Dietary influences on insulin sensitivity: A randomised crossover study

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Table of Contents

LIST OF FIGURES VI

LIST OF TABLES VII

ABBREBIATIONS IX

EXECUTIVE SUMMMARY XIII

DECLARATION XVI

ACKNOWLEDGEMENTS XVII

PUBLICATIONS ARISING FROM THIS THESIS XVIII

CONFERENCE PRESENTATIONS XIX

DESCRIPTION OF THESIS XX

1 INTRODUCTION 1

1.1 Diabetes prevalence and its implication 1

1.2 Diagnosis 1

1.3 Insulin resistance and type 2 diabetes mellitus 2

1.4 Risk factors in the pathogenesis of type 2 diabetes mellitus 3

1.4.1 Non-modifiable factors 3

1.4.2 Modifiable factors 3

1.4.2.1 Obesity 3

1.4.2.2 Lifestyle modification and dietary patterns 4

1.5 Summary 6

2 LITERATURE REVIEW: RED AND PROCESSED MEAT AND INSULIN SENSITIVITY 7

2.1 Preface 7

2.2 Published article 7

3 LITERATURE REVIEW: DAIRY AND INSULIN SENSITIVITY 8

3.1 Association between dairy and insulin resistance 8

3.1.1 Epidemiological studies 8

3.1.2 Intervention studies 8

3.1.3 Acute studies 9

3.2 Effects of dairy components on insulin sensitivity 9

4 LITERATURE REVIEW: WHOLE GRAINS, NUTS AND LEGUMES AND INSULIN SENSITIVITY 11
4.1 Association between the dietary pattern rich in whole grains, nuts, legumes and insulin resistance 11
4.2 Association between whole grains and insulin resistance 11
4.2.1 Epidemiological studies 12
4.2.2 Intervention studies 12
4.2.3 Acute studies 12
4.3. Association between nuts and insulin resistance 12
4.3.1 Epidemiological studies 12
4.3.2 Intervention studies 12
4.3.3 Acute studies 13
4.4 Association between legumes and insulin resistance 14
4.4.1 Epidemiological studies 14
4.4.2 Intervention studies 14
4.4.3 Acute studies 15
4.5 Components found in whole grains, nuts and legumes 15
4.6 Association between fruits and vegetables and insulin resistance 15
5 LITERATURE REVIEW: POLYPHENOLS AND GLYCEMIC CONTROL 17
5.1 Preface 17
5.2 Published article 17
6 LITERATURE REVIEW: PROBIOTICS, PREBIOTICS, SYNBIOTICS AND INSULIN SENSITIVITY 18
6.1 Preface 18
6.2 Introduction 19
6.3 Gut microbiota in individuals with T2DM and Obesity 19
6.4 Probiotics and Effects of Probiotics on Glucose Metabolism in Human Interventions 22
6.5 Other Fermented Food 24
6.6 Potential Mechanisms of Action of Probiotics 26

6.7 Prebiotics and Effects of Prebiotics on Glucose Metabolism in Human Interventions 28
6.7.1 Fructooligosaccharides 29
6.7.2 Inulin 30
6.7.3 Oligofructose-Enriched Inulin 30
6.7.4 Resistant Starch 30
6.7.5 Other Potential Prebiotics 31
6.8 Potential Mechanisms of Action of Prebiotics-derived SCFAs in Insulin Sensitivity 31
6.8.1 SCFAs and free fatty acid receptors (FFAR2 and FFAR3) 32
6.8.2 Anti-Inflammatory Effects 33
6.8.3 SCFAs and angiopoietin-like protein 4 34
6.8.4 SCFAs and Intestinal Gluconeogenesis 36
6.9 Synbiotics and Effects of Synbiotics on Glucose Metabolism in Human Interventions 36
6.10 Conclusion 37
7 LITERATURE REVIEW: METHODS TO ASSESS INSULIN SENSITIVITY 50
7.1 Hyperinsulinemic-euglycemic clamp 50
7.2 Hyperglycaemic clamp 51
7.3 Minimal Model Analysis of Frequently sampled intravenous glucose tolerance test 51
7.4 Oral glucose tolerance test 52
7.5 Homoeostasis model assessment 52
7.6 Quantitative insulin sensitivity check index 53
7.7 CONTINUOUS LOW DOSE INSULIN AND GLUCOSE INFUSION TEST 53
8 SUMMARY AND SCOPE OF THESIS 54
9 CONSUMPTION OF RED AND PROCESSED MEAT AND REFINED GRAINS FOR 4 WEEKS DECREASES INSULIN SENSITIVITY IN INSULIN-RESISTANT ADULTS: A RANDOMIZED CROSSOVER STUDY 56
9.1 Preface 56
9.2 Published article 56

10 DIFFERENTIAL EFFECTS OF RED MEAT/REFINED GRAIN DIET AND DAIRY/CHICKEN/NUTS/WHOLE GRAIN DIET ON GLUCOSE, INSULIN AND TRIGLYCERIDE IN A RANDOMIZED CROSSOVER STUDY 57

10.1 Preface 57

10.2 Published Article 57

11 EFFECTS OF TWO DIFFERENT DIETARY PATTERNS ON INFLAMMATORY MARKERS, ADVANCED GLYCATION END PRODUCTS AND LIPIDS IN SUBJECTS WITHOUT TYPE 2 DIABETES: A RANDOMISED CROSSOVER STUDY 59

11.1 Preface 59

11.2 Published Article 59

12 SUMMARY AND CONCLUSIONS 61

12.1 Discussion 61

12.2 Limitations 63

12.3 Future Directions 64

12.4 Conclusion 65

13 REFERENCES 67

14 APPENDICES 86

14.1 Statement of Authorship: A review of potential metabolic etiologies of the observed association between red meat consumption and development of type 2 diabetes mellitus 86

14.2 Statement of Authorship: Response to the comment by Kuipers and Pruiboom. 88

14.3 Statement of Authorship: Polyphenols and Glycemic Control 90

14.4 Statement of Authorship: Consumption of red and processed meat and refined grains for 4 weeks decreases insulin sensitivity in insulin-resistant adults: A randomized crossover study 92

14.5 Statement of Authorship: Differential Effects of Red Meat/Refined Grain Diet and Dairy/Chicken/Nuts/Whole Grain Diet on Glucose, Insulin and Triglyceride in a Randomized Crossover Study 94

14.6 Statement of Authorship: Effects of Two Different Dietary Patterns on Inflammatory Markers, Advanced Glycation End Products and Lipids in Subjects without Type 2 Diabetes: A Randomised Crossover Study 96

14.7 Examples of Weighed Food Diary and Food Checklists.....98

LIST OF FIGURES

2.1: The summary of possible mechanisms linking red and processed meat metabolites to etiology of type 2 diabetes

2.2: Metal Catalyzed Haber–Weiss reaction

4.1: Associations between diet score and fasting glucose and insulin concentration

5.1: Chemical structures and dietary sources of different groups of polyphenols

5.2: The summary of potential mechanisms linking dietary polyphenol metabolites to improved glucose homeostasis

9.1: Flow chart of participant recruitment and withdrawal from the study

9.2: Mean ± SE changes in glucose concentrations over the course of the 2.5-h LDIGIT assessed at the end of each diet period

10.1: A participant flow chart

10.2: Mean ± standard deviation (SD) changes in postprandial glucose concentrations after two diets

10.3: Individual data for glucose AUC and iAUC

10.4: Mean ± SD changes in postprandial insulin concentrations after two diets

10.5: Individual data for insulin AUC and iAUC. The bold black line shows the mean and SD

10.6: Individual data for C-peptide AUC and iAUC

10.7: Individual data for triglyceride AUC and iAUC

LIST OF TABLES

1.1: 2006 WHO recommendations for the diagnosis of diabetes and intermediate hyperglycaemia

1.2: 2016 ADA recommendations for the diagnosis of diabetes and prediabetes

2.1: Meta-analyses of the association between red and processed meat intake and risk of T2DM

2.2: Studies investigating the association between AGE-restricted diets and markers of oxidative stress, inflammation, endothelial function and insulin sensitivity

2.3: Meta-analyses of serum ferritin or dietary heme iron and risk of T2DM

6.1: The summary of probiotic human intervention studies

6.2: The summary of prebiotic human intervention studies

6.3: The summary of synbiotic human intervention studies

9.1: The recommended menus for each of the diets

9.2: Baseline characteristics of participants

9.3: Reported dietary intake assessed by weighed food records

9.4: Effects of diets on insulin, glucose, ISI and cholesterol measured at the end of each diet period

9.5: Effects of diets on fasting glucose, fasting insulin and C-peptide measured at the end of each diet period

10.1: The recommended menus for each of the diets

10.2: Nutrient composition of the test meals

10.3: Baseline characteristics of a total of participants and participants by sequence order

10.4: Reported dietary intake of key foods assessed by weighed food records

10.5: Reported dietary intake of nutrients assessed by weighed food records

10.6: Effects of meals on glucose, insulin and TG measured at the end of each diet period

10.7 Effects of the two diets on postprandial glucose, insulin and triglyceride responses divided into the two insulin sensitivity subgroups

11.1: Baseline characteristics of participants

11.2: Insulin sensitivity, lipids, inflammatory and fibrinolytic markers, and AGEs measured at the end of each dietary period in all participants (n = 51)

11.3: Effects of the two diets on blood IL-6, PAI-1, fluorescent AGEs and CML divided into the two insulin sensitivity subgroups

ABBREBIATIONS

AAS α-Amino Adipic Semi-Aldehyde

ACO–1 Acyl CoA Oxidase-1

ADA American Diabetes Association

AGE Advanced Glycation End Products

AGE-BSA Advanced Glycation End Product of Bovine Serum Albumin

Aix Arterial Stiffness

AMPK 5' Adenosine Monophosphate-activated Protein Kinase

ANGPTL4 Angiopoietin-Like Protein 4

ATP Adenosine Triphosphate

AUC Area Under the Curve

BAC Blood Alcohol Concentration

BCAA Branched Chain Amino Acids

BMI Body Mass Index

BPA Bisphenol A

cAMP Cyclic Adenosine Monophosphate

CHD Coronary Heart Disease

CHO Carbohydrate

CI Confidence Interval

CML Carboxymethyllysine

COX2 Cyclooxygenase-2 Protein

CPT-1 Carnitine Palmitoyl Transferase-1

CV Coefficient of Variation

CVD Cardiovascular Disease

DASH Dietary Approaches to Stop Hypertension

DBP Diastolic Blood Pressure

DBP Dibutyl Phthalate

DEHP Di-2-Ethylhexyl Phthalate

DEXA Dual Energy X-ray Absorptiometry

EDCs Endocrine Disrupting Chemicals

EFSA European Food Safety Authority

EGCG Epigallocatechin-3-O-Gallate

Fe^{2+} Ferrous

Fe^{3+} Ferric

FFA Free Fatty Acid

FFAR3 Free Fatty Acid Receptor 3

FIAF Fasting-Induced Adipose Factor

FOS Fructo-Oligosaccharide

FOXO1 Forkhead Box Protein O1

FSIGT Frequently Sampled Intravenous Glucose Tolerance Test

GGS γ-Glutamic Semi-aldehyde

GK Glucokinase

GL Glycemic Load

GS Glycogen Synthase

Gss Steady-State Blood Glucose

GI Glycemic Index.

Ginf Glucose Infusion Rate

GIP Glucose-dependent Insulinotropic Polypeptide

GLP-1 Glucagon-Like Peptide-1

GLUT Glucose Transporter

GP Glycosylated Blood Protein

G6Pase Glucose-6-Phosphatase

GPR G-Protein-Coupled Receptor

GPx Glutathione Peroxidase;

GSH Glutathione

HbAlc Hemoglobin A1c

HCF High-Carbohydrate moderate Fat

HDAC Histone Deacetylase

HDL High-Density Lipoprotein

HMD Diet High in Red and Processed Meat and Refined Grains

H_2O_2 Hydrogen Peroxide

HOMA-IR Homeostasis Model Assessment-Insulin Resistance

HR Hazard Ratio

hs-CRP high sensitive C-Reactive Protein

HWD Diet High in Whole Grains, Nuts, Dairy and Legumes

iAUC Incremental Area Under the Curve

ICAM-1 Intercellular Adhesion Molecule 1

IFG Impaired Fasting Glucose

IFN-γ Interferon Gamma

IGF Insulin-like Growth Factor

IGN Intestinal Gluconeogenesis

IGT Impaired Glucose Tolerance

IL-1 β Interleukin-1 beta

IL-6 Interleukin-6

IRAS Insulin Resistance Atherosclerosis Study

IRS Insulin Receptor Substrate

ISI Insulin Sensitivity Index

ISS Steady-State Serum Insulin

KATP channels ATP-sensitive K+ channels

LAB Lactic Acid–Producing Bacteria

LDIGIT Low-Dose Insulin and Glucose Infusion Test

LDL Low-Density Lipoprotein

LFA-3 Lymphocyte Function-Associated Antigen 3

LPS Lipopolysaccharide

LPL Lipoprotein Lipase

MCP-1 Monocyte Chemoattractant Protein-1

MLD–STZ Multiple Low Dose Streptozocin

mTORC Mammalian Target of Rapamycin Complex

MTT Meal Tolerance Test

MUFA Monounsaturated Fatty Acid

Neu5GC ***N***-Glycolylneuraminic Acid

NF-Kb Nuclear Factor Kappa-light-chain-enhancer of activated B cells

NGF Nerve Growth Factor

NGT Normal Glucose Tolerance

NHS Nurses' Health Study

NO Nitric Oxide

NOx Nitric Oxide Metabolic Pathway Products

O_2 Oxygen

O_2^- Superoxide

OEI Oligofructose-Enriched Inulin

OGTT Oral Glucose Tolerance Test

OH^- Hydroxyl ion

•OH Hydroxyl radical

$ONOO^-$ Peroxynitrite

PAD Peripheral Artery Disease

PAI-1 Plasminogen Activator Inhibitor-1

PBMC Peripheral Blood Mononuclear Cell

PCs Procyanidins

PEPCK Phosphoenolpyruvate Carboxykinase

PGC1 α Peroxisomal Proliferator Activated Receptor Gamma Coactivator 1 α

PGE2 Prostaglandin E2

PI3K Phosphatidylinositol 3-Kinase

PPAR Peroxisome Proliferator Activated Receptor

PUFA Polyunsaturated Fatty Acid

PYY Peptide Tyrosine-Tyrosine

QUICKI Quantitative Insulin Check Index

RAGE Receptor for AGE

RAS Renin Angiotensin System

ROS Reactive Oxygen Species

RR Relative Risk

SBP Systolic Blood Pressure

SCFAs Short Chain Fatty Acids

SD Standard Deviation

SE Standard Error

SF Saturated Fat

SFA Saturated Fatty Acid

SGLT 1 Sodium-dependent Glucose T ransporter 1

SMD Standardised Mean Difference

SML Specific Migration Limit

SOD Superoxide Dismutase

S6k1 Ribosomal Protein S6 Kinase β 1

STZ Streptozotocin

TBARS Thiobarbituric Acid Reactive Substances

TCA Tricarboxylic Acid Cycle

T1D Type 1 diabetes

T2D Type 2 Diabetes

T2DM Type 2 Diabetes Mellitus

TDI Tolerable Daily Intake

TG Triglyceride

TLR4 Toll-Like Receptor 4

TMA Trimethylamine

TMAO Trimethylamine N-oxide

TNF-α Tumor Necrosis Factor Alpha

TZDs Thiazolidinediones

UA Uric Acid

USDA United States Department of Agriculture

VAT Visceral Adipose Tissue

VCAM-1 Vascular Cell Adhesion Molecule 1

VLDL Very Low Density Lipoprotein

WHO World Health Organization

EXECUTIVE SUMMMARY

The increased prevalence of diabetes and its complications pose a serious threat to human health and put an enormous burden on global healthcare costs. Type 2 diabetes mellitus (T2DM) is caused by the interaction between modifiable risk factors including diet, weight and activity levels, and non-modifiable risk factors including genes, ageing and ethnicity.

Improvement in dietary quality, increased physical activity levels, energy restriction and weight loss have been shown to be associated with a lower risk of T2DM. However, the role of particular dietary patterns or particular foods in the absence of weight loss is not clear.

Epidemiological studies indicate that consumption of red and processed meat and refined grains are associated with an increased risk of T2DM but intervention studies with these foods are few in number. On the other hand, consumption of whole grains, nuts, legumes and dairy products have been shown to have protective or neutral effects on the risk of T2DM in epidemiological and a small number of intervention studies. No intervention study investigating the effect of red and processed meat on insulin sensitivity without weight loss has been performed.

Therefore, the aim of the research presented in this thesis was to examine if a dietary pattern high in red and processed meat and refined grains reduces insulin sensitivity, compared with a dietary pattern high in whole grains, nuts, legumes, dairy and devoid of red and processed meat in a randomised crossover clinical trial with a weight-stable design.

The secondary endpoint was to analyse possible biomarkers associated with insulin resistance and investigate the correlation between these markers and changes in insulin sensitivity.

The key findings from this randomised crossover trial consisting of two 4-week weight-stable dietary intervention periods as follows:

A dietary pattern high in red and processed meat and refined grains reduced insulin sensitivity compared with a dietary pattern high in whole grains, nuts, legumes and dairy only in relatively insulin-resistant individuals and not in insulin-sensitive individuals. Insulin sensitivity in the insulin-resistant group was correlated with BMI and fat mass. Insulin sensitivity index (ISI) on the dietary pattern high in whole grains, nuts, legumes and dairy was positively correlated with the amount of carbohydrates in this dietary pattern after

adjustment for ISI on the alternative dietary pattern indicating the importance of carbohydrate quality and quantity.

A dietary pattern high in red and processed meat and refined grains resulted in a significant increase in serum total cholesterol and triglyceride compared with the alternative dietary pattern.

The dietary pattern high in red and processed meat and refined grains significantly elevated plasminogen activator inhibitor-1 (PAI-1), a good marker of fibrinolytic activity, compared with a dietary pattern high in whole grains, nuts, dairy and legumes. PAI-1 was inversely correlated with ISI, and positively correlated with serum total cholesterol and triglycerides only after the dietary pattern high in red and processed meat and refined grains. PAI-1 on the dietary pattern high in whole grains, nuts, legumes and dairy was inversely correlated with reported whole grains consumption after adjustment for PAI-1 on the alternative dietary pattern. There were no significant differences in inflammatory markers (hs-CRP and IL-6) and serum AGEs [total fluorescent AGEs and carboxymethyllisine (CML)] between two dietary patterns.

An acute meal tolerance test performed at the end of each 4-week dietary intervention period demonstrated that a red meat/refined grain meal resulted in significant increases in total area under the curve (AUC) and incremental area under the curve (iAUC) for insulin, total area under the curve for C-peptide, as well as incremental glucose AUC in comparison with a dairy/chicken/nuts/whole grain meal. These findings were consistent with the main study of the chronic dietary intervention which showed decreased insulin sensitivity in relatively insulin-resistant individuals as well as reflecting the differences in carbohydrate quality and quantity between two different types of meals.

In summary, the primary study end-point was negative, and effects of the diet on insulin resistance were only detected in a post-hoc subgroup analysis. A dietary pattern high in red and processed meat and refined grains for 4 weeks significantly decreased insulin sensitivity only in relatively insulin-resistant individuals compared with a dietary pattern high in whole grains, nuts, legumes and dairy. Moreover, a dietary pattern high in red and processed meat and refined grains negatively affected lipid profiles by increasing total cholesterol and triglycerides and significantly increased serum PAI-1 levels compared with a diet high in whole grains, nuts, legumes and dairy. Elevated serum PAI-1 was associated with insulin resistance.

These findings from the human intervention trial provide significant evidence that insulin-resistant people are more susceptible to a dietary pattern high in red and processed meat and refined grains leading to increased insulin resistance. Insulin resistance increases the risk for cardiovascular disease. The most important factor influencing insulin sensitivity appears to be the quality and quantity of carbohydrate.

DECLARATION

I, Yoona Kim declare that this work contains no material which has been submitted or 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.

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Signed

Date 23/02/2017

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PUBLICATIONS ARISING FROM THIS THESIS

Published Articles

Kim Y, Keogh JB, Clifton PM. Consumption of red and processed meat and refined grains for 4 weeks decreases insulin sensitivity in insulin-resistant adults: A randomized crossover study. Metabolism. 2017; 68:173-83.

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Kim Y, Keogh JB, Clifton PM. Polyphenols and Glycemic Control. Nutrients 2016; 8(1). doi: 10.3390/nu8010017.

Kim Y, Keogh J, Clifton PM. Response to the comment by Kuipers and Pruiboom. Metabolism 2016; 65(1): e5. doi: 10.1016/j.metabol.2015.09.003.

Kim Y, Keogh J, Clifton P. A review of potential metabolic etiologies of the observed association between red meat consumption and development of type 2 diabetes mellitus. Metabolism 2015; 64(7):768-79. doi: 10.1016/j.metabol.2015.03.008.

Kim Y, Keogh JB, Clifton PM. Effects of Two Different Dietary Patterns on Inflammatory Markers, Advanced Glycation End Products and Lipids in Subjects without Type 2 Diabetes: A Randomised Crossover Study. Nutrients. 2017; 9.

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Published Conference Abstracts

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CONFERENCE PRESENTATIONS

‘Effect of a dietary pattern on insulin sensitivity’, Australian Atherosclerosis Society, December 2016, Hobart, Tasmania – Poster Presentation

‘Effect of red and processed meat and refined grains on insulin sensitivity in insulin resistant subjects’, Nutrition Society of Australia National Conference, November 2016, Melbourne, Victoria – Oral Presentation * *Awarded Second Prize for Oral Presentation*

‘Effect of red and processed meat and refined grains on insulin sensitivity in insulin resistant subjects’, Nutrition Society of Australia Student Presentation Night, November 2016, Adelaide, South Australia – Oral Presentation

‘Dietary influences on insulin sensitivity: a randomised, crossover study’, American Diabetes Association Scientific Sessions, June 2016, New Orleans, LA, USA – Oral Presentation

‘Dietary influences on insulin sensitivity: a randomized crossover study’ University of South Australia School of Pharmacy Symposium, October 2015, Adelaide, South Australia – Oral Presentation

DESCRIPTION OF THESIS

This thesis is submitted to a Thesis containing Published Research in accordance with UniSA Academic Regulations for Higher Degrees by Research Clause 16.1.1.b. Chapters from 2 to 7 provide a literature review. Chapters 2, 5, 9 10 and 11 have been accepted for publication prior to completion of the thesis. Chapter 6 is under review. A brief preface for each chapter provides context for the publication within the thesis. Each publication is formatted to the style of the journal in which it is published. The methodologies are included within the related chapters. The discussion in chapter 12 is a general summary to integrate and reconcile the various results contained in the thesis publications. Author contribution statements are included in the appendices.

1 INTRODUCTION

1.1 Diabetes prevalence and its implication

Diabetes prevalence is increasing worldwide. The number of people with diabetes was 382 million in 2013 and 592 million people are predicted to have diabetes by 2035 [1]. Australia experienced a 2-fold increase in prevalence between 1981 and 2000 in men and women over the age of 25. During this period, 8% of men and 6.8% of women had diabetes and an additional 17.4% of men and 15.4% of women had impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) [2].

Diabetes can be a very significant disease leading to complications such as coronary heart disease (CHD), cerebrovascular disease, peripheral artery disease (PAD), retinopathy, nephropathy, neuropathy, dental disease and psychosocial problems [3]. Sixty-five percent of all cardiovascular disease (CVD) deaths occur in those with diabetes, newly diagnosed diabetes, IFG or IGT [4]. Individuals with diabetes have more than a 2-fold higher mortality risk than those with normal glucose tolerance (NGT) [hazard ratio (HR) 2.6, 95% confidence interval (CI): 1.4, 4.7] and individuals with IFG (HR 2.5, 95% CI: 1.2, 5.1) after adjustment for age, sex, and other traditional CVD risk factor, compared with those with normal glucose tolerance [4].

The medical and socioeconomic burden of diabetes is enormous. Diabetes-associated costs have been markedly rising [5]. In the U.S. 174 billion dollars were spent on diagnosed diabetes in 2007 and the cost increased to 245 billion dollars in 2012 [6, 7].

1.2 Diagnosis

Diabetes is diagnosed in accordance with the World Health Organization (WHO) guidelines incorporating a 2-h, 75 g oral glucose tolerance test (OGTT) criteria. **TABLE 1** shows the most recent WHO diagnostic criteria for diabetes [8].

TABLE 1: 2006 WHO recommendations for the diagnosis of diabetes and intermediate hyperglycaemia

	Fasting plasma glucose	2-h plasma glucose*	HbA1c
Diabetes	≥ 7.0 *mmol/L* (126 *mg/dL*)	Or ≥ 11.1 *mmol/L* (200 *mg/dL*)	Or ≥ 6.5% (48 *mmol/mol*)
Impaired Glucose Tolerance (IGT)	< 7.0 *mmol/L* (126 *mg/dL*)	And ≥ 7.8 and <11.1 *mmol/L* (140-200 *mg/dL*)	
Impaired Fasting Glucose (IFG)	6.1 to 6.9 *mmol/L* (110-125 *mg/dL*)	And (if measured) < 7.8 *mmol/L* (140 *mg/dL*)	

*Venous plasma glucose 2-h after ingestion of 75 g oral glucose load

* If 2-h plasma glucose is not measured, status is uncertain as diabetes or IGT cannot be excluded

Diabetes may be diagnosed based on the 2016 Standards of Medical Care in Diabetes (Standards) published by the American Diabetes Association (ADA). **TABLE 2** shows the most recent ADA diagnostic criteria for diabetes [9].

TABLE 2: 2016 ADA recommendations for the diagnosis of diabetes and prediabetes

	Fasting plasma glucose	2-h plasma glucose[#]	HbA1c	Random plasma glucose level
Diabetes	7.0 *mmol/L* ≥126 *mg/dL*	11.1 *mmol/L*[†] ≥ 200 *mg/dL*	≥ 6.5%	11.1 *mmol/L* ≥ 200 *mg/dL*[‡]
Prediabetes	5.6-6.9 *mmol/L* 100-125 *mg/dL*	7.8-11.0 *mmol/L* 140-199 *mg/dL*	5.7-6.4%	

[#] 2-h plasma glucose level after 75 g oral glucose tolerance test

[†] In the absence of unequivocal hyperglycemia, results should be confirmed by repeated testing.

[‡] Only diagnostic in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis.

1.3 Insulin resistance and type 2 diabetes mellitus

Insulin resistance exerts a key role in the pathogenesis of T2DM [10] and it is a condition where target tissues including skeletal muscle, adipocytes and liver cells have attenuated responses to insulin [10, 11]. The condition of peripheral insulin resistance in muscle and

adipocytes, pancreatic β cells increases insulin secretion to maintain normal glucose levels, resulting in compensatory hyperinsulinaemia [11]. Over time, pancreatic β-cells gradually lose the capacity to secrete sufficient insulin leading to elevated blood glucose (hyperglycaemia) and consequently the development of T2DM [12]. Pancreatic function may be slowly lost with time in the absence of significant insulin resistance and these lean individuals with a high genetic risk for pancreatic failure have a higher mortality than those with obesity-driven insulin resistance [12].

Insulin-resistant individuals are likely to have abnormalities such as impaired glucose tolerance or impaired fasting glucose, dyslipidemia (increased triglycerides and decreased high-density lipoprotein cholesterol), endothelial dysfunction, increased inflammatory markers, increased procoagulant factors (plasminogen activator inhibitor-1 and fibrinogen), abnormal uric acid metabolism, increased sympathetic nerve system activity and renal sodium retention and hypertension [13].

1.4 Risk factors in the pathogenesis of type 2 diabetes mellitus

Risk factors including aging, genetics and ethnicity are not modifiable, while others such as poor eating habits, physical inactivity, smoking and obesity are modifiable [14]. T2DM results from the interaction among those factors.

1.4.1 Non-modifiable factors

Genome studies have shown the association between many loci and the risk of T2DM [15]. Adults aged over 60 years had concordance rates of 35-58% for diabetes in monozygotic twins compared with 17-20% in dizygotic twins [16, 17]. People with a first-degree relative with diabetes (a parent or sibling) had a 4-fold higher risk of diabetes than those without a family history after adjustments for gender, age, race, poverty income ratio and body mass index (BMI) [18]. Offspring of one parent with T2DM had the prevalence of T2DM of 38% at age 80 years, while offspring of both parents with T2DM had the prevalence of T2DM of 60% by the age of 60 years [19, 20]. A high genetic risk score (the top quintile of 1000 SNPs) can increase the risk of incident T2DM by 3.45 compared with the bottom quintile after adjustment for BMI and other known predictors [21].

1.4.2 Modifiable factors

1.4.2.1 Obesity

Obesity is the most powerful modifiable risk factor for T2DM [22-24]. In the Nurse Health Survey with a 16-year follow-up period, women with a BMI above 35.0 kg/m^2 had a relative

risk (RR) of 38.8 for T2DM, while women with a BMI between 30.0 and 34.9 kg/m^2 had a RR of 20.1 for T2DM, compared with women with a BMI of less than 23.0 kg/m^2 [22]. In people with a BMI above 27 kg/m^2, the risk of incident T2DM elevated by 20% for each 1 kg/m^2 increase in the BMI compared with people with normal weight (BMI≤ 25 kg/m^2). The risk increased by 100% at a BMI between 27.2 and 29.4 kg/m^2 and by 300% at a BMI above 29.4 kg/m^2 [24]. The combination of a sedentary lifestyle and fast food consumption is likely to contribute to obesity [25].

1.4.2.2 Lifestyle modification and dietary patterns

Numerous intervention studies with follow-up periods of 3-6 years have shown that intensive lifestyle modification in individuals with IGT can prevent or delay the incidence of T2DM [26-31].

Two lifestyle intervention studies found a 58% reduction in the incidence of T2DM among individuals with IGT during a mean 3-4 year intervention period [28, 32].

The Finnish Diabetes Prevention Study has shown that lifestyle changes including low in total fat and saturated fat, high fiber, weight loss and increased physical activity decreased the risk of T2DM by 58 % over a median of 4 years [28]. The mean reduction in body weight was 4.2 ± 5.1 kg (4.7 ± 5.4 percent) in the intervention group and 0.8 ± 3.7 kg (0.9 ± 4.2 percent) in the control group during the first year, and the mean body loss by the end of year 2 significantly remained with 3.5 ± 5.5 kg in the intervention group and 0.8 ± 4.4 kg in the control group. [28]. The US Diabetes Prevention Program aiming at weight loss of at least 7% of initial body weight, moderate physical activity (at least 150 minutes per week), a low-calorie and low-fat diet postponed the onset of T2DM much more effectively than treatment with metformin. The intensive lifestyle-intervention lowered the incidence of T2DM by 58% and metformin (plus standard lifestyle recommendations) by 31%, compared with placebo and standard lifestyle recommendations after a mean 2.8 years [32].

The follow-up studies of the Finnish Diabetes Prevention Study have reported that the incidence of T2DM was still reduced by 43% after a median 7 years of follow-up [31] and by 38% after 13 years from baseline [33]. These findings were also observed in the China Da Qing Diabetes Prevention Study showing a 43% reduction over 20 years [34] and in the US Diabetes Prevention Program Outcomes Study showing a 34% reduction over 10 years [35].

