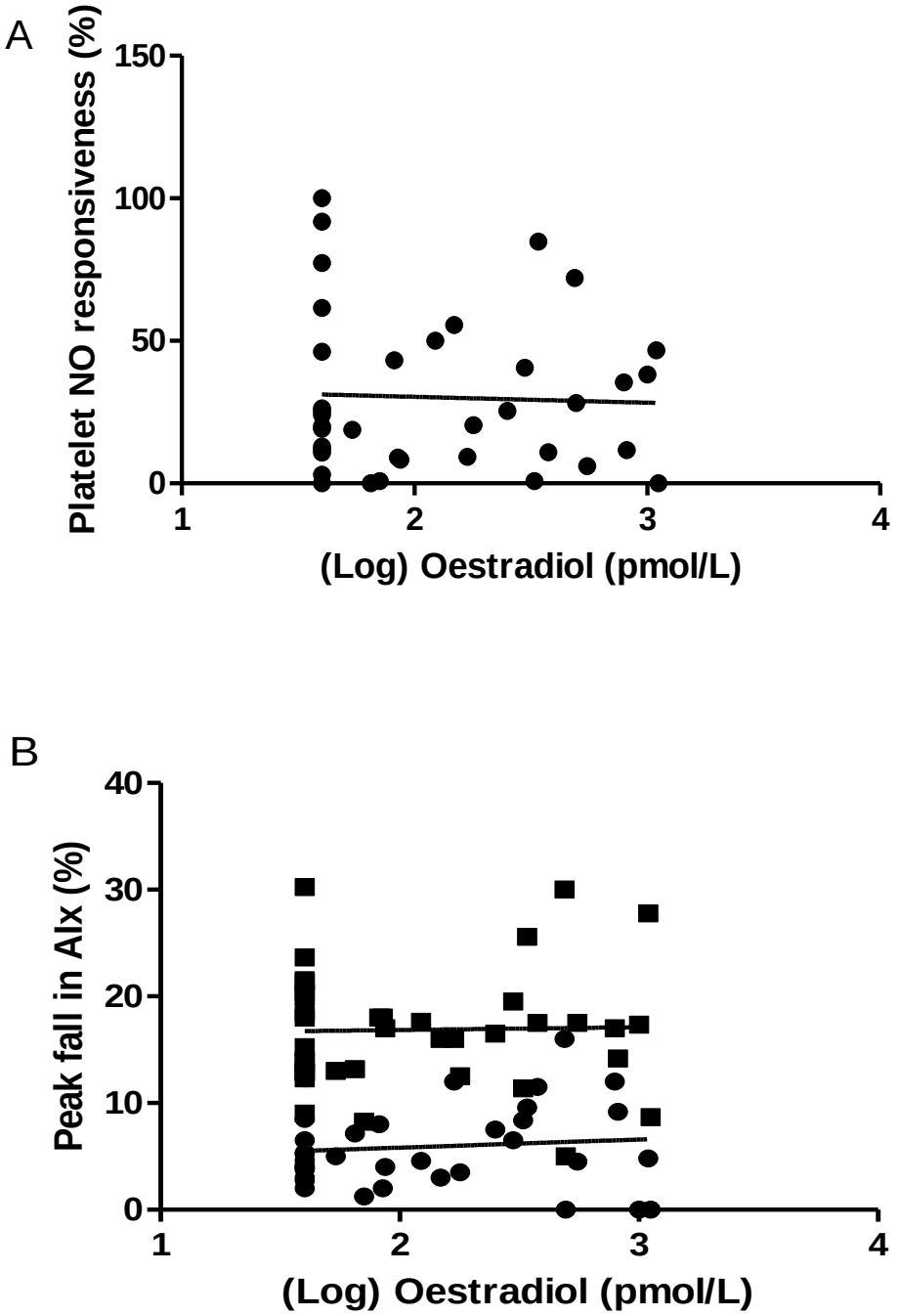

Figure 3.18 **Correlation of platelet NO responses (A) and vascular responses, salbutamol (-●-) and NTG (-■-) with oestradiol levels.** There was no relationship between platelet NO (r=-0.039, p=0.8), salbutamol (r=0.024, p=0.9) and NTG (r=0.099, p=0.6) responses with plasma oestradiol levels.

3.6 Discussion

This study was undertaken (1) to evaluate the impact of ageing on platelet NO and vascular NO signalling in normal women, (2) to compare these changes with PCOS subjects and (3) to assess the impact of menopause on these parameters.

In normal women, ageing led to progressive impairment of a number of parameters related to NO signalling: platelet NO response and endothelial function decreased while plasma ADMA concentrations increased. PCOS was associated with both accentuation of these anomalies and with their appearance by young adult life. The additional finding that OC use in normal women was associated with impaired NO response was a post hoc observation. Furthermore there was no correlation between changes in oestradiol levels with these anomalies.

The implication of these findings is that ageing in women is associated with progressive endothelial dysfunction, which accords with previous investigations in a mixed gender population (Celermajer, Sorensen et al. 1994). Furthermore, the results indicate that the basis for this impairment is both inhibition of NO synthesis (for example via the effects of increased ADMA concentrations) and also impairment of the NO signalling "cascade", as evidenced by the phenomenon of NO resistance. We have previously demonstrated that NO resistance reflects both "scavenging "of NO by superoxide and dysfunction of soluble guanylate cyclase (sGC) (Chirkov, Holmes et al. 1999).

As regards PCOS, these findings extend our previous observations (Rajendran, Willoughby et al. 2009) in a group of young women. It appears that the cardiovascular abnormalities associated with ageing are present in young adult life in PCOS, and that there may be a trend towards convergence of data from normal and PCOS groups with ageing. Comparison of ADMA concentrations, in particular, shows that differences persist between PCOS and control subjects throughout the period from 18 to 60 years.

The impact of increased age on ADMA kinetics has been previously evaluated in a number of studies which in general demonstrated an increase in ADMA concentrations with increasing age in both animals (Xiong, Yuan et al. 2001) and humans (Kielstein, Bode-Boger et al. 2003; Sydow, Fortmann et al. 2010). Importantly, ADMA concentrations may rise because of renal insufficiency (Vallance, Leone et al. 1992) or with coronary risk factors (Boger, Bode-Boger et al. 1998; Abbasi, Asagmi et al. 2001). However, these do not appear to fully account for the observed age-related increase in this control population. Indeed, on multivariate analysis, age, obesity and elevated AIx were the only significant correlates of ADMA concentrations within this cohort. Previously postulated mechanisms for the rise in ADMA concentrations with age include reduction in clearance of ADMA via decreased dimethylarginine dimethylaminohydrolase (DDAH) activity with age and oestrogen levels (Holden, Cartwright et al. 2003) and/or increased synthesis of ADMA by the upregulation of protein arginine methyltransferases (PRMT) which is activated by increasing oxidative stress with age (Boger, Sydow et al. 2000). Given that ADMA, but not SDMA, is a DDAH substrate, and that SDMA concentrations were not elevated, the

strongest implication is that of reduced DDAH activity. However, evidence for activation of PRMTs can also be inferred within the PCOS group, given the correlation between ADMA and SDMA concentrations (Table 3B) in this group, which is consistent with the results of the Bruneck Study (Kiechl, Lee et al. 2009). Interestingly, one previous evaluation of determinants of ADMA kinetics in ageing individuals found no differences in either endothelial cell ADMA concentrations or vascular DDAH II expression (Gates, Boucher et al. 2007).

PCOS has been reported to be associated with ADMA elevation (Heutling, Schulz et al. 2008; Rajendran, Willoughby et al. 2009; Toulis, Goulis et al.). All previous reports were generated in relatively young populations. The current data suggest some attenuation of differences between PCOS and control populations with ageing (although this trend did not reach statistical significance). The finding that PCOS was associated with reduced plasma arginine concentrations raised the issue that there might also be increased activity of arginases, which might also contribute to the observed endothelial dysfunction (Durante, Johnson et al. 2007). While plasma arginase activity did not differ between PCOS and control subjects, it remains possible that PCOS might be associated with activation of tissue arginases. On the other hand, the observed changes may result from systematic inflammatory activation in PCOS subjects:- van der Zwan et al (2011), have observed an inverse correlation between CRP and arginine levels while a direct association between ADMA and inflammatory activation has also been previously documented (Ngo, Sverdlov et al. 2010; van der Zwan, Scheffer et al. 2011).

The correlation between ADMA with other markers of impaired NO bioavailability (vascular endothelial dysfunction) and function (platelet NO responsiveness) further strengthens the notion that PCOS is associated with a relative "NO-deficient state". This is in keeping with previous studies suggesting NO deficiency in PCOS individuals, although not related to ADMA concentrations (Turkcuoglu, Engin-Ustun et al. 2010). While the correlation between ADMA elevation and physiological measures of endothelial dysfunction is in accordance with theoretical expectations, the association with tissue responsiveness to NO represents a novel finding without an obvious underlying biochemical mechanism. However, we have previously demonstrated [see (Chirkov and Horowitz 2007) for review] that impaired platelet responsiveness to NO results from oxidative changes in soluble guanylate cyclase, together with superoxide anion-mediated "scavenging "of NO. Potentially similar mechanisms may contribute to DDAH inactivation (Lin, Ito et al. 2002). Previous reports have suggested, in this clinically heterogeneous disorder, that the subset of PCOS individuals who are anovulatory and hyperandrogenic are at particularly high risk of cardiovascular disease (Moran, Cameron et al.; Wild, Carmina et al. 2010). Intriguingly, we found that ADMA concentrations were also particularly elevated in the presence of obesity and higher FAI, consistent with the results of previous studies (Heutling, Schulz et al. 2008).

We also measured circulating endothelial progenitor cells as a marker of NO-mediated progenitor cell mobilization from the bone marrow. We observed that there was a progressive rise in EPC numbers till the age 50, and then a subsequent decline. There have been multiple studies demonstrating a decline in EPC numbers and/or function with increasing age

(Scheubel, Zorn et al. 2003; Heiss, Keymel et al. 2005). More importantly, Tao et al (2006) demonstrated a significant linear relationship between EPC numbers and arterial stiffness, with other studies showing also a correlation with endothelial function (Hill, Zalos et al. 2003), suggesting a pathophysiological role of vascular regenerative capacity in endothelial dysfunction and therefore cardiovascular risk . Although PCOS women demonstrated impaired NO signalling, their EPC counts were similar to those of control women. Furthermore, it might have been expected from the fact that plasma ADMA concentrations are substantially elevated on PCOS subjects that this would also be associated with low EPC counts, analogous with the previous findings of Thum et al (2005). However, EPC counts are controlled by multiple mechanisms, rather than just NO signalling (Takahashi, Kalka et al. 1999; Hamada, Kim et al. 2006; Cesari, Caporale et al. 2008; Herrler, Leicht et al. 2009). In this regard, inflammatory activation in PCOS (as reflected by higher hs-CRP levels) may provide a stimulus to EPC proliferation (George, Goldstein et al. 2004), although there is evidence that chronic inflammation is associated with depressed EPC numbers and function.

In normal women, two additional observations were evident:

(1) In post hoc analysis, there is a suggestion that oral contraceptive pills may modulate platelet NO responsiveness in young healthy females. This effect is only partially explained by the evidence of platelet hyperaggregability in OC users. Platelet hyperaggregability in response to

ADP has long been described (Montanari, Vittoria et al. 1979), although there have been some conflicting results (Saleh, Ginsburg et al. 1995). In animal models, this effect has been shown to be mediated in part by oxidative stress (Durand and Blache 1996) and can be reversed with antioxidant therapy such as Vitamin E (Renaud, Ciavatti et al. 1987). Effects on NO responsiveness have yet to be reported, although there have been studies evaluating the impact of OC use on endothelial function (John, Jacobi et al. 2000; Torgrimson, Meendering et al. 2007) which again showed conflicting results. John et al (2000) did not find any difference in forearm blood flow responses to acetylcholine and nitroglycerine infusion between premenopausal women using oral contraceptives compared to those who were not OCs. Furthermore, OCs seem to enhance basal NO production based on evidence of enhanced vasodilatation in response to N (G)-monomethyl-L –arginine infusion. Trogrimson et al (2007) on the other hand, found impaired endothelium-dependent vasodilatation in those on low dose combination oral contraceptives. In PCOS women, no effect of OC on platelet NO responsiveness was observed despite some evidence that combination OCs (oestrogen and anti-androgen) may improve NO signalling by reducing inflammation and ADMA concentrations (Charitidou, Farmakiotis et al. 2008).