The PREMIER study examined the effect of lifestyle interventions with and without the Dietary Approaches to Stop Hypertension (DASH) diet on insulin sensitivity as well as blood

pressure. The control group received only advice on the basis of the National High Blood Pressure Educational Program while the established intervention group received counselling on low-sodium (2.4 g/day or less)/low-fat diets (30% of calories from fat), weight loss, moderate alcohol intake and 180 min of moderate physical activity per week. The DASH group received a similar counselling plus the DASH diet (25% of calories from fat, ≤ 7% of calories from saturated fat, 9–12 servings/day of fruits and vegetables and 2–3 servings/day of low-fat dairy). Only the DASH group had significantly improved insulin sensitivity compared with the control group [36]. In the ENCORE study combining the DASH diet with caloric restriction and aerobic exercise improved insulin sensitivity and lipid profiles in comparison with the DASH diet alone and usual diet, while the DASH diet alone did not significantly improve insulin sensitivity compared with usual diet [37].

In the Shanghai Women's Health Study, a dietary pattern high in milk reduced the risk of T2DM by 22% compared with a dietary pattern high in staple foods (rice and noodles) after 6.9 years of follow-up. A dietary pattern high in milk and with a modest reduction in staple foods combined with other risk factors (BMI ≤ 23, waist circumference < 0.85 and exercise) showed an 86% lower risk of T2DM [38].

A high consumption of red and processed meat, refined grains, sweetened fruit juice, fast-food (hamburgers, pizza and sausages) was positively associated with weight gain and insulin resistance [39, 40]. Moreover, combining low physical activity with a dietary pattern high in red and processed meat, refined grains, French fries and sweets resulted in a high risk of T2DM during 12 years of follow-up in U.S. men [41].

In a meta-analysis of 10 prospective studies with follow-up periods ranging from 2 to 23 years, 10 studies have shown that dietary patterns with high intakes of whole grains, fish, poultry, fruit and vegetables, and low intakes of red and processed meat, sugar-sweetened beverages and starch foods decreased the risk of T2DM by 15 to 83% [42].

In addition, a meta-analysis of 9 prospective cohort studies examined the association between *a posteriori* dietary patterns and the risk of T2DM. This analysis included subjects aged 24 to 84 years and a follow-up period ranging from 4 to 23 years. The Unhealthy/Western dietary pattern contained red meat, processed meat, refined grains, sweets/sugary drinks and fried foods, whereas the Healthy/Prudent dietary pattern was characterized by whole grains, legumes, fish, and poultry, fruit and vegetables. People in the highest category of Healthy/Prudent dietary pattern showed a 15% reduction in the risk of T2DM compared with those in the lowest category (95% CI: 0.80, 0.91; P < 0.0001), whereas those in the highest

category of Unhealthy/Western dietary pattern showed a 41% increase in the risk of T2DM (95% CI: 1.32, 1.52; P < 0.0001) compared with those in the lowest category [43].

1.5 Summary

The global burden of diabetes is increasing. Diabetes and its complication put a substantial socioeconomic burden on the health care system. Type 2 diabetes mellitus develops as a consequence of the interaction between non-modifiable factors (genes, age and ethnicity) and modifiable factors (dietary patterns, weight, sedentary lifestyle and activity levels). T2DM can be preventable in people at high risk despite the influence of genetic predisposition. An improved dietary pattern in conjunction with weight loss and increased physical activity lowers the risk of diabetes. The Western dietary patterns which include red meat, processed meat, refined grains and sweets have been shown to increase the risk of T2DM, whereas the dietary patterns rich in whole grains, legumes, fish, and poultry, fruit and vegetables have been shown to decrease the risk of T2DM. However, there are few interventions investigating the effect of those two different dietary patterns on the risk of T2DM without concomitant weight loss. This thesis investigated the effect of a dietary pattern high in red and processed meat and refined grains on insulin resistance compared with a dietary pattern high in whole grains, nuts, legumes and dairy in weight stability.

2 LITERATURE REVIEW: RED AND PROCESSED MEAT AND INSULIN SENSITIVITY

2.1 Preface

This chapter contains a review article and a letter to the editor. In the review article published in 2015, meta-analyses of prospective studies are summarised. Possible mechanisms linking several metabolites of red and processed meat to the incidence of type 2 diabetes are suggested. The letter published in 2016 is a response to the comment on trimethylamine N-oxide (TMAO) addressed in this review article.

2.2 Published article

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Kim Y, Keogh J, Clifton PM. Response to the comment by Kuipers and Pruiboom. Metabolism 2016; 65(1): e5. doi: 10.1016/j.metabol.2015.09.003.

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Reviews

A review of potential metabolic etiologies of the observed association between red meat consumption and development of type 2 diabetes mellitus

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ABSTRACT

Epidemiological studies suggest that red and processed meat consumption is related to an increased risk of type 2 diabetes. However, it is not clearly understood which components of red and processed meat contribute to this increased risk. This review examines potential mechanisms addressing the role of saturated fatty acid, sodium, advanced glycation end products (AGEs), nitrates/nitrites, heme iron, trimethylamine N-oxide (TMAO), branched amino acids (BCAAs) and endocrine disruptor chemicals (EDCs) in the development of type 2 diabetes based on data from published clinical trials and animal models. TMAO which is derived from dietary carnitine and choline by the action of bacterial enzymes followed by oxidation in the liver may be a strong candidate molecule mediating the risk of type 2 diabetes. BCAAs may induce insulin resistance via the mammalian target of rapamycin complex 1 (mTORC1) and ribosomal protein S6 kinase β 1 (S6k1)-associated pathways. The increased risk associated with processed meat compared with red meat suggests that there are interactions between the saturated fat, salt, and nitrates in processed meat and iron, AGEs and TMAO. Intervention studies are required to clarify potential mechanisms and explore interactions among components, in order to make firm recommendations on red and processed meat consumption.

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Abbreviations: AGEs, advanced glycation end products; TMAO, trimethylamine N-oxide; BCAAs, branched amino acids; EDCs, endocrine disrupting chemicals; mTORC1, mammalian target of rapamycin complex 1; S6k1, ribosomal protein S6 kinase β 1; T2DM, type 2 diabetes mellitus; CVD, cardiovascular disease; HR, hazard ratio; 95% CI, 95% confidence interval; SFA, saturated fatty acid; BMI, body mass index; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; FSIGT, frequently sampled intravenous glucose tolerance test; IRS-1, insulin receptor substrate-1; PI3K, phosphatidylinositol 3-kinase; SF, saturated fat; RR, relative risk; OGTT, oral glucose tolerance test; HOMA, homeostatic model assessment; RAS, renin–angiotensin system; RAGE, receptor for AGE; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells;-; AAS, α-amino adipic semi-aldehyde; GGS, γ-glutamic semi-aldehyde; VCAM-1, vascular cell adhesion molecule 1; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1 beta; IL-6, interleukin 6; CRP, C-reactive protein; ROS, reactive oxygen species; AGE-BSA, advanced glycation end product of bovine serum albumin; NO, nitric oxide; ATP, adenosine triphosphate; ICAM-1, intercellular adhesion molecule 1; TBARS, thiobarbituric acid reactive substances; $ONOO^-$, peroxynitrite; LDL, low-density lipoprotein; NOx, nitric oxide metabolic pathway products; Fe^{3+}, ferric; O_2^-, superoxide; Fe^{2+}, ferrous; O_2, oxygen; H_2O_2, hydrogen peroxide; •OH, hydroxyl radicals, OH–, hydroxyl ion; GLUT4, glucose transporter type 4; FFA, free fatty acid; HOMA-IR, homeostasis model assessment of insulin resistance; CHD, coronary heart disease; TMA, trimethylamine; HbA1c, glycated hemoglobin; IRS-2, insulin receptor substrate 2; BPA, bisphenol A; DEHP, di-2-ethylhexyl phthalate; SML, specific migration limit; DBP, dibutyl phthalate; EFSA, the European Food Safety Authority; TDI, tolerable daily intake.

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1. Introduction

In 2011, an estimated 366 million people in the world had diabetes and this number is expected to increase to 552 million by 2030 [1]. Type 2 Diabetes Mellitus (T2DM) results in complications such as cardiovascular disease, retinopathy, nephropathy, neuropathy, leg ulcers and gangrene [2], which are very common, costly and shorten life expectancy [3]. In Australia, 65% of all cardiovascular disease (CVD) death occurs in those with T2DM and pre diabetes [4]. In the USA, people with diabetes on average, spent 2.5 times more on medical care than people without diabetes [5].

Individual characteristics including age, family history, ethnicity, obesity, smoking, sedentary lifestyle and hypertension are well-known risk factors for T2DM [3]. There is a strong focus on translating what has been learned from controlled trials into community action for the prevention of T2DM [6,7]. Although diet quality is associated with risk of T2DM exactly which components are important is not clear [8–10]. Over the last decade there have been a considerable number of prospective studies and meta-analyses of prospective studies that show that a diet rich in red and processed meat is associated with an increased risk of T2DM. Although many mechanisms for this association have been proposed there is no clear consensus on which may be the most likely candidates. This review aims to describe potential mechanisms and their interactions, and the implications for the prevention of T2DM.

2. Meta-analyses of Red and Processed Meat Intake

The association between red and processed meat intake and risk of T2DM has been investigated by four meta-analyses [11–14] (Table 1) with risk estimates of 1.13 to 1.19 per 100 g per day of total red meat and 1.19 to 1.51 per 50 g processed meat per day. The recent EPIC-Interact Study included 12,403 cases of newly diagnosed T2DM among 340,234 participants over 11.7 years of follow-up. In this study the hazard ratio (HR) was 1.08 per 50 g per day increase for total meat (95% confidence interval (CI) 1.05–1.12), 1.08 (95% CI 1.03–1.13) for fresh red meat and 1.12 (95% CI 1.05–1.19) for processed meat, after multivariate analyses [15]. A meta-analysis showed no interactions between dietary patterns and diabetes risk genetic loci on glucose and insulin levels [16], suggesting no individual genetic differences in susceptibility to the effect of diet on the risk of T2DM. However the association between red meat and diabetes may be confounded by other imperfectly measured or unmeasured lifestyle attributes.

3. Potential Mechanistic Pathways

A summary of all the possible mechanistic pathways is shown in Fig. 1.

4. Saturated Fatty Acids

Meat is a source of saturated fatty acids (SFA) and cholesterol that may increase insulin resistance [13]. In order to evaluate the potential impact of SFA intake on the risk of T2DM in this review, we approached evidence coming from i) association studies ii) interventional studies in model (i.e., animal) systems and iii) interventional studies in humans. A correlation between a higher SFA intake and decreased insulin sensitivity has been demonstrated in several cross-sectional studies [17]. Four of eight epidemiological studies showed a relationship between a high SFA intake and risk of T2DM independently of body mass index (BMI) [18–21]. In a 20-year follow-up study, 389 men with newly diagnosed diabetes were reported to have a higher intake of total, saturated, monounsaturated fatty acid (MUFA) and dietary cholesterol 20 years before diagnosis than men with no diabetes [18]. An association between total and saturated fat intake and fasting insulin concentrations was found in a non-diabetic community sample after adjustment for confounding factors such as age, sex, ethnicity, BMI, waist circumference, total energy intake and physical activity [19]. Similar results were found in a study of healthy female twins but there was no adjustment for obesity [22].

Three studies showed no significant association between total and saturated and insulin sensitivity, after adjustment for body fat [23] or BMI [24,25]. Five intervention studies all with a small sample size showed no effect of dietary

Table 1 – Meta-analyses of the association between red and processed meat intake and risk of T2DM.

Reference	Studies	RRs of red meat	RRs of processed meat
Aune et al. [11]	10 of 12 cohorts for red meat 9 of 12 cohorts for processed meat	1.21 for > 600 g/week vs <100 g/week (95% CI 1.07–1.38, I^2 = 58.5 %)	1.41 for > 250 g/week vs <50 g/week (95% CI 1.25–1.60, I^2 = 53.2 %)
Micha et al. [12]	7 cohorts (3 common to Aune's analysis)	1.16 for 100 g/day (95% CI 0.92–1.46, P = 0.25)	1.19 for 50 g/day (95% CI 1.11–1.27, P < 0.001)
Pan et al. [14]	79,570 women in NHS I 87,504 women in NHS II 37,083 men in HPFUS	1.19 for 100 g/day (95% CI 1.04–1.37) 1.34 for 1166.2 g/week vs 220.5 g/week (95% CI 1.26–1.43, P < 0.001)	1.51 for 50 g/day (95% CI 1.25–1.83) 1.32 for 583.1 g/week vs 3.15 g/week (95% CI 1.24–1.40, P < 0.001)
Feskens et al. [13]	14 cohorts for red meat 21 cohorts for processed meat	1.13 per 100 g (95% CI 1.03–1.23, I^2 = 36 %)	1.32 per 50 g (95% CI 1.19–1.48, I^2 = 89 %)

HPFUS, Health Professional Follow-Up Study; NHS I, Nurses' Health Study I; NHS II, Nurses' Health Study II; RR, relative risk; 95% CI, 95% confidence interval; I^2, Indicator of heterogeneity between studies.

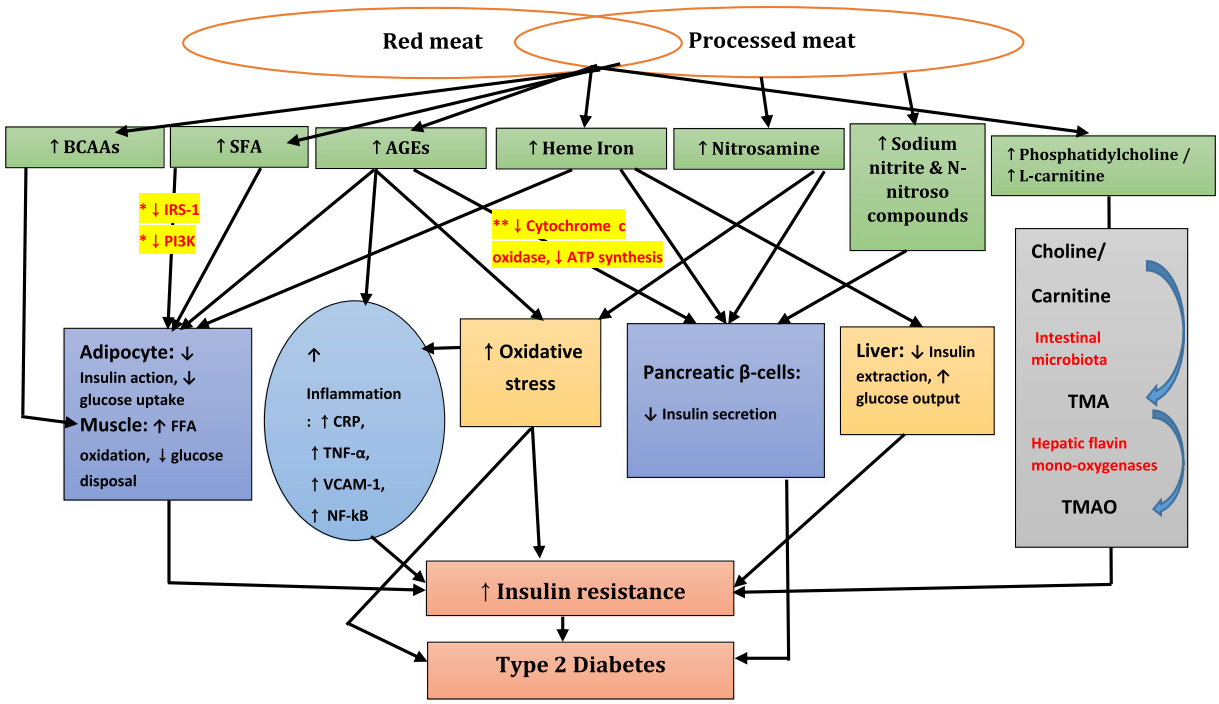


Fig. 1 – The summary of possible mechanisms linking red and processed meat metabolites to etiology of type 2 diabetes. * Increased SFA intake elevates intracellular fatty acyle-CoA and diacylglyceride, which in turn, results in decreased insulin activation of IRS-1-associated PI3K in Muscle, leading to decreased insulin-stimulated glucose transport activity. ** Elevated AGEs increase the formation of nitric oxide by inducing the expression of nitric oxide synthase and in turn, impair glucose-stimulated insulin secretion through nitric oxide-dependent inhibition of cytochrome c oxidase and ATP synthesis. See text for other details. BCAAs, branched amino acids; SFA, saturated fatty acid; AGEs, advanced glycation end products; IRS-1, insulin receptor substrate 1; PI3K, phosphatidylinositol 3-kinase; FFA, free fatty acid; CRP, C-reactive protein; TNF-α, tumor necrosis factor-alpha; VCAM-1, vascular cell adhesion 1; TMA, trimethylamine; TMAO, trimethylamine N-oxide; ATP, adenosine triphosphate.

saturated fat versus unsaturated fat on insulin sensitivity in healthy or type 2 diabetic individuals [26–30].

In the KANWU study [31], 162 healthy volunteers were randomized to one of two isocaloric diets — a high SFA diet (17% of energy (E) from SFA, 14% from MUFA, 6% from PUFA) or a high MUFA diet (23%E from MUFA, 8%E from SFA, and 6% E from PUFA). A beneficial effect of MUFA on insulin sensitivity assessed by a frequently sampled intravenous glucose tolerance test (FSIGT) was seen in subjects with a total fat intake below the median (37%E), but not in subjects with a higher total fat intake (>37%E) although this split in fat intake was only a post hoc observation. Substituting dietary mono-unsaturated for saturated fat did not influence insulin secretion [31]. Consistent with the KANWU study, two controlled intervention studies, with 59 healthy and 11 insulin resistant subjects with a family history of diabetes, demonstrated that an SFA-rich diet impaired insulin sensitivity compared with a MUFA-rich diet. The test diets had a total fat content of 37%E [32,33]. A significant reduction in insulin sensitivity (40%–50% reduction) was observed in six healthy subjects undergoing an SFA-rich lipid intravenous infusion, compared with those who received a PUFA-rich lipid infusion (20%–27% reduction) [34]. In animal models an increase in intracellular fatty acyl-CoA and diacylglycerol concentrations decreased insulin-stimulated glucose transport activity by inhibiting insulin activation of insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI3K) activity [35]. In a recent large sample of overweight and obese adults [36], SFAs did not affect insulin sensitivity assessed by FSIGT. One hundred and fifty eight adults underwent a 4-week baseline diet [55%E carbohydrate, 15%E protein, 30%E fat (7% E saturated fat (SF)] and were then randomized to 4 weeks of either the baseline diet or 1 of 4 test diets containing 35% E carbohydrate and either 20%E or 30%E protein and either 7%E or 15%E SF mostly from dairy. No significant effects of SFAs on insulin sensitivity or insulin secretion were observed [36].

In summary, given the mixed results from interventions it is still unclear to what extent SFA from red and processed meat contributes to insulin resistance.

5. Intracellular Lipid

Increased intramuscular lipid content lowers the rate of insulin-stimulated glucose uptake. [37,38]. Vegans with high intakes of fiber and carbohydrates and a lower intake of protein and fat had lower intramuscular lipid content compared with omnivores matched for sex, age, BMI, waist measurement,

percentage body fat, activity levels and energy intake. The SFA intake of omnivores was twice as high as vegans (P <0.001) [39]. These studies suggest that meat fat might contribute to enhanced intracellular lipid storage, which contributes to increased insulin resistance [40]. However, other foods high in SFA also contribute to intracellular lipid storage to a similar extent as red meat and there is no evidence linking increased intracellular lipid particularly to red meat.

6. Sodium

Sodium in processed meat may be one of the contributors to an increased risk of T2DM. The Alpha-Tocopherol, Beta-carotene Cancer Prevention study of 24,845 participants with 1098 incident cases of diabetes during 12 years of follow-up [41] found a relative risk (RR) of 1.37 (95% CI 1.11–1.71, P = 0.001) for 139 g of processed meat per day compared with 28 g per day in a multivariate model. This association was markedly attenuated by an adjustment for sodium (RR 1.19; 95% CI 0.95–1.49, P = 0 · 10). This finding led the authors to conclude that the sodium content of processed meat increased the risk of T2DM more than SFA, protein, cholesterol, heme Fe, Mg and nitrate [41]. Processed meat was found to contain on average of 400 % more sodium than unprocessed red meats in the USA [12].

The association between high salt intake and insulin sensitivity is not still clearly understood, as there have been much conflicting data. Severe salt restriction showed a 20% increase in insulin sensitivity assessed by a low dose euglycemic insulin clamp 5 days after high (200 mmol/d)- and low (10 mmol/d)-sodium diets [42]. Others however [43–45] reported no change in insulin sensitivity in healthy subjects using an oral glucose tolerance test (OGTT) (20 mmol/d vs 260 mmol/d for 6 days) [44], a two-step euglycemic clamp (40 mmol/d vs 220 mmol/d for 6 days) [43], and a 3 h insulin-suppression test (25 mmol/d vs 200 mmol/d for 5 days) [45], while in hypertension, salt restriction reduced insulin sensitivity as assessed by an OGTT [46] and a homeostatic model assessment (HOMA) [47]. Increased salt improved insulin sensitivity in men but not women [48]. Inhibition of the renin-angiotensin system (RAS) prevents type 2 diabetes so a low salt diet should increase risk as it stimulates RAS. Overall it appears unlikely that the increased salt content of processed meat contributes to insulin resistance.

7. Advanced glycation End Products

Advanced glycation end products (AGEs) derived from meat and processed meat may contribute to the pathogenesis of T2DM. Exposure to dry heat, high temperature and elevated pH over a short time can generate high levels of AGEs in foods. When meat is roasted or seared, meat is browned through a Maillard reaction involving breakdown of tetrapyrrole rings in the muscle protein myoglobin [49]. Intestinal absorption of about 10%–30% of dietary AGEs occurs and one-third of ingested AGEs are excreted in urine and feces and two-thirds are retained in tissues [50].

Lowering dietary AGEs lowers insulin resistance in healthy people [51], and restricting dietary AGEs and advanced lipoxidation end products improves insulin sensitivity and pancreatic structure in the db/db mouse model [52]. The interaction between an AGE and its receptor (RAGE) activates nuclear factor kB (NF-kB), producing inflammation, oxidative stress, thrombogenesis, potentially enhancing the development of atherosclerosis [53].

7.1. *Proposed AGE Formation in Red Meat*

The question of whether the formation of AGEs within foods rich in animal fats and meat is facilitated in the presence of elemental iron has been raised [54]. Carbonyl derivatives from lysine, arginine, threonine and proline side chains can be formed during metal catalyzed oxidation of polyunsaturated fat [55]. Threonine is converted into α-amino-3-keto butyric acid, lysine into α-amino adipic semi-aldehyde (AAS), and arginine and proline into γ-glutamic semi-aldehyde (GGS). AAS and GGS account for between 23% and 60% of the total carbonyl compounds in oxidized plasma and liver proteins after metal catalyzed oxidation [55]. In addition the combination of transition metals with H_2O_2 generates protein semi-aldehydes [56]. An in-vitro study induced the carbonylation of meat proteins using a variety of oxidation systems [57]. Myoglobin content is a good predictor of protein carbonyl formation, but the relative contributions of the other iron forms to protein oxidation in meat and meat products have not been determined [58]. Because of the involvement of myoglobin in the formation of AGEs, red meat and particularly processed red meat have the highest AGE values with raw beef containing 707 kU per 100 g, boiled beef frankfurters 7484 kU per 100 g and broiled beef frankfurters 11,270 kU per 100 g [59]. However, it is noted that carbohydrate-rich foods contribute most AGEs to the human diet due to the higher amount of carbohydrates eaten, despite the high AGE content of red and processed meat [60].

7.2. *AGEs in Animal and Human Studies*

Dietary AGEs provoke the production of vascular cell adhesion molecule-1 (VCAM-1), tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin 6 (IL-6) and C-reactive protein (CRP) by generating reactive oxygen species (ROS) [61]. In patients with type 2 diabetes, a 6-week high-AGE diet increased serum AGEs, CRP, TNF-α and VCAM-1 and more glycated [61] and oxidized LDL and significantly activated NF-kB [62].

Animal [52,63,64] and human studies [65] suggest that food-derived AGEs are associated with progression to T2DM. Higher post meal glucose levels and lower insulin levels were observed in a dose-dependent manner in the mice treated with an advanced glycation end product of bovine serum albumin (AGE-BSA). However fasting glucose levels and peripheral insulin sensitivity were unchanged by AGE-BSA treatment compared with controls [63]. Fresh islets isolated from 6-month-old normal C57BL/6 mice were then treated with AGE-BSA for 48 h. Elevated AGE levels induced expression of nitric oxide synthases and the generation of nitric oxide (NO). Elevated levels of NO inhibited cytochrome c oxidase activity and cytochrome c oxidase-dependent

adenosine triphosphate (ATP) production leading to impaired insulin secretion [63]. In another study low AGE-fed mice showed lower fasting insulin levels, 13% weight reduction and 2-fold higher glucose uptake by adipose tissue compared with high AGE-fed mice despite almost similar nutritional and caloric intake [52]. In normal C57/BL6 mice a high-fat and a high AGE diet for 6 months increased visceral fat by 2–4 fold compared with a high fat and a low AGE diet and a control diet. 75% of the high-fat and high AGE-fed mice developed diabetes [64].

In humans, AGE-restricted diets are associated with decreased markers of oxidative stress, inflammation and improved insulin sensitivity and endothelial function in subjects with diabetes [61,66], as well as in healthy subjects [51,66–68] (Table 2). A systematic review of 12 studies that examined if an AGE-restricted diet attenuates insulin resistance concluded that the generalizability was limited due to the low quality of evidence and suggested additional long-term high-quality randomized controlled [65]. A crossover study in 20 subjects with diabetes found that a single high AGE meal induced significant endothelial dysfunction and oxidative stress compared with an identical meal with about one sixth of the AGE content. There were significant changes in intercellular adhesion molecule 1 (ICAM-1) and VCAM-1 at 2 h and 4 h. Thiobarbituric acid reactive substances (TBARS) increased by 23.3% ($P = 0.003$) 2 h after the high-AGE meal [69].

In summary, dietary AGEs are absorbed and directly affect plasma AGE concentration. It is still unclear if dietary AGEs influence insulin sensitivity. More studies are required with more comprehensive and dynamic measures of insulin resistance.

8. Nitrates, Nitrites and Nitrosamine

Dietary nitrates and nitrites improve endothelial dysfunction, hypertension, microvascular dysfunction and insulin sensitivity and enhance NO production [70]. Vegetables are a good source of nitrates [70]. However when NO and superoxide are both present, a strong oxidant, peroxynitrite ($ONOO^-$) is formed, which is cytotoxic in many experimental systems and thus can potentially influence cardiovascular disease, neurodegenerative disease, diabetes and its complications [71]. Peroxynitrite can modify tyrosine, tryptophan, cysteine, and methionine residues [72]. Modified proteins may damage catalytic and structural integrity or interrupt regulatory pathways in cellular function [72]. No differences in protein oxidation (measured as o-tyrosine) between diabetics and non-diabetic controls have been observed [73]. However, higher levels of advanced oxidation protein products, AGEs, oxidized low-density lipoprotein (LDL) and nitric oxide metabolic pathway products (NOx) were found in impaired fasting glucose and T2DM groups compared with a healthy group [74].

Nitrites and nitrates used in the preservation of processed meat are converted into nitrosamines by binding to amino compounds, either in the stomach or within the food [14]. Sodium nitrite and two N-nitroso compounds N-nitrosomethylurea and N-nitrosomethylurethane decrease insulin secretion in adult rats [75]. Nitrosamines are toxic to pancreatic beta-cells and increase the risk of T2DM in animals [76]. Nitrosamine plays a key role in DNA damage and generation of ROS involved in lipid peroxidation, protein adduct formation, and pro-inflammatory cytokine activation [76]. The correlation between a Western dietary pattern high in red and processed meat and the risk of T2DM persisted after adjustments for both saturated and total fat and other factors such as BMI [77] suggesting that nitrosamine and AGEs in processed meat, rather than fatty acids, might account for the association between processed meat intake and T2DM [78]. Processed meat contained an average of 50 % more nitrates than unprocessed red meat [12].

9. Iron

Red meat is an important source of heme iron. Heme iron is more easily absorbed than non-heme iron; absorption of heme iron and non-heme iron is 25% and 5-15% respectively [79]. Lacto-ovo vegetarians have greater insulin sensitivity independent of gender, BMI and age — possibly via low iron status, compared with meat-eaters [80].

Iron may potentially play a role in glucose metabolism because of its action as a pro-oxidant.

Iron causes cellular oxidative stress which can inhibit insulin binding [81]. Insulin increases ferritin synthesis and iron uptake by the cell through the translocation of transferrin receptors from the intracellular compartment to the cell surface. Free iron also induces ferritin synthesis, while oxidative stress increases the release of iron from ferritin. Protein glycation accelerates the insulin resistance-induced or increased oxidative stress-induced iron release from transferrin [81].

Iron is reversibly oxidized and reduced and participates in the formation of ROS, such as hydroxyl radical. Superoxide and hydrogen peroxide are the primary species generated. These produce the highly reactive hydroxyl radical in which iron plays a catalytic role in a metal catalyzed Haber–Weiss reaction. The general effect of catalytic iron is to convert poorly reactive free radicals, such as H_2O_2, into highly reactive ones, such as OH– and O_2[82] (Fig. 2).

ROS can damage pancreatic beta cells and also impair insulin-stimulated IRS 1 tyrosine phosphorylation, reduce activated PI3K and inhibit glucose transporter type 4 (GLUT4) translocation to the plasma membrane [83].

Elevated levels of iron may contribute to increased T2DM by increasing glucose production and decreasing glucose utilization [79]. Elevated hepatic iron stores interfere with hepatic insulin extraction and increased hepatic glucose output [84]. In adipocytes, iron inhibits insulin action and glucose uptake [85]. Increased muscle iron stores facilitate free fatty acid (FFA) oxidation and thus can impede glucose disposal [86]. Therefore the relationship between iron and the development of T2DM can be explained by both increased hepatic glucose output and decreased glucose disposal while iron deposition in the pancreatic β-cells leads to impaired insulin secretion [87].

9.1. *Meta-Analyses of Serum Ferritin and Dietary Heme Iron*

Meta-analyses showed the association between serum ferritin (a biomarker of iron stores) (Table 3) and T2DM incidence.

Table 2 – Studies investigating the association between AGE-restricted diets and markers of oxidative stress, inflammation, endothelial function and insulin sensitivity.