(2) Hormonal changes during the perimenopausal period do not appear to play a major role in the changes in platelet and vascular NO signalling, contrary to previous findings by Celermajer et al (1994). In this study of 283 subjects (103 men, 135 women), the investigators found that flow-

mediated dilatation was significantly correlated with age ($r = -0.34$, $p < 0.0001$). Endothelial dependent vasodilatation in women declined about a decade later than that in men and at a much steeper rate of 0.49%/year compared to 0.21%/year ($p=0.002$), suggesting a protective effect of oestrogen on the vasculature during their premenopausal years.

3.7 Implications of study findings

These findings have obvious therapeutic implications, given in particular our recent demonstration that NO resistance is ameliorated by therapy with ACE inhibitors (Willoughby, Rajendran et al. 2012). In the first instance, it might be proposed that such therapy might attenuate ageing-associated deterioration in platelet and vascular NO signalling. Given that increasing age represents a major independent risk factor for cardiovascular morbidity and mortality in both males and females (Castelli 1984), these data extend previous observations in a mixed-gender population (Gerhard, Roddy et al. 1996; Taddei, Virdis et al. 2001) by suggesting that the decline in NO signalling is a progressive one, rather than a process that begins abruptly in the post-menopausal years. Similarly, the PCOS subject data provide a potential rationale for "lifelong" therapy to normalize NO signalling, as distinct from the current predominantly symptom-orientated approach towards treatment of PCOS subjects. In this regard, the recent reports that metformin, an agent used essentially for symptomatic control in a subset of patients with PCOS, may improve endothelial function in such patients (Diamanti-Kandarakis, Alexandraki et al. 2005; Orio, Palomba et al. 2005; Heutling, Schulz et al. 2008; Agarwal, Rice et al. 2009) provides one possible basis for further therapeutic evaluation.

3.8 Study limitations

There are several major limitations of this study. First, the PCOS and normal populations are somewhat heterogeneous. For example, 3.7% of the PCOS subjects were diabetic, while BMI varied widely. Furthermore, background pharmacotherapy varied within the groups. In this regard we made a post hoc observation that OC use was associated with increased ADP and decreased NO responsiveness, which should be regarded as hypothesis-forming only. Second, the precise mechanisms of impairment of NO signalling were not investigated: it is possible, for example that the relative contributors to NO "scavenging" and of sGC dysfunction vary between groups and or with age: such variation might affect the therapeutic options available. We did not directly measure oxidative stress as compared to our previous study (Rajendran, Willoughby et al. 2009), although there is evidence from previous studies of increased oxidative stress in PCOS individuals (Sabuncu, Vural et al. 2001; Rajendran, Willoughby et al. 2009), which may contribute to the altered NO signalling and ADMA kinetics seen in our study. Finally, the implications of our finding reside predominantly in the potential nexus between NO dysfunction and atherogenesis. Although this relationship has been investigated widely, including in the context of PCOS (Paradisi, Steinberg et al. 2001; Heutling, Schulz et al. 2008) this was not examined in this subject cohort.

While this study demonstrates disturbances of NO physiology with ageing and their premature emergence in PCOS, in order to test the Koch's postulates regarding the pathophysiological implications of these findings, a controlled intervention study with long-term follow-up would be necessary.

3.9 Conclusions

Both “normal” ageing in women and PCOS are associated with impairment of NO signalling in platelets and blood vessels. In PCOS, these changes appear by early adult life and persist at least until age 60. Because of the role of NO dysfunction in atherogenesis and as a risk factor for coronary events, these findings represent a potential basis for lifelong therapeutics in PCOS, and an additional therapeutic consideration even in “normal” women.

Chapter 4

CONCLUSIONS AND FUTURE DIRECTIONS

CHAPTER 4

Conclusions and future directions

Impairment in the NO signalling system appears to be a key initiating event in the pathogenesis of atherosclerosis, and therefore is also associated with adverse cardiovascular outcomes. As there are well-described gender differences in pathophysiology, epidemiology and clinical outcomes of cardiovascular disease, this study focuses primarily on women and the impact of several major factors on changes in NO signalling. The three factors explored were:

- Ageing, as it is a major independent risk factor for cardiovascular disease
- Polycystic ovary syndrome, which is associated with multiple cardiovascular risk factors and premature atherosclerosis
- Menopausal state and the influence of oestradiol on NO signalling

The major results and future implications of the experiments described in this thesis can be summarized as follows:

4.1 Ageing and NOS/cGMP system

In Chapter 3, Section 3.5, the impact of ageing on both tissue (platelet) and vascular responsiveness to NO was explored.

With increasing age, there was progressive decline in platelet NO responsiveness and vascular endothelial function (decline in salbutamol responses in blood vessels and raised plasma ADMA concentrations) which appeared to occur in parallel. There was no significant change in vascular NO responsiveness and EPC counts with increasing age. Although the changes in NO responses correlated with age, BMI, several inflammatory and metabolic markers on univarate analyses, multivariate analyses indicated that age is a major independent determinant of platelet NO responses, as is the degree of platelet aggregation.

These findings support the notion of an ageing NO system, which is independent of metabolic changes or inflammatory changes with age, which leads to the incremental risk of cardiovascular events with increasing age.

The main limitation of this section of the study was that we did not make any comparisons to any male population. Furthermore, from a mechanistic viewpoint, we did not measure markers of oxidative stress which also increases with increasing age and contributes to the dysfunction of NOS/cGMP pathway.

The overall implication of the findings is that ageing is associated with impairment in NO signalling, and that consideration of potential therapeutics in primary prevention should be addressed in the context of improving NOS/cGMP signalling pathway. At present there is clinical evidence for the use of aspirin, lipid-lowering agents and ACEI in secondary and possibly, primary prevention of cardiovascular events, but limited mechanistic data. In this regard, we have recently published evidence that ACEIs ameliorate NO resistance in high risk patients (Willoughby, Rajendran et al. 2012). However, these studies did not relate specifically to women, ageing or PCOS.

4.2 Ageing in women with polycystic ovary syndrome

After obtaining data in normal women, the changes in NO signalling with ageing were compared with women with polycystic ovary syndrome in Chapter 3, Section 3.6 and 5

Overall ageing in the entire cohort was associated with impairment of platelet NO responsiveness and vascular endothelial function. Two major findings were observed in PCOS women compared to control women:

- They demonstrated evidence of these anomalies as early as their 20s
- The anomalies may diminish relative to normal physiology above the age of 50 years.

The major limitations of the current study are as follows:

- The inclusion of PCOS women with multiple comorbidities and treatment in comparison to our previous study

- Limited insights into mechanistic of anomalies including the role of oxidative stress.

The results of this section of the study suggest that the anomalies in NO/cGMP pathway may provide the pathophysiological setting for premature atherosclerosis, which has been demonstrated in this cohort of women. However, we have not demonstrated if these anomalies do eventually lead to premature cardiovascular mortality/morbidity in PCOS individuals, nor would this be an easy undertaking.

The implications are therefore in therapeutics, and that we have already planned to explore the impact of a commonly used therapeutic agent in PCOS individuals (metformin), on platelet and vascular function in a cohort of PCOS subjects.

4.3 Menopause and NO/cGMP pathway

It is thought the relative cardioprotection seen in women is in part due to oestradiol levels, which appear to modulate the NO system as well. We investigated the impact of menopause on platelet NO responsiveness in Chapter 3, Section 3.7.

We did not find any specific impact of changes in oestradiol levels which occurred at menopause on platelet or vascular function. The implication of these findings is that the function of platelet NO signalling and endothelial function in general are not substantially affected by menopause.

Future directions

Two further issues are worthy of discussion and potential future exploration. First, it was observed that in control women, oral contraceptive use was associated with apparent diminution of platelet responses to NO, without significant reductions in salbutamol response. This pos-hoc observation might conceivably be coincidental, and should be regarded as hypothesis-generating only. However, this is an important issue with clear-cut therapeutic implications, which therefore needs to be re-evaluated in a prospective study.

Secondly, the evaluations of determinants of vascular and platelet signalling in this study were confined to measurement of responses to eNOS activation, and of platelet responsiveness to NO, as well as consideration of concentrations of ADMA. It has recently been shown that NO-sGC "cascade" can be modulated distally by changes in activation of the cGMP "target" protein kinase G (PKG), which can undergo oxidative activation in an NO-independent manner by oxidants such as H_2O_2 and by S-nitrosothols (Burgoyne, Madhani et al. 2007; Prysyazhna, Rudyk et al.). Since PKG controls both vasodilatation and inhibition of platelet aggregation, it is now appropriate to evaluate the integrity of PKG functioning in the clinical context of the study.

Conclusion

In conclusion, the main findings of the current experiments show that both normal ageing and the lifetime course of PCOS represent independent bases for disordered NO signalling in blood vessels and platelets, but do not advance the somewhat nebulous link between this and accelerated atherogenesis in the individuals concerned. It is conceded that other biochemical processes, such as NO-independent PKG signalling (Prysyazhna, Rudyk et al.), perturbation of the TRX/TxNIP system (Yamawaki, Haendeler et al. 2003) and/or TSP-1 signalling (Isenberg, Frazier et al. 2008) represent additional modulators of abnormal physiology and indeed of atherogenesis, Furthermore, the therapeutic implications of these findings remain to be explored.

References

(2002). "Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report." Circulation **106**(25): 3143-421.

(2004). "Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome." Fertil Steril **81**(1): 19-25.

Abbasi, F., T. Asagmi, et al. (2001). "Plasma concentrations of asymmetric dimethylarginine are increased in patients with type 2 diabetes mellitus." Am J Cardiol **88**(10): 1201-3.

ABS (2008). "Causes of death Australia 2006. " AIHW 2008 Australia's Health 2008 op. cit. **ABS Cat No 3303.0, Canberra: AIHW**.

Adams, M. R., J. Robinson, et al. (1998). "Smooth muscle dysfunction occurs independently of impaired endothelium-dependent dilation in adults at risk of atherosclerosis." J Am Coll Cardiol **32**(1): 123-7.

Agarwal, N., S. P. Rice, et al. (2009). "Metformin reduces arterial stiffness and improves endothelial function in young women with polycystic ovary syndrome: a randomized, placebo-controlled, crossover trial." J Clin Endocrinol Metab **95**(2): 722-30.

Aicher, A., C. Heeschen, et al. (2003). "Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells." Nat Med **9**(11): 1370-6.

AIHW (2011). "Cardiovascular disease: Australian facts 2011." Cardiovascular disease series **Cat. no. CVD 53. Canberra: Australian Institute of Health and Welfare**

Alvarez-Blasco, F., J. I. Botella-Carretero, et al. (2006). "Prevalence and characteristics of the polycystic ovary syndrome in overweight and obese women." Arch Intern Med **166**(19): 2081-6.

Anderson, K. M., P. M. Odell, et al. (1991). "Cardiovascular disease risk profiles." Am Heart J **121**(1 Pt 2): 293-8.

Anderson, K. M., P. W. Wilson, et al. (1991). "An updated coronary risk profile. A statement for health professionals." Circulation **83**(1): 356-62.

Anderson, R. A., G. R. Ellis, et al. (2005). "Platelet nitrate responsiveness in fasting and postprandial type 2 diabetes." Diab Vasc Dis Res **2**(2): 88-93.

Anderson, T. J., A. Uehata, et al. (1995). "Close relation of endothelial function in the human coronary and peripheral circulations." J Am Coll Cardiol **26**(5): 1235-41.

Anfossi, G., E. M. Mularoni, et al. (1998). "Platelet resistance to nitrates in obesity and obese NIDDM, and normal platelet sensitivity to both insulin and nitrates in lean NIDDM." Diabetes Care **21**(1): 121-6.

Apridonidze, T., P. A. Essah, et al. (2005). "Prevalence and characteristics of the metabolic syndrome in women with polycystic ovary syndrome." J Clin Endocrinol Metab **90**(4): 1929-35.