Reference	Participants	Study design	Dietary design	Results
Vlassara et al. [61]	11 diabetic participants. Mean age: 52 y, mean HbA1c: 7.8 ± 0.7%, mean BMI: 28 kg/m^2	2-week randomized crossover study	A low-AGE diet (3,670 kU CML/day) vs a high-AGE diet (16,300 kU CML)/day)	↑fasting serum AGE (P = 0.02) on high-AGE diet, ↓fasting serum AGE (P = 0.02) on a low-AGE diet. The values of inflammatory markers corresponding to changes of serum AGE. AGE-LDL changed within 2 weeks (P = 0.03).
	13 diabetic participants. Mean age: ~62 y, mean HbA1c: 7.2 ± 0.65%, mean BMI: 29.5 kg/m^2	6-week randomized parallel study		↑TNF-α, ↑CRP, ↑VCAM-1, ↑serum AGEs, ↑AGE-LDL on a high-AGE diet, ↓CRP (P = 0.014), ↓VCAM-1 (P < 0.01), ↓serum AGEs (P = 0.02), ↓AGE-LDL (P < 0.05) on a low-AGE diet.
Uribarri et al. [66]	18 participants with type 2 diabetes and 18 participants without diabetes. Mean age: 64 y, mean BMI: 29.8 kg/m^2	16-week randomized parallel study	A standard diet (>20,000 kU CML/day) or an isocaloric AGE-restricted diet (<10,000 kU CML/day)	In diabetic subjects, ↓serum AGE, ↓8-isoprostanes (P = 0.006), ↓insulin, ↓HOMA, ↓leptin on an AGE restricted diet. In healthy subjects, ↔ HOMA, ↓ sCML (P = 0.016), ↓8-isoprostanes (P = 0.006) on an AGE restricted diet.
Vlassara et al. [67]	30 healthy participants	16-week randomized parallel study	A regular diet (>13, 000 kU CML/day) or a low-AGE diet (<5500kU CML/day)	In both healthy (P <0.05) and CKD subjects (P <0.05), ↓sCML, ↓8-isoprostanes, ↓TNF-α, ↓VCAM-1 on a low-AGE diet.
	9 CKD participants	4-week randomized parallel study		
Birlouez-Aragon et al. [51]	64 healthy participants (32 M & 32 F). Mean age: 19 y, mean BMI: 21.8 kg/m^2	4-week randomized cross over study	A low-MRP diet (2.2 ± 0.9 mg CML/day) vs a standard MRP diet (5.4 ± 2.3 mg CML/day)	On a low-MRP diet, ↑insulin sensitivity (P = 0.002), compared with a standard MRP diet.
Tahara et al. [68]	322 nondiabetic Japanese participants	cross-sectional study		*Mean serum AGE levels were 8.96 ± 2.57 U/mL and serum AGE correlated with insulin resistance evaluated by HOMA-IR index (r = 0.281, P < 0.001).
Mark et al. [119]	74 overweight women aged 20–50 y, BMI 25–40 kg/m^2	A two-factorial design, 4-week parallel study	A high-AGE diet (fried, baked, roasted, or grilled cooking) or a low-AGE diet (boiled or steamed cooking), together with either fructose or glucose drinks	On a low-AGE diet, ↓urine AGEs, ↓fasting insulin, ↓HOMA-IR, compared with a high-AGE diet.

HbA1c, glycated hemoglobin; BMI, body mass index; CML, Nε-carboxy-methyl-lysine; AGE, advanced glycation end products; LDL, low-density lipoprotein; VCAM-1, vascular cell adhesion 1; TNF-α, tumor necrosis factor alpha; HOMA, homeostatic model assessment; CKD, kidney disease; sCML, serum carboxy-methyl-lysine MRP, Maillard reaction product; HOMA-IR, homeostasis model assessment of insulin resistance, y, years; M, male; F, female; ↑, increase; ↓, decrease; ↔, no change, r, correlation coefficient.
* Data are described as mean ± standard deviation (SD).

Meta-analyses provided some evidence for the association of dietary heme iron and the risk of T2DM (Table 3) although some studies linking red meat intake and T2DM have adjusted for heme iron intake [14].

9.2. *Lowering Body Iron Stores*

The association between an increased number of blood donations and a lower prevalence of diabetes in men (4.1% among non-donors vs 2.7% among those with more than 30 lifetime donations) was reported [88] but this was not confirmed on later follow up [89]. Increased insulin sensitivity and decreased insulin secretion were observed in blood donors compared with non-donors [90]. Decreased serum ferritin in highly frequent blood donors (≥8 per 2 years) was associated with increased flow-mediated dilation, in comparison with infrequent donors (1-2 per 2 years), but no improvement of insulin sensitivity was observed [91]. Ten healthy blood donors receiving 4-weekly phlebotomy (500 ml) showed significantly reduced insulin and glucose by 39% and 19% respectively in an OGTT. This study unfortunately had no control group [92]. A phlebotomized group showed improved homeostasis model assessment of insulin resistance (HOMA-IR) compared with nutritional counseling alone. This outcome was independent of

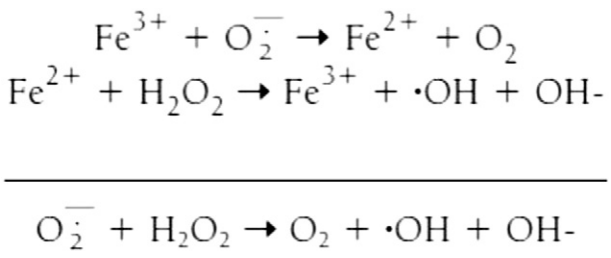


Fig. 2 – Metal Catalyzed Haber–Weiss reaction. Highly reactive •OH could be generated from an interaction between O_2^- snd H_2O_2. This net reaction is catalyzed by reduction of ferric ion to ferrous (the first step) and involves the second step of the Fenton reaction. Fe^{3+}, ferric; O_2^-, superoxide; Fe^{2+}, ferrous; O_2, oxygen; H_2O_2, hydrogen peroxide; •OH, hydroxyl radicals, OH–, hydroxyl ion.

changes in BMI [93]. Monthly or bimonthly phlebotomy (500 ml) in a group with glucose intolerance showed reduced fasting glucose and insulin levels using an OGTT but again there was no control group [94] while 28 subjects with diabetes randomized to three phlebotomies of 500 ml each at 2 week intervals, showed increased insulin sensitivity, assessed by an insulin tolerance test, at 12 months [95].

In summary, data from meta-analyses confirm that increased dietary heme iron and increased iron status measured by serum ferritin are associated with an increased risk of T2DM. Currently no controlled, randomized intervention studies to reduce iron levels with appropriate outcome measures [96] have been performed.

10. Trimethylamine N-Oxide from L-Carnitine and Phosphatidylcholine

Different food intake patterns can alter the colonization and establishment of gut microbiota at birth and alter the microbiota in adults [97]. L-carnitine and phosphatidylcholine from red meat are associated with metabolic disorders and coronary heart disease (CHD) [98,99]. Dietary phosphatidylcholine is broken down to choline in the intestine. Intestinal microbiota change this choline into trimethylamine (TMA), and then TMA is metabolized to TMAO after absorption by hepatic flavin mono-oxygenases [99,100]. L-carnitine is similarly metabolized to TMAO by the microbiota. Dietary TMAO increased fasting insulin levels and HOMA-IR and produced impaired glucose tolerance in high fat diet-fed mice [98]. Vegans or vegetarians were reported to have lower fasting plasma levels of L-carnitine and to produce less TMAO from dietary L-carnitine compared with omnivorous subjects [99]. A study investigating the association between fasting plasma TMAO levels and incident cardiovascular events over 3 years in 4007 primarily male patients undergoing elective coronary angiography showed an HR of 2.54 (95% CI 1.96–3.28, P < 0.001) for death, MI or stroke in the highest TMAO quartile, compared with the lowest TMAO quartile after 3 years. After adjustment for traditional risk factors and log-transformed high-sensitivity C-reactive protein levels, elevated plasma levels of TMAO remained a significant predictor of the risk of major adverse cardiovascular events (HR: 1.88, 95% CI 1.44 – 2.44, P <0.001) [101].

11. Elevated Protein Intake

Increased plasma branched amino acids (BCAAs) such as leucine, isoleucine and valine have been associated with insulin resistance [102,103] and levels of glycated hemoglobin (HbA1c) [102,104]. Increased BCAA concentration observed in obese children and adults were associated with the development of T2DM [105]. Non-diabetic individuals with a high protein intake of 1.97 ± 0.3 g/kg/day over 6 months showed reduced glucose disposal during an intravenous glucose

Table 3 – Meta-analyses of serum ferritin or dietary heme iron and risk of T2DM.

Reference	Studies included	Participants	Comparison Highest quintile vs Lowest quintile	RR	95% CI	P-trend	I^2 (%)	Q
Serum ferritin								
Zhao et al. [120]	6 prospective studies	4366 diabetic patients and 41,091 controls	Highest (–) vs Lowest (–)	1.66	1.15–2.39	0.01	66.3	14.84
	6 cross-sectional studies		Highest (–) vs Lowest (–)	2.29	1.48–3.54	< 0.001	88.7	44.16
Bao et al. [121]	6 studies	10,016 participants and 2198 cases	Highest (136 μg/L) vs Lowest (34 μg/L)	1.63	1.03–2.56	0.036	–	–
Kunutsor et al. [122]	9 studies	26,339 participants and 3391 cases	Highest (222 μg/L) vs Lowest (0 μg/L)	1.73	1.35–2.22	0.014	58	–
Heme iron								
Zhao et al. [120]	4 prospective cohort studies	9246 diabetic patients and 179,689 controls	Highest (–) vs Lowest (–)	1.31	1.21–1.43	0.71	0	1.39
Bao et al. [121]	5 studies	197,488 participants and 9269 cases	Highest (2.39 mg/day) vs Lowest (0.56 mg/day)	1.33	1.19–1.48	< 0.001	27.4	–
Kunutsor et al. [122]	3 studies	159,123 participants and 7688 cases	Highest (–) vs Lowest (–)	1.28	1.16–1.41	–	–	–

T2DM, type 2 diabetes mellitus; RR, relative risk; 95% CI, 95% confidence interval; I^2, indicator of heterogeneity between studies; Q, a statistical test of heterogeneity; vs, versus; –, not given.

tolerance test and decreased insulin sensitivity as assessed by a euglycemic hyperinsulemic clamp, compared with a normal dietary protein group with 0.74 g/kg/day [106]. In seven healthy men, a 2.1-fold elevated amino acid concentrations (from intravenous delivery) decreased whole-body glucose disposal by 25% ($P < 0.01$) leading to a 64% reduction in muscle glycogen synthesis ($P < 0.01$) [107]. This was confirmed by another group [108]. Leucine, as does insulin, activates the mammalian target of rapamycin complex 1 (mTORC1) and ribosomal protein S6 kinase β 1 (S6k1) [103]. Persistently activated mTORC1 and S6k1 result in serine phosphorylation of IRS-1 and IRS-2 which interrupts signaling and might target IRS 1 for proteolysis via a proteasomal pathway [103]. Weight-stable diets high in either dairy protein or dairy saturated fat did not influence insulin sensitivity in overweight and obese adults although BCAA levels were related to insulin clearance [36]. In a randomized crossover weight-stable study of 15 postmenopausal women with abdominal obesity, partly replacing meat protein with soy protein increased insulin sensitivity ($P = 0.048$) as assessed by a FSIGT, possibly via increased isoflavones [109]. Epidemiological studies showed no clear inverse association between soy intake and the incidence of T2DM [110]. Soy protein was associated with a reduced risk of metabolic syndrome in Chinese women and with an elevated risk in men [111].

12. Endocrine Disruptors

Endocrine disrupting chemicals (EDCs) such as dioxins, phthalates and bisphenol A (BPA) are industrial chemicals used in plastic products, medical devices and food packaging [112] and increase T2DM risk by inducing both insulin resistance and impaired pancreatic β-cell function [113]. A review [112] of dietary phthalate exposures on the basis of all available data from North America, Europe and Asia, found that di-2-ethylhexyl phthalate (DEHP) concentrations in beef ranged from the limit of detection in samples from the USA to 1100 μg/kg in Canada. When beef, poultry, pork, other meats were combined, DEHP concentrations were high (175.8–758.3 μg/kg). Highest DEHP intakes in adolescents (90.6 μg/kg-day) and infants (21.6 μg/kg-day) occurred on a diet high in meat and dairy, compared with other dietary patterns. DEHP content in red meat and processed meat varies from region to region [112]. Meat and meat products were the major source of phthalates and the second source of BPA in Norway, but the levels were considerably lower than tolerable daily intake (TDI) values [114]. Even though phthalate concentrations mostly declined after cooking at home [115], long term exposure to EDCs at a predicted 'safe' (50 μg/kg per day) dose of BPA derived from red and processed meat might alter glucose metabolism as seen in rats [116].

13. Conclusion

Meta-analyses of prospective studies indicate a positive correlation between red and processed meat consumption and risk of T2DM. Red and processed meat has several components potentially linked to an increased risk of T2DM. SFA, AGEs, nitrites, heme iron, TMAO, elevated BCAAs and EDCs are potential contributors to insulin resistance. Even though red and processed meat is a good source of iron, zinc and vitamin B12, certainly red and processed meat contains detrimental components. It is hard to single out the most likely candidates. However, heme iron, AGEs and TMAO have strong mechanistic links with insulin resistance. Excessive dietary AGEs are closely associated with insulin resistance. Increased heme iron stores can facilitate the oxidation of free fatty acids producing increased free radicals which damage beta cells. The interaction among components may play a key role in accelerating the development of T2DM. However more intervention studies are required to clarify these relationships and to draw a firm conclusion about their roles in T2DM. There are mixed results from interventions related to the effects of SFA intake on insulin resistance, very few dietary AGE clinical intervention studies with dynamic measures of insulin resistance, and few studies of dietary heme iron intake with good measures of insulin sensitivity.

The strengths of the data linking red meat with T2DM are a series of very plausible mechanisms which we have outlined. The weakness of the case against red meat is the complete lack of any short or long term intervention data showing that red meat intake increases insulin resistance or reduces pancreatic function. This is a severe limitation and without this the case against red meat is relatively weak.

Until there is stronger evidence available fresh meat only trimmed of fat, is probably the best dietary option. Cooking techniques such as boiling, steaming, stewing and poaching are recommended rather than using high, dry heat such as broiling, deep-frying, baking, roasting, and grilling to prevent AGES formation.

In light of the evidence that people who consume high red and processed meat are more likely to consume higher refined grains, desserts, sweets, high fat dairy and less fiber than a prudent diet [117], and to have a higher BMI [118], red and processed meat consumption is closely associated with high risk behaviors. Therefore, global preventive measures are required to minimize the risk of diabetes [6,7]. These include increased physical activity and a healthy diet (high fiber, low saturated fat and appropriate energy intake). Systematic prevention programs using evidence-based recommendations from lifestyle intervention programs and population based prevention programs with active cooperation with local and national government, organizations and services are required [7].

Author Contribution

All authors conceived of the manuscript structure and contributed to the writing and editing.

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Conflict of Interest

The authors have no conflicts of interest related to this manuscript.

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Letter to the Editor

Response to the comment by Kuipers and Pruiboom

We thank Kuipers and Pruiboom [1] for their comments on our article [2] and agree that Neu5GC may well be an explanation for the association of red meat with diabetes and CVD and that red meat per se may significantly alter the microbiome and produce effects independently of TMA production. However there is data in the literature that omnivores have higher urinary TMAO excretion and higher plasma TMAO compared to vegans [3]. The former was at least 4 fold higher and latter was about 25–30% higher. In addition feeding carnitine or steak increases plasma TMAO up to 6 μmol/L in omnivores with no changes in vegans and the plasma TMAO was related to microbiota composition. There were significant differences in microbiota composition between vegans/vegetarians and omnivores

Yours sincerely

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3 LITERATURE REVIEW: DAIRY AND INSULIN SENSITIVITY

3.1 Association between dairy and insulin resistance

3.1.1 Epidemiological studies

Most meta-analyses have shown that total dairy is inversely associated with the risk of T2DM [44-46], while one study has shown no association [47].

In the latest and largest dose-response meta-analysis of 22 cohort studies [44], total and low-fat dairy were linearly associated with a lower risk of T2DM (RR: 0.97 per 200 g/d increment; 95% CI: 0.95, 1.00; P = 0.04; I^2 = 66% and RR: 0.96 per 200 g/d; 95% CI: 0.92, 1.00; P = 0.072; I^2 = 68%). High-fat dairy was not associated with the risk of T2DM (RR: 0.98 per 200 g/d; 95% CI: 0.93, 1.04; P = 0.52). Total milk, low-fat milk, high-fat milk, cheese and cream were not associated with the risk of T2DM. Yoghurt (at 80 g/d of yoghurt, RR: 0.86 compared with 0 g/d of yoghurt; 95% CI: 0.83, 0.90; P < 0.001; I^2 = 73%) and ice cream (at 10 g/d of ice cream, RR: 0.81; 95% CI: 0.78, 0.85; P < 0.001; I^2 = 86%) showed nonlinear inverse associations, with no added incremental benefits observed at a higher intake [44].

Total dairy [48], low fat dairy [45, 49, 50] and full fat dairy in Japanese participants [51] have all been associated with decreased insulin resistance.

The PREDIMED study in a high fat consuming Spanish population showed that low-fat dairy products (HR: 0.65; 95% CI: 0.45, 0.94; P < 0.05; highest *vs.* lowest), mainly low-fat milk (HR: 0.67; 95% CI: 0.46, 0.95; P < 0.05; highest *vs*. lowest) and both low-fat and whole-fat yoghurt (HR: 0.60; 95% CI: 0.42, 0.86; P = 0.002; highest *vs*. lowest) were associated with a lower risk of T2DM after multivariate adjustment in older Mediterranean adults at high cardiovascular risk during a median follow-up of 4.1 years [52].

3.1.2 Intervention studies

Dairy products have shown mixed results in interventions. A randomised crossover study of 23 overweight and obese subjects showed that the consumption of 4 servings/d of low-fat milk and yoghurt for 6 months lowered insulin resistance as assessed by Homeostasis Model Assessment-Insulin Resistance (HOMA-IR), compared with 2 servings/d for 6 months with no changes in weight and lipids [53]. Compliance was poor as there was no difference in macronutrient or calcium intake and there were many dropouts (40%). In contrast, Turner et al. [54] showed that 4–6 servings/d of low-fat dairy for 4 weeks decreased insulin sensitivity

compared with ≥ 200 g/d of red meat and a control diet with no dairy or red meat in 47 overweight and obese subjects [54]. A systematic review of intervention studies showed no consistent positive or negative effects of dairy [55].

3.1.3 Acute studies

In a 3-h acute meal setting, 43 subjects without T2DM consumed two isoenergetic meals which were equal in 31 g protein, 46 g carbohydrate and 20g fat contents (total ~ 2090kJ; 36% carbohydrate; 36% fat; 18 % saturated fat; 25% protein). One meal consisted of lean red meat, wholemeal bread and orange juice and the other contained skim milk, low-fat yoghurt, cheese and wholemeal bread. The two test meals showed a similar metabolic response with no differences in the insulin AUC and iAUC, and the glucose AUC, except the glucose iAUC was higher after the dairy meal than after the red meat meal [56].

3.2 Effects of dairy components on insulin sensitivity

The Insulin Resistance Atherosclerosis Study of 1,036 individuals with no diabetes at baseline with a 5-year follow-up period showed a positive association between magnesium and calcium intake and insulin sensitivity, but no association between dairy intake and insulin sensitivity [57].

A meta-analysis of observational studies examining the association between dairy products consumption and the risk of T2DM found that the highest intake of combined vitamin D and calcium was inversely associated with the risk of T2DM [58], suggesting that inadequate intake vitamin D and calcium may negatively affect glucose metabolism [58].

Milk proteins are predominantly comprised of 20% whey and 80% casein [59]. In overweight subjects, the consumption of whey lowered fasting insulin concentrations and insulin resistance as assessed by HOMA-IR [60, 61]. Whey protein also acutely decreased the postprandial glycemia [62-64]. Higher consumption of whey and casein elevated insulin release as assessed by plasma C-peptide concentrations [59]. Whey protein contains branched-chain amino acids (BCAAs: leucine, isoleucine and valine) which could exert an insulinotropic effect [65-67] with potential mechanisms including the activations of the mammalian rapamycin (mTOR) signalling pathway in beta cells, especially by leucine [68, 69] and the incretin system. The mTOR pathway regulates cell growth, metabolism, proliferation and survival in response to growth factors, cellular oxygen or energy status and amino acid availability [70, 71]. The incretin system includes postprandial responses of glucose-dependent insulinotropic polypeptide and glucagon-like peptide 1 (GLP-1) [64, 65].

However, elevated fasting BCAAs concentrations have been correlated with insulin resistance and are associated with an increased risk of T2DM [72-75]. Higher concentrations of BCAAs chronically activate mTOR complex 1 (mTORC1) leading to insulin resistance via phosphorylation of insulin receptor substrate 1 (IRS-1) [70, 76].

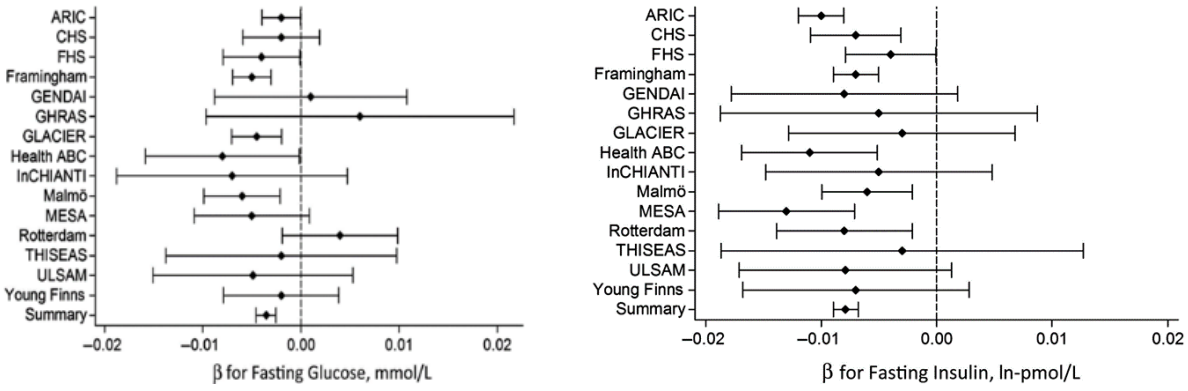
Dairy fat contains predominantly 70% saturated fatty acids (SFAs) followed by 25% monounsaturated fat and 2.3% polyunsaturated fat [77]. Cows' milk is comprised of short- and medium chain fatty acids with 4-10 carbon atoms and odd numbered fatty acids including pentadecanoic acid (C15:0) and margaric acid (17:0) [77]. A prospective case-cohort study of 3,737 adults with a 4-year follow-up period found that higher concentrations of plasma phospholipid 15:0 were associated with a reduced incidence of T2DM [78].

4 LITERATURE REVIEW: WHOLE GRAINS, NUTS AND LEGUMES AND INSULIN SENSITIVITY

4.1 Association between the dietary pattern rich in whole grains, nuts, legumes and insulin resistance

A meta-analysis of 7 prospective studies reported that a high consumption of whole grains, nuts and beans abundant in magnesium reduced the risk of T2DM by 14% (95% CI: 0.77, 0.95) [79]. A meta-analysis of 15 cohort studies also showed that a diet high in whole grains, fish, fruits, vegetables and nuts/seeds and low in red and processed meat, sweets, sugar-sweetened beverages and fried potatoes was associated with lower fasting glucose and insulin concentrations [80].

FIGURE 1: Associations between diet score and fasting glucose and insulin concentration



The two forest plots in FIGURE 1 showed associations between the healthy diet score and fasting glucose and insulin concentration. Regression coefficients (β) represented the difference in fasting glucose or in fasting insulin concentration per 1-unit increase in diet score after adjustment for the covariates: energy intake, age, sex, field centre, population substructure, smoking, physical activity level, educational level, alcohol consumption and body mass index [80].

4.2 Association between whole grains and insulin resistance

4.2.1 Epidemiological studies

Whole grains have been shown to have a favourable effect on the risk of T2DM [81, 82]. A meta-analysis of prospective studies showed that 3 servings/day of whole grains was related to a 32% lower risk of T2DM, but no association between refined grain consumption and the risk of T2DM was observed [81]. A recent prospective study with a median 7.9-year follow-up period, showed that one serving of whole grain per day reduced the risk of T2DM by 27% and 2 servings of whole grain per day lowered the risk of T2DM by 43% [82].

4.2.2 Intervention studies

Intervention studies with a weight-stable design have shown inconsistent findings. Several studies found a favourable effect of whole grain on insulin sensitivity or postprandial glucose and insulin [83-87], but others found no effect [88, 89].

4.2.3 Acute studies

A randomised crossover meal study of 15 overweight (BMI $\geq$ 23 kg/m^2) Asian Indians showed that 200 g/day of brown rice and 200 g/day of brown rice with 50 g/day of legumes significantly attenuated 24-h glycaemic- and insulin responses, compared with 200 g/day of white rice alone [90].

4.3. Association between nuts and insulin resistance

4.3.1 Epidemiological studies

Nuts have been shown to be protective [91] or neutral [92, 93] against the risk of T2DM. The Nurses' Health Study with a16-year follow-up period found that women who consumed nuts 5 times or more per week decreased incident T2DM by 27% (95% CI: 0.60, 0.89; P < 0.001), compared with women who rarely or never consumed nuts [91]. In contrast, two meta-analyses of prospective studies showed no significant association between nut consumption and the risk of T2DM [92, 93].

4.3.2 Intervention studies

Most interventions have shown a favourable effect of nuts on the risk of T2DM or glycemic control in T2DM [94-99]. A meta-analysis of randomised controlled interventions comparing a diet supplemented with tree nuts and an isocaloric diet without tree nuts in subjects with T2DM, showed that a diet with tree nuts at a median dose of 56 g/d significantly attenuated HbA1c (P = 0.0003) and fasting glucose (P = 0.03) compared with a diet without tree nuts, while fasting insulin and HOMA-IR did not differ [94]. A Mediterranean diet enriched with mixed nuts (30 g/day) decreased plasma glucose concentrations and insulin resistance after 3

months in 257 adults with a high risk of cardiovascular disease compared with a low nut diet [95]. In the Predimed-Reus population that had an annual oral glucose tolerance test, a Mediterranean diet supplemented with mixed nuts (30 g/day) decreased the incidence of T2DM by 52% after a median follow-up of 4 years in 145 adults with a high risk of cardiovascular disease compared with a low nut diet (a low-fat diet where all types of fat, from both animal and vegetable sources were advised to be reduced but overall fat differences achieved were minimal) [96]. A low-fat diet consumed with walnuts (30 g/day) for 1 year significantly lowered fasting insulin concentrations in subjects with T2DM, compared with a low-fat diet alone [97]. A healthy diet supplemented with nuts (30 g/day: 15 g walnuts, 7.5 g almonds and 7.5 hazelnuts) for 3 months significantly decreased fasting insulin concentrations and insulin resistance as assessed by HOMA-IR in subjects with the metabolic syndrome, compared with a healthy diet alone [98].

In contrast, diets supplemented with almonds (100 g/day) consumed for 4 weeks did not alter insulin sensitivity in either healthy subjects or subjects with T2DM [100]. A modified low-fat diet supplemented with walnuts (30 g/day) did not alter HbA1c concentrations compared with a modified low-fat or a low-fat diet [101]. A diet advised for each group was as follows: (1) a low fat diet group received dietary advice targeting ≤30% energy as fat and <10% saturated fat; (2) a modified low fat diet group received dietary advice targeting ≤30% energy as fat and <10% saturated fat plus an exchange list; (3) a modified-fat diet and walnut group received dietary advice targeting ≤30% energy as fat and <10% saturated fat plus an exchange list (30 g of walnuts/day as one exchange). The dietary advice for three groups included recommended serving size of carbohydrate foods, the type of protein-rich and low-fat foods, oils/spreads rich in monounsaturated fatty acid or polyunsaturated fatty acid and fish consumption (2 fish meals/week) [101].

4.3.3 Acute studies

In a 3-h acute meal study with a randomised crossover design [102], 20 subjects with metabolic syndrome consumed 5 test meals [3 meals containing 50 g of available carbohydrate (CHO): white bread, white bread + butter + cheese and white bread + 3 oz pistachios; 2 meals containing 12 g of available CHO: white bread and 3 oz pistachios]. The white bread (50 g of available CHO) consumed with 3 oz pistachios significantly lowered the glycemic response compared with the white bread alone, and lowered insulin concentrations

during the second hour of the test compared with the white bread consumed with butter and cheese [102].

Fifteen healthy subjects ingested 2 test meals (97 g white bread, 97 g white bread + 60 g almonds). Almonds decreased postprandial glycemic and insulinemic responses to white bread [103]. The consumption of 28 g of almonds prior to a high-starch meal attenuated postprandial glycemia in subjects with well-controlled T2DM, but not in healthy subjects without T2DM [104].

4.4 Association between legumes and insulin resistance

4.4.1 Epidemiological studies

Two studies have shown that legumes appear to decrease the risk of T2DM [105, 106]. One study has shown no protective effect of soy consumption on T2DM [107].

A prospective cohort study of middle-aged Chinese women has shown an inverse association between legumes and the incidence of T2DM. The multivariate-adjusted RR for the upper quintile was 0.62 (95% CI: 0.51, 0.74) compared with the lower quintile. This study also has shown an inverse association between soybeans and the risk of T2DM with a RR in the upper quintile of 0.53 (95% CI: 0.45, 0.62) compared with the lower quintile [105]. The Japan Public Health Centre-Based Prospective Study has shown that soy products overall were not associated with T2DM. However, a higher intake of soy products was associated with a lower risk of T2DM in overweight women (BMI $\geq$ 25 kg/m^2) [106]. Unlike these studies [105, 106], no association between soy consumption and the risk of T2DM was found in the Hawaii component of the Multiethnic Cohort study [107].

4.4.2 Intervention studies

Legumes have shown mixed results in intervention studies. The consumption of legumes (peas, lentils, beans, chickpeas) was favourably associated with improved glycaemic control [108, 109]. A meta-analysis of 41 randomised controlled studies in people with and without diabetes found that legumes alone (peas, lentils, beans, chickpeas; 11 studies) attenuated fasting blood glucose [standardised mean difference (SMD): -0.82; 95% CI: -1.36, -0.27] and insulin (SMD: -0.49; 95% CI: -0.93, -0.04), while legumes as part of low-GI diets (19 studies) lowered fructosamine or HbAlc (SMD: -0.28; 95% CI: -0.42, -0.14), while legumes as part of high-fiber diets (11 studies) attenuated fasting blood glucose and fructosamine or HbA1c [109]. Whole soy foods or soy diets had a beneficial effect on fasting glucose, but purified

isoflavones and isolated soy protein did not significantly alter fasting glucose and insulin concentrations in a later meta-analysis of randomized controlled studies of soy [110].

In contrast, two randomised cross-over weight stable studies showed that daily consumption of half a cup of legumes for 8 weeks did not favourably affect glucose, insulin, HbA1c and hs-CRP (high sensitivity C-reactive protein) in hyperinsulinaemic adults [111] or hypercholesterolaemic adults [112].

4.4.3 Acute studies

In acute meal studies, legumes decreased postprandial glucose responses in healthy people [113-115]. A randomised crossover meal study in 17 subjects with T2DM showed that three test meals (pinto or dark red kidney or black beans with white rice, which were matched for 50 g carbohydrate) attenuated the postprandial glycaemic response compared with the white rice control [116].

4.5 Components found in whole grains, nuts and legumes

Whole grains are good sources of non-digestible complex polysaccharides such as soluble and insoluble fibers including inulin, β-glucan, and resistant starches, and non-carbohydrate components including phenolic acids (ferulic acid, vanillic acid, caffeic acid, syringic acid and P-cumaric acid), carotenoids, tocopherols, phytoestrogens and phytates [117, 118]. Nuts are high-energy foods rich in total fat and mono- and polyunsaturated fatty acids. They have proteins, peptides, fiber, phytosterols, polyphenols, α and γ tocopherols, other antioxidant vitamins and minerals (magnesium) [117, 119]. Legumes (beans, peas and lentils) are abundant in dietary protein, oligosaccharides, dietary fiber and resistant starches [117]. Linoleic acid, α-linolenic acid, isoflavones (daidzein, genistein, glycitein), phenolic acids, saponins and phytic acid; polyphenols (pelargonidin, cyanidin, delphinidin, malvidin) could also be bioactive components in legumes [117].

4.6 Association between fruits and vegetables and insulin resistance

Most prospective studies have shown no effect of fruits and vegetables on insulin resistance or the risk of T2DM [120-122]. In a meta-analysis of 5 cohort studies, the RR for the daily consumption of at least 5 servings of fruits and vegetables was 0.96 (95% CI: 0.79, 1.17) compared with the lowest intake or non- consumption. When fruits and vegetables were analysed separately, (4 cohort studies each), the RR for either more than 3 servings of fruits or more than 3 servings of vegetables alone was 1.01 (95% CI: 0.88, 1.15) and 0.97 (95% CI:

0.86, 1.10) respectively, compared with less than 3 servings [122]. Other meta-analysis also showed no association between increased consumption of vegetables, fruits, or vegetables and fruits combined (HR: 1.00; 95% CI: 0.92, 1.09; highest *vs.* lowest) [120]. Only a high consumption of green leafy vegetables was associated with a lower risk of T2DM [120, 121].

5 LITERATURE REVIEW: POLYPHENOLS AND GLYCEMIC CONTROL

5.1 Preface

This chapter is the second part of chapter 4 consisting of a review article. As mentioned in the first part of chapter 4, whole grains, nuts and legumes are good sources of polyphenols, which may exert an anti-diabetic effect. Therefore, we conducted an extensive literature review based on animal and human studies. The aim of this article published in 2016 was to clarify the association between dietary polyphenols and glucose metabolism and explore potential mechanisms of action.

5.2 Published article

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Review

Polyphenols and Glycemic Control

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Abstract: Growing evidence from animal studies supports the anti-diabetic properties of some dietary polyphenols, suggesting that dietary polyphenols could be one dietary therapy for the prevention and management of Type 2 diabetes. This review aims to address the potential mechanisms of action of dietary polyphenols in the regulation of glucose homeostasis and insulin sensitivity based on *in vitro* and *in vivo* studies, and to provide a comprehensive overview of the anti-diabetic effects of commonly consumed dietary polyphenols including polyphenol-rich mixed diets, tea and coffee, chocolate and cocoa, cinnamon, grape, pomegranate, red wine, berries and olive oil, with a focus on human clinical trials. Dietary polyphenols may inhibit α-amylase and α-glucosidase, inhibit glucose absorption in the intestine by sodium-dependent glucose transporter 1 (SGLT1), stimulate insulin secretion and reduce hepatic glucose output. Polyphenols may also enhance insulin-dependent glucose uptake, activate 5′ adenosine monophosphate-activated protein kinase (AMPK), modify the microbiome and have anti-inflammatory effects. However, human epidemiological and intervention studies have shown inconsistent results. Further intervention studies are essential to clarify the conflicting findings and confirm or refute the anti-diabetic effects of dietary polyphenols.

Keywords: dietary polyphenols; insulin sensitivity; glucose homeostasis; clinical trials

1. Introduction

Type 2 diabetes (T2D) is a chronic disease of metabolic dysregulation, most notably abnormal glucose metabolism, accompanied by complications including cardiovascular disease, retinopathy, nephropathy, neuropathy, leg ulcers and gangrene [1]. In 2013, 382 million people were estimated to have diabetes globally and this number is projected to be 592 million by 2035 [2]. Identification of modifiable lifestyle factors including dietary factors that reduce the incidence of T2D is a vital area of research [3,4]. One dietary factor of interest is consumption of polyphenol-rich foods. Dietary polyphenols have been suggested to lower the risk of T2D [5–7].

This review aims to summarize relevant epidemiological and clinical studies linking polyphenol-rich foods to the risk of T2D, and to describe the multiple mechanisms of action of polyphenol-rich foods through which these effects are mediated.

2. Polyphenols

Polyphenols are a large and heterogeneous group of phytochemicals containing phenol rings [6]. Several hundred different polyphenols are found in plant-based foods including vegetables (particularly, broccoli, onion and cabbage), fruits (grapes, pears, apples, cherries and various berries contain up to 200–300 mg polyphenols per 100 g fresh weight), legumes (soybean), cereals, plant-derived beverages and chocolate [5,8,9]. Approximately 100 mg of polyphenols are identified in a cup of coffee or tea or a glass of red wine [8,9]. Polyphenols are divided into flavonoids, phenolic acids, stilbenes, and lignans [9]. Flavonoids include flavones, flavonols flavanols, flavanones,

isoflavones, and anthocyanins [7,8,10]. The most common phenolic acids are caffeic acid (present in many fruits and vegetables, most often esterified with quinic acid as in chlorogenic acid, which is a major phenolic compound in coffee) and ferulic acid present in cereals which is esterified to hemicelluloses in the cell wall. The best studied stilbene is resveratrol in grapes, grape products and red wine [10]. The richest source of lignans is linseed present mainly as secoisolariciresinol (up to 3.7 g/kg dry weight) with low quantities of matairesinol, while minor sources are cereals, lentils, fruits (pears, prunes) and vegetables (garlic, asparagus, carrots) [10]. Chemical structures and dietary sources of different groups of polyphenols are shown in Figure 1.

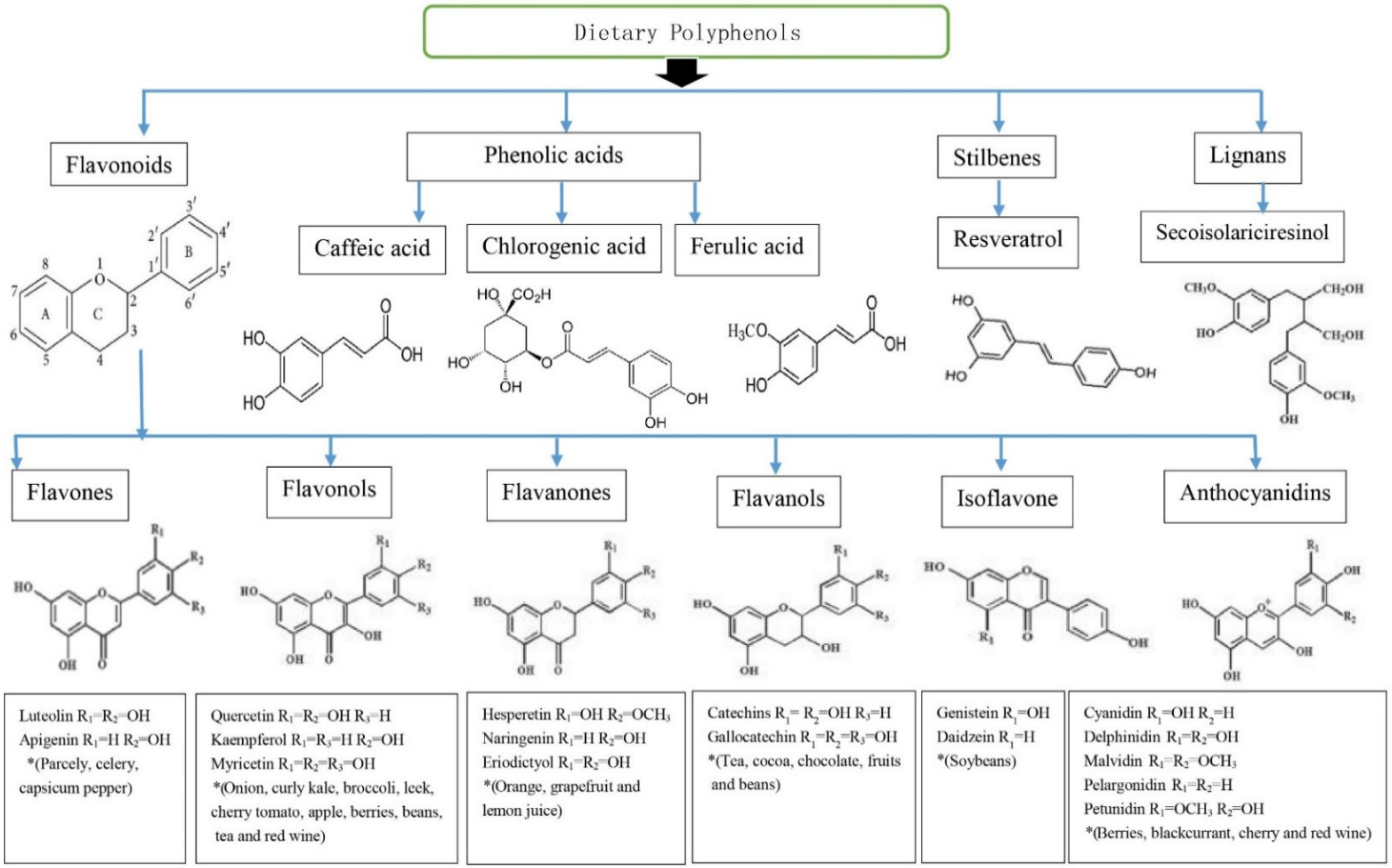


Figure 1. Chemical structures and dietary sources of different groups of polyphenols. Flavonoids are most abundant in four groups of dietary polyphenols and share a basic structure. Resveratrol is one of a subclass of stilbenes. Some phenolic acids are caffeic acid, chlorogenic acid and ferulic acid. Flavones, flavonols, flavanones, flavanols, isoflavone and anthocyanidins are main subclasses of flavonoids. Individual compound of those are characterised in accordance with the arrangement and the number of the hydroxyl groups and their extent of alkylation and/or glycosylation [9]. * Food sources from six main subclasses of flavonoids are briefly described. Typically, quercetin is rich in onion, tea, and apple. Hesperetin is rich in citrus fruits, genistein and daidzein are rich in soybeans. Anthocyanins including cyanidins contribute colour to many red fruits such as strawberry, raspberry and blackcurrant [8,10].

3. Epidemiological Studies of Dietary Polyphenols

Epidemiological studies support favourable effects of polyphenol-rich diets in preventing and managing T2D [6].

3.1. Flavonoids

A prospective study in 1111 T2D case-control pairs in the Nurses' Health Study I and II (NHS and NHSII) investigating urinary excretion of eight polyphenol metabolites found that flavanones (naringenin and hesperetin) and flavonols (quercetin and isorhamnetin), as well as the phenolic acid, caffeic acid, were associated with a 39%–48% lower T2D risk during early follow-up (median of ⩽4.6 years since urine sample collection), but not during later follow-up which ranged from 4.6 to 11.4 years [11]. Metabolites of flavan-3-ols and ferulic acid were not associated with T2D risk in either period [11]. This is in accordance with the Women's Health Study of 38,018 US women aged ⩾45 years with an average 8.8 years of follow-up which reported that none of total flavonols and flavones, or quercetin, kaempferol, myricetin, apigenin, and luteolin were significantly associated with the risk of T2D [12]. However, apple consumption of ⩾1 apple/day showed a 28% lower T2D risk compared with no apple consumption (relative risk—RR 0.72; 95% confidence interval (CI) 0.56–0.92; $p = 0.006$) [12]. A Finnish study of 10,054 men and women with 526 T2D cases showed that intakes of apples (hazard ratio—HR 0.73; 95% CI 0.57–0.92; $p = 0.003$) and berries (HR 0.74; 95% CI 0.58–0.95; $p = 0.03$) were significantly associated with a lower risk of T2D [13]. These findings are consistent with prospective studies of 159,560 women in the NHS and NHS II, and 41,334 men in the Health Professionals Follow-Up Study [14], which also found that higher intakes of anthocyanins were significantly associated with a lower risk of T2D (HR 0.85; 95% CI 0.80–0.91; $p < 0.001$) [14]. Apples/pears (HR 0.77; ⩾5 servings/week *vs.* <1 serving/month; 95% CI 0.65–0.83; $p < 0.001$) and blueberries (HR 0.77; ⩾2 servings/week *vs.* <1 serving/month; 95% CI 0.68–0.87; $p < 0.001$) were inversely associated with T2D [14], but total flavonoid intake or other flavonoid subclasses were not associated with a lower risk of T2D [14]. An inverse association remained after adjustment for body mass index (BMI) or weight [13,14].

The Iowa Women's Health Study of mainly white, postmenopausal women did not observe any T2D-protective effect of total flavonoid intake or any of the flavonoid subclasses, including anthocyanins. On the other hand, regular red wine consumption of ⩾1 occasion/week was associated with a 16% lower T2D risk than wine consumption <1 occasion/week (HR 0.84; 95% CI 0.71–0.99), with similar findings for white wine, beer, and liquor [15], suggesting it is the alcohol *per se* and not the polyphenols which is associated with protection. Inconsistent findings might be explained due to the use of the older, less complete versions of the United States Department of Agriculture (USDA) database [12,15] and misclassification of intake in the baseline questionnaires [14]. Much of the epidemiological data is based on reported food intake rather than blood or urine measures of polyphenol metabolites and is thus relatively unreliable compared with more objective measures.

3.2. Phenolic Acids

Epidemiological studies showed a fairly consistent association between coffee (caffeinated and decaffeinated) [16–20] or green tea [21] consumption and a lower risk of T2D in a dose-response manner in most studies [16–21] except [22].

A meta-analysis of 28 prospective studies with 1,109,272 participants and 45,335 T2D cases showed an inverse association of coffee consumption with T2D in a dose-response manner (1–6 cups/day) [16]. Coffee consumption of six cups/day was associated with a 33% lower risk of T2D, compared with no coffee consumption (RR 0.67; 95% CI 0.61–0.74; $p < 0.001$). Both caffeinated and decaffeinated coffee were associated with a lower risk of T2D [16]. A retrospective cohort study of 17,413 Japanese men and women aged 40–65 years, based on completed five-year follow-up questionnaire found RRs of 0.67 (95% CI 0.47–0.94) for green tea intake of ⩾6 cups/day and 0.58 (95%

CI 0.37–0.90) for coffee intake of ⩾3 cups/day, compared with less than one cup/week [21]. The consumption of black or oolong teas was not associated with the risk of T2D [21]. Total caffeine intake was associated with a 33% lower risk of T2D [21]. These inverse associations were more prominent in women and in overweight men [21]. Similarly, another study [17] also observed a stronger inverse association between coffee, decaffeinated coffee and caffeine intake, and T2D incidence in women than in men as well as a stronger inverse association between coffee intake and T2D incidence in non-smokers and subjects with BMI <25 kg/m^2 [17] compared with smokers and overweight and obese people. Three other meta-analyses showed that tea intakes of ⩾3 cups/day (RR 0.84; 95% CI 0.73–0.97) [23] or ⩾4 cups/day [24] had a 16% reduction in the risk of developing T2D, compared with the lowest intake (RR 0.84; 95% CI 0.73–0.94) but a third meta-analysis [25] showed no effect.

A prospective cohort of 40,011 participants with a mean follow-up of 10 years and 918 cases of T2D [22] reported potential beneficial effects of both coffee and tea intakes, but there was no dose-response as coffee and/or tea intakes of at least three cups/day reduced the risk of T2D by 42%. Coffee intake of 4.1–6.0 cups/day and tea intake of >5.0 cups/day reduced the risk of T2D by 23% (HR 0.77; 95% CI 0.63–0.95; $p = 0.033$) and 37% (HR 0.63; 95% CI 0.47–0.86; $p = 0.002$), respectively [22]. The relative amount of coffee *vs.* tea was not important [22].

Positive effects of coffee and tea on a lower risk of T2D were independent of weight or BMI [16,18,20–22,24].

Observational studies have shown that higher coffee consumption was associated with decreased fasting insulin [26] and 2-h post-load glucose [27], and homeostatic model assessment levels [26,28,29], increased insulin sensitivity [30], but other studies have shown no association of coffee consumption with fasting insulin [27,31] or homeostasis model assessment of insulin resistance (HOMA–IR) [31]. Green tea consumption was positively associated with HOMA–IR ($p = 0.02$) with no dose-response [28]. The Whitehall II study, a British cohort study, showed no association between coffee, tea and the risk of T2D, although there was an association between combined intake of tea and coffee (⩾2 cups/day of both beverages) and the risk of T2D (HR 0.68; 95% CI 0.46–0.99; $p < 0.05$) [32].

4. Potential Mechanisms of Action of Polyphenols in Diabetes Risk

The hypoglycemic effects of dietary polyphenolic compounds may be related to inhibition of carbohydrate digestion by inhibiting salivary and pancreatic α-amylase and α-glucosidase in the small intestinal brush border, inhibition of glucose absorption, and stimulation of insulin secretion and protection of pancreatic β-cells against glucotoxicity. Polyphenols may suppress glucose release from the liver, and improve glucose uptake in peripheral tissues by modulating intracellular signalling [6]. Polyphenols have antioxidant activity and can inhibit advanced glycation endproduct (AGE) formation [5].

4.1. Carbohydrate Digestion and Glucose Absorption in the Intestine

The key enzymes involved in digestion of dietary carbohydrate are α-amylase and α-glucosidase. Maltose, maltotriose and α-dextrins are the three major products of α-amylase digestion. Further digestion occurs in the small intestine by α-glucosidase which is a class of brush-border bound enzymes which hydrolyse the terminal α-1, 4-linked glucose residues [6,33]. Glucose is taken into cells via transporters, predominately sodium-dependent glucose transporter (SGLT1) located in the brush border membrane at the apical side of enterocytes [34].

Inhibition of α-amylase and α-glucosidase activities *in vitro* has been demonstrated with dietary polyphenols from berries (strawberries, raspberries, blueberries and blackcurrants) [35], vegetables (pumpkin, beans, maize and eggplant) [36,37], black rice [38], legumes [39], green and black tea [33], tea polyphenols [40] and red wine [35,41]. Inhibition of glucose transport has been shown with flavonoids and phenolic acids [42–46].

4.2. Tissue Uptake of Glucose

Enhanced insulin-mediated glucose uptake *in vitro*, a glucose transporter 4-mediated process, has been shown with dietary polyphenols including epicatechin [47], epigallocatechin-3-*O*-gallate (EGCG) [48], grape seed-derived procyanidins [49,50], bitter melon [51], blueberry [52], canna indica root [53] and black soy bean [54]. Black soybean seed rich in anthocyanins (cyanidin 3-glucoside) and procyanidins (PCs) lowered glucose levels and improved insulin sensitivity by activation of 5′ adenosine monophosphate-activated protein kinase (AMPK) in the skeletal muscle and liver of type 2 diabetic mice. This activation was accompanied by the up-regulation of glucose transporter 4 GLUT4 in skeletal muscle and the down-regulation of gluconeogenesis in the liver in type 2 diabetic mice [54]. EGCG maintained insulin sensitivity in rat L6 muscle cells exposed to dexamethasone and dose-dependently increased glucose uptake and GLUT4 translocation through activation of phosphoinositide 3-kinase (PI3K) signalling and AMPK and PI3K activation [48]. Cinnamon has shown potential to promote insulin signalling, and GLUT4 translocation in Swiss albino mouse embryo fibroblast line 3T3-L1 adipocytes [55], and increase glucose uptake in insulin resistant rats induced by a high fructose diet [56,57].

4.3. Gut Microbiota

Only 5%–10% of the total intake of dietary polyphenols are directly absorbed through the stomach and the small intestine [58]. The majority of the ingested polyphenols reach the colon, thereafter undergoing intensive metabolism prior to absorption [58,59]. Some polyphenols are thought to exert a prebiotic effect by stimulating the growth and activity of some bacteria in the digestive tract [59]. After absorption, polyphenols undergo phase I and II biotransformation (sulfation, glucuronidation, methylation and glycine-conjugation) by enterocytes in the liver to increase hydrophilicity favouring urinary secretion [58]. Polyphenol metabolites derived from liver metabolism interact with adipose tissue, pancreas, muscle and liver, and may exert anti-diabetic effects [59]. Absorption of polyphenols can be affected by dosage, size of phenolic compound, prior diet, food matrix, gender and differences in the gut microbial populations [59].An increased level of faecal *Bifidobacteria* has been associated with improved glucose tolerance and diminished inflammatory markers such as the interleukins IL-6, IL-1α and IL-1β, tumor necrosis factor α and monocyte chemoattractant protein-1 [59–61]. A few clinical trials [62–67] have shown the potential prebiotic effects of dietary polyphenols to increase the population of *Bifidobacteria*. In a randomized, controlled, double-blind, crossover study of 22 health volunteers [62], the daily consumption of 494 mg cocoa flavanols for four weeks significantly increased the growth of *Lactobacillus* spp. ($p < 0.001$) and *Bifidobacterium* spp. ($p < 0.01$) relative to that of a drink containing only 29 mg flavanols and matched for fibre and other potential prebiotics.A significant reduction in C-reactive protein (CRP) ($p < 0.05$) was observed and changes in CRP were associated with changes in *Lactobacilli* counts ($p < 0.05$; $R^2 = 0.20$) [62]. Similarly, the daily consumption of red wine polyphenols for four weeks modulated the growth of gut microbiota [66] by significantly increasing *Bifidobacterium* and *Enterococcus*, *Prevotella*, *Bacteroides*, *Bacteroides uniformis*, *Eggerthella lenta*, and *Blautia coccoides-Eubacterium rectale* groups compared with baseline but there was no control drink for comparison. Improvements in cholesterol and CRP levels were associated with changes in the *Bifidobacteria* amount [66]. Daily consumption of 25 g of wild blueberry powder drink for six weeks increased *Bifidobacterium* spp. compared with the placebo drink (250 mL of water, 7.5 g of fructose, 7 g of glucose, 0.5 g of citric acid, 0.03 g of blueberry flavour and colors) [63]. A proanthocyanidin-rich extract from grape seeds administered to nine healthy adults for two weeks significantly increased *Bifidobacterium* but no control material was provided to volunteers [64].

4.4. Pancreatic β-Cell Function

Glucose-stimulated insulin secretion from pancreatic β-cells involves glucose entrance via glucose transporter 2 (GLUT2), increased ATP content through glycolysis and tricarboxylic acid cycle (TCA) activity, and then inactivation of ATP-sensitive K^+ (KATP) channels on the cell membrane [6,68]. Rat pancreatic β-cells, RIN-m5F treated with EGCG and the buckwheat flavonoid, rutin showed reduced glucotoxicity with activation of insulin receptor substrate 2 (IRS2) and AMPK signalling and elevated adenosine triphosphate (ATP) levels [69]. Activation of AMPK plays a critical role in the protection of β-cell against glucolipotoxicity [70]. The anti-diabetic drugs, metformin and thiazolidinediones (TZDs) are known to activate AMPK [69]. Chronic high glucose exposure directly increased intracellular reactive oxygen species (ROS) generation and disturbed mitochondrial function, uncoupling ATP generation and impairing insulin secretion [69]. Moreover, chronic high glucose exposure suppressed AMPK activity leading to increased lipogenesis [69]. This consequently resulted in impairing normal β-cell functioning [69].

Quercetin, apigenin, and luteolin inhibited cytokine-induced pancreatic β-cell damage through suppression of nuclear factor kappaB (NF-κB) activation in RINmF5 cells [71]. Quercetin showed protective effects by decreasing oxidative stress with preservation of pancreatic β-cell integrity in streptozotocin (STZ)-induced diabetes in rats [72]. Isolated rat islets exposed to (−) epicatechin (0.8 mmol/L) or quercetin (0.01 mmol/L) showed enhanced insulin secretion by approximately 44%–70% in the presence of 20 mmol/L glucose [73].

4.5. Liver Glucose Homeostasis

The liver is a key regulator of blood glucose levels in coordination with muscle and adipose tissues [6]. In the postprandial state, the liver stores glucose as glycogen via the glycogenesis pathway involving glucokinase (GK) which is a key enzyme in the regulation of glucose utilization in the liver, and glycogen synthase (GS). Under fasting conditions, the liver produces glucose via two routes—either glycogenolysis or gluconeogenesis involving pyruvate carboxylase, phosphoenolpyruvatecarboxykinase (PEPCK), fructose-1,6-bisphosphatase, and glucose-6-phosphatase [6].

Suppressed hepatic glucose production appears to account for decreased glucose levels in EGCG and green tea feeding and *in vitro* studies [74–76]. In the rodent study [76] of EGCC supplementation of either 0.25%–1% for seven weeks, a decrease of glucose levels in EGCC-treated db/db mice was observed in a dose dependent manner, compared with placebo-treated mice [76]. The GK mRNA expression was increased in the livers of mice supplemented with EGCC for seven weeks in a dose dependent manner and a decrease of PEPCK mRNA expression was observed in adipose tissue of EGCC supplemented mice. High-dose EGCC supplementation also increased acyl CoA oxidase-1 (ACO–1) and carnitine palmitoyl transferase-1β (CPT-1β) in both livers and adipose tissues [76]. Moreover, in H4IIE hepatoma cells, EGCC downregulated genes involved in gluconeogenesis and the synthesis of fatty acid, triacylglycerol and cholesterol, whereas genes involved in glycolysis (phosphofructokinase) and glucose transport (glucose transporter 1) were increased [76]. Supplementation of the soy isoflavones genistein and daidzein (0.02% in diet) improved glucose homeostasis in NOD mice with an increase in insulin/glucagon ratio and C-peptide level with preservation of insulin staining β-cell of pancreas. In the liver, glucose-6-phosphatase (G6Pase), PEPCK activities and β-oxidation of fatty acid were suppressed and lipogenesis was increased compared with the control group [77]. In db/db mice, the citrus flavonoids (hesperidin and naringin) were also found to lower glucose levels through up-regulating the hepatic GK via peroxisome proliferator-activated receptor gamma (PPAR γ), and upregulating adipocyte GLUT4. Naringin also suppressed PEPCK and G6Pase. This mechanism of citrus flavonoid is very similar to the mechanism of thiazolidinediones [78]. Similarly, areca nut procyanidins fed 10 mg/kg to multiple low dose streptozocin (MLD–STZ) treated mice for four weeks reduced fasting blood glucose, PEPCK and G6Pase and activated AMPKα [79].

In the fed state, dietary cyanidin 3-glucoside was shown to downregulate G6Pase and PEPCK by enhancing the phosphorylation of Akt and, forkhead box protein O1 (FOXO1—an important transcription factor) and by decreasing the nuclear translocation of FOXO1 in the liver and adipose tissues of high-fat diet-fed and db/db mice [80].

4.6. Inflammation

Low-grade inflammation and obesity-induced oxidative stress in the mitochondria may be partly responsible for insulin resistance. Elevated proinflammatory cytokines can alter metabolic homeostatic control systems leading to T2D [81]. Grape-seed procyanidins inhibited the production of proinflammatory molecules such as CRP, IL-6 and TNF-α and enhanced the production of adiponectin in fat fed rats [82]. Dietary cyanidin 3-glucoside (0.2% in diet) improved insulin sensitivity in both high-fat diet fed and obese db/db mice [80], decreased mRNA abundance of inflammatory cytokines including TNF-α, IL-6 and MCP-1 and suppressed macrophage infiltration in adipose tissue [80]. Procyanidins reduced gene expression of NF-κB, cyclooxygenase-2 protein (COX2), CRP, IL-6 and TNF-α in adipocytes [83,84], muscle [84] and liver [85,86] in both rats and mice. In male C57Bl/6j mice, eight-week supplementation of a high-fat diet with an anthocyanin-rich, 4% blueberry powder attenuated gene expression in macrophages in adipose tissue, for TNF-α, IL-6, MCP-1, inducible nitric oxide synthase, and improved whole body insulin sensitivity [87]. Quercetin also showed anti-inflammatory effects in male C57Bl/6j mice [88] and in obese Zucker rats [89]. Supplementation of a high-fat diet with 0.8% quercetin for eight weeks decreased interferon-γ, interleukin-1α, and interleukin-4 in male C57Bl/6j mice [88]. Administration of both 2 and 10 mg/kg of body weight of quercetin for 10 weeks improved insulin sensitivity and blood pressure in obese Zucker rats, but only the dose of 10 mg/kg reduced NOx concentration and visceral adipose tissue (VAT) TNF-α production, increased adiponectin and downregulated fat iNOS expression [89].

A summary of possible mechanisms of action is shown in Figure 2.

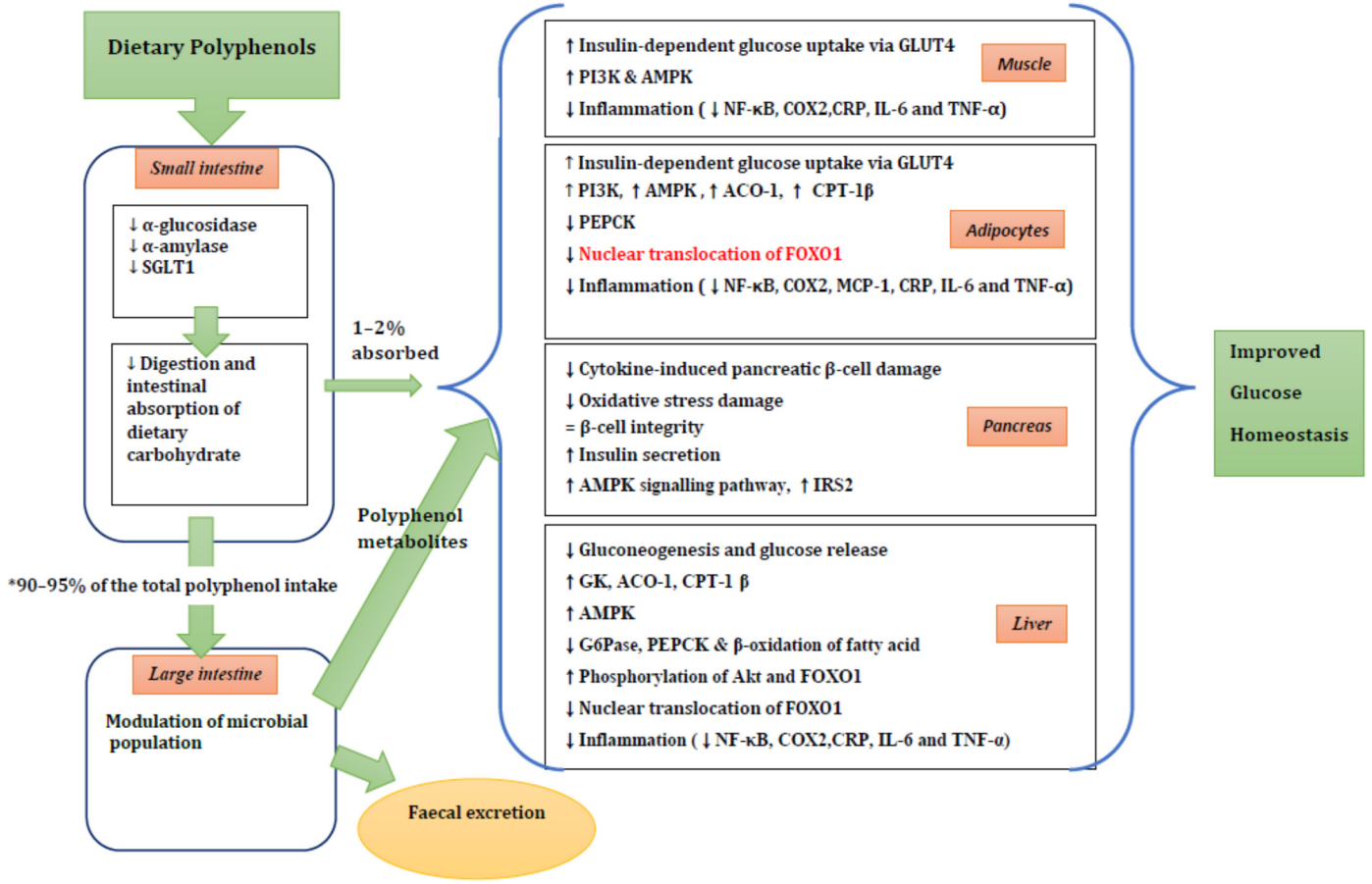


Figure 2. The summary of potential mechanisms linking dietary polyphenol metabolites to improved glucose homeostasis. ↑, increase; ↓, decrease; ↔, maintenance of stability. * 90%–95% of the ingested polyphenols reach the colon. See the text for more details. SGLT1, sodium-dependent glucose transporter; GLUT4, glucose transporter 4; PI3K, phosphoinositide 3-kinase; AMPK, 5′ adenosine monophosphate-activated protein kinase; NF-κB, nuclear factor kappaB; COX2, cyclooxygenase-2 protein; CRP, C-reactive protein; IL-6, interleukin 6; TNFα, tumor necrosis factor α; ACO-1, acyl CoA oxidase-1; CPT-1 β, carnitine palmitoyl transferase-1β; PEPCK, phosphoenolpyruvate carboxykinase; FOXO1, forkhead box protein O1; MCP-1, monocyte chemoattractant protein-1; IRS2, insulin receptor substrate 2; GK, glucokinase; AC0-1, acyl CoA oxidase-1; G6Pase, glucose-6-phosphatase.