Arikan, S., H. Akay, et al. (2009). "The evaluation of endothelial function with flow-mediated dilatation and carotid intima media thickness in young nonobese polycystic ovary syndrome patients; existence of insulin resistance alone may not represent an adequate condition for deterioration of endothelial function." Fertil Steril **91**(2): 450-5.

Asahara, T., H. Masuda, et al. (1999). "Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization." Circ Res **85**(3): 221-8.

athAdams, M. R., J. Robinson, et al. (1998). "Smooth muscle dysfunction occurs independently of impaired endothelium-dependent dilation in adults at risk of atherosclerosis." J Am Coll Cardiol **32**(1): 123-7.

Aviv, H., M. Y. Khan, et al. (2001). "Age dependent aneuploidy and telomere length of the human vascular endothelium." Atherosclerosis **159**(2): 281-7.

Avolio, A. P., K. M. Clyde, et al. (1986). "Improved arterial distensibility in normotensive subjects on a low salt diet." Arteriosclerosis **6**(2): 166-9.

Azevedo, G. D., J. M. Duarte, et al. (2006). "[Menstrual cycle irregularity as a marker of cardiovascular risk factors at postmenopausal years]." Arq Bras Endocrinol Metabol **50**(5): 876-83.

Azziz, R., K. S. Woods, et al. (2004). "The prevalence and features of the polycystic ovary syndrome in an unselected population." J Clin Endocrinol Metab **89**(6): 2745-9.

Balen, A. H., J. S. Laven, et al. (2003). "Ultrasound assessment of the polycystic ovary: international consensus definitions." Hum Reprod Update **9**(6): 505-14.

Barrett-Connor, E. and T. L. Bush (1991). "Estrogen and coronary heart disease in women." JAMA **265**(14): 1861-7.

Battinelli, E., S. R. Willoughby, et al. (2001). "Induction of platelet formation from megakaryocytoid cells by nitric oxide." Proc Natl Acad Sci U S A **98**(25): 14458-63.

Bauer, E. M., Y. Qin, et al. (2010). "Thrombospondin-1 supports blood pressure by limiting eNOS activation and endothelial-dependent vasorelaxation." Cardiovasc Res **88**(3): 471-81.

Bekaert, S., T. De Meyer, et al. (2007). "Telomere length and cardiovascular risk factors in a middle-aged population free of overt cardiovascular disease." Aging Cell **6**(5): 639-47.

Benjamin, N., A. Calver, et al. (1995). "Measuring forearm blood flow and interpreting the responses to drugs and mediators." Hypertension **25**(5): 918-23.

Berkowitz, D. E., R. White, et al. (2003). "Arginase reciprocally regulates nitric oxide synthase activity and contributes to endothelial dysfunction in aging blood vessels." Circulation **108**(16): 2000-6.

Bickerton, A. S., N. Clark, et al. (2005). "Cardiovascular risk in women with polycystic ovarian syndrome (PCOS)." J Clin Pathol **58**(2): 151-4.

Bishop, S. C., S. Basch, et al. (2009). "Polycystic ovary syndrome, depression, and affective disorders." Endocr Pract **15**(5): 475-82.

Bivalacqua, T. J., W. J. Hellstrom, et al. (2001). "Increased expression of arginase II in human diabetic corpus cavernosum: in diabetic-associated erectile dysfunction." Biochem Biophys Res Commun **283**(4): 923-7.

Blake, G. J. and P. M. Ridker (2001). "Novel clinical markers of vascular wall inflammation." Circ Res **89**(9): 763-71.

Bode-Boger, S. M., F. Scalera, et al. (2006). "Symmetrical dimethylarginine: a new combined parameter for renal function and extent of coronary artery disease." J Am Soc Nephrol **17**(4): 1128-34.

Boger, R. H. (2003). "The emerging role of asymmetric dimethylarginine as a novel cardiovascular risk factor." Cardiovasc Res **59**(4): 824-33.
Boger, R. H., S. M. Bode-Boger, et al. (1998). "Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia." Circulation **98**(18): 1842-7.
Boger, R. H., K. Sydow, et al. (2000). "LDL cholesterol upregulates synthesis of asymmetrical dimethylarginine in human endothelial cells: involvement of S-adenosylmethionine-dependent methyltransferases." Circ Res **87**(2): 99-105.
Bompais, H., J. Chagraoui, et al. (2004). "Human endothelial cells derived from circulating progenitors display specific functional properties compared with mature vessel wall endothelial cells." Blood **103**(7): 2577-84.
Born, G. V. (1962). "Aggregation of blood platelets by adenosine diphosphate and its reversal." Nature **194**: 927-9.
Boudreaux, M. Y., E. O. Talbott, et al. (2006). "Risk of T2DM and impaired fasting glucose among PCOS subjects: results of an 8-year follow-up." Curr Diab Rep **6**(1): 77-83.
Boyle, J. A., J. Cunningham, et al. (2012). "Prevalence of polycystic ovary syndrome in a sample of Indigenous women in Darwin, Australia." Med J Aust **196**(1): 62-6.
Bredt, D. S. and S. H. Snyder (1990). "Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme." Proc Natl Acad Sci U S A **87**(2): 682-5.
Brevetti, G., A. Silvestro, et al. (2003). "Endothelial dysfunction and cardiovascular risk prediction in peripheral arterial disease: additive value of flow-mediated dilation to ankle-brachial pressure index." Circulation **108**(17): 2093-8.
Brinkworth, G. D., M. Noakes, et al. (2006). "Flow-mediated dilatation in overweight and obese women with polycystic ovary syndrome." BJOG **113**(11): 1308-14.
Buckle A, D. L., Ng MKC (2007). "Hyperglycaemia Inhibits Thioredoxin-Mediated Angiogenesis: Implications for Impairment of Neovascularisation in Diabetes Mellitus." Heart, Lung and Circulation **16:** S214–S215.
Burgoyne, J. R., M. Madhani, et al. (2007). "Cysteine redox sensor in PKGIa enables oxidant-induced activation." Science **317**(5843): 1393-7.
Cardinal, D. C. and R. J. Flower (1980). "The electronic aggregometer: a novel device for assessing platelet behavior in blood." J Pharmacol Methods **3**(2): 135-58.
Carmina, E. and R. A. Lobo (2004). "Use of fasting blood to assess the prevalence of insulin resistance in women with polycystic ovary syndrome." Fertil Steril **82**(3): 661-5.
Castelli, W. P. (1984). "Epidemiology of coronary heart disease: the Framingham study." Am J Med **76**(2A): 4-12.
Celermajer, D. S., K. E. Sorensen, et al. (1992). "Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis." Lancet **340**(8828): 1111-5.
Celermajer, D. S., K. E. Sorensen, et al. (1994). "Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women." J Am Coll Cardiol **24**(2): 471-6.

Cernadas, M. R., L. Sanchez de Miguel, et al. (1998). "Expression of constitutive and inducible nitric oxide synthases in the vascular wall of young and aging rats." Circ Res **83**(3): 279-86.
Cesari, F., R. Caporale, et al. (2008). "NT-proBNP and the anti-inflammatory cytokines are correlated with endothelial progenitor cells' response to cardiac surgery." Atherosclerosis **199**(1): 138-46.
Cha, Y. J., E. A. Chang, et al. (2000). "Effects of endothelial cells and mononuclear leukocytes on platelet aggregation." Haematologia (Budap) **30**(2): 97-106.
Challah, M., S. Nadaud, et al. (1997). "Circulating and cellular markers of endothelial dysfunction with aging in rats." Am J Physiol **273**(4 Pt 2): H1941-8.
Charitidou, C., D. Farmakiotis, et al. (2008). "The administration of estrogens, combined with anti-androgens, has beneficial effects on the hormonal features and asymmetric dimethyl-arginine levels, in women with the polycystic ovary syndrome." Atherosclerosis **196**(2): 958-65.
Chen, H., M. E. Herndon, et al. (2000). "The cell biology of thrombospondin-1." Matrix Biol **19**(7): 597-614.
Chen, J., H. Cha-Molstad, et al. (2009). "Diabetes induces and calcium channel blockers prevent cardiac expression of proapoptotic thioredoxin-interacting protein." Am J Physiol Endocrinol Metab **296**(5): E1133-9.
Chen, J., G. Saxena, et al. (2008). "Thioredoxin-interacting protein: a critical link between glucose toxicity and beta-cell apoptosis." Diabetes **57**(4): 938-44.
Chen, S. J., H. Li, et al. (1996). "Estrogen reduces myointimal proliferation after balloon injury of rat carotid artery." Circulation **93**(3): 577-84.
Chirkov, Y. Y., A. S. Holmes, et al. (1999). "Nitrate resistance in platelets from patients with stable angina pectoris." Circulation **100**(2): 129-34.
Chirkov, Y. Y., A. S. Holmes, et al. (2004). "Effect of perindopril on platelet nitric oxide resistance in patients with chronic heart failure secondary to ischemic left ventricular dysfunction." Am J Cardiol **93**(11): 1438-40, A10.
Chirkov, Y. Y., A. S. Holmes, et al. (2002). "Association of aortic stenosis with platelet hyperaggregability and impaired responsiveness to nitric oxide." Am J Cardiol **90**(5): 551-4.
Chirkov, Y. Y., A. S. Holmes, et al. (2001). "Stable angina and acute coronary syndromes are associated with nitric oxide resistance in platelets." J Am Coll Cardiol **37**(7): 1851-7.
Chirkov, Y. Y. and J. D. Horowitz (2007). "Impaired tissue responsiveness to organic nitrates and nitric oxide: a new therapeutic frontier?" Pharmacol Ther **116**(2): 287-305.
Chou, T. C., M. H. Yen, et al. (1998). "Alterations of nitric oxide synthase expression with aging and hypertension in rats." Hypertension **31**(2): 643-8.
Christian, R. C., D. A. Dumesic, et al. (2003). "Prevalence and predictors of coronary artery calcification in women with polycystic ovary syndrome." J Clin Endocrinol Metab **88**(6): 2562-8.
Chung, H. Y., E. K. Lee, et al. (2011). "Molecular inflammation as an underlying mechanism of the aging process and age-related diseases." J Dent Res **90**(7): 830-40.

Cibula, D., R. Cifkova, et al. (2000). "Increased risk of non-insulin dependent diabetes mellitus, arterial hypertension and coronary artery disease in perimenopausal women with a history of the polycystic ovary syndrome." Hum Reprod **15**(4): 785-9.

Clarkson, P., M. R. Adams, et al. (1996). "Oral L-arginine improves endothelium-dependent dilation in hypercholesterolemic young adults." J Clin Invest **97**(8): 1989-94.

Collins, P., J. Shay, et al. (1994). "Nitric oxide accounts for dose-dependent estrogen-mediated coronary relaxation after acute estrogen withdrawal." Circulation **90**(4): 1964-8.

Conway, G. S., J. W. Honour, et al. (1989). "Heterogeneity of the polycystic ovary syndrome: clinical, endocrine and ultrasound features in 556 patients." Clin Endocrinol (Oxf) **30**(4): 459-70.

Cooke, J. P. (2004). "Asymmetrical dimethylarginine: the Uber marker?" Circulation **109**(15): 1813-8.

Cosentino, F. and Z. S. Katusic (1995). "Tetrahydrobiopterin and dysfunction of endothelial nitric oxide synthase in coronary arteries." Circulation **91**(1): 139-44.

Costa, L. O., M. P. dos Santos, et al. (2008). "Low-grade chronic inflammation is not accompanied by structural arterial injury in polycystic ovary syndrome." Diabetes Res Clin Pract **81**(2): 179-83.

Creager, M. A., S. J. Gallagher, et al. (1992). "L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans." J Clin Invest **90**(4): 1248-53.

Cussons, A. J., G. F. Watts, et al. (2008). "Cardiometabolic risk in polycystic ovary syndrome: a comparison of different approaches to defining the metabolic syndrome." Hum Reprod **23**(10): 2352-8.

Dahlgren, E., P. O. Janson, et al. (1992). "Polycystic ovary syndrome and risk for myocardial infarction. Evaluated from a risk factor model based on a prospective population study of women." Acta Obstet Gynecol Scand **71**(8): 599-604.