5. Clinical Studies of Dietary Polyphenols

There are a few clinical studies investigating the effects of specific polyphenols or food products rich in polyphenols in reducing the risk of insulin resistance [6].

5.1. Tea and Coffee

In a meta-analysis of green tea or green tea extract of seven randomised controlled trials, the consumption of green tea did not decrease the levels of fasting plasma glucose, fasting serum insulin, 2-h oral glucose tolerance test (OGTT), glucose, glycated hemoglobin (HbA1c) or HOMA–IR index [90]. However the authors stated that limitations of the studies included the small sample sizes, poor quality and low evidence levels of the included studies. In contrast, another meta-analysis of 17 randomised controlled trials including seven high quality studies and 10 low quality studies, demonstrated that green tea had favourable effects significantly reducing the fasting glucose and HbA1c by 0.09 mmol/L (95% CI 0.15–0.03 mmol/L; $p < 0.01$) and 0.30% (95% CI 0.37%–0.22%; $p < 0.01$), respectively. Subgroup analyses from studies with high Jadad scores showed a significant reduction in fasting insulin (−1.16 μIU/mL, 95% CI −1.91 to −0.40 μIU/mL; $p = 0.03$) [91]. This study attributed these findings to measures of glucose control or insulin sensitivity not coming from primary outcomes, limited high quality studies, and concealment of null findings in most selected studies [91].

A third meta-analysis of 22 randomized controlled trials with 1584 subjects showed that green tea catechins (EGCG capsules or powder, whole green tea or green tea extract powder) with or without caffeine reduced fasting glucose (−1.48 mg/dL; 95% CI −2.57 to −0.40 mg/dL), whereas fasting insulin, HbA1c, and HOMA–IR were not affected. In subgroup analyses, the glucose-lowering effect was observed when the duration of follow-up was over a median of 12 weeks. This finding suggested that longer trials were required to support favourable effects of green tea consumption on glycemic control [92].

In summary, given the mixed results from interventions in contrast to epidemiological studies, it is still unclear if polyphenol-rich green tea has anti-diabetic effects.

A randomized cross over study investigating the effect of a single dose of espresso caffeinated coffee, decaffeinated coffee, or water on glucose tolerance and insulin sensitivity (Matsuda index) during an OGTT in 18 habitual coffee drinkers with T2D showed no difference in area under the curve AUC insulin, glucose or insulin sensitivity [31]. However, a three-way, randomized, crossover study of nine healthy subjects [93] showed that chlorogenic acid in coffee acutely modulated gastrointestinal hormone secretion and glucose uptake [93]. The consumption of 25 g glucose in either 400 mL water (control) or 400 mL caffeinated or decaffeinated coffee (equivalent to 2.5 mmol chlorogenic acid/L), did not affect glycemia or insulinemia but attenuated the postprandial increase of glucose-dependent insulinotropic-polypeptide (GIP) ($p < 0.005$) while glucagon-like peptide 1 secretion increased 0–120 min ($p < 0.01$) with a delayed intestinal glucose absorption compared with the control [93]. This finding suggested that chlorogenic acid in coffee decreased the rate of intestinal absorption of glucose or slowed gastric empting [93].

Overall, there is little evidence available to assess the effect of coffee on glucose homeostasis.

5.2. Chocolate and Cocoa

Meta-analyses of acute or short-term, chronic (⩽18 weeks), randomized controlled trials showed that chocolate or cocoa reduced fasting insulin (−2.65 μU/mL) and insulin after glucose challenge (−17 μU/mL at 30 min; 95% CI −20.7 to −13.4 μU/mL) and improved insulin resistance (HOMA–IR −0.67; 95% CI −0.98 to −0.36). There was no effect on fasting glucose and HbA1c [94]. Dark chocolate (100 g bar containing approximately 500 mg of polyphenols) for 15 days decreased blood pressure and improved insulin sensitivity in healthy individuals as demonstrated by decreasing HOMA–IR (0.94 ± 0.42 *vs.* 1.72 ± 0.62; $p < 0.001$) and increasing quantitative insulin sensitivity

check index (QUICKI) (0.398 ± 0.039 *vs.* 0.356 ± 0.023; p = 0.001), compared with 90 g white chocolate bar containing no polyphenols [95], and similar results were shown in hypertensive [96] and glucose-intolerant hypertensive individuals [97]. A four-week consumption of dark chocolate containing 500 mg polyphenols reduced blood pressure (BP), fasting glucose and HOMA–IR in lean and overweight females [98] compared with 20 g of placebo dark chocolate containing negligible polyphenol but similar nutritional composition. Consumption of cocoa flavanols (902 mg) for 12 weeks also improved insulin sensitivity in overweight and obese individuals compared with a low-flavanol cocoa drink [99]. In contrast, consumption of flavanol-rich cocoa drink (150 mL twice a day, containing approximately 900 mg flavanols) for two weeks did not improve insulin resistance or blood pressure in people with essential hypertension [100]. Daily consumption of 25 g dark chocolate for eight weeks did not improve fasting glucose, insulin and HbA1c levels in hypertensive diabetic subjects compared with 25 g white chocolate consumption [101]. Similarly, in acute studies [102,103], dark chocolate containing 450 mg of polyphenols did not improve insulin and glucose in hypertensive diabetic subjects given a 75 g oral glucose load 60 min after ingestion of either low (control) or a high polyphenol dark chocolate [102], and cocoa beverage containing 960 mg total polyphenols and 480 mg flavanols showed no effect on glucose and increased insulin levels in obese subjects with T2D given a high-fat breakfast (766 kcal; 50 g fat) compared with a placebo (110 mg total polyphenols; <0.1 mg flavanols) [103].

In summary, given conflicting results in interventions, current data is insufficient to recommend chocolate and cocoa for glycemic control. Therefore, large placebo controlled trials with good quality designs are required to clarify the effects of cocoa polyphenols.

5.3. Cinnamon

Cinnamon is known to contain polyphenols such as catechin, procyanidin, cinnamtannin trans-cinnamic acid and flavones (cinnamaldehyde and trans-cinnamaldehyde) [5,104,105]. Eleven studies have shown positive effects of cinnamon on glycemic control. Three studies [106–108] included subjects with T2D with baseline HbA1c levels >8%. Seven studies [109–115] included healthy normal subjects [111–114], subjects with impaired fasting glucose [109] and subjects with metabolic syndrome [115]. One study showed beneficial effects on improved insulin sensitivity in patients with polycystic ovary syndrome [105]. Two studies [116,117] showed positive effects on fasting blood glucose levels, but did not report baseline HbA1c levels [116] and no significant changes of HbA1c, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL) or TG [117]. Five studies [118–122] showed no significant changes in fasting glucose, lipids, HbA1c, or insulin levels in 25 postmenopausal women with T2D taking 1.5 g of cinnamon daily for six weeks [120], in 43 subjects with T2D receiving 1 g of cinnamon daily for three months [118], in 60 subjects with T2D taking 1 g of cinnamon daily for 12 weeks [121], in 72 adolescents with Type 1 diabetes (T1D) taking 1 g of cinnamon daily [122] and in 11 healthy subjects taking 3 g of cinnamon daily for four weeks [119]. A randomized, controlled trial [108] (no placebo used with the concern that the strong taste of cinnamon capsules would preclude any blinding), investigating whether daily cinnamon plus usual care *versus* usual care alone lowers HbA1c in 109 patients with T2D (HbA1c > 7.0), showed that supplementation with 1 g of daily cassia cinnamon did not significantly lower HbA1c over 90 days [108]. A randomized, placebo-controlled, double-blind clinical trial of 58 subjects with T2D found that intake of 2 g daily of cinnamon for 12 weeks significantly reduced HbA1c (−0.36%; $p < 0.005$), systolic blood pressure—SBP (−4 mm Hg; $p < 0.001$) and diastolic blood pressure—DBP (−4 mmHg; $p < 0.001$) among 58 poorly controlled patients with T2D, compared with the placebo [107]. A randomized, double-blinded clinical study of 66 Chinese with T2D treated with gliclazide found significant reductions in HbA1c in those receiving 360 mg of cassia cinnamon extract (−0.92%; p = 0.0004) and 120 mg of cassia cinnamon extract (−0.67%; p = 0.003) after 90 days. No significant reductions were seen in the placebo group but the groups were not compared statistically. Fasting glucose levels were also significantly reduced over time in both treatment groups [106].

A study [116] in 60 subjects with T2D treated with sulfonylureas found significant reductions in fasting glucose (18%–29%), triglycerides (23%–30%), LDL (7%–27%), and total cholesterol (12%–26%), following supplementation with either 1, 3, or 6 g of cassia cinnamon daily for 40 days, while no change was observed in the placebo group but the groups again were not compared statistically. Significant effects were observed even after 20 days of washout [116]. A double-blind study [117] found that intake of 3 g daily cinnamon powder for 120 days significantly decreased fasting glucose but not HbA1c in 65 patients with T2D treated with oral antidiabetics or diet alone. Fasting glucose was reduced by 10.3% ± 13.2% in the treatment group and 3.37% ± 14.2% in the placebo group (p = 0.046). No significant changes in HbA1c, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL) or triglycerides (TG) were observed. Baseline HbA1c values were below 7% [117]. Two recently updated meta-analyses showed that the intake of cinnamon/cinnamon extracts lowered fasting glucose levels [123,124]. A meta-analysis of 10 randomized controlled trials (543 patients) reported that the consumption of cinnamon ranging from 120 mg/day to 6 g/day for 4–18 weeks decreased levels of fasting glucose, total cholesterol, LDL, and triglyceride and increased levels of HDL. No significant effect on HbA1c was observed [124]

In summary, the consumption of cinnamon may have glycaemic-lowering effects, but more intervention studies covering different types and amounts of cinnamon and types of subjects, are required as not all studies have shown positive effects of cinnamon on insulin resistance and many have been poorly analysed. The usual dose of cinnamon used was 1–2 g/day but some studies used larger doses of 6 g/day which would be difficult to maintain long term.

5.4. Grape Polyphenols

A double-blinded, randomized, crossover trial of 32 subjects with T2D showed positive effects of grape seed extract intake of 600 mg/day for four weeks on fructosamine, whole blood glutathione (GSH; $p < 0.01$), high sensitivity C reactive protein hsCRP, (p = 0.0006) and total cholesterol concentration (p = 0.05), but did not show significant changes in HOMA–IR [125]. Another study showed protective effects of grape polyphenols on fructose-induced oxidative stress and insulin resistance in first-degree relatives of patients with T2D. Subjects were randomised to eight weeks of supplementation with 2 g/day grape polyphenols or placebo and then ingested 3 g fructose/kg fat free mass/day as a 20% fructose solution with the three meals during the six days preceding muscle biopsies and a two-step hyperinsulinemia-euglycemic clamp. After six-day fructose loading, the placebo group showed a 20% decrease in hepatic insulin sensitivity (11.9 ± 1.5 *vs.* 9.6 ± 0.9; $p < 0.05$) associated with an 11% decrease in glucose infusion rate ($p < 0.05$). However, the grape polyphenol group did not show these deleterious effects of fructose. Moreover, grape polyphenol supplementation showed protective effects against fructose-induced oxidative stress markers of urinary F2-isoprostanes, muscle thiobarbituric acid reactive substances (TBARS), while fructose load induced a downregulation of mitochondrial genes and decreased mitochondrial respiration in permeabilized muscle fibers [126]. In a randomized, controlled study of 38 males with at least one component of metabolic syndrome [127], the daily consumption of 20 g of wine grape pomace flour containing 10 g of dietary fiber, 822 mg of polyphenols for 16 weeks showed a significant reduction ($p < 0.05$) in postprandial insulin and fasting glucose levels compared with the baseline, but there were no significant differences in fasting insulin, postprandial glucose, insulin, glycosylated haemoglobin and HOMA–IR between a wine grape pomace flour group and a control group [127].

In summary, there is little persuasive evidence of an effect of mixed grape polyphenols on glucose homeostasis.

Resveratrol (3, 5, 4′-trihydroxy-trans-stilbene) is a phytoallexin naturally synthesized by plants in response to infection and injury pathogens such as bacteria or fungi. Berries, grape skins, red wine, Japanese knotweed, peanuts, and the roots of rhubarb are sources of resveratrol. Resveratrol exists in two isomeric forms. The main form is trans-resveratrol mainly found in red grape juice

(3.38 mg/L) [128]. The levels of resveratrol in food vary. Its levels in red wine are higher (5.8 mg/L *vs.* 0.2 mg/L) than white wine as red wine is fermented with skins [129].

A randomized, double-blind, crossover study of 11 healthy, obese men showed that resveratrol supplementation of 150 mg/day for 30 days reduced glucose, insulin, HOMA–IR and leptin, decreased inflammatory markers (TNF-α, leukocyte count, alanine aminotransferase) and SBP by 5 mmHg, improved muscle mitochondrial respiration on a fatty acid-derived substrate from the vastus lateralis muscle biopsy (~30 mg), and decreased adipose tissue lipolysis and plasma fatty acid and glycerol in the postprandial state [130]. However, there was no control group or placebo period. Nineteen subjects with T2D took 5 mg trans-resveratrol daily for four weeks and showed improved insulin sensitivity as assessed by HOMA–IR, decreased glucose levels and a delayed glucose peak following a standard meal. There was no control group or placebo used. This study considered these favourable effects were attributed to decreased oxidative stress leading to more efficient insulin signalling via the Akt pathway, with an increase in the ratio of phosphorylated *vs.* total Akt (pAkt: Akt) ratio in platelets [131]. However, a combination of other polyphenolic compounds found in red wine may contribute to the many beneficial effects, as resveratrol is known to have low bioavailability [104].

In summary, it is hard to conclude that resveratrol has anti-diabetic effects. More clinical trials of resveratrol are required to confirm the findings described above in this article.

5.5. Red Wine

In a randomized, controlled study, red wine significantly improved insulin sensitivity, lipid profiles and endothelial function compared with other alcoholic beverages [132,133].

A study [134] comparing the acute effects of red wine, beer and vodka on oxidative stress reported that only red wine demonstrated protection against oxygen-induced oxidative stress, while all three alcoholic beverages provided similar protection against oxygen-induced (via hyperoxia) increase in arterial stiffness. Ten males randomly consumed one of four tested beverage of red wine, vodka, beer (0.32 g ethanol/kg body weight) and water (control). Every beverage was consumed once, a week apart in a cross-over design. The volunteers breathed 100% normobaric O2 between 60th and 90th min of 3-h study protocol. Plasma lipid peroxides (LOOH—biochemical marker of oxidative stress) and uric acid (UA) concentration, blood alcohol concentration (BAC) and arterial stiffness—indicated by augmentation index (AIx)—were measured before and 30, 60, 90, 120 and 180 min after beverage consumption [134]. Moreover, 2 g/day of grape seed extract (1 g of polyphenols) also improved endothelial function in subjects with high vascular risk [135].

However, overall, there is little evidence of an effect of wine polyphenols on glucose and insulin.

5.6. Pomegranate

Fresh pomegranate juice is comprised of phenolic acids, including gallic acid, chlorogenic acid, caffeic acid, ferulic acid, and coumaric acids, as well as flavonoids, such as catechin, phloridzin, and quercetin. Other flavan-3-ols such as epicatechin and epigallocatechin are present in pomegranate [136]. Various *in vivo* and *in vitro* studies have demonstrated favourable effects of pomegranate on insulin sensitivity, inhibition of a-glucosidase and reduced oxidative stress and lipid peroxidation [136,137]. Very limited human studies have focused on the effects of pomegranate juice on T2D [137]. One hundred and twenty milliliters of pomegranate juice/day in obese subjects did not modify insulin secretion and sensitivity after one month of administration in a fasted state [138]. Similarly, 50 mL of pomegranate juice/day for four weeks in subjects with T2D induced no changes in fasting glucose, insulin and HbA1c [139]. Overall, there appears to be little benefit of pomegranate juice.

5.7. Berries

In a randomized, cross-over, double-blind, placebo-controlled acute meal study of 20 healthy subjects, no significant effects were observed on plasma glucose, plasma triacylglycerol, plasma non esterified fatty acids, as well as markers of vascular inflammation, intercellular adhesion molecule-1 (sICAM-1) and vascular cell adhesion molecule-1 (sVCAM-1) after consumption of a black currant (20% juice), compared with consumption of placebo juice devoid of polyphenols and Vitamin C. Both drinks contained 50% citric acid solution, 1% aspartame and 8% acesulfame K. However, an increase in plasma insulin levels was detected [140]. Freeze-dried blueberry beverage containing 50 g of blueberry powder (equivalent to approximately 350 g fresh blueberries) was given daily to 48 individuals with metabolic syndrome for eight weeks in a randomized controlled trial. The serum glucose concentration, lipid profiles, fasting plasma hs-CRP, IL-6, adiponectin, sICAM-1 and sVCAM-1 were not changed significantly, whereas blood pressure decreased in the blueberry-supplemented group compared with the 960 mL water control group [141]. Insulin sensitivity improved more in a 22.5 g blueberry group (22.2% $\pm$ 5.8%) than in the placebo group (4.9% $\pm$ 4.5%) ($p = 0.02$) as measured by a high-dose hyperinsulinemic-euglycemic clamp [142]. The thirty-two obese, nondiabetic, and insulin-resistant participants of this study showed no significant changes in adiposity, energy intake, and inflammatory biomarkers.

Polyphenol-rich berries may reduce sucrose digestion and absorption leading to delayed glycaemic response [143,144]. In a randomised, controlled, cross-over study of two 3-h meal tests in healthy subjects, a berry puree (150 g) made of equal amounts (37.5 g) of bilberries, blackcurrants, cranberries and strawberries, and sweetened with 35 g sucrose lowered glucose levels at 15 ($p < 0.05$) and 30 min ($p < 0.01$) compared with the control meal including 250 mL water, 35 g sucrose, 4.5 g glucose and 5.1 g fructose which are similar in glycemic profile and amounts of available carbohydrates. The berry meal and the control meal showed the peak glucose concentration at 45 and 30 min, respectively. No significant difference in the 3-h area under the glucose response curve was observed. These findings indicated that berries rich in polyphenols delayed digestion of sucrose and/or absorption of glucose from the berry meal or slowed gastric empting [144]. In three randomised, controlled, cross-over, 2-h meal studies [143], healthy female volunteers underwent four 2-h meal tests on separate visits, at least three days apart with a mix of berries and bread strawberries, and a berry mix reduced insulin but not glucose to both wheat and rye breads [143].

A randomized, single-blind, placebo-controlled, cross-over trial in an acute-meal setting showed beneficial effects of polyphenolic- and antioxidant-rich strawberry (Fragaria) on postprandial inflammation and insulin sensitivity. Twenty-four overweight subjects consumed two isocaloric meals of high-carbohydrate, moderate-fat (HCF) accompanied by either a strawberry or a strawberry-flavoured beverage that served as a placebo. The strawberry beverage significantly attenuated the postprandial inflammatory response as measured by hsCRP and IL-6 ($p < 0.05$) and reduced postprandial insulin response ($p < 0.05$). In addition to polyphenolic compounds, Vitamin C and glutathione were also suggested to contribute to the antioxidant activity of strawberry. The test meals including strawberry and placebo beverage did not contain any soluble fibre [145]. The same team conducted an extended study [146] to investigate the effects of six weeks of strawberry consumption (~95 mg total strawberry phenols/day) on HCF meal-induced increases in glucose, insulin and indicators of inflammation and homeostasis over 6 h. Twenty-four overweight subjects consumed either a strawberry beverage or a placebo beverage for six weeks after a seven-day run-in of low/no berry consumption. Strawberry consumption attenuated postprandial plasminogen activator inhibitor-1,plasminogen activator inhibitor-1 (PAI-1) ($p = 0.002$), IL-1 β ($p = 0.05$) with moderate suppression of IL-6, but no significant changes in platelet aggregation, hsCRP, TNF-α, insulin, or glucose were observed compared with the placebo [146]. Consumption of a beverage of 50 g freeze-dried strawberries (2006 mg polyphenols) for eight weeks decreased total cholesterol and LDL-C, small LDL particles and VCAM-1, whereas waist circumference, SBP/DBP, fasting glucose, triglycerides, and HDL-C were not affected, compared with control (equivalent amounts of water)

in 27 obese subjects with metabolic syndrome after eight weeks [147]. Daily consumption of two cups of a strawberry drink for four weeks in women with metabolic syndrome (each cup had 25 g of freeze-dried strawberry powder equivalent to approximately 3.5 cups or 500 g fresh strawberries) decreased total cholesterol and LDL-C levels by 5% and 6%, respectively ($p < 0.05$) and decreased lipid peroxidation in the form of malondialdehyde and hydroxynonenal by 14% ($p < 0.01$) at four weeks *versus* baseline. There was no control group or control period. This study suggested that phytosterol, dietary fiber (8 g/day), or polyphenolic flavonoids content of strawberries might contribute to the hypocholesterolemic effects of freeze dried strawberry powder [148].

A small study suggested that dried cranberries might lower the glycemic response for people with T2D [149], but other studies did not find glucose-lowering effects, despite antioxidant, anti-inflammatory, and HDL-raising and LDL-lowering effects of cranberry juice supplementation [150–153].

In summary, berries appear to reduce the insulin response to a glucose load and have anti-inflammatory effects. However, the results are very mixed and there is limited data.

5.8. Extra Virgin Olive Oil

In the PREDIMED study of 3541 patients with high cardiovascular risk with a 4.1-year follow-up period, a Mediterranean diet rich in extra virgin oil showed a 40% reduction (95% CI 15–57) in the risk of T2D compared with the control group [154]. An olive-rich Mediterranean diet also improved glucose metabolism and reduced body weight in subjects with T2D. Insulin resistance was measured by HOMA–IR, adiponectin/leptin and adiponectin/HOMA–R ratios after one year of follow-up, but HbA1c was not measured [155]. Moreover, the Mediterranean diet supplemented with virgin olive oil or nuts showed an anti-inflammatory effect by decreasing CRP, IL-6 and adhesion molecules, chemokines and T-lymphocytes (CD49d) and monocytes (CD 11b, CD49d, CD40) [156]. In contrast, in a study of a six-week olive oil supplementation in healthy adults, fasting glucose and HDL-C increased compared with baseline in both the low- and high-phenolic olive oil group. There was no change in fructosamine, or plasma total phenols within either group. No significance in a proteomic biomarker score for diabetes was observed with either low- or high-phenolic olive oil [157]. Phenolic components such as oleuropein and hydroxytyrosol abundant in olive leaves as well as oil may protect against diabetes and metabolic disorders [158]. Only two intervention studies of olive leaf polyphenols [159,160] have been conducted so far. Supplementation with olive leaf polyphenols (51.1 mg oleuropein and 9.7 mg hydroxytyrosol per day) for 12 weeks significantly improved insulin sensitivity and pancreatic β-cells secretory capacity after oral glucose challenge in overweight, middle-aged men at the risk of developing the metabolic syndrome [159]. Similarly, in a randomized, placebo-controlled trial in subjects with T2D, supplementation of a 500 mg olive leaf extract tablet once daily for 14 weeks was shown to significantly lower HbA1c and fasting insulin with no significant changes in postprandial insulin levels [159,160].

More clinical studies evaluating the potential effects of olive oil or leaf polyphenols on insulin sensitivity and glycaemic control should be conducted to confirm these beneficial effects.

5.9. Whole Grains, Nuts and Seeds

Whole grains such as wheat, rye, soy and flaxseed are a good source of polyphenols as are nuts such as chestnuts, hazelnuts, pecans and almonds [161]. Whole grain intake is associated with a reduced risk of T2D but the mechanism of the protection is not clear [162]. Whole grain intake is also associated with a lower body weight, lower waist circumference and lower fasting insulin in the Framingham offspring study [163]. However, interventions comparing whole grain with refined grain cereals for 12 weeks have shown no effect on insulin sensitivity or insulin release in 146 individuals with the metabolic syndrome [164] although 2- and 3-h postprandial insulin and triglyceride was lower after the whole grain intervention [165] compared with a standard test meal during the run-in period. A large study with 316 individuals compared usual diet (<30 g/day

of whole grains) to 60 and 120 g/day for four months. No effects were seen on glucose, insulin, inflammatory, coagulation or endothelial markers [166]. It would appear despite the epidemiology that increasing whole grain intake with more fibre and polyphenols has little effect on metabolic risk. However, in the Predimed study, nuts reduced the incidence of T2D assessed annually by OGTT by 52% in the Reus Substudy over four years [167].

5.10. Fruits and Vegetables

Most prospective studies have shown no effect of vegetables and fruits on insulin resistance or the risk of T2D [168–171]. Only a high daily consumption of green leafy vegetables was inversely associated with T2D [169,170], while a high intake of berries was associated with a 35% lower risk of T2D (HR 0.65; 95% CI 0.49–0.88; $p = 0.15$) [171].

5.11. Dietary Patterns

5.11.1. Polyphenol-Rich Diets

Polyphenol-rich diets have been shown to reduce blood glucose in a 3-h OGTT by increasing early insulin secretion and improving insulin sensitivity. Eighty-six participants aged 35–70 years with a BMI of 27–35 kg/m^2 at high cardiometabolic risk were randomized to one of four eight-week isoenergetic diets with a 2 × 2 factorial design [90]: (1) high in polyphenols and high in long chain n-3 polyunsaturated fatty acids (2861 mg of polyphenols/day); (2) high in polyphenols and low in long chain n-3 polyunsaturated fatty acids (2903 mg/day); (3) low in polyphenols and high in long chain n-3 polyunsaturated fatty acids from fish (363 mg/day); (4) low in polyphenols and low in long chain n-3 polyunsaturated fatty acids (365 mg/day). Polyphenols came from decaffeinated green tea and coffee, dark chocolate, blueberry jam, extra-virgin olive oil and polyphenol rich vegetables such as steamed artichokes, onions and spinach, and raw rocket. The polyphenols were 57% flavonoids (41% flavanols) and 43% phenolic acids. The high polyphenol diet decreased plasma glucose area under the curve (AUC; $p = 0.038$) and increased the early phase of insulin secretion (AUC 0–30 min; $p = 0.048$) [172].

5.11.2. Nordic Diet

Scandinavian Nordic diets are considered healthy diets as they are rich in high-fibre plant foods (such as vegetables, fruits and berries). They also include whole grains, fish, rapeseed oils, nuts and low-fat dairy but are low in salt, added sugars and saturated fats [173–175]. This diet appears to be related to a lower risk of T2D.

Cross-sectional studies [176] and randomized clinical trials [175,177,178] of this Nordic diet showed weight loss compared with controls. In a randomized controlled trial of 88 mildly hypercholesterolaemic subjects [175], subjects who consumed a Nordic diet for six weeks had decreased insulin (−9%; $p = 0.01$) and SBP by -6.6 ± 13.2 mmHg (−5%; $p < 0.05$) compared with the control group following their usual Western diet. However, the six-week Nordic diet caused a 4% weight loss compared with the control group ($p < 0.001$). After adjustment for weight change, no significant differences in DBP or triglyceride or glucose was observed although blood lipids were still improved [175]. Similarly, a weight-stable Nordic diet for 18–24 weeks [177] did not alter insulin sensitivity and blood pressure in people with metabolic syndrome. Significant changes between the groups were found in non-HDL-C, LDL-C to HDL-C and apolipoprotein B to apolipoprotein A1 ratios favouring the Nordic diet.

In contrast, a prospective study of 10-year follow-up showed no association between a healthy Nordic diet and the risk of T2D [179]. In a systematic review [173] of prospective observational and randomized intervention studies investigating the association between five food groups (red and processed meat, whole grains, berries, milk products and potatoes) commonly consumed in Nordic countries and the risk of T2D, only wholegrain was inversely associated with the risk of T2D [173].

The association between a Nordic diet and a lower risk of increased hs-CRP levels was observed in a meta-analysis of three cross-sectional Finnish studies [180]. Nevertheless, high adherence to this diet was associated with a lower risk of low HDL-C levels, especially in women [180].

5.11.3. Mediterranean Diet

There have been no interventions comparing a Mediterranean diet to a Western control diet for the prevention of T2D but there have been many interventions in people with T2D. A recent meta-analysis of nine interventions showed that a Mediterranean diet reduced HbA1c by 0.3% and also improved many other metabolic parameters [181].

6. Conclusions

In vitro and *in vivo* studies have shown that dietary polyphenolic compounds improved glucose homeostasis through potential multiple mechanisms of action in the intestine, liver, muscle adipocytes and pancreatic β-cells, as well as through prebiotic effects in the digestive tract. Overall, most epidemiological studies showed that dietary polyphenols were associated with a lower risk of T2D, but this association was not entirely consistent. Berries and cassia cinnamon may be promising candidates for diabetes prevention and management. There have only been limited clinical studies of dietary polyphenols in relation to glucose and insulin homeostasis and these have not been as successful as *in vitro* and *in vivo* studies. The mixed results may be related to poor statistical analyses, small sample size (many are acute studies) and no dynamic measures of glucose metabolism. Larger and better designed studies are required before any recommendations can be made.

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6 LITERATURE REVIEW: PROBIOTICS, PREBIOTICS, SYNBIOTICS AND INSULIN SENSITIVITY

6.1 Preface

This chapter 6 is a review article with the aim of investigating the association between probiotics or/and prebiotics and insulin sensitivity, as well as the potential mechanisms of actions mediated by the probiotic metabolites.

Epidemiological studies in chapter 3 indicated that yoghurt (fermented milk) was positively associated with insulin sensitivity. In chapter 4, the non-digestible complex polysaccharides abundant in whole grains and legumes prompted us to examine the effects of probiotics and/or prebiotics on glucose metabolism.

This chapter is currently under review for publication and therefore written per the journal style guidelines.

6.2 Introduction

The global diabetic population is rapidly growing from 382 million in 2013 to an estimated 592 million by 2035 [1]. This situation imposes a great socio economic burden on public health [123]. Type 2 Diabetes Mellitus (T2DM) is a chronic metabolic disorder of abnormal glucose and lipid metabolism, resulting in cardiovascular disease, retinopathy, nephropathy, neuropathy, leg ulcers and gangrene [124]. The risk factors for T2DM include obesity, age, genetics, smoking, sedentary lifestyle and hypertension [123]. Recently, it has been proposed that changes in gut microbiota composition resulting from obesity could contribute to the pathogenesis of T2DM [125-129].

Probiotics, and prebiotics may exert anti-diabetic effects through changes in microbiota [130-134]. Beneficial modification of the gut flora by probiotic and/or prebiotic treatment could be one dietary therapy for the prevention and treatment of T2DM.

PubMed, EMBASE, Cochrane and Scopus online database were searched for human intervention studies using the following terms: probiotic OR fermented OR yogurt OR cheese OR prebiotic OR inulin OR fructo-oligosaccharide OR synbiotic OR resistant starch OR gut microbiota, PLUS glucose OR glycemic OR hyperglycemia OR insulin OR insulin sensitivity OR type 2 diabetes Plus Human trial. Reviews were also utilised to clarify the potential mechanisms by which probiotics, prebiotics and synbiotics may alter insulin sensitivity.

6.3 Gut microbiota in individuals with T2DM and Obesity

Most gut microorganisms inhabit the large intestine that contains an estimated 10^{11-12} bacteria per gram [135]. Gut microbiota can influence host adiposity and regulate fat storage [125, 128, 136-138].