Dai, S., Y. He, et al. (2009). "Endothelial-specific expression of mitochondrial thioredoxin promotes ischemia-mediated arteriogenesis and angiogenesis." Arterioscler Thromb Vasc Biol **29**(4): 495-502.

Davi, G., G. Ciabattoni, et al. (1999). "In vivo formation of 8-iso-prostaglandin f2alpha and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation." Circulation **99**(2): 224-9.

Davis, K. B., B. Chaitman, et al. (1995). "Comparison of 15-year survival for men and women after initial medical or surgical treatment for coronary artery disease: a CASS registry study. Coronary Artery Surgery Study." J Am Coll Cardiol **25**(5): 1000-9.

Dawes, M., P. J. Chowienczyk, et al. (1997). "Effects of inhibition of the L-arginine/nitric oxide pathway on vasodilation caused by beta-adrenergic agonists in human forearm." Circulation **95**(9): 2293-7.

Dayoub, H., V. Achan, et al. (2003). "Dimethylarginine dimethylaminohydrolase regulates nitric oxide synthesis: genetic and physiological evidence." Circulation **108**(24): 3042-7.

de Resende, M. M., L. Y. Huw, et al. (2007). "Role of endothelial nitric oxide in bone marrow-derived progenitor cell mobilization." Handb Exp Pharmacol(180): 37-44.
Dereli, D., G. Ozgen, et al. (2003). "Platelet dysfunction in lean women with polycystic ovary syndrome and association with insulin sensitivity." J Clin Endocrinol Metab **88**(5): 2263-8.
Deshaies, Y., A. Dagnault, et al. (1997). "Interaction of corticosterone and gonadal steroids on lipid deposition in the female rat." Am J Physiol **273**(2 Pt 1): E355-62.
DeUgarte, C. M., A. A. Bartolucci, et al. (2005). "Prevalence of insulin resistance in the polycystic ovary syndrome using the homeostasis model assessment." Fertil Steril **83**(5): 1454-60.
DeVan, A. E., D. Umpierre, et al. (2011). "Habitual resistance exercise and endothelial ischemia-reperfusion injury in young adults." Atherosclerosis **219**(1): 191-3.
Diamanti-Kandarakis, E., K. Alexandraki, et al. (2006). "Inflammatory and endothelial markers in women with polycystic ovary syndrome." Eur J Clin Invest **36**(10): 691-7.
Diamanti-Kandarakis, E., K. Alexandraki, et al. (2005). "Metformin administration improves endothelial function in women with polycystic ovary syndrome." Eur J Endocrinol **152**(5): 749-56.
Dimmeler, S. and A. M. Zeiher (2000). "Reactive oxygen species and vascular cell apoptosis in response to angiotensin II and pro-atherosclerotic factors." Regul Pept **90**(1-3): 19-25.
Dunaif, A., A. R. Hoffman, et al. (1985). "Clinical, biochemical, and ovarian morphologic features in women with acanthosis nigricans and masculinization." Obstet Gynecol **66**(4): 545-52.
Dunn, L. L., A. M. Buckle, et al. (2010). "The emerging role of the thioredoxin system in angiogenesis." Arterioscler Thromb Vasc Biol **30**(11): 2089-98.
Durand, P. and D. Blache (1996). "Enhanced platelet thromboxane synthesis and reduced macrophage-dependent fibrinolytic activity related to oxidative stress in oral contraceptive-treated female rats." Atherosclerosis **121**(2): 205-16.
Durante, W., F. K. Johnson, et al. (2007). "Arginase: a critical regulator of nitric oxide synthesis and vascular function." Clin Exp Pharmacol Physiol **34**(9): 906-11.
Dutta, K. K., Y. Nishinaka, et al. (2005). "Two distinct mechanisms for loss of thioredoxin-binding protein-2 in oxidative stress-induced renal carcinogenesis." Lab Invest **85**(6): 798-807.
Ebrahimian, T. and R. M. Touyz (2008). "Thioredoxin in vascular biology: role in hypertension." Antioxid Redox Signal **10**(6): 1127-36.
Egashira, K., T. Inou, et al. (1993). "Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans." Circulation **88**(1): 77-81.
Ehrmann, D. A. (2005). "Polycystic ovary syndrome." N Engl J Med **352**(12): 1223-36.
Ehrmann, D. A., R. B. Barnes, et al. (1999). "Prevalence of impaired glucose tolerance and diabetes in women with polycystic ovary syndrome." Diabetes Care **22**(1): 141-6.

Ehrmann, D. A., D. R. Liljenquist, et al. (2006). "Prevalence and predictors of the metabolic syndrome in women with polycystic ovary syndrome." J Clin Endocrinol Metab **91**(1): 48-53.
Elting, M. W., T. J. Korsen, et al. (2001). "Prevalence of diabetes mellitus, hypertension and cardiac complaints in a follow-up study of a Dutch PCOS population." Hum Reprod **16**(3): 556-60.
Epel, E. S., E. H. Blackburn, et al. (2004). "Accelerated telomere shortening in response to life stress." Proc Natl Acad Sci U S A **101**(49): 17312-5.
Esler, M. D., A. G. Turner, et al. (1995). "Aging effects on human sympathetic neuronal function." Am J Physiol **268**(1 Pt 2): R278-85.
Faloia, E., P. Canibus, et al. (2004). "Body composition, fat distribution and metabolic characteristics in lean and obese women with polycystic ovary syndrome." J Endocrinol Invest **27**(5): 424-9.
Ferrari, A. U., A. Radaelli, et al. (2003). "Invited review: aging and the cardiovascular system." J Appl Physiol **95**(6): 2591-7.
Fichtlscherer, S., G. Rosenberger, et al. (2000). "Elevated C-reactive protein levels and impaired endothelial vasoreactivity in patients with coronary artery disease." Circulation **102**(9): 1000-6.
Fischer, D., S. Rossa, et al. (2005). "Endothelial dysfunction in patients with chronic heart failure is independently associated with increased incidence of hospitalization, cardiac transplantation, or death." Eur Heart J **26**(1): 65-9.
Fitzpatrick, A. L., R. A. Kronmal, et al. (2007). "Leukocyte telomere length and cardiovascular disease in the cardiovascular health study." Am J Epidemiol **165**(1): 14-21.
Fleg, J. L., S. Schulman, et al. (1994). "Effects of acute beta-adrenergic receptor blockade on age-associated changes in cardiovascular performance during dynamic exercise." Circulation **90**(5): 2333-41.
Franks, S. (1989). "Polycystic ovary syndrome: a changing perspective." Clin Endocrinol (Oxf) **31**(1): 87-120.
Gao, A. G., F. P. Lindberg, et al. (1996). "Thrombospondin modulates alpha v beta 3 function through integrin-associated protein." J Cell Biol **135**(2): 533-44.
Garg, U. C. and A. Hassid (1989). "Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells." J Clin Invest **83**(5): 1774-7.
Gates, P. E., M. L. Boucher, et al. (2007). "Impaired flow-mediated dilation with age is not explained by L-arginine bioavailability or endothelial asymmetric dimethylarginine protein expression." J Appl Physiol **102**(1): 63-71.
George, J., E. Goldstein, et al. (2004). "Circulating endothelial progenitor cells in patients with unstable angina: association with systemic inflammation." Eur Heart J **25**(12): 1003-8.
Gerhard, M., M. A. Roddy, et al. (1996). "Aging progressively impairs endothelium-dependent vasodilation in forearm resistance vessels of humans." Hypertension **27**(4): 849-53.
Gesquiere, L., N. Loreau, et al. (1999). "Oxidative stress leads to cholesterol accumulation in vascular smooth muscle cells." Free Radic Biol Med **27**(1-2): 134-45.

Gilligan, D. M., D. M. Badar, et al. (1994). "Acute vascular effects of estrogen in postmenopausal women." Circulation **90**(2): 786-91.
Gilligan, D. M., A. A. Quyyumi, et al. (1994). "Effects of physiological levels of estrogen on coronary vasomotor function in postmenopausal women." Circulation **89**(6): 2545-51.
Goldberg, S. F., M. E. Miele, et al. (2003). "Melanoma metastasis suppression by chromosome 6: evidence for a pathway regulated by CRSP3 and TXNIP." Cancer Res **63**(2): 432-40.
Goldstein, L. J., K. A. Gallagher, et al. (2006). "Endothelial progenitor cell release into circulation is triggered by hyperoxia-induced increases in bone marrow nitric oxide." Stem Cells **24**(10): 2309-18.
Goon, P. K. and G. Y. Lip (2007). "Endothelial progenitor cells, endothelial cell dysfunction and much more: observations from cardiac syndrome X." Heart **93**(9): 1020-1.
Goon, P. K., G. Y. Lip, et al. (2009). "Circulating endothelial cells and circulating progenitor cells in breast cancer: relationship to endothelial damage/dysfunction/apoptosis, clinicopathologic factors, and the Nottingham Prognostic Index." Neoplasia **11**(8): 771-9.
Goubareva, I., E. Gkaliagkousi, et al. (2007). "Age decreases nitric oxide synthesis and responsiveness in human platelets and increases formation of monocyte-platelet aggregates." Cardiovasc Res **75**(4): 793-802.
Grady, D., D. Herrington, et al. (2002). "Cardiovascular disease outcomes during 6.8 years of hormone therapy: Heart and Estrogen/progestin Replacement Study follow-up (HERS II)." JAMA **288**(1): 49-57.
Greving, J. P., E. Buskens, et al. (2008). "Cost-effectiveness of aspirin treatment in the primary prevention of cardiovascular disease events in subgroups based on age, gender, and varying cardiovascular risk." Circulation **117**(22): 2875-83.
Griendling, K. K. and G. A. FitzGerald (2003). "Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS." Circulation **108**(16): 1912-6.
Gries, A., B. W. Bottiger, et al. (1997). "Inhaled nitric oxide inhibits platelet aggregation after pulmonary embolism in pigs." Anesthesiology **86**(2): 387-93.
Gulati, M., R. M. Cooper-DeHoff, et al. (2009). "Adverse cardiovascular outcomes in women with nonobstructive coronary artery disease: a report from the Women's Ischemia Syndrome Evaluation Study and the St James Women Take Heart Project." Arch Intern Med **169**(9): 843-50.
Guzick, D. S., E. O. Talbott, et al. (1996). "Carotid atherosclerosis in women with polycystic ovary syndrome: initial results from a case-control study." Am J Obstet Gynecol **174**(4): 1224-9; discussion 1229-32.
Guzik, T. J., N. E. West, et al. (2000). "Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors." Circ Res **86**(9): E85-90.
Haendeler, J., J. Hoffmann, et al. (2003). "Hydrogen peroxide triggers nuclear export of telomerase reverse transcriptase via Src kinase family-dependent phosphorylation of tyrosine 707." Mol Cell Biol **23**(13): 4598-610.

Halcox, J. P., W. H. Schenke, et al. (2002). "Prognostic value of coronary vascular endothelial dysfunction." Circulation **106**(6): 653-8.
Hamada, H., M. K. Kim, et al. (2006). "Estrogen receptors alpha and beta mediate contribution of bone marrow-derived endothelial progenitor cells to functional recovery after myocardial infarction." Circulation **114**(21): 2261-70.
Hamilton, C. A., M. J. Brosnan, et al. (2001). "Superoxide excess in hypertension and aging: a common cause of endothelial dysfunction." Hypertension **37**(2 Part 2): 529-34.
Hammond, B. and M. L. Hess (1985). "The oxygen free radical system: potential mediator of myocardial injury." J Am Coll Cardiol **6**(1): 215-20.
Harman, S. M., E. A. Brinton, et al. (2005). "KEEPS: The Kronos Early Estrogen Prevention Study." Climacteric **8**(1): 3-12.
Hayward, C. S., M. Kraidly, et al. (2002). "Assessment of endothelial function using peripheral waveform analysis: a clinical application." J Am Coll Cardiol **40**(3): 521-8.
Hein, T. W., U. Singh, et al. (2009). "Human C-reactive protein induces endothelial dysfunction and uncoupling of eNOS in vivo." Atherosclerosis **206**(1): 61-8.
Hein, T. W., C. Zhang, et al. (2003). "Ischemia-reperfusion selectively impairs nitric oxide-mediated dilation in coronary arterioles: counteracting role of arginase." FASEB J **17**(15): 2328-30.
Heiss, C., S. Keymel, et al. (2005). "Impaired progenitor cell activity in age-related endothelial dysfunction." J Am Coll Cardiol **45**(9): 1441-8.
Heitzer, T., H. Just, et al. (1996). "Antioxidant vitamin C improves endothelial dysfunction in chronic smokers." Circulation **94**(1): 6-9.
Heitzer, T., T. Schlinzig, et al. (2001). "Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease." Circulation **104**(22): 2673-8.
Heresztyn, T., M. I. Worthley, et al. (2004). "Determination of l-arginine and NG, NG - and NG, NG' -dimethyl-L-arginine in plasma by liquid chromatography as AccQ-Fluor fluorescent derivatives." J Chromatogr B Analyt Technol Biomed Life Sci **805**(2): 325-9.
Herrington, D. M., D. M. Reboussin, et al. (2000). "Effects of estrogen replacement on the progression of coronary-artery atherosclerosis." N Engl J Med **343**(8): 522-9.
Herrler, T., S. F. Leicht, et al. (2009). "Prostaglandin E positively modulates endothelial progenitor cell homeostasis: an advanced treatment modality for autologous cell therapy." J Vasc Res **46**(4): 333-46.
Heutling, D., H. Schulz, et al. (2008). "Asymmetrical dimethylarginine, inflammatory and metabolic parameters in women with polycystic ovary syndrome before and after metformin treatment." J Clin Endocrinol Metab **93**(1): 82-90.
Hickey, M. J., D. N. Granger, et al. (2001). "Inducible nitric oxide synthase (iNOS) and regulation of leucocyte/endothelial cell interactions: studies in iNOS-deficient mice." Acta Physiol Scand **173**(1): 119-26.
Hill, J. M., G. Zalos, et al. (2003). "Circulating endothelial progenitor cells, vascular function, and cardiovascular risk." N Engl J Med **348**(7): 593-600.

Hishikawa, K., T. Nakaki, et al. (1995). "Up-regulation of nitric oxide synthase by estradiol in human aortic endothelial cells." FEBS Lett **360**(3): 291-3.
Ho, K. K., J. L. Pinsky, et al. (1993). "The epidemiology of heart failure: the Framingham Study." J Am Coll Cardiol **22**(4 Suppl A): 6A-13A.
Hoffmann, J., J. Haendeler, et al. (2001). "Aging enhances the sensitivity of endothelial cells toward apoptotic stimuli: important role of nitric oxide." Circ Res **89**(8): 709-15.
Holden, D. P., J. E. Cartwright, et al. (2003). "Estrogen stimulates dimethylarginine dimethylaminohydrolase activity and the metabolism of asymmetric dimethylarginine." Circulation **108**(13): 1575-80.
Hornig, B., C. Kohler, et al. (1997). "Role of bradykinin in mediating vascular effects of angiotensin-converting enzyme inhibitors in humans." Circulation **95**(5): 1115-8.
Horowitz, A., C. B. Menice, et al. (1996). "Mechanisms of smooth muscle contraction." Physiol Rev **76**(4): 967-1003.
Horowitz, J. D. and T. Heresztyn (2007). "An overview of plasma concentrations of asymmetric dimethylarginine (ADMA) in health and disease and in clinical studies: methodological considerations." J Chromatogr B Analyt Technol Biomed Life Sci **851**(1-2): 42-50.
Hulley, S., D. Grady, et al. (1998). "Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group." JAMA **280**(7): 605-13.
Ii, M., H. Nishimura, et al. (2005). "Endothelial progenitor cells are rapidly recruited to myocardium and mediate protective effect of ischemic preconditioning via "imported" nitric oxide synthase activity." Circulation **111**(9): 1114-20.
Ingerman-Wojenski, C., J. B. Smith, et al. (1983). "Evaluation of electrical aggregometry: comparison with optical aggregometry, secretion of ATP, and accumulation of radiolabeled platelets." J Lab Clin Med **101**(1): 44-52.
Isenberg, J. S., W. A. Frazier, et al. (2008). "Thrombospondin-1: a physiological regulator of nitric oxide signaling." Cell Mol Life Sci **65**(5): 728-42.
Isenberg, J. S., Y. Jia, et al. (2007). "Thrombospondin-1 inhibits nitric oxide signaling via CD36 by inhibiting myristic acid uptake." J Biol Chem **282**(21): 15404-15.
Isenberg, J. S., G. Martin-Manso, et al. (2009). "Regulation of nitric oxide signalling by thrombospondin 1: implications for anti-angiogenic therapies." Nat Rev Cancer **9**(3): 182-94.
Isenberg, J. S., L. A. Ridnour, et al. (2005). "Thrombospondin-1 inhibits endothelial cell responses to nitric oxide in a cGMP-dependent manner." Proc Natl Acad Sci U S A **102**(37): 13141-6.
Ito, A., P. S. Tsao, et al. (1999). "Novel mechanism for endothelial dysfunction: dysregulation of dimethylarginine dimethylaminohydrolase." Circulation **99**(24): 3092-5.
Jensen, J., B. J. Riis, et al. (1989). "Long-term and withdrawal effects of two different oestrogen-progestogen combinations on lipid and lipoprotein profiles in post-menopausal women." Maturitas **11**(2): 117-28.

Jensterle, M., M. Sebestjen, et al. (2008). "Improvement of endothelial function with metformin and rosiglitazone treatment in women with polycystic ovary syndrome." Eur J Endocrinol **159**(4): 399-406.
Jia, L., C. Bonaventura, et al. (1996). "S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control." Nature **380**(6571): 221-6.
John, S., J. Jacobi, et al. (2000). "Effects of oral contraceptives on vascular endothelium in premenopausal women." Am J Obstet Gynecol **183**(1): 28-33.
Johnson, F. K., R. A. Johnson, et al. (2005). "Arginase inhibition restores arteriolar endothelial function in Dahl rats with salt-induced hypertension." Am J Physiol Regul Integr Comp Physiol **288**(4): R1057-62.
Jones, G. L., J. M. Hall, et al. (2008). "Health-related quality of life measurement in women with polycystic ovary syndrome: a systematic review." Hum Reprod Update **14**(1): 15-25.
Katz, S. D., L. Biasucci, et al. (1992). "Impaired endothelium-mediated vasodilation in the peripheral vasculature of patients with congestive heart failure." J Am Coll Cardiol **19**(5): 918-25.
Kazakov, A., P. Muller, et al. (2011). "Endothelial nitric oxide synthase of the bone marrow regulates myocardial hypertrophy, fibrosis, and angiogenesis." Cardiovasc Res.
Keaney, J. F., Jr., A. Xu, et al. (1995). "Dietary probucol preserves endothelial function in cholesterol-fed rabbits by limiting vascular oxidative stress and superoxide generation." J Clin Invest **95**(6): 2520-9.
Kelly, C. C., H. Lyall, et al. (2001). "Low grade chronic inflammation in women with polycystic ovarian syndrome." J Clin Endocrinol Metab **86**(6): 2453-5.
Kelly, C. J., A. Speirs, et al. (2002). "Altered vascular function in young women with polycystic ovary syndrome." J Clin Endocrinol Metab **87**(2): 742-6.
Kelm, M., S. Schafer, et al. (1997). "Nitric oxide induced contractile dysfunction is related to a reduction in myocardial energy generation." Cardiovasc Res **36**(2): 185-94.
Ketel, I. J., C. D. Stehouwer, et al. (2010). "Greater arterial stiffness in polycystic ovary syndrome (PCOS) is an obesity--but not a PCOS-associated phenomenon." J Clin Endocrinol Metab **95**(10): 4566-75.
Kiechl, S., T. Lee, et al. (2009). "Asymmetric and symmetric dimethylarginines are of similar predictive value for cardiovascular risk in the general population." Atherosclerosis **205**(1): 261-5.
Kielstein, J. T., S. M. Bode-Boger, et al. (2003). "Asymmetric dimethylarginine, blood pressure, and renal perfusion in elderly subjects." Circulation **107**(14): 1891-5.
Kirsch, T., A. Woywodt, et al. (2010). "Endothelial-derived thrombospondin-1 promotes macrophage recruitment and apoptotic cell clearance." J Cell Mol Med **14**(7): 1922-34.
Krempl, T. K., R. Maas, et al. (2005). "Elevation of asymmetric dimethylarginine in patients with unstable angina and recurrent cardiovascular events." Eur Heart J **26**(18): 1846-51.
Krentz, A. J., D. von Muhlen, et al. (2007). "Searching for polycystic ovary syndrome in postmenopausal women: evidence of a dose-effect

association with prevalent cardiovascular disease." Menopause **14**(2): 284-92.
Kurose, I., R. Wolf, et al. (1995). "Microvascular responses to inhibition of nitric oxide production. Role of active oxidants." Circ Res **76**(1): 30-9.
Labinskyy, N., A. Csiszar, et al. (2006). "Vascular dysfunction in aging: potential effects of resveratrol, an anti-inflammatory phytoestrogen." Curr Med Chem **13**(9): 989-96.
Lakatta, E. G. and D. Levy (2003). "Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part I: aging arteries: a "set up" for vascular disease." Circulation **107**(1): 139-46.
Lambiase, P. D., R. J. Edwards, et al. (2004). "Circulating humoral factors and endothelial progenitor cells in patients with differing coronary collateral support." Circulation **109**(24): 2986-92.
Legro, R. S., A. R. Kunselman, et al. (1999). "Prevalence and predictors of risk for type 2 diabetes mellitus and impaired glucose tolerance in polycystic ovary syndrome: a prospective, controlled study in 254 affected women." J Clin Endocrinol Metab **84**(1): 165-9.
Leiper, J., M. Nandi, et al. (2007). "Disruption of methylarginine metabolism impairs vascular homeostasis." Nat Med **13**(2): 198-203.
Lekakis, J., P. Abraham, et al. (2011). "Methods for evaluating endothelial function: a position statement from the European Society of Cardiology Working Group on Peripheral Circulation." Eur J Cardiovasc Prev Rehabil **18**(6): 775-89.
Lerman, A. and A. M. Zeiher (2005). "Endothelial function: cardiac events." Circulation **111**(3): 363-8.
Lesniewski, L. A., J. R. Durrant, et al. (2011). "Aerobic exercise reverses arterial inflammation with aging in mice." Am J Physiol Heart Circ Physiol **301**(3): H1025-32.
Leung, W. H., C. P. Lau, et al. (1993). "Beneficial effect of cholesterol-lowering therapy on coronary endothelium-dependent relaxation in hypercholesterolaemic patients." Lancet **341**(8859): 1496-500.
Levy, D. (1988). "Left ventricular hypertrophy. Epidemiological insights from the Framingham Heart Study." Drugs **35 Suppl 5**: 1-5.
Li, X., Y. Rong, et al. (2009). "Up-regulation of thioredoxin interacting protein (Txnip) by p38 MAPK and FOXO1 contributes to the impaired thioredoxin activity and increased ROS in glucose-treated endothelial cells." Biochem Biophys Res Commun **381**(4): 660-5.
Lin, K. Y., A. Ito, et al. (2002). "Impaired nitric oxide synthase pathway in diabetes mellitus: role of asymmetric dimethylarginine and dimethylarginine dimethylaminohydrolase." Circulation **106**(8): 987-92.
Lockette, W., Y. Otsuka, et al. (1986). "The loss of endothelium-dependent vascular relaxation in hypertension." Hypertension **8**(6 Pt 2): II61-6.
Lu, T. M., Y. A. Ding, et al. (2003). "Plasma levels of asymmetrical dimethylarginine and adverse cardiovascular events after percutaneous coronary intervention." Eur Heart J **24**(21): 1912-9.
Ludmer, P. L., A. P. Selwyn, et al. (1986). "Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries." N Engl J Med **315**(17): 1046-51.
Luque-Ramirez, M., C. Mendieta-Azcona, et al. (2007). "Androgen excess is associated with the increased carotid intima-media thickness observed in