The *Bacteroidetes* and the *Firmicutes* are groups of bacteria dominant in the human gut [129]. A correlation between changes in gut microbiota composition and obesity was reported in obese humans [129, 139] and ob/ob mice [140], with lower microbial diversity, increased *Firmicutes* and decreased *Bacteroidetes* and this obesity-associated gut microbiota had an increased capacity for energy harvest from the diet [140]. Germ-free wild-type C57BL/6J mice colonized with caecal microbiota from obese donors showed a significant increase (47 ± 8.3%) in body fat, compared with 27 ± 3.6% in mice colonized with a microbiota from lean donors over 2 weeks. During the 2 weeks, obese microbiota recipients consumed 55.4 ± 2.5 g chow and gained 1.3 ± 0.2 g fat, while the lean microbiota recipients consumed 54.0 ± 1.2 g chow and gained 0.86 ± 0.1 g fat a difference of 2% of total calories consumed [140].

Furthermore, composition of the intestinal microbiota in adults with T2DM was different from non-diabetic adults. The proportion of the *Firmicutes, Clostridia* and *Bifidobacteria* was significantly lower in diabetic adults than non-diabetic adults [141, 142].

Mucin-degrading bacteria such as *Akkermansia muciniphila* and *Desulfovirbrio* were enriched in samples derived from T2DM patients [143]. In contrast, several recent studies showed that in mice, direct administration of *Akkermansia muciniphila* [144-148] or specific proteins isolated from the outer of membrane of *Akkermansia muciniphila* could prevent obesity, insulin resistance as well as atherosclerosis and a human study also showed that the abundance of *Akkermansia muciniphila* was associated with glucose homeostasis and body fat composition [149].

Vrieze et al [150] investigated the effect of altering the gut microbiota on insulin sensitivity in subjects with metabolic syndrome. Obese subjects given a small intestinal infusion of faeces from lean donors (n = 9) have shown improved peripheral insulin sensitivity after 6 weeks (median rate of glucose disappearance changed from 26.2 to 45.3 μmol/kg/min; $P < 0.05$), as

assessed by 2-step hyperinsulinemic euglycemic clamp. These subjects also have shown a 2.5-fold increase in butyrate-producing gut microbiota, *Roseburia intestinalis*, compared with obese subjects reinfused with their own feces (n = 9). The faecal microbiota of obese subjects had lower microbial diversity, and contained higher amounts of *Bacteroidetes* and lower amounts of *Clostridium* cluster XIVa compared with faecal microbiota after lean donor infusion at 6 weeks [150].

Germ-free mice which are protected from diet-induced obesity, had increased phosphorylated 5' adenosine monophosphate–activated protein kinase (AMPK) in skeletal muscle and liver and increased fatty acid oxidation enzymes (acetylCoA carboxylase; carnitine palmitoyltransferase), while germ-free knockout mice deficient in fasting-induced adipose factor (FIAF), a circulating lipoprotein lipase (LPL) inhibitor were not resistant to diet-induced obesity. Germ free FIAF deficient animals fed a Western diet showed decreased expression of the peroxisomal proliferator activated receptor gamma coactivator 1 α (PGC1α) which is known to increase genes encoding regulators of mitochondrial fatty acid oxidation in gastrocnemius muscle compared with germ-free FIAF +/+ littermates, while there were no differences in phosphorylated AMPK levels between both groups. Consequently, germ free mice were protected from diet-induced obesity through increased FIAF by inducing PGC-l α and also through elevated AMPK activity, implicating that obese gut microbiota can be responsible for decreased fatty acid oxidation and decreased FIAF/AMPK within the adipose tissue and liver [151].

The lipopolysaccharides (LPS) endotoxins, found in the outer membrane of some species of gram-negative bacteria (e.g. *Neisseria spp*. and *Haemophilus spp*.), induce signalling by binding to Toll-like receptor 4 (TLR4) present on endothelial cells, macrophages and monocytes. This promotes pro-inflammatory cytokines, chemokines, adhesion molecules and reactive oxygen species (ROS). An increase in LPS has been directly associated with insulin

resistance [152]. Cani et al [153] found in a mouse model that high-fat feeding changes gut microbiota with a marked reduction in some bacteria (*Lactobacillus* spp., and *Bacteroides-Prevotella* spp.) leading to an increased intestinal permeability, and LPS absorption. This increased metabolic endotoxemia initiates adipose tissue inflammation [plasminogen activator inhibitor-1 (PAI-1) and interleukin-1 (IL-1) mRNA], macrophage infiltration markers [macrophage chemoattractant protein-1 (MCP-1) mRNA, F4/80 mRNA] and oxidative stress, [NADPHox mRNA, visceral adipose tissue and six transmembrane protein of prostate 2 (STAMP2: known to regulate nutrient-derived and inflammatory signals co-ordinately for metabolic homeostasis [154, 155])].

Even though intestinal microbiota may play a role in the etiology of obesity and insulin resistance, the relation between the bacteria and these metabolic disorders remains a matter of debate and most publications merely report associations between intestinal microbial composition and metabolic disorders such as obesity and T2DM [156].

6.4 Probiotics and Effects of Probiotics on Glucose Metabolism in Human Interventions

According to the FAO/WHO, probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host"[157]. The requirement of adequate amounts differs between countries: products must contain at least 10^7CFU (colony forming unit)/g of probiotic bacteria in Japan, at least 10^8 CFU/g probiotic bacteria in USA and 10^9 CFU/g probiotic bacteria in Canada. In general, $>10^6$-10^8 CFU/g, or $>10^8$-10^{10} CFU/d of viable cells are regarded efficacious [158, 159], but the cell count levels recognised do not guarantee a health effect [159, 160]. Moreover, it is suggested that the recommendation for CFU determination should be established using accurate and frequent assessments because the number of viable cells is reduced during production, processing and formulation [159].

Some of the species are 1) lactic acid–producing bacteria (LAB): *Lactobacillus, Bifidobacterium, Streptococcus*; 2) non-lactic acid producing bacterial species: *Bacillus, Propionibacterium*; 3) non-pathogenic yeasts: *Saccharomyces* (e.g. *Saccharomyces boulardii*: a non-colonizing lactic acid-producing yeast); 4) non-spore-forming and non-flagellated rod or coccobacilli [161]. Over 100 *Lactobacillus* and over 30 *Bifidobacterium* species have been identified [161]. Lactic-producing *Bifidobacterium* and *Lactobacillus* are the predominant and subdominant probiotic groups [162].

Human interventions with probiotics are shown in **Table 1**. Ten interventions [131, 133, 163-170] have shown positive effects of probiotics on glucose control. Ten interventions [127, 171-179] have shown no effect and 2 interventions have shown negative effects [180].

Patients withT2DM supplemented with probiotic yogurt experienced attenuated fasting glucose and glycated haemoglobin (HbA1c) concentrations and increased erythrocyte superoxide dismutase (SOD), glutathione peroxidase (GPx) activities and total antioxidants, compared with the control group [131]. Pregnant women given a probiotic supplement (*Bifidobacterium lactis* Bb12 and *Lactobacillus rhamnosus* GG) and dietary counselling together had improved glycaemic control during and after pregnancy compared with the control/placebo group [163].

In sixty women with gestational diabetes, the daily supplement of a probiotic capsule, containing three viable freeze-dried strains including *Bifidobacterium bifidum* (2×10^9 CFU/g), *Lactobacillus acidophilus* (2×10^9 CFU/g) and *L. casei* (2×10^9 CFU/g) for 6 weeks showed improved insulin sensitivity as assessed by homoeostasis model assessment for insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI), compared with a placebo capsule (cellulose). This probiotic supplementation also lowered triglyceride (TG) and very low density lipoprotein (VLDL) levels, but this study did not measure HbA1c

[169]. Moreover, oral supplementation of *L. acidophilus* NCFM (progenitor of the strain being used for complete chromosome sequencing in order to identify the relationship between genetics and probiotic functionality [181]) for 4 weeks improved insulin sensitivity as assessed by hyperinsulinaemic-euglycaemic clamp, compared with a placebo group, without changes in inflammatory markers such as TNF-α, IL-6, IL-Ira and high-sensitivity C-reactive protein (hs-CRP) [164].

On the other hand, one study of overweight adults [180] has shown conflicting results in that the intake of probiotic yogurt increased HOMA-IR (P = 0.038) and probiotic capsules significantly increased fasting glucose (P = 0.037) with no change in HOMA-IR [180]. (n = 77 for the probiotic group and n = 79 for the probiotic capsule; the specific study design shown in **Table 1**). The probiotics used were *Lactobacillus acidophilus* La5 and *Bifidobacterium animalis subsp lactis* Bb12 dose of 3.0×10^9 CFU/d) [180].

A single-blinded clinical trial of 34 subjects with T2DM showed no effects on glycemic control, lipid profiles and inflammatory markers between a placebo group (n = 18; 1000 mg magnesium stearate) and a treatment group (n = 16) who received 1500 mg probiotic capsules containing *Lactobacillus acidophilus, L. bulgaricus, L. bifidum and L. casei* twice daily for 6 weeks [175]. However, this study provided no counts of probiotics used.

In summary, given the mixed results from human interventions it is still unclear if probiotics favourably influence glucose control. More human interventions are needed with more comprehensive and dynamic measures of insulin sensitivity as most studies did not use these. It is also required to investigate the treatment effects of specific strain (s) at different dosages and durations on insulin resistance.

6.5 Other Fermented Food

Kimchi, made with napa cabbage and various ingredients (garlic, red pepper powder, onion, ginger, radish, fermented fish sauce and starch syrup), is a fermented traditional Korean food. Kimchi can give health benefits due to its high nutritional value and abundant bioactive compounds including dietary fibers, minerals, amino acids, vitamins, carotenoids, glucosinolates, and polyphenols. Kimchi can be improved by additional ingredients and altered fermentation conditions [182]. Fermented Kimchi mostly contains lactic acid producing bacteria including *Lactobacillus plantarum, Lactobacillus brevis, Pediococcus cerevisiae, Streptococcus faecalis* and *Leuconostoc mesenteroides* which could exert a probiotic effect [182].

Studies have shown beneficial effects of fermented kimchi on glucose metabolism in obese [183] and prediabetic individuals [184].

Fermented kimchi intake for 4 weeks decreased fasting glucose, fasting insulin, total cholesterol, MCP-1, leptin and the waist-hip ratio compared with fresh kimchi in a crossover design of 22 overweight and obese patients. Fresh kimchi was defined as 1-day-old kimchi and fermented kimchi was defined as 10-day-old kimchi. The number of *Lactobacilli* in fermented kimchi was higher than fresh kimchi ($4.3 \times 10^9 \pm 1.2 \times 10^9$/mL *vs.* $1.4 \times 10^7 \pm 3 \times 10^6$/mL) [183]. The consumption of fermented kimchi for 8 weeks decreased HbA1c, fasting insulin, HOMA and increased QUICKI and β-cell function compared with prior to fermented kimchi intake, in 22 adults with prediabetes [184].

A randomized controlled clinical trial in 24 obese women showed that fermented kimchi intake (180 g/day) for 8 weeks altered gut microbiota composition with a decrease in genus *Blautia* and an increase in *Prevotella* and *Bacteroides*, compared with fresh kimchi intake and upregulated expression of genes related to metabolic syndrome such as Acyl-CoA synthetase long-chain family member 1 (ACSL1 is involved in enhancing fatty acid degradation) and

aminopeptidase N (ANPEP is involved in the regulation of pain, angiogenesis, inflammation and apoptosis) [185].

Very recently, Shin et al [186] demonstrated metabolic pathways of Kimchi action based on in silico modelling of published data. A total of 4,351 genes were associated with kimchi metabolites. Of these, 283 genes were associated with carbohydrate metabolism. Three hundred and nine genes were associated with lipid metabolism and 27 genes [especially, *GNAS, CTNNB1, EDN1, RAC1, and* adenyl cyclases (*ADCY1, ADCY2, ADCY5*) known to act as regulators of metabolic and cardiovascular function] are directly related with cardiovascular disease. Twenty-three genes [especially, *PTPRC, LCK, JAK3, ZAP70, and VEGFA*] were related to immune diseases and 25 genes were related to endocrine and metabolic diseases [186]. In summary, these inconsistent findings with probiotic interventions might result from heterogeneity in probiotic strains and populations. Intervention studies should be designed with a specific group and a specific strain [187].

6.6 Potential Mechanisms of Action of Probiotics

One potential mechanism of anti-diabetic effects is that certain probiotics facilitate production of short chain fatty acids [SCFAs: acetate (C_2), propionate (C_3) and butyrate (C_4)] leading to the secretion of incretin hormones which may influence glucose levels [130, 188]. Yadav et al [130] have demonstrated a potential mechanism of probiotics through butyrate-induced secretion of glucagon-like peptide 1 (GLP-1) in mice models. In this study [130], VSL#3 consisting of *Lactobacillus casei, L. plantarum, L. acidophilus* and *L. delbrueckii subsp. bulgaricus, Bifidobacterium longum, B. breve* and *B. infantis,* and *Streptococcus salivarius subsp. thermophilus was used.* The levels of the SCFA butyrate in the mouse faecal samples significantly increased after VSL#3 (daily oral dose of 5 mg/kg body weight), as measured by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).

Significantly increased plasma butyrate levels were observed in VSL#3-treated mice compared with PBS-treated control mice. For the measurement of butyrate-producing bacteria, gene expression of butyrate kinase was assayed after 2 and 4 weeks of oral administration. Gene expression of butyrate kinase increased at 2 weeks in VSL#3-treated mice. An increase in GLP-1 was observed in the human intestinal L-cell line NCI-H716 treated with butyrate [130]. GLP-1, an incretin hormone secreted by L-cells mainly in the ileum and large intestine, increases insulin secretion while glucagon is suppressed. GLP1 secretion results in delayed gastric emptying and reduced appetite, food intake and body weight gain [189]. Lactic acid produced by lactic acid producing bacteria such as LAB can be converted to acetate or propionate by *Clostridium propionicum, Propionibacterium* ssp, *Desulfovibrio* ssp, *Veillonella* ssp and *Selenomonas* ssp [190] via methylmalonyl-CoA or acrylyl-CoA, and then to butyrate via acetyl-CoA by *Eubacterium hallii* (butyrate-producing specie) [188].

Other potential mechanisms of anti-diabetic effects of probiotics could be associated with enhanced immunity and increased anti-inflammatory cytokine production, reduced intestinal permeability and reduced oxidative stress [131, 132, 191, 192]. In a randomized, double-blind, controlled intervention, patients with T2DM consumed 300 g/d of yogurt (*L. acidophilus* La5, *B. lactis* Bb12 with dose of 3.98 x 10^9 CFU) for 6 weeks experienced a reduction in fasting glucose and HbA1c and an increase in GPx and erythrocyte SOD activities and total antioxidant status (TAS), compared with the control (300 g/d of conventional yogurt). GPx and SOD are scavengers of ROSs [131].

Pre incubation of HeLa cells with live *Lactobacillus reuteri* cells for 1-2 h inhibited translocation of nuclear factor kappa B (NF-kB) to the nucleus, inhibited degradation of IkB and prevented expression of pro-inflammatory cytokines under NF-kB regulation. Live *L. reuteri* upregulated nerve growth factor (NGF) and inhibited constitutive synthesis and secretion of IL-8 induced by tumor necrosis factor-α (TNF-α) in T84 and HT29 cells (human

colonic adenocarcinoma) [191]. NGF is known to play roles in the regulation of inflammation [193, 194] and proliferation of pancreatic β-cells [195]. Metabolites of *Lactobacillus plantarum* 2142 downregulated peroxide-induced elevation in proinflammatory cytokines IL-8 and TNF-α in IPEC-J2 Cell Line (jejunal epithelia isolated from neonatal piglet) [192]. In streptozotocin (STZ)-induced diabetic rats, probiotic Dahi containing *Lactobacillus casei* and *Lactobacillus acidophilus* suppressed STZ-induced oxidative stress in pancreatic tissues by preventing the depletion of glutathione (GSH), GPx and SOD, as well as by decreasing thiobarbituric acid reactive substances (TBARS) and nitrite [132]. This finding implicates that probiotic Dahi could delay STZ-induced alteration in glucose homeostasis by exerting an antioxidant effect on β-cells [132].

6.7 Prebiotics and Effects of Prebiotics on Glucose Metabolism in Human Interventions

Prebiotics are non-digestible food ingredients that are not metabolised or absorbed while passing through the upper gastrointestinal tract and are fermented by bacteria in the colon and selectively enhance the growth and/or activity of one or potential beneficial bacteria (e.g. *Bifidobacterium* and *Lactobacillus*) in the digestive system [196-198].

Food sources of prebiotics are seeds, whole grains, legumes, chicory roots, Jerusalem artichokes, onions, garlic and some vegetables. Some prebiotics can be produced during the process of enzymatic action or alcohol or cooking [198].

Prebiotics include fructooligosaccharides (FOS), galactooligosaccharides, lactulose, and large polysaccharides (inulin, resistant starches, cellulose, hemicellulose, pectin, and gum) [196]. Of these, researchers have given more attention to FOS [198]. Synthetic oligosaccharides such as galactooligosaccharies have shown better effects and fewer side effects than natural forms [198]. Oligofructose-enriched inulin (OEI) can act across the whole colon. Oligofructose is a short chain fructan (a polymer of fructose molecules) containing 3-10 monosaccharides linked

together. It is quickly fermented and completely metabolized in the ascending part of the colon, whereas inulin is a long-chain fructan containing 9-64 monosaccharides linked together. It is fermented and metabolised in the descending colon [198, 199].

Inulin-type fructans of 10-20 g/day can normalise glucose tolerance or lipid profiles [200-205]. FOS or inulin of 4 g/day is minimal requirement for the enhancement of bifidobacteria growth but 14 g/day or more of inulin can cause intestinal discomfort [198].

In various animal studies, prebiotics have shown improved glucose metabolism [206, 207]. However, a few human studies have demonstrated inconsistent findings. Human interventions of prebiotics are shown in **Table 2**.

6.7.1 Fructooligosaccharides

Seven studies have shown a favourable effect of FOS [208-214] on glycemic control, while three studies of FOS [215-217] have shown no effect.

Forty-eight overweight or obese adults [body mass index (BMI) > 25 kg/m^2] in a randomized, double-blind, placebo-controlled trial received 21 g oligo-fructose per day or a placebo (maltodextrin) for 12 weeks. FOS supplementation decreased ghrelin, glucose and insulin, and increased peptide YY (PYY) compared with a placebo [211]. Yamashita et al [208] have also demonstrated a beneficial effect of supplementation of 8 g FOS per day for 14 days on glucose metabolism in people with T2DM. They found reductions in fasting glucose, total cholesterol and LDL-C. The intake of short–chain FOS-of 10.6 g/day for 2 months reduced postprandial insulin response with no significant alteration in postprandial responses of glucose, FFA and TG in mild hypercholesterolaemic adults, compared with placebo [209]. In a double-blind crossover design, a daily consumption of 20 g FOS for 4 weeks decreased basal hepatic glucose production with no change in insulin-suppressed hepatic glucose production nor insulin-stimulated glucose uptake using a hyperinsulinemic clamp, compared with a daily consumption

of 20 g sucrose in 12 healthy subjects [210]. However, in subjects with T2DM, supplementation of 20 g FOS had no effect on basal hepatic glucose production, fasting glucose and insulin concentrations [216], and on blood glucose, serum lipids [215].

6.7.2 Inulin

Effects of inulin on glycemic control have shown mixed results with 3 interventions [201-203] showing a positive effect and 4 interventions [204, 205, 218, 219] showing no effect.

In a parallel study of 54 subjects receiving 10 g inulin (n = 27) or maltodextrin (n = 27) daily for 8 weeks, insulin concentrations were lower at 4 weeks within the inulin group compared with baseline, but no differences were observed at weeks 4 and 8 in comparison with a placebo. No effect of inulin on fasting glucose concentrations was observed compared with a placebo [201]. However, in a crossover study of 12 men with hypercholesterolemia, a diet supplemented with one pint of vanilla ice cream made with 20 g inulin for 3 weeks decreased total cholesterol and TG but did not alter glucose and insulin, compared with the same diet supplemented with one pint of vanilla ice cream made with sucrose [219].

6.7.3 Oligofructose-Enriched Inulin

Three interventions have shown favourable effects on glycemic control when combined FOS and inulin was used [199, 213, 220]. In a randomized controlled study of 52 women with T2DM, 10 g FOS-enriched inulin per day (n = 27) for 8 weeks lowered fasting glucose and glycosylated haemoglobin and improved inflammatory markers (IL-6, TNF-α) and decreased lipopolysaccharide, compared with a placebo (maltodextrin; n = 25) [199]. Further research is necessary to clarify the effects of oligofructose-enriched inulin on glucose metabolism.

6.7.4 Resistant Starch

Consumption of resistant starch improved insulin sensitivity in healthy subjects [221-223] or in subjects with metabolic syndrome [224, 225], and lowered postprandial glucose or insulin

in people with T2DM [226] and women [227]. One study [228] showed no difference in glycemic control. The 3-day intake of barley kernel-based bread rich in resistant starch and NSP increased fasting SCFAs levels, gut hormones (fasting GLP-1, postprandial PYY and GLP-2) secretion and breath H_2 excretion, and improved insulin sensitivity (the Matsuda index) after consuming a standardised breakfast, compared with white wheat bread [222].

In summary, the effects of prebiotics (inulin or FOS or OEI administration) on glucose and lipid metabolism are not clear, but resistant starch appears to have a favourable effect on insulin sensitivity.

6.7.5 Other Potential Prebiotics

Costabile et al [229] suggested that whole-grain wheat could exert a prebiotic effect on gut microbiota composition. This double-blind, randomised, crossover trial comparing 100 % whole-grain breakfast cereal of 48 g/day with wheat bran breakfast cereal of 48 g/day for 3 weeks showed that whole grain significantly increased the number of faecal bifidobacteria and lactobacilli compared with the wheat bran cereal. However, there were no significant differences in faecal SCFA, fasting glucose, insulin, total cholesterol, triacylglycerol or HDL-C on whole grain intake compared with whole bran [229].

6.8 Potential Mechanisms of Action of Prebiotics-derived SCFAs in Insulin Sensitivity

Microbial fermentation of prebiotics facilitates the production of SCFAs (essential end-products of carbohydrate metabolism) and enhances gut barrier function [230]. In a mouse model, a prebiotic treatment decreased intestinal permeability and increased GLP-2 secretion, and reducing the hepatic expression of inflammatory and oxidative stress markers and decreasing LPS during obesity and diabetes [230].

6.8.1 SCFAs and free fatty acid receptors (FFAR2 and FFAR3)

SCFAs in the intestine activate G-protein-coupled receptors (GPRs), such as GPR41 [namely, free fatty acid receptor 3 (FFAR3)] and GPR43 [namely, free fatty acid receptor 2 (FFAR2)]. These receptors are present on ileal and colonic enteroendocrine L cells, adipocytes and immune cells [231]. Both GPR41 and GPR43 on intestinal epithelia L cells trigger the secretion of gut hormones (GLP-1 and PYY). Leptin is also released from adipocytes when SCFAs bind to GPR41. PYY, GLP1 and leptin can decrease appetite [232-235]. GLP-1 increases insulin secretion from pancreatic β cells and decrease glucagon secretion from the pancreatic islets, which leads to lower glucose output from the liver and enhanced peripheral uptake of glucose. GLP1 may supress appetite and food intake via the autonomic nervous system (ANS) or the brain [196, 236, 237]. It is known that FFAR3 is activated by butyrate and propionate while FFAR2 is activated by acetate and propionate. [238]. FFAR3 knockout mice showed that butyrate and propionate inhibited food intake, reduced high-fat diet-induced weight gain and glucose intolerance and enhanced gut hormone release. FFAR3 was necessary for the maximal induction of GLP-1 by butyrate, whereas FFAR3 was unnecessary for the effects on body weight and glucose-dependent insulinotropic peptide (GIP) secretion [239]. FFAR3 and FFAR2 can be expressed in several cells such as adipocytes, endocrine cells (e.g. pancreatic islets), and immune cells [240]. FFAR2 is highly expressed in immune cells (neutrophils and monocytes) and hematopoietic tissues, compared with FFAR3 [241]. SCFAs can exert potent roles in inhibiting lipolysis and inflammation, and regulating energy metabolism [240-243]. Ge et al [242] demonstrated that when FFAR2 on adipocytes was activated by SCFAs (acetate and propionate), adipocyte lipolysis and differentiation were inhibited, while in GPR43 knockout animals, this was not observed, suggesting that prebiotic fermentation could be detrimental with regard to obesity [242]. However, obese mice and human studies of prebiotics (especially, inulin-type fructans) have not shown this [244, 245]. SCFAs inhibited the

production of MCP-1 and LPS-induced IL-10 in human monocytes, as well as LPS-induced TNF-α and Interferon gamma (IFN-γ) in human peripheral blood mononuclear cells [PBMC: monocytes and lymphocytes (T cells, B cells and NK cells)][241].

6.8.2 Anti-Inflammatory Effects

Elevated pro inflammatory makers such as hs-CRP, TNF-α and IL-6 are increased in T2DM [199]. SCFAs can suppress these inflammatory mediators [241, 246-249]. SCFAs (acetate, propionate, and butyrate) decrease nitric oxide (NO) [246]. NO is produced by nitric oxide synthase which converts oxygen and arginine to citrulline and NO. NO acts as a vasodilator with beneficial effects on vascular health [250-253] and has an anti-inflammatory effect under normal physiological conditions [254]. However, NO participates in immune responses by cytokine-activated macrophages which produces NO in high concentrations [254].

SCFAs suppressed LPS-stimulated TNF-α [247] from neutrophils and also suppressed TNF-α, IL-1β, IL-6, and NO in RAW264.7 murine macrophage cells [248]. Moreover, SCFAs (0.2–20 mmol/L) lowered LPS-induced production of TNF-α and IFN-γ in human peripheral blood mononuclear cells in a dose-dependent manner [241]. Butyrate suppressed IL-6 and TNF-α in IFN-γ-stimulated RAW264.7 murine macrophage cells [249]. Studies [246, 247, 249] showed that anti-inflammatory effects of SCFAs could be mediated by inhibiting the activation of NF-κB (a transcriptional factor involved in the inflammatory response and cell proliferation and TNF-α production [255]). Butyrate is a histone deacetylase (HDAC) inhibitor [255, 256]. SCFAs (propionate and butyrate) suppressed the release of LPS-stimulated TNF-α and downregulated NF-κB by facilitating prostaglandin E2 (PGE2) levels and cyclooxygenase-2 (COX-2) activities through inhibiting HDAC in PBMCs [255] and murine macrophage cell line RAW 264.7 cells [256]. Therefore, SCFAs, especially butyrate, exert an anti-inflammatory effect via two potential signalling pathways NF-κB and HDAC inhibition.

Butyrate also decreased the levels of MCP-1 in a dose-dependent manner with or without LPS in human PBMC [241]. SCFAs inhibited the expression of vascular cell adhesion molecule-1 (VCAM-1) induced by TNF-α and IL-1β in human umbilical vein endothelial cells (HUVEC) [257-259]. Butyrate suppressed T-cell activation stimulated by antigen-presenting cell (APC) by downregulating the expression of intracellular cell adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen 3 (LFA-3) in monocytes [260].

6.8.3 SCFAs and angiopoietin-like protein 4

Angiopoietin-like protein 4 (ANGPTL4) is a 50 kD pro-hormone secreted from brown and white adipose tissues, liver, skeletal muscle, intestine and heart. Human ANGPTL4 is mainly expressed in the liver. It is known as fasting induced adipose factor because ANGPTL4 is upregulated in white adipose tissue and liver during fasting [231, 261], while human plasma ANGPTL4 concentrations are reduced after meal consumption [261]. ANGPTL4 is a LPL inhibitor and thus causes decreased uptake of fatty acids into tissue [231]. In mice, overexpression of ANGPTL4 decreased clearance of TG-rich lipoproteins and increased circulating TG levels [262]. A very recent study showed that inhibition of or a lower level of ANGPTL4 is associated with lower risk of cardiovascular disease in mice and nonhuman primate models [263]. The supressed ANGPTL4 may result in increased LPL-activity and lipolysis [196, 236].

On the other hand, lower serum ANGPTL4 levels are observed in subjects with T2DM compared with normal subjects. An inverse association between plasma glucose levels and HOMA-IR, and serum ANGPTL4 levels was found. These findings suggest that decreased ANGPTL4 could play a role in glucose tolerance [264].

ANGPTL4 induced by fatty acids via peroxisome proliferator activated receptors (PPARs) in various tissues appears to reduce cellular lipid overload, oxidative stress and inflammation

[265, 266]. The SCFAs, especially propionate, inhibited lipid synthesis in the presence of acetate as a source of acetyl-CoA in hepatocytes [267]. Propionate and/or butyrate, but not acetate, activated ANGPTL4 production in intestinal (Caco-2, HT-29 and HCT-116) and hepatic (HepG2) cancer cell lines [268] and the entero-endocrine cell line HuTu-80 from the human small intestine [261].

ANGPTL4 is a downstream target gene of PPARs [196]. PPARs, transcription factors with three isoforms (α,β and γ) is a superfamily of nuclear receptors [269]. Fatty acids and lipid-derived substrates are their ligands. PPAR γ agonists are used as T2DM treatment drugs. PPAR-α present in liver, heart and skeletal muscle, promotes primarily hepatic fatty acid oxidation, ketone body synthesis and glucose sparing, while PPAR γ expressed in lower intestine, adipose tissue and immunity cells, facilitates an increase in fatty acid storage in adipocytes [269].

Thiazolidinediones (TZDs) are strong activators of PPAR γ which improve insulin sensitivity and facilitate insulin-mediated suppression of gluconeogenesis in the liver and glucose uptake in the skeletal muscle [269, 270]. However, PPAR γ is expressed in adipose tissues but not in muscle, the main insulin-sensitive tissue [269]. Activation of PPAR γ causes release of adiponectin from mature adipocytes, which stimulates AMP involved in the upregulation of glucose transporter (especially, GLUT4) in skeletal muscle, the stimulation of increased fatty acid oxidation in mitochondria [269], as well as the downregulation of gluconeogenesis in the liver [271], consequently leading to improved insulin sensitivity in skeletal muscle [269] and in the liver [270]. Metformin, a T2DM treatment medicine, is a stimulator of AMPK [269]. It is suggested that the combined use of PPAR ligands (e.g. TZDs) and SCFAs could minimise weight gain from TZDs releasing ANGPTL4 [272].

6.8.4 SCFAs and Intestinal Gluconeogenesis

One potential mechanism for SCFAs to prevent T2DM involves intestinal gluconeogenesis (IGN) which is mediated by signalling of the periportal nervous system [273]. Hepatic and intestinal gluconeogenesis play opposite roles in glucose homeostasis. IGN might be inversely associated with risk of T2DM as beneficial effects of IGN on a reduction in food intake, weight gain and hepatic glucose output, and on improvement in glycaemic control, have been shown [274-276]. In contrast, increased hepatic gluconeogenesis is related to the risk of T2DM [277, 278]. The intestine produces approximately 20-25% of total endogenous glucose in the fasted state [279]. Glucose produced by intestine is sent to the portal vein. The periportal neural system in the portal vein walls detects glucose and sends a signal to the brain for the modulation of energy and glucose metabolism [279]. Interestingly, butyrate directly promotes IGN gene expression in enterocytes by increasing intracellular cyclic adenosine monophosphate (cAMP) levels in an FFAR2-independent manner [273]. Propionate binding to FFAR3 present in the portal nerves increases IGN gene expression through a portal hypothalamic neural circuit [273]. The benefit of this gut-brain neural circuits has been shown for portal glucose sensing initiated by protein-enriched diet resulting in decreased food intake [275].