young women with polycystic ovary syndrome." Hum Reprod **22**(12): 3197-203.
Lyons, D., S. Roy, et al. (1997). "Impaired nitric oxide-mediated vasodilatation and total body nitric oxide production in healthy old age." Clin Sci (Lond) **93**(6): 519-25.
Mancini, G. B., G. C. Henry, et al. (1996). "Angiotensin-converting enzyme inhibition with quinapril improves endothelial vasomotor dysfunction in patients with coronary artery disease. The TREND (Trial on Reversing ENdothelial Dysfunction) Study." Circulation **94**(3): 258-65.
Martens-Lobenhoffer, J. and S. M. Bode-Böger (2006). "Measurement of asymmetric dimethylarginine (ADMA) in human plasma: from liquid chromatography estimation to liquid chromatography-mass spectrometry quantification." European Journal of Clinical Pharmacology **62** (Supplement 1): 61-68.
Mather, K. J., S. Verma, et al. (2000). "Normal endothelial function despite insulin resistance in healthy women with the polycystic ovary syndrome." J Clin Endocrinol Metab **85**(5): 1851-6.
Matthews, D. R., J. P. Hosker, et al. (1985). "Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man." Diabetologia **28**(7): 412-9.
McCord, J. M. (1985). "Oxygen-derived free radicals in postischemic tissue injury." N Engl J Med **312**(3): 159-63.
Mehta, J. L., L. Y. Chen, et al. (1995). "Identification of constitutive and inducible forms of nitric oxide synthase in human platelets." J Lab Clin Med **125**(3): 370-7.
Michel, J. B., D. Heudes, et al. (1994). "Effect of chronic ANG I-converting enzyme inhibition on aging processes. II. Large arteries." Am J Physiol **267**(1 Pt 2): R124-35.
Minuz, P., P. Patrignani, et al. (2002). "Increased oxidative stress and platelet activation in patients with hypertension and renovascular disease." Circulation **106**(22): 2800-5.
Montanari, C. M., A. Vittoria, et al. (1979). "Human platelet aggregation curve and oral contraception." Acta Haematol **61**(4): 230-2.
Morales, D. E., K. A. McGowan, et al. (1995). "Estrogen promotes angiogenic activity in human umbilical vein endothelial cells in vitro and in a murine model." Circulation **91**(3): 755-63.
Moran, L. J., J. D. Cameron, et al. "Vascular function in the diagnostic categories of polycystic ovary syndrome." Hum Reprod.
Moran, L. J., S. K. Hutchison, et al. (2009). "A comprehensive assessment of endothelial function in overweight women with and without polycystic ovary syndrome." Clin Sci (Lond) **116**(10): 761-70.
Morel, D. W., P. E. DiCorleto, et al. (1984). "Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation." Arteriosclerosis **4**(4): 357-64.
Moro, M. A., R. J. Russel, et al. (1996). "cGMP mediates the vascular and platelet actions of nitric oxide: confirmation using an inhibitor of the soluble guanylyl cyclase." Proc Natl Acad Sci U S A **93**(4): 1480-5.
Morrow, J. D., B. Frei, et al. (1995). "Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers. Smoking as a cause of oxidative damage." N Engl J Med **332**(18): 1198-203.

Mukai, Y., H. Shimokawa, et al. (2002). "Inhibition of renin-angiotensin system ameliorates endothelial dysfunction associated with aging in rats." Arterioscler Thromb Vasc Biol **22**(9): 1445-50.
Murad, F., C. K. Mittal, et al. (1978). "Guanylate cyclase: activation by azide, nitro compounds, nitric oxide, and hydroxyl radical and inhibition by hemoglobin and myoglobin." Adv Cyclic Nucleotide Res **9**: 145-58.
Murphy-Ullrich, J. E. and M. Poczatek (2000). "Activation of latent TGF-beta by thrombospondin-1: mechanisms and physiology." Cytokine Growth Factor Rev **11**(1-2): 59-69.
Murphy, C., G. S. Kanaganayagam, et al. (2007). "Vascular dysfunction and reduced circulating endothelial progenitor cells in young healthy UK South Asian men." Arterioscler Thromb Vasc Biol **27**(4): 936-42.
Nagai, Y., E. J. Metter, et al. (1998). "Increased carotid artery intimal-medial thickness in asymptomatic older subjects with exercise-induced myocardial ischemia." Circulation **98**(15): 1504-9.
Ndolo, R. A., M. L. Forrest, et al. (2010). "The role of lysosomes in limiting drug toxicity in mice." J Pharmacol Exp Ther **333**(1): 120-8.
Nelson, M. R., D. Liew, et al. (2005). "Epidemiological modelling of routine use of low dose aspirin for the primary prevention of coronary heart disease and stroke in those aged > or =70." BMJ **330**(7503): 1306.
Ngo, D. T., A. L. Sverdlov, et al. (2010). "Does vitamin D modulate asymmetric dimethylarginine and C-reactive protein concentrations?" Am J Med **123**(4): 335-41.
Niebauer, J., J. Dulak, et al. (1999). "Gene transfer of nitric oxide synthase: effects on endothelial biology." J Am Coll Cardiol **34**(4): 1201-7.
Nijveldt, R. J., T. Teerlink, et al. (2003). "Asymmetrical dimethylarginine (ADMA) in critically ill patients: high plasma ADMA concentration is an independent risk factor of ICU mortality." Clin Nutr **22**(1): 23-30.
Nishinaka, Y., A. Nishiyama, et al. (2004). "Loss of thioredoxin-binding protein-2/vitamin D3 up-regulated protein 1 in human T-cell leukemia virus type I-dependent T-cell transformation: implications for adult T-cell leukemia leukemogenesis." Cancer Res **64**(4): 1287-92.
Norman, R. J. (2001). "Obesity, polycystic ovary syndrome and anovulation--how are they interrelated?" Curr Opin Obstet Gynecol **13**(3): 323-7.
Norman, R. J., S. Masters, et al. (1996). "Hyperinsulinemia is common in family members of women with polycystic ovary syndrome." Fertil Steril **66**(6): 942-7.
O'Rourke, M. F. and D. E. Gallagher (1996). "Pulse wave analysis." J Hypertens Suppl **14**(5): S147-57.
O'Rourke, M. F., A. Pauca, et al. (2001). "Pulse wave analysis." Br J Clin Pharmacol **51**(6): 507-22.
Oemar, B. S., M. R. Tschudi, et al. (1998). "Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis." Circulation **97**(25): 2494-8.
Orio, F., Jr., S. Palomba, et al. (2004). "Early impairment of endothelial structure and function in young normal-weight women with polycystic ovary syndrome." J Clin Endocrinol Metab **89**(9): 4588-93.
Orio, F., Jr., S. Palomba, et al. (2005). "Improvement in endothelial structure and function after metformin treatment in young normal-weight women

with polycystic ovary syndrome: results of a 6-month study." J Clin Endocrinol Metab **90**(11): 6072-6.
Paganini-Hill, A., R. K. Ross, et al. (1988). "Postmenopausal oestrogen treatment and stroke: a prospective study." BMJ **297**(6647): 519-22.
Pamuk, B. O., A. N. Torun, et al. (2010). "Asymmetric dimethylarginine levels and carotid intima-media thickness in obese patients with polycystic ovary syndrome and their relationship to metabolic parameters." Fertil Steril **93**(4): 1227-33.
Paradisi, G., H. O. Steinberg, et al. (2001). "Polycystic ovary syndrome is associated with endothelial dysfunction." Circulation **103**(10): 1410-5.
Parikh, H., E. Carlsson, et al. (2007). "TXNIP regulates peripheral glucose metabolism in humans." PLoS Med **4**(5): e158.
Patvardhan, E., K. S. Heffernan, et al. (2011). "Augmentation index derived from peripheral arterial tonometry correlates with cardiovascular risk factors." Cardiol Res Pract **2011**: 253758.
Peichev, M., A. J. Naiyer, et al. (2000). "Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors." Blood **95**(3): 952-8.
Perticone, F., R. Ceravolo, et al. (2001). "Prognostic significance of endothelial dysfunction in hypertensive patients." Circulation **104**(2): 191-6.
Petrie, J. R., S. Ueda, et al. (1998). "How reproducible is bilateral forearm plethysmography?" Br J Clin Pharmacol **45**(2): 131-9.
Pierpoint, T., P. M. McKeigue, et al. (1998). "Mortality of women with polycystic ovary syndrome at long-term follow-up." J Clin Epidemiol **51**(7): 581-6.
Polson, D. W., S. Franks, et al. (1987). "The distribution of oestradiol in plasma in relation to uterine cross-sectional area in women with polycystic or multifollicular ovaries." Clin Endocrinol (Oxf) **26**(5): 581-8.
Prysyazhna, O., O. Rudyk, et al. (2012). "Single atom substitution in mouse protein kinase G eliminates oxidant sensing to cause hypertension." Nat Med **18**(2): 286-90.
Pulichino, A. M., I. M. Wang, et al. (2008). "Identification of transforming growth factor beta1-driven genetic programs of acute lung fibrosis." Am J Respir Cell Mol Biol **39**(3): 324-36.
Puurunen, J., T. Piltonen, et al. (2011). "Unfavorable Hormonal, Metabolic, and Inflammatory Alterations Persist after Menopause in Women with PCOS." J Clin Endocrinol Metab **96**(6): 1827-34.
Quyyumi, A. A., N. Dakak, et al. (1997). "Nitric oxide activity in the atherosclerotic human coronary circulation." J Am Coll Cardiol **29**(2): 308-17.
Raitakari, O. T., J. P. Seale, et al. (2001). "Impaired vascular responses to nitroglycerin in subjects with coronary atherosclerosis." Am J Cardiol **87**(2): 217-9, A8.
Rajagopalan, S., X. P. Meng, et al. (1996). "Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability." J Clin Invest **98**(11): 2572-9.
Rajendran, S., S. R. Willoughby, et al. (2009). "Polycystic ovary syndrome is associated with severe platelet and endothelial dysfunction in both obese and lean subjects." Atherosclerosis **204**(2): 509-14.

Reckelhoff, J. F., J. A. Kellum, et al. (1994). "Changes in nitric oxide precursor, L-arginine, and metabolites, nitrate and nitrite, with aging." Life Sci **55**(24): 1895-902.

Renaud, S., M. Ciavatti, et al. (1987). "Influence of vitamin E administration on platelet functions in hormonal contraceptive users." Contraception **36**(3): 347-58.

Riba, R., N. G. Oberprieler, et al. (2006). "Von Willebrand factor activates endothelial nitric oxide synthase in blood platelets by a glycoprotein Ib-dependent mechanism." J Thromb Haemost **4**(12): 2636-44.

Ridker, P. M. (2001). "High-sensitivity C-reactive protein: potential adjunct for global risk assessment in the primary prevention of cardiovascular disease." Circulation **103**(13): 1813-8.

Ridker, P. M., C. H. Hennekens, et al. (2000). "C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women." N Engl J Med **342**(12): 836-43.

Ridker, P. M., M. J. Stampfer, et al. (2001). "Novel risk factors for systemic atherosclerosis: a comparison of C-reactive protein, fibrinogen, homocysteine, lipoprotein(a), and standard cholesterol screening as predictors of peripheral arterial disease." JAMA **285**(19): 2481-5.

Rifai, N., R. P. Tracy, et al. (1999). "Clinical efficacy of an automated high-sensitivity C-reactive protein assay." Clin Chem **45**(12): 2136-41.

Rossouw, J. E., G. L. Anderson, et al. (2002). "Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial." JAMA **288**(3): 321-33.

Rywik, T. M., M. R. Blackman, et al. (1999). "Enhanced endothelial vasoreactivity in endurance-trained older men." J Appl Physiol **87**(6): 2136-42.

Sabuncu, T., H. Vural, et al. (2001). "Oxidative stress in polycystic ovary syndrome and its contribution to the risk of cardiovascular disease." Clin Biochem **34**(5): 407-13.

Saleh, A. A., K. A. Ginsburg, et al. (1995). "Hormonal contraception and platelet function." Thromb Res **78**(4): 363-7.