IGN-deficient mice (with disruption of the glucose 6-phosphatase (G6Pase) catalytic subunit in the intestine) fed SCFA- or FOS-rich diet showed no favourable effect on glucose and insulin with no change in body weight, compared with normal mice fed SCFA- or FOS-rich diet [273]. Moreover, normal mice fed a high-fat/high sucrose diet supplemented with FOS showed improved glucose and insulin tolerance and decreased fat mass, whereas these metabolic benefits were absent in IGN-deficient mice fed the same diet with FOS [273]. Therefore, IGN appears to be essential for the effect of SCFAs on glucose homeostasis.

6.9 Synbiotics and Effects of Synbiotics on Glucose Metabolism in Human Interventions

A combination of probiotics and prebiotics is described as a synbiotic [198]. Lactobacillus acidophilus DSM20079 induced 14.5 fold more butyrate in the presence of inulin or pectin than in the presence of glucose [280].

Human interventions of synbiotics are shown in **Table 3**. Eleven of twelve studies of synbiotics have shown favourable effects on glucose metabolism [134, 138, 281-290]. Three [138, 289, 290] of four [138, 288-290] studies in subjects with non-alcoholic fatty liver disease have shown a positive effect on glucose control. The Asemi research group conducted a randomised, double-blind, placebo-controlled trial in subjects with T2DM; the consumption of synbiotic bread [containing the probiotic *Lactobacillus sporogenes* (1 X 10^8 CFU) and 0.07 g inulin per 1 g as prebiotic] for 8 weeks improved insulin metabolism, lipid profiles and plasma NO and malondialdehyde levels, compared with the probiotic alone [*Lactobacillus sporogenes* (1 X 10^8 CFU)] and a control bread [285, 291, 292].

In summary, a very limited number of interventions have shown beneficial effects on glucose metabolism. More pronounced effects of synbiotics on glycemic control and inflammation have been observed than with the use of probiotics alone [136].

6.10 Conclusion

People with obesity or T2DM have been observed to have a different composition of gut microbiota. Altered gut microbiota may contribute to the development of T2DM. The composition of gut microbiota can be beneficially modified by probiotics and/or prebiotics to maintain glucose homeostasis. The potential mechanisms of action could involve insulinotropic and satiety effects mediated by gut hormones, GLP-1 and PYY, a β-cell protective effect by reduced oxidative stress and lowered pro-inflammatory cytokines, anti-lipolytic activities and enhanced insulin sensitivity via GLUT4 through the upregulation of AMPK signalling in tissues. An additional role of SCFAs in glycemic control through intestinal

gluconeogenesis and mediated by the periportal nervous system. The antidiabetic effects of SCFAs require further research. Use of resistant starch and synbiotics may become a diabetic nutritional strategy. Overall human interventions of probiotic and prebiotics showed mixed findings so further work is required.

Table 1. The summary of probiotic human intervention studies

Type	Probiotic stains and dose used	Design	Subjects	Period	Outcomes	No effects	Ref
Drink	400 mL/d of a rose-hip drink containing *L. plantarum* 299 v (5 x 10^7 CFU/mL) *vs*. 400 mL/d of a rose-hip drink without bacteria	C, PC, P RD, DB,	36 healthy smokers aged 35-45 years Treatment (n = 18) Control (n = 18)	6 wk	↓ SBP, fibrinogen & IL-6 within the treatment group *vs.* baseline	glucose, insulin, BMI, BP, lipids	Narusz ewicz et al 2002 [171]
Yogurt	Low-fat yogurt of 100 ml/3 x day containing *S. thermophilus* and *L. delbrueckii subsp*. *bulgaricus plus B. longum BL1 vs.* low-fat yogurt of 100 ml/3 x day containing *S. thermophilus* and *L. delbrueckii subsp. bulgaricus*	RD, PC, SB, P	32 adults with a total cholesterol ranging from 220 to 280 mg/dl Treatment (n = 16) Control (n = 16)	4 wk	↓ TC, ↓ LDL-C, ↓ TG	glucose	Xiao et al 2003 [172]
Capsule	Freeze-dried capsule (2 x 10^9 CFU/tab) containing *L. fermentum vs.* placebo twice daily	DB, PC, P	44 subjects with HC aged 30-75 years Treatment (n = 23) Placebo (n = 21)	10 wk		glucose, TC, HDL-C, LDL-C, TG,	Simons et al 2006 [173]
Capsule	a) diet supplemented with probiotics containing *L. rhamnosus GG* and *B. lactis Bb12* dietary counselling b) diet/placebo c) placebo	RD, P, DB, PC	196 pregnant women aged 25-35 years a) (n = 66) b) (n = 70) c) (n = 60)	20 wk	↓ glucose, ↓ insulin, ↓ HOMA, ↑ QUICKI	HbA1c	Laitine n et al 2009 [163]
Capsule	Capsule with freeze-dried *L. acidophilus* [NCFM-American Type Culture Collection (ATCC) 700396 (1 g; about 10^{10} CFU)] *vs*. placebo (a mixture of silicium dioxide and lactose, ratio 1:1)	RD, P DB, PC	45 males Treatment (n = 21: 10 NGT, 3 IGT & 8 T2DM) Placebo (n = 24: 12 NGT, 2 IGT & 10 T2DM)	4 wk	↑ insulin sensitivity by hyperinsulinaemic-euglycaemic clamp	TNF-α, IL-6, IL-Ira, hs-CRP	Andrea sen et al 2010 [164]
Capsule	2.9 × 10^9 CFU of *L. reuteri* NCIMB 30242 capsule *vs*. placebo maltodextrin capsule twice daily	DB, PC, P	124 adults with HC Treatment (n = 62) Placebo (n = 62)	9 wk		fasting glucose, BMI	Jones et al 2012 [174]
Yoghurt	300 g/d of yogurt containing *L. acidophilus* La5*, B. lactis* Bb12 with dose of 3.98 x 10^9 CFU *vs.* conventional yoghurt	RD, P, DB, PC	60 patients with T2DM aged 30-60 years, BMI <35 kg/m^2 Treatment (n = 30) Control (n = 30)	6 wk	↓fasting glucose, ↓ HbA1c, ↑ antioxidant status	insulin	Ejtahe d et al.201 2 [131]

Capsule	10^{10} CFU of *Lactobacillus salivarius* Ls-33 *vs.* placebo daily	RD, PC, DB, P	50 obese adolescents aged 12 to 15 years Treatment (n = 27) Placebo (n = 23)	12 wk		fasting glucose, insulin, HOMA-IR, C-peptide, TC, TG,FFA, hs-CRP, IL-6, TNF-α	Gøbel et al 2012 [178]
Capsule	1500 mg probiotic capsule containing *L. acidophilus, L. bulgaricus, L. bifidum,* and *L. casei* twice daily *vs*. 1500 mg placebo capsule containing 1000 mg magnesium stearate twice daily	SB, PC, P	34 subjects with T2DM Treatment (n = 16) Placebo (n = 18)	6 wk		glycemic control, lipids, inflammatory markers	Yousefinejad et al 2013 [175]
Capsule	Capsule containing 10^{10} CFU *L. gasseri* BNR17 & filler powder (50% trehalose, 25% skim milk, and 25% FOS) *vs.* placebo capsule containing filler powder. 6 capsules per day taken	RD, PC, DB, P	50 obese adults with BMI ≥ 23 kg/m^2 Treatment (n = 22) Placebo (n = 28)	12 wk		fasting glucose, insulin, BMI,	Jung et al 2013 [176]
Cheese	Hypocaloric diet (1512 kcal) supplemented with 50 g probiotic cheese made with *L. plantarum* TENSIA added to the cheese milk in amounts of 1.5 x 10^{11} CFU/g before renneting *vs*. 50 g control Edam-type cheese made with a starter C92. 50 g of each cheese was 175 kcal	RD, DB, PC, P	36 obese hypertensive subjects Treatment (n = 25) Control (n = 11)	3 wk	↓ BMI, ↓ BP	glucose, lipids	Sharafedtinov et al 2013 [177]
Yoghurt	Probiotic yoghurt of 200 g/d containing *S. thermophiles*, *L. bulgaricus*, *L. acidophilus* LA5 and *B. animalis* BB12 with dose of 1 × 10^7 CFU *vs*. conventional yoghurt of 200 g/d containing only starter cultures of *S. thermophilus* and *L. bulgaricus*	RD, SB, PC, P	70 primigravida pregnant women with singleton pregnancy at their third trimester Treatment (n = 37) Control (n = 33)	9 wk	↓ insulin, ↓ HOMA	fasting glucose, BP	Asemi et al 2013 [166]
Yoghurt	300 g/d of probiotic yogurt containing a total of *L. delbrueckii subsp. Bulgaricus*, *S. thermophilus. B. animalis subsp. lactis* Bb12 and *L.acidophilus* La5 with dose of 1.11 x 10^9 CFU/d *vs.* 300 g/d conventional yogurt containing *L. delbrueckii subsp. bulgaricus* and *S. thermophilus*	RD, P, DB, PC	40 obese adults with T2DM, BMI ⩾ 25 kg/m^2 Treatment (n = 20) Control (n = 20)	8 wk	↓ HbA1c, ↓ TNF-α, ↓ hs-CRP, ↓ IL-6	glucose	Mohamadshahi et al 2014 [165]

Capsule	Probiotic capsule containing *L. salivarius* with dose of 1x 10^9 CFU/d *vs.* placebo capsule	DB, PC, RD, P	138 pregnant obese women with mean BMI 33.6 kg/m^2 Treatment (n = 63) Placebo (n = 75)	4 wk		fasting glucose, insulin, HOMA-IR, lipids, hs-CRP,	Lindsay et al 2014 [127]
Capsule/ Yoghurt	a) Probiotic yoghurt + probiotic capsules b) Probiotic yoghurt + placebo capsules c) Control milk + probiotic capsules d) Control milk + placebo capsules Probiotic yogurt and a probiotic capsule contained *L. acidophilus La5* and *B. animalis subsp lactis* Bb12 *with* dose of 3.0 x 10^9 CFU/d.	DB, P, PC	156 overweight adults aged over 55 years (mean: 67 ± 8 years old) a) (n = 40) b) (n = 37) c) (n = 39) d) (n = 40)	6 wk	↑ HOMA-IR in probiotic yoghurt, ↑ fasting glucose in probiotic capsules	insulin, HbA1c	Ivey et al. 2014 [180]
Capsule	12.5 × 10^9 CFU/capsule VSL#3 containing *B. longum, B. infantis, B. breve, L. acidophilus, L. paracasei, L. delbrueckii subsp. bulgaricus, L. plantarum and S. salivarius subsp. thermophilus. vs.* omega-3 capsules *vs.* omega-3 + VSL#3 *vs*. placebo (cellulose)	RD, DB, PC, P	60 overweight (BMI > 25 kg/m^2), healthy adults, aged 40–60 years Treatment 1 (n = 15) Treatment 2 (n = 15) Placebo (n = 15)	6 wk	↓ lipids, ↓ HOMA, ↑ hsCRP within VSL#3 and VSL#3 + omega-3 groups *vs.* baselines ↑ *lactobacilli* and *bifidobacteri* in a VSL#3 group		Rajkumar et al 2014 [167]
Yoghurt	80 mL/d of fermented milk with *L. plantarum* (1.25 x 10^7 CFU/g) *vs.* 80 mL/d of non-fermented milk	SB, PC,P	24 postmenopausal women with MetS Treatment (n = 12) Placebo (n = 12)	90 d	↓ fasting glucose, ↓ homocysteine	insulin, HOMA-IR	Barreto et al 2014 [170]
Yoghurt	200 g/d of fermented milk with 5 × 10^{10} CFU/100 g of *L. gasseri* SBT2055 (LG2055) *vs.* fermented milk without LG2055	SB, PC,P	20 subjects with hypertriacylglycerolemia	4 wk	↑ HbA1c, ↓ postprandial and fasting serum NEFA levels,		Ogawa et al 2014 [179]
Yoghurt	600 ml/d of probiotic fermented milk containing *Lactobacillus casei, Lactobacillus acidophilus* and *Bifidobacteria* with dose of 3.4 x 10^9 CFU/d (at day 21) *vs.* 600 ml/d of placebo conventional fermented milk	RD, P, DB, PC	60 diabetic patients aged 35-65 years Treatment (n = 30) Placebo (n = 30)	8 wk	↓ HbA1c, ↓ fasting glucose	TG, TC, LDL-C, HDL-C	Ostadrahimi et al 2015 [168]

Yoghurt	300ml of Cardi04 yogurt containing *Lactobacillus helveticus* once daily	RD, P, DB, PC	41 patients with T2DM Treatment (n = 23) Placebo (n = 18)	12 wk	↓ fasting glucose	HbA1c, lipids, hs-CRP, TNF-α	Hove et.al 2015 [133]
Capsule	Probiotic capsule containing three viable freeze-dried strains including *L. acidophilus* (2 x 10^9 CFU/g), *L. casei* (2 x 10^9 CFU/g) and *B. bifidum* (2 x 10^9 CFU/g) *vs*. placebo (cellulose) once daily.	RD, P, DB, PC	Primigravida 60 pregnant women with gestational diabetes aged 18-40 years Treatment (n = 30) Placebo (n = 30)	6 wk	↓ fasting glucose, ↓ insulin, ↓ HOMA-IR, ↓ HOMA for β-cell function, ↑ QUICKI, ↓ TG, ↓ VLDL-C		Karam ali et al 2016 [169]

BMI, body mass index; BP, blood pressure; C, control; CFU, colony forming unit; DB, double blind; FFA, free fatty acid; FOS, fructooligosaccharide; HbA1c, glycated haemoglobin; HC, hypercholesterolemia; HDL-C, high density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-C, low density lipoprotein cholesterol; IL-6, interleukin 6; IL-Ira, interleukin-1 receptor antagonist; MetS, metabolic syndrome; NEFA, non-esterified fatty acid; P, parallel; PC, placebo control; QUICKI, quantitative insulin sensitivity check index; RD, randomised; SB, single blind; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride; TNF-α, tumor necrosis factor-alpha; T2DM, type 2 diabetes mellitus; VLDL-C, very low density lipoprotein cholesterol.

Table 2. The summary of prebiotic human intervention studies

Source	Type and Dose	Subjects	Design	Period	Outcomes	No effects	Ref
Inulin	50 g/d of rice-based ready-to-eat cereal containing (18%) inulin *vs*. 50 g/d of rice-based ready-to-eat cereal placebo	12 healthy men with mean BMI 25.7 kg/m^2	CO	4 wk	↓ TC, ↓ TG, ↑ breath H_2, ↑ faecal lactic acid, ↑ bifidobacteria	glycemic responses, faecal and bile acid output, faecal SCFA, faecal PH	Brighenti et al 1999 [204]
Inulin	10 g/d of inulin *vs.* 10 g/d of maltodextrin placebo	54 middle aged subjects Treatment (n = 27) Placebo (n = 27)	RD, DB, PC, P	8 wk	↓ insulin at wk 4 within the inulin group *vs.* baseline. ↓ TAG in the inulin group *vs.* the placebo group at wk 8	fasting glucose	Jackson et al 1999 [201]
Inulin	Diet supplemented with 10 g/d of inulin *vs.* 10 g/d of maltodextrin placebo	8 healthy subjects aged 23-31 years with BMI 19-25 kg/m^2	RD, DB, CO, PC	3 wk	↓ TAG, ↓ hepatic lipogenesis	glucose, insulin, glucagon, NEFA, lipids	Letexier et al 2003 [205]
Inulin	Diet supplemented 11% inulin-enriched pasta (100 g/d) *vs*. wheat pasta (100 g/d) placebo diet	15 healthy males	RD, DB, CO	5 wk	↓ HbA1c, ↓ HOMA-IR, ↓ fasting glucose, ↓ fructosamine, ↓ gastric empting, ↓ TC, ↑ HDL, ↓TAG		Russo et al 2010 [202]
Inulin	Low-energy diet plus one of following treatments: a) partial meal replacement (PMR) alone; b) PMR+10 g/d of inulin; c) 10 g/d of inulin alone; d) control (no inulin or PMR)	110 obese women aged 18-50 years with BMI ≥ 25 kg/m^2 a) (n = 28), b) (n = 23), c) (n = 30), d) (n = 29)	RD, C, L, P	12 wk	↓ TAG	glucose, TC, HDL-C	Tovar et al 2012 [218]
Chicory root inulin	Diet supplemented with one pint of low-fat vanilla ice cream made with 20 g/d of inulin *vs.* the same diet supplemented with one pint of low-fat vanilla ice cream made with sucrose	12 men with HC	RD, DB, CO	3 wk	↓ TC, ↓ TG	glucose, insulin	Causey et al 2000 [219]
Chicory inulin	Diet supplemented with 16 g/d of chicory-derived fructan/d *vs.* diet with16 g/d of maltodextrin placebo	10 healthy adults with mean BMI 21.6 kg/m^2 Treatment (n = 5) Placebo (n = 5)	RD, DB, P, PC	2 wk	↓ postprandial glucose, ↑ breath H2, ↓ hunger, ↑ GLP-1, ↑ PYY	fasting glucose, insulin	Cani et al 2009 [203]

Inulin & FOS	Yacon syrup with about 12.5 g/d of FOS *vs*. placebo syrup containing 2.5% tartaric acid, 1.8%, carboxymethylcellulose, 2.5% saccharine & 10% glycerine	35 obese women with mean BMI 33.5 kg/m^2 Treatment (n = 20) Placebo (n = 15)	DB, PC, P	17 wk	↓ fasting insulin, ↓ HOMA-IR, ↓ LDL-C, ↑ satiety, ↓ BMI, ↓ waist circumference	fasting glucose, TC, HDL-C, TAG	Genta et al 2009 [213]
Inulin & FOS	16 g/d mixture of inulin and FOS (50/50) *vs.* placebo maltodextrin. Dietary instruction given to all participants for weight loss	30 obese women Treatment (n = 15) Placebo (n = 15)	DB, PC, P	12 wk	↓ post-OGTT glycaemia within the treated group, ↓ LPS ↓ *Bacteroides intestinalis*, *Bacteroides vulgatus* and *Propionibacterium* ↑ *Bifidobacterium* and *Faecalibacterium prausnitzii* ↓ *Bacteroides intestinalis, Bacteroides vulgatus* and *Propionibacterium*	HbA1c, fasting glycaemia, insulinaemia, post-OGTT insulinaemia, HOMA, adiponectinaemia, hs-CRP	Dewulf et al 2013 [220]
FOS enriched inulin	10 g/d of FOS-enriched inulin *vs.* 10 g/d of maltodextrin placebo	52 diabetic women with BMI >25 but < 35 kg/m^2 Treatment (n = 27) Placebo (n = 25)	RD, PC, P	8 wk	↓ fasting glucose ↓ glycosylated haemoglobin ↓ IL-6, ↓ TNF-α, ↓ LPS	hs-CRP, IL-10, interferon γ	Dehghan et al 2014 [199]
FOS	8 g/d of FOS *vs*. 5 g/d of placebo sucrose	28 patients with T2DM Treatment (n = 18) Placebo (n = 10)	RD, PC, P	2 wk	↓ fasting glucose, ↓TC, ↓ LDL-C	FFA, TG, HDL-C	Yamashita et al 1984 [208]
FOS	20 g/d FOS *vs.* 20 g/d sucrose	12 healthy subjects	RD, DB, CO	4 wk	↓ basal hepatic glucose production	fasting glucose, insulin, lipids	Luo et al 1996 [210]
FOS	15 g/d of FOS *vs*. 4 g/d of placebo glucose	20 patients with T2DM	RD, SB, CO	20 d		glucose, lipids, TAG, FFA, acetate	Alles et al 1999 [215]
FOS	20 g/d of FOS *vs.* 20 g/d of placebo sucrose	10 patients with T2DM	DB, CO, no wash out	4 wk		basal hepatic glucose production, fasting glucose, insulin, lipids	Luo et al 2000 [216]
sc-FOS	10.6 g/d of sc-FOS *vs.* 15 g/d of placebo maltodextrine & aspartame with tea and/or coffee	30 adults with mild HC	RD, DB, CO	8 wk	↓ postprandial insulin response	postprandial effects on glucose, FFA, TG	Giacco et al 2004 [209]

FOS	Diet supplemented with 16 g/d of FOS *vs.* diet with placebo 16 g/d of maltodextrin	7 male adults with nonalcoholic steatohepatitis (mean BMI 29.1 kg/m^2)	RD, DB, CO	8 wk	↓ aminotransferases ↓ AST after 8 wk ↓ insulin at 4 wk	insulin after 8 wk	Daubioul et al 2005 [212]
FOS	a) FOS rich yacon syrup (0.29 g FOS/body weight/d) b) FOS rich yacon syrup (0.14 g FOS/body weight/d) c) placebo	55 obese slightly dyslipidemic pre-menopausal women Treatment (n = 40) Placebo (n = 15)	Db, PC, P	120 d	↓ fasting insulin, HOMA-IR, BMI & LDL-C within the yacon syrup at 0.14 g FOS/body weight/d *vs.* baseline	fasting glucose, TC, HDL-C, TAG	Genta et al 2009 [213]
FOS	21 g/d of FOS *vs.* 7.9 g/d of placebo maltodextrin	39 overweight or obese adults with BMI > 25 kg/m^2 Treatment (n = 21) Placebo (n = 18)	RD, DB, PC, P	12 wk	↓ ghrelin, ↑ PYY, ↓ postprandial insulin, ↓ body weight, ↓ fat mass ↓ energy intake	postprandial glucose, GLP-1, GIP, leptin, lipids	Parnell et al 2009 [211]
FOS	30 g/d of FOS *vs.* 30 g/d of placebo cellulose	22 healthy obese subjects with BMI 25-35 kg/m^2 aged 20-50 years Treatment (n = 12) Placebo (n = 10)	P, SB, PC,	6 wk	↑ PYY ↓ appetite	AST, ALT, γGT, glucose, insulin, HOMA-IR, HOMA % B, lipids, GLP-1	Daud et al 2014 [217]
FOS	18 g/d of freeze-dried powdered yacon containing 7.4 g/d of FOS *vs.* placebo (18 g/d of maltodextrin)	72 elderly subjects aged > 60 years Treatment (n = 37) Placebo (n = 35)	RD, DB, PC, P	9 wk	↓ serum glucose within the treatment group *vs.* baseline	Insulin, HOMA-IR, lipids, hs-CRP	Scheid et al 2014 [214]
RS	60 g/d of RS (Novelose 260) *vs.* placebo (0 g/d of RS)	10 healthy adults	SB, CO, PC, P	24 h	↓ postprandial glucose and insulin ↑ insulin sensitivity by a minimal model approach.	TAG	Robertson et al 2003 [223]
RS	30 g/d of RS *vs.* placebo (0 g/d of RS)	10 healthy adults with mean BMI 23.4 kg/m^2	SB, CO, PC, P	4 wk	↑ insulin sensitivity by hyperinsulinemic-euglycemic clamp at 3 wk ↓ insulin AUC at 4 wk ↑ insulin sensitivity by MTT at 4 wk	fasting glucose and insulin by MTT at 4 wk	Robertson et al 2005 [221]
RS	Low β-glucan (low, medium and high RS) *vs.* medium β-glucan (low, medium and high RS) *vs.* high β-glucan (low, medium and high RS) *vs.* glucose	10 normal-weight and 10 overweight women	MTT		↓ glucose and insulin AUC		Behall et al 2006 [227]

RS	40 g/d of RS *vs.* placebo (0 g/d of RS)	20 insulin resistant subjects	SB, RD, PC, P	12 wk	↑ insulin sensitivity by euglycaemic-hyperinsulinaemic clamp	adiposity, lipids, inflammatory markers	Johnston et al 2010 [224]
RS	15 g/d of HAM-RS2 *vs.* 30 g/d HAM-RS21 *vs.* control (0 g/d of HAM-RS2)	Overweight and obese adults (11 men and 22 women)	RD, DB, CO, C	4 wk	↑insulin sensitivity in men by insulin-modified i.v. glucose tolerance test	fasting glucose, insulin, HOMA-IR, hs-CRP, fructosamine adiponectin	Maki et al 2012 [225]
RS	24 g/d of RS vs. control (0 g/d of RS)	86 subjects both with MetS and without MetS	DB, CO, PC, P	12 wk	↓ cholesterol in subjects with MetS ↓ body fat composition	fasting glucose, postprandial glucose, HbA1c	Nichena metla et al 2014 [228]
RS	40 g/d of type 2 RS (HAM-RS2) *vs.* placebo (0 g/d of RS)	17 diabetic adults with mean BMI 30.6 kg/m^2 aged mean 55 years	SB, RD, CO	12 wk	↓ postprandial glucose by MTT, ↓ TG, ↑ GLP-1, ↓ TNF α	fasting glucose, insulin, HOMA-IR, insulin sensitivity, HbA1c, C-peptide, lipids, IL-6	Bodinham et al 2014 [226]
RS	Barley kernel-based bread high in RS and NSP (37.6 g/d of total dietary fiber) *vs.* white wheat bread (9.1 g/d of total dietary fiber)	20 healthy middle-aged subjects	RD, CO	3 d	↑ fasting GLP-1, ↑ postprandial PYY and GLP-2, ↑ breath H_2, ↑ fasting SCFA, ↑ insulin sensitivity, ↓ b-glucose and s-insulin responses to the standardised breakfast	fasting glucose, insulin, HOMA-IR, inflammatory markers	Nilsson et al 2015 [222]

AUC, area under the curve; AST, aspartate aminotransferase; BMI, body mass index; C, control; CO, crossover; DB, double blind; FFA, free fatty acid; FOS, fructooligosaccharide; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide-1; γGT, gamma-glutamyltranspeptidase; HAM-RS2, high-amylose maize type 2 resistant starch; HbA1c, glycated haemoglobin; HC, hypercholesterolemia; HDL-C, high density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; hs-CRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; L, longitudinal; LDL-C, low density lipoprotein cholesterol; IL-10, interleukin 10; LPS, lipopolysaccharide; MetS, metabolic syndrome; MTT, meal tolerance test; NEFA, non-esterified fatty acid; OGTT, oral glucose tolerance test; P, parallel; PC, placebo control; PYY, peptide YY; RD, randomised; RS, resistant starch; SB, single blind; sc-FOS, short chain fructo-oligosaccharide; SCFA, short chain fatty acid; TAG, triacylglycerol; TC, total cholesterol; T2DM, type 2 diabetes mellitus; TG, triglyceride; TNF-α, tumor necrosis factor-alpha.

Table 3. The summary of synbiotic human intervention studies

Dietary synbiotic intervention	Subjects	Design	period	Outcomes	No effects	Ref
200 mL of synbiotic shake containing 10^8 UFC/mL *L. acidophilus*,10^8 UFC/mL *B. bifidum* and 2 g FOS *vs.* 200 mL of the synbiotic shake without bacteria	20 adults with T2DM aged 50-60 years, 10/group	RD, P, DB, PC	15 d preingestion and 30 d ingestion	↑ HDL-C ↓ glycemia	TC, TG	Moroti et al 2012 [281]
14 x 10^9 CFU/tablet of probiotic capsule containing 7 viable and freeze-dried strains of *L. acidophilus* (2 × 10^9 CFU), *L. casei* (7 × 10^9 CFU), *L. rhamnosus* (1.5 × 10^9 CFU), *L. bulgaricus* (2 × 10^8 CFU), *B. breve* (2 × 10^{10} CFU), *B. longum* (7 × 10^9 CFU), *S. thermophilus* (1.5 × 10^9 CFU) and 100 mg FOS *vs.* placebo containing the same substance without bacteria	54 adults with T2DM aged 35–70 years, 27/group	RD DB, PC, P	8 wk	↓ hs-CRP ↑ GSH ↓fasting glucose		Asemi et al 2013 [282]
Two tablets/d of 500 mg metformin + two tablets/d of protexin containing *L. acidophilus* (1 × 10^8 CFU), *L. casei* (5 × 10^8 CFU), *L. bulgaricus* (1.5 × 10^8 CFU), *L. rhamnosus* (7.5 × 10^7 CFU), *B. longum* (2.5 × 10^7 CFU), *B. breve* (5 × 10^7 CFU), *S. thermophiles* (5 × 10^7 CFU) and 350 mg FOS *vs.* two tablets/d of 500 mg metformin + two tablets/d of placebo (120 mg of starch)	63 subjects with NASH	RD, DB,PC, P	24 wk	↓ fasting glucose ↓ TG, ↓ cholesterol ↓ BMI ↓ ALT ↓ AST		Shavakhi et al 2013 [138]
Two sachets/d of Lepicol containing 2 × 10^8 CFU of strains of *L. plantarum, L. delbrueckii ssp.bulgaricus, L. acidophilus, L. rhamnosus* , *B. bifidum* and 3 g FOS/d *vs.* control (usual care)	20 subjects with NASH	OL, RD, C	26 wk	↓ liver fat within the synbiotic group *vs.* baseline ↓ AST within and between groups	fasting glucose, TC, TG, HDL-C, LDL-C, ALT, liver stiffness	Wong et al 2013 [288]
Two tablets/d of protexin containing 2 x 10^8 CFU of strains (*L. casei, L. rhamnosus, S. thermophilus, B. breve, L. acidophilus, B. longum and L. bulgaricus*) and 250 mg FOS *vs*. two tablets/d of placebo (250 mg maltodextrin)	52 subjects with NAFLD	RD, DB,PC, P	28 wk	↓ fasting glucose, ↓ insulin, ↓ HOMA-IR, ↓ ALT, ↓ AST, ↓ GGT, ↓ hs-CRP, ↓ TNF-α, ↓ NF-kB		Eslamparast et al 2014 [289]
Two tablets/d of protexin containing 2 x 10^8 CFU of strains (*L. casei, L. rhamnosus, S. thermophilus, B. breve, L. acidophilus, B. longum and L. bulgaricus*) and 250 mg FOS *vs*. two tablets/d of placebo (250 mg maltodextrin)	38 subjects with MetS	RD, DB,PC, P	28 wk	↓ fasting glucose, insulin & HOMA-IR, ↑ QUICKI	BMI, LDL-C, waist	Eslamparast et al 2014 [284]

				within the treatment group *vs.* baseline	circumstance	
a) 120 g/d of synbiotic bread containing *L. sporogenes* (1×10^8 CFU) and 0.07 g inulin as prebiotic per 1 g b) probiotic c) control bread	78 (or 81)patients with T2DM aged 35-70 years, 26 (or 27)/group	RD, P, DB, PC	8 wk	↓ insulin ↓ HOMA-IR ↓ HOMA for β-cell function ↓ TAG ↓ VLDL-C ↓ TC/HDL-C ↑ HDL-C ↑ NO ↓ MDA *vs*. probitic and control breads	fasting glucose, QUICKI. hs-CRP, TC, LDL-C, TAC, GSH, Catalase, Liver enzymes ,Calcium, Magnesium, BP	Asemi et al 2014, 2016 [285, 291, 292]
Synbiotic food containing *L. sporogenes* ($27 \text{ X } 10^7$ CFU) and 1.08 g inulin per day *vs*. control food (the same substance without probiotic bacteria and prebiotic inulin	62 diabetic patients aged 35-70 years	RD, DB, CO, C	6 wk	↓ insulin ↓ hs-CRP ↑ GSH ↑ uric acid	fasting glucose, HOMA-IR, TG, HDL-C, TAC,	Asemi et al 2014 [286]
Synbiotic food containing *L. sporogenes* ($18 \text{ X } 10^7$ CFU) and 0.72 g inulin per day *vs*. control food (the same substance without probiotic bacteria and prebiotic inulin	52 pregnant women, primigravida, aged 18-35 years in their third trimester	RD, PC	9 wk	↓ insulin ↓ HOMA-IR ↑ QUICKI	fasting glucose, hs-CRP	Taghizadeh et al 2014 [287]
a) synbiotic group ($4 \text{ x } 10^9$ CFU *L. salivarius* UBL S22 and 10 g/d of FOS) b) probiotic ($4 \text{ x } 10^9$ CFU *L salivarius*) c) placebo capsule (gelatin)	45 healthy adults aged 20-25 years, 15/group	RD, PC, P, SB	6 wk	↓ fasting glucose within all groups *vs*. baselines, ↓ insulin & ↓ HOMA-IR in probiotic & synbiotic groups *vs.* placebo		Rajkumar et al 2015 [283]
Synbiotic capsule containing *L. acidophilus* ($2 \text{ x } 10^9$, *L. casei* $2 \text{ x } 10^9$, *B. bifidum* $2 \text{ x } 10^9$ CFU/g and 800 mg inulin	60 overweight diabetic patients	RD DB, PC, P	12 wk	↓ fasting glucose,		Tajabadi-

	with CHD aged 40–85 years, 30/group			↓ insulin, ↓ HOMA for β-cell function, ↑ QUICKI, ↓ HDL-C		Ebrahimi et al 2016 [134]
Two tablets/d of protexin containing 2 x 10^8 CFU of strains (*L. casei, L. rhamnosus, S. thermophilus, B. breve, L. acidophilus, B. longum and L. bulgaricus*) and 250 mg FOS *vs*. two tablets/d of placebo (250 mg maltodextrin)	50 lean subjects with NAFLD	RD DB, PC, P	28 wk	↓ fasting glucose ↓ TAG ↓ TC ↓ hs-CRP ↓ TNF-α ↓ NF-kB p65 ↓ hepatic steatosis ↓ fibrosi ↓ AST	HOMA-IR, insulin, QUICKI, HDL-C, LDL-C, ALT, GGT	Mofidi et al 2017 [290]

ALT, alanine aminotransferase; AST, aspartate aminotransferase activity; BP, blood pressure; C, control; CO, crossover; CFU, colony forming unit; CHD, coronary heart disease; DB, double blind; FOS, fructooligosaccharide; GGT, g-glutamyltransferase; GSH, glutathione; HDL-C, high density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-C, low density lipoprotein cholesterol; MDA, malondialdehyde; MetS, metabolic syndrome; NASH, non-alcoholic steatohepatitis; NAFLD, nonalcoholic fatty liver disease; NF-kb, nuclear factor kappa B; OL, open label; P, parallel; PC, placebo control; QUICKI, quantitative insulin sensitivity check index; RD, randomised; SB, single blind; TAC, total antioxidant capacity; TAG, triacylglycerol; TC, total cholesterol; TG, triglycerides; TNF-α, tumor necrosis factor-α; T2DM, type 2 diabetes mellitus; UFC, urinary free cortisol; VLDL-C, very low density lipoprotein cholesterol.