Sanmuganathan, P. S., P. Ghahramani, et al. (2001). "Aspirin for primary prevention of coronary heart disease: safety and absolute benefit related to coronary risk derived from meta-analysis of randomised trials." Heart **85**(3): 265-71.

Sarabi, M., J. Millgard, et al. (1999). "Effects of age, gender and metabolic factors on endothelium-dependent vasodilation: a population-based study." J Intern Med **246**(3): 265-74.

Sase, K. and T. Michel (1995). "Expression of constitutive endothelial nitric oxide synthase in human blood platelets." Life Sci **57**(22): 2049-55.

Schachinger, V., M. B. Britten, et al. (2000). "Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease." Circulation **101**(16): 1899-906.

Schachinger, V. and A. M. Zeiher (1995). "Quantitative assessment of coronary vasoreactivity in humans in vivo. Importance of baseline vasomotor tone in atherosclerosis." Circulation **92**(8): 2087-94.

Scheubel, R. J., H. Zorn, et al. (2003). "Age-dependent depression in circulating endothelial progenitor cells in patients undergoing coronary artery bypass grafting." J Am Coll Cardiol **42**(12): 2073-80.

Schmidt, K., E. R. Werner, et al. (1992). "Tetrahydrobiopterin-dependent formation of endothelium-derived relaxing factor (nitric oxide) in aortic endothelial cells." Biochem J **281 (Pt 2)**: 297-300.

Schnabel, R., S. Blankenberg, et al. (2005). "Asymmetric dimethylarginine and the risk of cardiovascular events and death in patients with coronary artery disease: results from the AtheroGene Study." Circ Res **97**(5): e53-9.

Schulman, S. P., E. G. Lakatta, et al. (1992). "Age-related decline in left ventricular filling at rest and exercise." Am J Physiol **263**(6 Pt 2): H1932-8.

Schulze, P. C., G. W. De Keulenaer, et al. (2002). "Vitamin D3-upregulated protein-1 (VDUP-1) regulates redox-dependent vascular smooth muscle cell proliferation through interaction with thioredoxin." Circ Res **91**(8): 689-95.

Schulze, P. C., H. Liu, et al. (2006). "Nitric oxide-dependent suppression of thioredoxin-interacting protein expression enhances thioredoxin activity." Arterioscler Thromb Vasc Biol **26**(12): 2666-72.

Schulze, P. C., J. Yoshioka, et al. (2004). "Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein." J Biol Chem **279**(29): 30369-74.

Sesso, H. D., J. E. Buring, et al. (2008). "Vitamins E and C in the prevention of cardiovascular disease in men: the Physicians' Health Study II randomized controlled trial." JAMA **300**(18): 2123-33.

Shalev, A., C. A. Pise-Masison, et al. (2002). "Oligonucleotide microarray analysis of intact human pancreatic islets: identification of glucose-responsive genes and a highly regulated TGFbeta signaling pathway." Endocrinology **143**(9): 3695-8.

Shaw, L. J., C. N. Bairey Merz, et al. (2008). "Postmenopausal women with a history of irregular menses and elevated androgen measurements at high risk for worsening cardiovascular event-free survival: results from the National Institutes of Health--National Heart, Lung, and Blood Institute sponsored Women's Ischemia Syndrome Evaluation." J Clin Endocrinol Metab **93**(4): 1276-84.

Shaw, L. J., C. N. Bairey Merz, et al. (2006). "Insights from the NHLBI-Sponsored Women's Ischemia Syndrome Evaluation (WISE) Study: Part I: gender differences in traditional and novel risk factors, symptom evaluation, and gender-optimized diagnostic strategies." J Am Coll Cardiol **47**(3 Suppl): S4-S20.

Shaw, L. J., C. N. Merz, et al. (2006). "The economic burden of angina in women with suspected ischemic heart disease: results from the National Institutes of Health--National Heart, Lung, and Blood Institute--sponsored Women's Ischemia Syndrome Evaluation." Circulation **114**(9): 894-904.

Shaw, L. J., R. E. Shaw, et al. (2008). "Impact of ethnicity and gender differences on angiographic coronary artery disease prevalence and in-hospital mortality in the American College of Cardiology-National Cardiovascular Data Registry." Circulation **117**(14): 1787-801.

Shimada, T., Y. Takeshita, et al. (2004). "Angiogenesis and vasculogenesis are impaired in the precocious-aging klotho mouse." Circulation **110**(9): 1148-55.
Shioji, K., C. Kishimoto, et al. (2002). "Overexpression of thioredoxin-1 in transgenic mice attenuates adriamycin-induced cardiotoxicity." Circulation **106**(11): 1403-9.
Singh, N., J. Graves, et al. (2002). "Effects of a 'healthy' diet and of acute and long-term vitamin C on vascular function in healthy older subjects." Cardiovasc Res **56**(1): 118-25.
Solomon, C. G., F. B. Hu, et al. (2002). "Menstrual cycle irregularity and risk for future cardiovascular disease." J Clin Endocrinol Metab **87**(5): 2013-7.
Stamler, J. S., D. I. Simon, et al. (1992). "S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds." Proc Natl Acad Sci U S A **89**(1): 444-8.
Stamler, J. S., D. J. Singel, et al. (1992). "Biochemistry of nitric oxide and its redox-activated forms." Science **258**(5090): 1898-902.
Stampfer, M. J., G. A. Colditz, et al. (1991). "Postmenopausal estrogen therapy and cardiovascular disease. Ten-year follow-up from the nurses' health study." N Engl J Med **325**(11): 756-62.
Stasch, J. P., P. Pacher, et al. (2011). "Soluble guanylate cyclase as an emerging therapeutic target in cardiopulmonary disease." Circulation **123**(20): 2263-73.
Stein, L. (1935). "Amennorhoea associated with nilaterla polycystic ovaries." Am J Obstet Gynecol **29**: 181-91
Stenina, O. I., I. Krukovets, et al. (2003). "Increased expression of thrombospondin-1 in vessel wall of diabetic Zucker rat." Circulation **107**(25): 3209-15.
Stepanian, A., M. Cohen-Moatti, et al. (2011). "Von Willebrand factor and ADAMTS13: a candidate couple for preeclampsia pathophysiology." Arterioscler Thromb Vasc Biol **31**(7): 1703-9.
Stepien, J. M., R. M. Prideaux, et al. (2003). "Pilot study examining the effect of cholesterol lowering on platelet nitric oxide responsiveness and arterial stiffness in subjects with isolated mild hypercholesterolaemia." Clin Exp Pharmacol Physiol **30**(7): 507-12.
Stroes, E., J. Kastelein, et al. (1997). "Tetrahydrobiopterin restores endothelial function in hypercholesterolemia." J Clin Invest **99**(1): 41-6.
Sudhir, K., G. L. Jennings, et al. (1996). "Estrogen enhances basal nitric oxide release in the forearm vasculature in perimenopausal women." Hypertension **28**(3): 330-4.
Sueda, S., H. Kohno, et al. (2004). "Clinical impact of selective spasm provocation tests: comparisons between acetylcholine and ergonovine in 1508 examinations." Coron Artery Dis **15**(8): 491-7.
Sullivan, T. R., Jr., R. H. Karas, et al. (1995). "Estrogen inhibits the response-to-injury in a mouse carotid artery model." J Clin Invest **96**(5): 2482-8.
Swift, M. E., H. K. Kleinman, et al. (1999). "Impaired wound repair and delayed angiogenesis in aged mice." Lab Invest **79**(12): 1479-87.
Sydow, K., S. P. Fortmann, et al. (2010). "Distribution of asymmetric dimethylarginine among 980 healthy, older adults of different ethnicities." Clin Chem **56**(1): 111-20.

Taddei, S., A. Virdis, et al. (1998). "Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension." Circulation **97**(22): 2222-9.
Taddei, S., A. Virdis, et al. (1998). "Effects of angiotensin converting enzyme inhibition on endothelium-dependent vasodilatation in essential hypertensive patients." J Hypertens **16**(4): 447-56.
Taddei, S., A. Virdis, et al. (2001). "Age-related reduction of NO availability and oxidative stress in humans." Hypertension **38**(2): 274-9.
Taddei, S., A. Virdis, et al. (2000). "Endothelial dysfunction in hypertension." J Nephrol **13**(3): 205-10.
Taddei, S., A. Virdis, et al. (1995). "Aging and endothelial function in normotensive subjects and patients with essential hypertension." Circulation **91**(7): 1981-7.
Takahashi, T., C. Kalka, et al. (1999). "Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization." Nat Med **5**(4): 434-8.
Talbott, E., D. Guzick, et al. (1995). "Coronary heart disease risk factors in women with polycystic ovary syndrome." Arterioscler Thromb Vasc Biol **15**(7): 821-6.
Talbott, E. O., D. S. Guzick, et al. (2000). "Evidence for association between polycystic ovary syndrome and premature carotid atherosclerosis in middle-aged women." Arterioscler Thromb Vasc Biol **20**(11): 2414-21.
Talbott, E. O., J. V. Zborowski, et al. (2004). "The relationship between C-reactive protein and carotid intima-media wall thickness in middle-aged women with polycystic ovary syndrome." J Clin Endocrinol Metab **89**(12): 6061-7.
Talbott, E. O., J. V. Zborowski, et al. (2004). "Evidence for an association between metabolic cardiovascular syndrome and coronary and aortic calcification among women with polycystic ovary syndrome." J Clin Endocrinol Metab **89**(11): 5454-61.
Tanaka, H., F. A. Dinenno, et al. (2000). "Aging, habitual exercise, and dynamic arterial compliance." Circulation **102**(11): 1270-5.
Tao, J., Y. Wang, et al. (2006). "Circulating endothelial progenitor cell deficiency contributes to impaired arterial elasticity in persons of advancing age." J Hum Hypertens **20**(7): 490-5.
Tarkun, I., B. C. Arslan, et al. (2004). "Endothelial dysfunction in young women with polycystic ovary syndrome: relationship with insulin resistance and low-grade chronic inflammation." J Clin Endocrinol Metab **89**(11): 5592-6.
Tarkun, I., B. Cetinarslan, et al. (2005). "Effect of rosiglitazone on insulin resistance, C-reactive protein and endothelial function in non-obese young women with polycystic ovary syndrome." Eur J Endocrinol **153**(1): 115-21.
Tell, G. S., J. F. Polak, et al. (1994). "Relation of smoking with carotid artery wall thickness and stenosis in older adults. The Cardiovascular Health Study. The Cardiovascular Health Study (CHS) Collaborative Research Group." Circulation **90**(6): 2905-8.
Thum, T., D. Tsikas, et al. (2005). "Suppression of endothelial progenitor cells in human coronary artery disease by the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine." J Am Coll Cardiol **46**(9): 1693-701.

Timmermans, F., J. Plum, et al. (2009). "Endothelial progenitor cells: identity defined?" J Cell Mol Med **13**(1): 87-102.
Ting, H. H., F. K. Timimi, et al. (1997). "Vitamin C improves endothelium-dependent vasodilation in forearm resistance vessels of humans with hypercholesterolemia." Circulation **95**(12): 2617-22.
Toprak, S., A. Yonem, et al. (2001). "Insulin resistance in nonobese patients with polycystic ovary syndrome." Horm Res **55**(2): 65-70.
Torgrimson, B. N., J. R. Meendering, et al. (2007). "Endothelial function across an oral contraceptive cycle in women using levonorgestrel and ethinyl estradiol." Am J Physiol Heart Circ Physiol **292**(6): H2874-80.
Toulis, K. A., D. G. Goulis, et al. (2011). "Meta-analysis of cardiovascular disease risk markers in women with polycystic ovary syndrome." Hum Reprod Update.
Tousoulis, D., C. Xenakis, et al. (2005). "Effects of vitamin C on intracoronary L-arginine dependent coronary vasodilatation in patients with stable angina." Heart **91**(10): 1319-23.
Trakakis, E., A. Balanika, et al. (2008). "Hemodynamic alterations and wall properties in large arteries of young, normotensive, and non-obese women with polycystic ovary syndrome." J Endocrinol Invest **31**(11): 1001-7.
Trial, T. W. G. f. t. P. (1995). "Effects of estrogen or estrogen/progestin regimens on heart disease risk factors in postmenopausal women. The Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial. ." JAMA **273**(3): 199-208.
Tsao, P. S., N. P. Lewis, et al. (1995). "Exposure to shear stress alters endothelial adhesiveness. Role of nitric oxide." Circulation **92**(12): 3513-9.
Tsikas, D., R. H. Boger, et al. (2000). "Endogenous nitric oxide synthase inhibitors are responsible for the L-arginine paradox." FEBS Lett **478**(1-2): 1-3.
Turkcuoglu, I., Y. Engin-Ustun, et al. (2010). "Evaluation of asymmetric dimethylarginine, nitric oxide levels and associated independent variables in obese and lean patients with polycystic ovarian syndrome." Gynecol Endocrinol.
Turturro, F., E. Friday, et al. (2007). "Hyperglycemia regulates thioredoxin-ROS activity through induction of thioredoxin-interacting protein (TXNIP) in metastatic breast cancer-derived cells MDA-MB-231." BMC Cancer **7**: 96.
Valkonen, V. P., H. Paiva, et al. (2001). "Risk of acute coronary events and serum concentration of asymmetrical dimethylarginine." Lancet **358**(9299): 2127-8.
Vallance, P., A. Leone, et al. (1992). "Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure." Lancet **339**(8793): 572-5.
Valtonen, P., J. Karppi, et al. (2005). "Comparison of HPLC method and commercial ELISA assay for asymmetric dimethylarginine (ADMA) determination in human serum." J Chromatogr B Analyt Technol Biomed Life Sci **828**(1-2): 97-102.
van der Zwan, L. P., P. G. Scheffer, et al. (2011). "Systemic inflammation is linked to low arginine and high ADMA plasma levels resulting in an

unfavourable NOS substrate-to-inhibitor ratio: the Hoorn Study." Clin Sci (Lond) **121**(2): 71-8.
Varady, K. A., S. Bhutani, et al. (2011). "Improvements in vascular health by a low-fat diet, but not a high-fat diet, are mediated by changes in adipocyte biology." Nutr J **10**: 8.
Vasa, M., S. Fichtlscherer, et al. (2001). "Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease." Circ Res **89**(1): E1-7.
Venugopal, S. K., S. Devaraj, et al. (2002). "Demonstration that C-reactive protein decreases eNOS expression and bioactivity in human aortic endothelial cells." Circulation **106**(12): 1439-41.
Verma, S., C. H. Wang, et al. (2002). "A self-fulfilling prophecy: C-reactive protein attenuates nitric oxide production and inhibits angiogenesis." Circulation **106**(8): 913-9.
Vlachopoulos, C., K. Aznaouridis, et al. (2010). "Prediction of cardiovascular events and all-cause mortality with central haemodynamics: a systematic review and meta-analysis." Eur Heart J **31**(15): 1865-71.
Vural, B., E. Caliskan, et al. (2005). "Evaluation of metabolic syndrome frequency and premature carotid atherosclerosis in young women with polycystic ovary syndrome." Hum Reprod **20**(9): 2409-13.
Waddell, T. K., C. Rajkumar, et al. (1999). "Withdrawal of hormonal therapy for 4 weeks decreases arterial compliance in postmenopausal women." J Hypertens **17**(3): 413-8.
Wakatsuki, A. and Y. Sagara (1995). "Lipoprotein metabolism in postmenopausal and oophorectomized women." Obstet Gynecol **85**(4): 523-8.
Walford, G. and J. Loscalzo (2003). "Nitric oxide in vascular biology." J Thromb Haemost **1**(10): 2112-8.
Wang, G. R., Y. Zhu, et al. (1998). "Mechanism of platelet inhibition by nitric oxide: in vivo phosphorylation of thromboxane receptor by cyclic GMP-dependent protein kinase." Proc Natl Acad Sci U S A **95**(9): 4888-93.
Wang, J. X., M. J. Davies, et al. (2001). "Polycystic ovarian syndrome and the risk of spontaneous abortion following assisted reproductive technology treatment." Hum Reprod **16**(12): 2606-9.
Wassmann, S., U. Laufs, et al. (2001). "HMG-CoA reductase inhibitors improve endothelial dysfunction in normocholesterolemic hypertension via reduced production of reactive oxygen species." Hypertension **37**(6): 1450-7.
Weber, T., J. Auer, et al. (2004). "Arterial stiffness, wave reflections, and the risk of coronary artery disease." Circulation **109**(2): 184-9.
Weis, M., S. Pehlivanli, et al. (2001). "Simvastatin treatment is associated with improvement in coronary endothelial function and decreased cytokine activation in patients after heart transplantation." J Am Coll Cardiol **38**(3): 814-8.
Werner, N., S. Junk, et al. (2003). "Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury." Circ Res **93**(2): e17-24.
Werner, N., S. Kosiol, et al. (2005). "Circulating endothelial progenitor cells and cardiovascular outcomes." N Engl J Med **353**(10): 999-1007.

White, R. E., D. J. Darkow, et al. (1995). "Estrogen relaxes coronary arteries by opening BKCa channels through a cGMP-dependent mechanism." Circ Res **77**(5): 936-42.
White, R. P., P. Vallance, et al. (2000). "Effect of inhibition of nitric oxide synthase on dynamic cerebral autoregulation in humans." Clin Sci (Lond) **99**(6): 555-60.
Wild, R. A. and M. J. Bartholomew (1988). "The influence of body weight on lipoprotein lipids in patients with polycystic ovary syndrome." Am J Obstet Gynecol **159**(2): 423-7.
Wild, R. A., E. Carmina, et al. (2010). "Assessment of cardiovascular risk and prevention of cardiovascular disease in women with the polycystic ovary syndrome: a consensus statement by the Androgen Excess and Polycystic Ovary Syndrome (AE-PCOS) Society." J Clin Endocrinol Metab **95**(5): 2038-49.
Wild, R. A., B. Grubb, et al. (1990). "Clinical signs of androgen excess as risk factors for coronary artery disease." Fertil Steril **54**(2): 255-9.
Wild, S., T. Pierpoint, et al. (2000). "Cardiovascular disease in women with polycystic ovary syndrome at long-term follow-up: a retrospective cohort study." Clin Endocrinol (Oxf) **52**(5): 595-600.
Wilkinson, I. B., J. R. Cockcroft, et al. (1998). "Pulse wave analysis and arterial stiffness." J Cardiovasc Pharmacol **32 Suppl 3**: S33-7.
Wilkinson, I. B., S. A. Fuchs, et al. (1998). "Reproducibility of pulse wave velocity and augmentation index measured by pulse wave analysis." J Hypertens **16**(12 Pt 2): 2079-84.
Wilkinson, I. B., I. R. Hall, et al. (2002). "Pulse-wave analysis: clinical evaluation of a noninvasive, widely applicable method for assessing endothelial function." Arterioscler Thromb Vasc Biol **22**(1): 147-52.
Willoughby, S., A. Holmes, et al. (2002). "Platelets and cardiovascular disease." Eur J Cardiovasc Nurs **1**(4): 273-88.
Willoughby, S. R., S. Rajendran, et al. (2012). "Ramipril Sensitizes Platelets to Nitric Oxide: Implications for Therapy in High-Risk Patients." J Am Coll Cardiol **60**(10): 887-94.
Willoughby, S. R., S. Stewart, et al. (2005). "Platelet nitric oxide responsiveness: a novel prognostic marker in acute coronary syndromes." Arterioscler Thromb Vasc Biol **25**(12): 2661-6.
Wolf, P. A., R. D. Abbott, et al. (1991). "Atrial fibrillation as an independent risk factor for stroke: the Framingham Study." Stroke **22**(8): 983-8.
Woods, J. D., J. S. Edwards, et al. (1993). "Inhibition by nitroprusside of platelet calcium mobilization: evidence for reduced sensitivity to nitric oxide in essential hypertension." J Hypertens **11**(12): 1369-73.
Xia, Y., A. L. Tsai, et al. (1998). "Superoxide generation from endothelial nitric-oxide synthase. A Ca2+/calmodulin-dependent and tetrahydrobiopterin regulatory process." J Biol Chem **273**(40): 25804-8.
Xiong, Y., L. W. Yuan, et al. (2001). "Elevated serum endogenous inhibitor of nitric oxide synthase and endothelial dysfunction in aged rats." Clin Exp Pharmacol Physiol **28**(10): 842-7.
Xu, S., Y. He, et al. (2009). "Endothelial cells negatively modulate reactive oxygen species generation in vascular smooth muscle cells: role of thioredoxin." Hypertension **54**(2): 427-33.

Xu, W., F. T. Kaneko, et al. (2004). "Increased arginase II and decreased NO synthesis in endothelial cells of patients with pulmonary arterial hypertension." FASEB J **18**(14): 1746-8.

Yamaguchi, F., M. Takata, et al. (2008). "Rare sugar D-allose induces specific up-regulation of TXNIP and subsequent G1 cell cycle arrest in hepatocellular carcinoma cells by stabilization of p27kip1." Int J Oncol **32**(2): 377-85.

Yamamoto, M., G. Yang, et al. (2003). "Inhibition of endogenous thioredoxin in the heart increases oxidative stress and cardiac hypertrophy." J Clin Invest **112**(9): 1395-406.

Yamawaki, H., J. Haendeler, et al. (2003). "Thioredoxin: a key regulator of cardiovascular homeostasis." Circ Res **93**(11): 1029-33.

Yang, Y., T. Ago, et al. (2011). "Thioredoxin 1 negatively regulates angiotensin II-induced cardiac hypertrophy through upregulation of miR-98/let-7." Circ Res **108**(3): 305-13.

Yao, M., D. D. Roberts, et al. (2011). "Thrombospondin-1 inhibition of vascular smooth muscle cell responses occurs via modulation of both cAMP and cGMP." Pharmacol Res **63**(1): 13-22.

Yasue, H., K. Matsuyama, et al. (1990). "Responses of angiographically normal human coronary arteries to intracoronary injection of acetylcholine by age and segment. Possible role of early coronary atherosclerosis." Circulation **81**(2): 482-90.

Yoshida, T., H. Nakamura, et al. (2005). "The involvement of thioredoxin and thioredoxin binding protein-2 on cellular proliferation and aging process." Ann N Y Acad Sci **1055**: 1-12.

Yusuf, S., G. Dagenais, et al. (2000). "Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators." N Engl J Med **342**(3): 154-60.

Zhang, F., J. L. Ram, et al. (1994). "17 beta-Estradiol attenuates voltage-dependent Ca2+ currents in A7r5 vascular smooth muscle cell line." Am J Physiol **266**(4 Pt 1): C975-80.

Zoccali, C., S. Bode-Boger, et al. (2001). "Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study." Lancet **358**(9299): 2113-7.

Appendices

Chan, W.P., Sverdlov, A.L. & Horowitz, J.D. (2008) Management of the metabolic syndrome in cardiovascular disease.
Current treatment options in cardiovascular medicine, v. 10 (1), pp. 27-38.

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Rajendran, S., Willoughby, S.R., Chan, W.P., Liberts, E.A., Heresztyn, T., Saha, M., Marber, M.S., Norman, R.J. & Horowitz, J.D. (2009) Polycystic ovary syndrome is associated with severe platelet and endothelial dysfunction in both obese and lean subjects.
Atherosclerosis, v. 204 (2), pp. 509-14.

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This publication is included on pages 211-216 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1016/j.atherosclerosis.2008.09.010

Ngo, D.T., Chan, W.P., Rajendran, S., Heresztyn, T., Amarasekera, A., Sverdlov, A.L., O'Loughlin, P.D., Morris, H.A., Chirkov, Y.Y., Norman, R.J. & Horowitz, J.D. (2011) Determinants of insulin responsiveness in young women: Impact of polycystic ovarian syndrome, nitric oxide, and vitamin D. *Nitric Oxide, v. 25 (3), pp. 326-30*

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Willoughby, S.R., Rajendran, S., Chan, W.P., Procter, N., Leslie, S., Liberts, E.A., Heresztyn, T., Chirkov, Y.Y. & Horowitz J.D. (2012) Ramipril Sensitizes Platelets to Nitric Oxide: Implications for Therapy in High-Risk Patients.
Journal of the American College of Cardiology, v. 60 (10), pp. 887-894.

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