7 LITERATURE REVIEW: METHODS TO ASSESS INSULIN SENSITIVITY

There are a wide variety of methods to assess insulin sensitivity [293]. All methods have their advantages and limitations [294]. A method could be chosen in accordance with the size, type and purposes of studies, available resources (equipment, researcher expertise, funds) [293-295].

7.1 Hyperinsulinemic-euglycemic clamp

The hyperinsulinemi-euglycemic clamp is referred to as the gold-standard method and directly assesses insulin-mediated glucose utilization under steady-state conditions [294]. This test is used to assess the sensitivity of body tissues (muscle, fat, and liver) to insulin [296]. The hyperinsulinemic-euglycemic glucose clamp was first developed by Andres, Swerdloff, Pozefsky, and Coleman in 1965 in response to perceived limitations of the prevailing tests, the oral glucose tolerance test and the rapid intravenous glucose tolerance test [297]. After an overnight fast, a cannula is placed in an antecubital vein for glucose and insulin infusion. The other is inserted into a hand vein in a retrograde manner while the forearm is kept in a heated box which opens arteriovenous anastomoses and "arterialises" the venous blood [296]. Insulin is exogenously infused at a constant rate (calculated as a dose per unit of surface area rather than body weight to avoid over-insulinisation of overweight subjects) [293], while 20% glucose is infused at a variable rate to maintain stable blood glucose concentrations between 5 and 5.5 mmol/L, determined at 5 min intervals [298]. Samples for insulin measurements are collected during the last 30-60 min of the test when a steady state is reached and the glucose infusion rate then equals the total glucose disposal rate or the metabolic clearance rate. Insulin-sensitive subjects will need a much greater glucose infusion rate while insulin-resistant subjects will need a lower glucose infusion rate [298].

A hyperinsulinemic-euglycemic glucose clamp has several limitations. It is costly, labour-intensive, time consuming and technically difficult to carry out. It requires a highly-experienced technician. It has a potential risk of hypoglycaemia. It suppresses hepatic glucose and free fatty acids output. The problems of the clamp include artificiality because of its steady state, the supra-physiological plasma levels of insulin achieved, stability of the glucose and insulin measures and relatively high coefficient of variation of the outcome measures [293, 294, 296].

7.2 Hyperglycaemic clamp

The hyperglycaemic clamp allows researchers to primarily quantify β-cell insulin secretion in response to glucose, but it is also used to assess insulin resistance [294]. The plasma glucose concentration is acutely raised to 7 mmol/L above basal levels by a continuous glucose infusion after a loading dose of glucose [294]. Hyperglycemic stability is achieved and maintained by adjusting the glucose infusion upward or downward at 5 min intervals for 2 hours [293, 294]. Hyperglycaemia induces insulin release from the pancreatic β-cell leading to increased glucose disposal [294]. The glucose infusion rate equals glucose uptake by all the tissues under the steady-state conditions. Insulin sensitivity is calculated by dividing the glucose infusion rate (mg/kg/min) by the average insulin concentrations over the last 20-30 min of the test [299]. Loss of glucose in the urine also needs to be accounted for. This test has several limitations. It is time-consuming, labour-intensive, expensive, and usually inappropriate for a large sample size. Prevailing glucose is higher than that found in normal physiology [293, 294].

7.3 Minimal Model Analysis of Frequently sampled intravenous glucose tolerance test

The frequently sampled intravenous glucose tolerance test (FSIGT) can be used to assess β-cell function and insulin sensitivity. Various modified protocols for this test exists [294]. One cannula is inserted for blood collections and the other is placed for infusion of glucose or glucose and insulin or tolbutamide (used for a modified FSIGT). Glucose (50% dextrose in water) with a dosage of 0.3 g/kg of body weight is infused over a 1-2 min period starting at time 0. Blood collections for insulin and glucose measurements are performed at -10, -1, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 20, 22, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, 120, 160, and 180 min. The frequency of blood collections vary depending on protocols but 26-30 blood samples are collected [300]. The FSIGT only requires glucose infusion while the modified FSIGT also infuses insulin or tolbutamide at 20 minutes to ensure that subjects have an adequate insulin level for calculation of insulin sensitivity [300-302]. The usual doses are insulin (0.02-0.03 U/kg of body weight) or tolbutamide (4.3 mg/kg). Glucose during the first 7-10 min of the test stimulates insulin secretion from the pancreatic β-cells and suppresses hepatic glucose. During the first 20 min of the test, glucose disposal is mediated by glucose and endogenous insulin injection [296]. The advantage of this test is to clarify all components of glucose disposal [296]. This test is less labour-intensive and less expensive than the hyperinsulinemic-euglycemic clamp [296]. However, it is still costly, time-consuming (3 hours) and needs an experienced technician and a large number of blood sample collections

[294, 296]. It requires a computer software program to calculate insulin sensitivity and glucose effectiveness indices [303-307].

7.4 Oral glucose tolerance test

The OGTT is a simple test used in clinical practice for the diagnosis of glucose intolerance and T2DM [9]. After an overnight fast, subjects consume a 75 g glucose drink after fasting blood collections. Blood samples are collected at 30 min intervals for 2 or 3 hours for glucose and insulin measurements [308]. This test is simpler and lower in cost than the hyperinsulinemic-euglycemic clamp or the FSIGT [296]. Insulin sensitivity and β-cell function can be calculated by using the methods of Matsuda and DeFronzo [308] and Stumvoll et al [309] by collecting blood samples at -30, -15, 0, 30, 60, 90 and 120 min following a 75 g glucose ingestion after an overnight fast.

Insulin Sensitivity Index $_{Matsuda}$ = 10^4/(fasting glucose $_{mg/dL}$ × fasting insulin $_{\mu IU/mL}$ × mean glucose OGTT $_{mg/dL}$ × mean insulin OGTT μIU/mL)$^{0.5}$

Insulin Sensitivity Index $_{Stumvoll}$ = 0.226 – (0.0032 × BMI $_{kg/m2}$) – (0.0000645×120 min Insulin $_{pmol/L}$) – (0.00375 × 90 min glucose $_{mmol/L}$)

β-cell function Index $_{derived\ from\ the\ OGTT}$ = (iAUC insulin $_{30min}$ / iAUC glucose $_{30min}$)

Several indices derived from the OGTT are available [310-313]. However, the results of the OGTT are likely to be influenced by variable rates of gastric emptying and glucose absorption leading to the poor reproducibility even in the same subject. Moreover, it does not provide sufficient information with regard to dynamics of glucose and insulin action (insulin sensitivity *per se*) [294, 296].

7.5 Homoeostasis model assessment

The HOMA is the most widely used index derived from fasting plasma glucose and insulin concentrations [314]. HOMA formulas can be described as follows:

HOMA = (fasting insulin $_{\mu U/mL}$ × fasting glucose $_{mmol/L}$) / 22.5

HOMA-β cell function = 20 × insulin $_{\mu U/mL}$ / (fasting glucose $_{mmol}$ – 3.5)

HOMA index correlates with indices derived from the hyperinsulinemic-euglycemic clamp, the hyperglycaemic clamp and the FSIGT. HOMA has been proposed as a simple, inexpensive alternative method for large-scale or epidemiological studies [314]. However, it should be noted that the coefficient of variation (CV) for HOMA varies resulting in low precision of estimates [294, 314-317]. This index does not include post-load insulin and glucose concentrations and this assumes that hepatic and peripheral insulin sensitivity are the same which is not realistic [294].

7.6 Quantitative insulin sensitivity check index

The QUICKI is also widely used index derived from fasting parameters [318].

QUICKI formula is proportional to 1/log (HOMA) as follows:

QUICKI = 1 / [log (fasting insulin $_{\mu U/mL}$) + log (fasting glucose $_{mg/dL}$)]

The HOMA and QUICKI are appropriate for the studies where insulin sensitivity assessment is only a secondary endpoint [319].

7.7 CONTINUOUS LOW DOSE INSULIN AND GLUCOSE INFUSION TEST

Borai et al [294, 295] suggested simplifications to the gold standard hyperinsulinemic-euglycemic clamp will still provide precise data with less expensive methods. The continuous low dose insulin and glucose infusion test (LDIGIT) is considered as a simple, less labour-intensive and accurate method to assess insulin sensitivity and secretion [320]. The LDIGIT is a dynamic measure of insulin sensitivity that has been validated against the gold standard clamp although it has not been used prognostically [320]. The LDIGIT is described in chapter 9. LDIGIT requires fewer blood collections, less complex techniques and it is less expensive and less complex than hyperinsulinemic-euglycemic clamp, hyperglycaemic clamp and FSIGT. Therefore the LDIGIT was applied in this present intervention study where there was a large sample size and the primary endpoint was assessment of insulin sensitivity. In addition to the LDIGIT, the HOMA was used to assess insulin sensitivity in the fasting state.

8 SUMMARY AND SCOPE OF THESIS

The global prevalence of diabetes is expected to increase from 382 million people in 2013 to 592 million by 2035 [1]. A strategy for the prevention of diabetes is fundamental to restraining the health expenditure on diabetes and its complications [321].

An improved dietary pattern, a reduced energy intake, weight loss along with regular physical activity can lower the risk of T2DM. Optimal dietary patterns without caloric restriction have been little investigated in intervention studies.

Epidemiological studies have indicated an association between consumption of red and processed meat diet and an increased risk of T2DM but interventions are very limited.

Dairy consumption, particularly yoghurt has shown to be inversely associated with the risk of T2DM in most meta-analyses. Interventions however have had very mixed results with most being negative. Mixed findings for whole grains, legumes and nuts are observed in both epidemiological studies and intervention studies.

The objective of this study therefore was to determine if a dietary pattern rich in red, processed meat and refined grains decreases insulin sensitivity compared with a dietary pattern rich in whole grains, nuts, legumes, dairy and devoid of red and processed meat, in the absence of weight changes and this is presented in chapter 9.

The primary endpoint of this study was to assess insulin sensitivity and the secondary endpoint was to analyse possible mediators of insulin sensitivity.

The weakness of the case against red meat as a promoter of type 2 diabetes is the complete lack of any short or long term intervention data showing that red meat intake increases insulin resistance or reduces pancreatic function. This is a severe limitation and without this the case against red meat is relatively weak. As a result, this submitted thesis aims to address an important gap in published literature in the context of red meat intake in an isocaloric diet, albeit with unbalanced macronutrient intake. It is designed to be the first weight-stable intervention study of the effect of a combination of red and processed meat and refined grains on insulin sensitivity.

Chapter 10 reports on the acute effects of a meal high red and processed meat and refined grains on postprandial glucose, insulin and lipid responses in comparison with a meal high in

dairy, whole grains, legumes and nuts. This acute study was conducted at the end of two 4 week diets and is thus an acute on chronic intervention.

Chapter 11 reports on biomarkers involved in insulin resistance to explore potential mechanisms of action for our findings reported in chapters.

9 CONSUMPTION OF RED AND PROCESSED MEAT AND REFINED GRAINS FOR 4 WEEKS DECREASES INSULIN SENSITIVITY IN INSULIN-RESISTANT ADULTS: A RANDOMIZED CROSSOVER STUDY

9.1 Preface

Chapters from 2 to 5 indicate that there have been a limited number of studies investigating the effects of a diet high in red and processed meat and refined grains on insulin sensitivity in comparison to a diet high in whole grains, nuts, dairy and legumes and insulin sensitivity, without confounding weight changes. We therefore conducted a 4-week weight-stable dietary intervention with a randomized crossover design to examine the effect of consuming a diet high in red and processed meat and refined grains on insulin sensitivity compared with a diet high in whole grains, nuts, dairy and legumes with no red meat.

9.2 Published article

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Consumption of red and processed meat and refined grains for 4 weeks decreases insulin sensitivity in insulin-resistant adults: A randomized crossover study☆,☆☆

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ABSTRACT

Background. Red and processed meat and refined grains are associated with an increased risk of type 2 diabetes. Interventions are limited. We hypothesized that a diet high in red and processed meat and refined grains (HMD) would decrease insulin sensitivity compared to a diet high in whole grains, nuts, dairy and legumes with no red meat (HWD).

Methods. Forty-nine subjects without diabetes [15 men and 34 women, age, 35.6 ± 15.7 years, body mass index (BMI), 27 ± 5.9 kg/m^2] underwent two 4-week weight-stable dietary interventions in a randomized crossover design. The insulin sensitivity index (ISI) was calculated from the last 30 min of a continuous low-dose insulin (25 mU/kg·h) and glucose (4 mg/kg·min) infusion test (LDIGIT $_{120–150min}$) at the end of each diet.

Results. The population fell into two very discrete groups: those with a very low insulin response in the LDIGIT $_{120–150min}$ on HMD (Group 1 < 56 pmol/L, n = 24), and those with relatively normal insulin responses (Group 2 > 56 pmol/L, n = 25). Group 2 had significantly higher insulin concentrations [(median and interquartile range) 153, 180 for HMD vs. 123, 149 pmol/L for HWD; $P = 0.019$] and glucose concentrations [(mean ± standard deviation) 7.4 ± 1.3 for HMD vs.6.7 ± 1.2 mmol/L for HWD; $P = 0.05$], resulting in a significantly decreased ISI [(median and interquartile range) 21.1, 34.2 for HMD vs. 31.6, 39.4 for HWD; $P = 0.014$] compared to HWD. Log ISI after HMD was significantly correlated with BMI ($r = −0.5$; $P = 0.009$), fat mass ($r = −0.55$; $P = 0.004$) and self-reported activity levels ($r = −0.45$; $P = 0.024$).

Abbreviations: AGE, advanced glycation end product; AUC, area under the curve; BMI, body mass index; DBP, diastolic blood pressure; DXA, dual energy X-ray absorptiometry; GI, glycemic index; GLP-1, glucagon like peptide-1; GL, glycemic load; Ginf, glucose infusion rate; Gss, steady-state blood glucose; HDL-C, high density lipoprotein cholesterol; HMD, a diet high in red and processed meat and refined grains; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high sensitivity C-reactive protein; HWD, a diet high in whole grains, nuts, dairy and legumes; iAUC, incremental area under the curve; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; ISI, insulin sensitivity index; ISS, steady-state serum insulin; LDIGIT, low-dose insulin and glucose infusion test; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; PYY, peptide tyrosine-tyrosine; SBP, systolic blood pressure; SCFAs, short chain fatty acids; T2DM, type 2 diabetes mellitus.

☆ This trial is registered with the Australian New Zealand Clinical Trials Registry www.anzctr.org.au/ (ACTRN12614000519651).

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Conclusions. A dietary pattern high in red and processed meat and refined grains decreased insulin sensitivity compared to a dietary pattern high in whole grains, nuts, dairy products and legumes only in relatively insulin-resistant adults.

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1. Introduction

Insulin resistance is involved in the pathogenesis of type 2 diabetes mellitus (T2DM) [1,2]. Although certain dietary patterns are known to be associated with the development of T2DM independent of other lifestyle factors [3], exactly which components are able to influence the risk of T2DM is not clear. Meta-analyses of prospective studies have shown that processed meat consumption is associated with an increased risk of T2DM [4,5]. A limited number of intervention studies investigating the effect of lean red meat consumption on insulin sensitivity in the absence of weight loss have shown conflicting results [6–8]. There are no weight-stable intervention studies of a processed meat-rich diet. In a recent meta-analysis yoghurt and ice-cream were protective although the association between total dairy intake and T2DM was relatively weak with a small effect size [9,10], consistent with the contradictory results on insulin sensitivity of randomized controlled trials of dairy-rich diets [6,11]. Epidemiological studies have shown that whole-grain consumption is associated with a reduced risk of T2DM [12,13] but weight-stable interventions of a whole grain-rich diet have shown mixed results [14–17]. The Predimed study has shown a reduced incidence of T2DM with 30 g/d of mixed nuts (almonds, walnuts and hazelnuts) [18], but in another trial no differences were seen in HOMA-IR in participants who received walnuts and cashews compared to those who did not receive nuts [19]. Prospective studies have observed no association of T2DM with vegetable and fruit consumption, but an inverse association with consumption of green leafy vegetables has been found [20,21].

This study aimed to examine the effect of a diet high in red and processed meat and refined grains on insulin sensitivity compared to a diet high in dairy, whole grains, nuts and legumes containing no red meat, using sensitive measures of insulin sensitivity in weight stability. The primary hypothesis was that a diet high in red and processed meat and refined grains would induce greater insulin resistance compared to a diet high in dairy, whole grains, nuts and legumes, with a secondary hypothesis that a diet high in dairy, whole grains, nuts and legumes would improve lipid profiles compared to a diet high in red and processed meat and refined grains.

2. Methods

2.1. Ethical Approval and Registration

This study was approved by the University of South Australia Human Research Ethics committee and all study participants gave their written informed consent prior to participating. The trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12614000519651). AUD$ 240 was offered to the participants on completion of both diets. Recruitment began in June 2014 and ended in September 2015.

2.2. Study Participants

A total of 51 participants [body mass index (BMI) 18–45 kg/m^2], aged over 18 years, were recruited by public advertisement and a recruiting agency. An initial screening questionnaire was carried out via email or telephone. Exclusion criteria were diabetes, medication or supplements which could affect glucose metabolism, a history of metabolic illness including liver or kidney disease, pregnancy or breastfeeding, significant weight gain or weight loss over the last 3 months and food allergy or lactose intolerance. We planned to exclude people if weight gain or loss was 3 kg or more over the previous 3 months but no volunteers were excluded. Initially we aimed to recruit only overweight and obese participants but slow recruitment led us to widen the entry criteria to normal weight subjects knowing we might have to analyze the groups separately. We did not test HOMA-IR prior to randomization.

Responders who passed the screening criteria visited the Sansom Institute for Health Research Clinical Trial facility at the University of South Australia in the morning after an overnight fast. Written informed consent was obtained. Blood pressure was measured four times after sitting quietly for 10 min (Omron, Kyoto, Japan). Height was measured on a wall mounted stadiometer (Seca, Hamburg, Germany) and body weight was measured on electronic digital scales (Tanita, Tokyo, Japan) in light clothing and without shoes. Body composition was measured by whole-body dual-energy x-ray absorptiometry (DXA) (Luna Prodigy, Lunar Radiation, Wisconsin, USA). A 75 g oral glucose tolerance test (OGTT) was performed to exclude people with diabetes. A simple survey on activity levels was performed at baseline (sedentary or moderately active).

Details of study recruitment and participant flow are shown in Fig. 1.

2.3. Dietary Intervention

Eligible participants were randomly allocated to either a diet high in red and processed meat and refined grains (HMD) or a diet high in whole grains, nuts, legumes, dairy and devoid of red and processed meat (HWD) for 4 weeks, and then were crossed over to the alternative diet for 4 weeks. Randomization was performed via an online random number generator (www.randomization.com) by a researcher who was not involved in this study. Analyses were performed by an investigator blinded to the diet order. A minimum of a 2-week washout period (on average 3 weeks) between the two diets with a return to usual diet was planned.

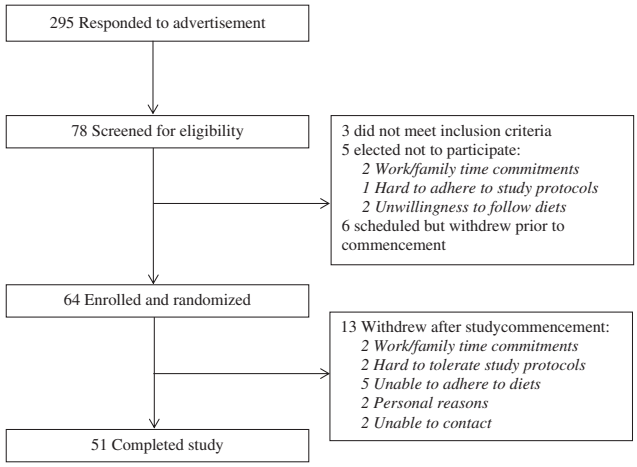


Fig. 1 – Flow chart of participant recruitment and withdrawal from the study.

Daily consumption of 200–300 g red meat, ≥50 g processed meat (ham, bacon and sausage) was recommended as this reflected the amount consumed in the highest quintile in the cohort studies [4,5,22]; in order to maximize the difference between two diets, 4–6 serves of refined grains (white bread, rice, noodles and pasta) based on volunteers' weights, 1–2 serves of vegetables, 1–2 serves of fruits and a minimal amount of dairy products (e.g. 1–2 teaspoons of milk) were allowed during the HMD. Total energy intake from those key foods in the HMD varied between 4500 and 6000 kJ. Two hundred to three hundred grams of potatoes, 1 serve of jam or marmalade, 3–9 serves of oil or spread, or 3–4 serves of indulgence food were recommended for additional energy in accordance with volunteers' weights. During the HWD, a daily consumption of 4 serves of low fat dairy products including 2 serves of yoghurt, 3–4 serves of whole grain breads, rice and pasta [1 slice (40 g) wholegrain or wholemeal bread, ½ cup (75–120 g) cooked brown rice, ½ cup (75–120 g) cooked wholemeal pasta, ¼ cup (30 g) muesli, ½ cup oats, $^2/_3$ cup (30 g) wheat cereal flakes, ½ cup (120 g) cooked wholegrain porridge, 3 (35 g) wholegrain crispbreads], 60–90 g unsalted nuts, either 70–150 g chicken or fish (or other seafood) or 150–225 g cooked legumes based on the volunteers' weights was advised. Total energy intake of those key foods in the HWD varied between 6000 and 8500 kJ. Two to seven serves of oil or spread and 1 serve of jam or marmalade were advised for additional energy intake in accordance with volunteers' weights. Planned composition of the two diets is shown in Table 1. Total energy was matched in the two diets. Vegetables and fruits were limited to 1–2 serves in both diets as epidemiology does not suggest that these components are related to the risk of type 2 diabetes [20, 21]. Serving sizes were defined according to the guidelines of the Australian National Health and Medical Research Council [23]. Food was not provided to participants but an AUD$ 80 voucher for red meat and an AUD$ 50 for nuts was given at the end of each diet period. Specific dietary instructions were provided for each diet along with eight different energy levels based on BMI and gender. For the HWD a healthy cooking method including boiling, steaming, stewing and poaching was advised rather

Table 1 – The recommended menus for each of the diets.

HMD		HWD	
Food	Daily Goal	Food	Daily Goal
Red meat	200–300 g	Chicken	80–150 g
		Fish	70–150 g
		Cooked legumes	150–225 g (l cup–1½ cups)
Processed meat	≥50 g	Nuts	60–90 g
Refined grains	4–6 serves	Whole grains	3–4 serves
Dairy products	minimal	Dairy products	4 serves
Potato	200–300 g		
Vegetables	1–2 serves	Vegetables	1–2 serves
Fruits	1–2 serves	Fruits	1–2 serves
Oil/spread	3–9 serves	Oil/spread	2–7 serves
Jam or marmalade	1 serve	Jam or marmalade	≤ 1 serve
Wine/Alcohol	Optional	Wine/Alcohol	Optional
Indulgence food	3–4 serves		
	To replace refined grains or potato if desired		

HMD, a high red and processed meat and refined grains diet; HWD, a high whole grain, nuts, dairy and legumes diet.

than deep-frying, grilling and roasting. Samples of recipes and daily meal plans for two diets were given. A kitchen scale (Homemaker Slimline Electronic Scale; Kmart Australia) was provided. Participants were encouraged to check their weight every day and maintain usual physical activity. Weight was measured at each visit and in the event of weight change, the diet was adjusted accordingly. A daily checklist and a 3-day weighed food diary every 2 weeks were used in order to monitor dietary compliance. Participants attended the clinic fortnightly and the 3-day weighed food diary and daily checklists were checked. The 3-day diaries were analyzed by Food Works Professional Edition 8.0 (Xyris, Australia). Dietary intake was not measured before the intervention or during the washout.

2.4. Assessment of Insulin Sensitivity

The LDIGITs were administered over 150 min after an overnight fast at the end of each diet period. LDIGIT was not performed at baseline or after washout so the primary endpoint was a comparison of insulin sensitivity at the end of the two diets. A fixed rate infusion of insulin (25 mU/kg·h) and glucose (4 mg/kg·min) was performed using an IMED Gemini PC-2 IV pump/controller (IMED, San Diego, CA, USA). A plastic cannula (BD Nexiva catheters; 20G 1.25 in. 1.1 × 32 mm) was inserted in large antecubital vein for glucose and insulin infusions. Another plastic was placed in the other antecubital vein for collecting blood samples. Blood samples were collected at −5, 0, 5, 10, 15, 20, 25, 30, 50, 70, 90, 110, 120, 125, 130, 135, 140, 145, 150 min into two tubes containing either no additives or sodium fluoride and EDTA. The tube with no additives for serum insulin and C-peptide was kept upright in a rack at room temperature for 30 min to ensure complete clot formation and then placed on ice, while the sodium fluoride EDTA tube for plasma glucose was placed immediately on ice until centrifugation and processing. Whole blood glucose concentration was monitored for safety reasons at each time-point using a glucometer (Abbott, Free Style Optium). The steady-state serum insulin (Iss) and steady-state glucose (Gss) concentrations during the last 30 min of the LDIGIT were measured for assessment of insulin sensitivity. The insulin sensitivity index (ISI) was calculated by dividing the constant glucose infusion rate (Ginf) by Gss and normalizing this ratio to Iss and expressed as glucose clearance per plasma insulin concentration: $ISI_{LDIGIT} = Ginf/Gss \cdot Iss$ [24]. Insulin sensitivity under fasting conditions was calculated with homeostasis model assessment of insulin resistance (HOMA-IR) [7], using the following formula: HOMA = (fasting insulin (μU/ml) × fasting glucose (mmol/l))/22.5. Serum C-peptide concentrations during the first 30 min were measured to evaluate insulin secretion. Blood samples were centrifuged at 4000 RPM at 4 °C for 10 min (Universal 32R, Hettich Zentrifugen, Germany). Plasma glucose and serum high sensitivity C-reactive protein (hsCRP), total cholesterol, triglyceride and high density lipoprotein cholesterol (HDL-C) was analyzed by an automated spectrophotometric analyzer (Konelab 20XTi, Thermo Electron, Massachusetts USA) and serum insulin and C-peptide were assayed with commercial ELISA kits (Alpha Diagnostic, Texas USA, Kit # 0030N for insulin, Kit # 0040 for C-peptide).

2.5. Statistical Analyses

Data were analyzed with SPSS V22 (IBM, Chicago IL, USA). A sample size of 50 overweight and obese subjects without diabetes was determined to provide 80% power to detect a 20% change (alpha level = 0.05) in insulin sensitivity as assessed by LDIGIT [24]. The area under the curve (AUC) for glucose $_{(0–150min)}$ and incremental area under the curve (iAUC) C-peptide $_{(0–30min)}$ were calculated using the trapezoidal rule. The Shapiro–Wilk test, Q-Q plots and histograms were used to test for the normality of distribution. Non-normally distributed variables such as fasting glucose, fasting insulin, HOMA-IR, insulin $_{(120–150min)}$, ISI and average C-peptide $_{(-30min)}$ were log transformed before analysis but untransformed data are shown in the tables. Differences between groups were tested by repeated measures ANOVA. Linear regression with backward elimination was used to examine dietary predictors of log HWD ISI after adjustment for log HMD ISI. Wilcoxon signed rank nonparametric tests were also used as fasting insulin, fasting glucose, HOMA-IR, iAUC for C-peptide $_{(0–30min)}$ and hs CRP were still skewed after log transformation. Associations were examined using Pearson and Spearman correlation coefficients. An unpaired t test, a chi-square test and Mann–Whitney nonparametric test were performed to contrast variables between groups. Repeated measures of ANOVA was used to examine glucose concentrations at all time points during the LDIGIT. If the time by treatment interaction was significant then post-hoc tests were undertaken to examine when treatment differences occurred. Data are presented as the mean ± standard deviation (SD). Skewed variables such as fasting glucose, fasting insulin, HOMA-IR, insulin $_{(120–150min)}$, ISI, fasting C-peptide, average C-peptide $_{(5–30min)}$, iAUC for C-peptide $_{(0–30min)}$ and hs CRP are expressed as medians and interquartile ranges. The glucose concentrations during the LDIGIT in Fig. 2 are presented as mean ± standard error (SE). Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Study Participants

Of the 295 people that initially responded to advertising, 78 people were screened and 64 enrolled and randomized. Fig. 1 outlines the participant recruitment flow. Fifty-one participants were recruited but only 49 subjects were used due to failure of blood sample collections or cannulations. We found that the insulin values of LDIGIT $_{(120–150min)}$ for both diets were not normally distributed (Shapiro–Wilk $P < 0.0001$) and fell naturally into 2 distinct populations: those with very low insulin concentrations of about 20–40 pmol/L (group 1) and those with normal to very high insulin concentrations (group 2). Therefore, we divided the population a posteriori into two groups at the median of 56 pmol/L with a median insulin of 33 in group 1 and a median insulin of 122 in group 2. Despite this division, the data in both groups still required log transformation to achieve normality. Baseline characteristics of the two groups are shown in Table 2. Group 2 had a significantly

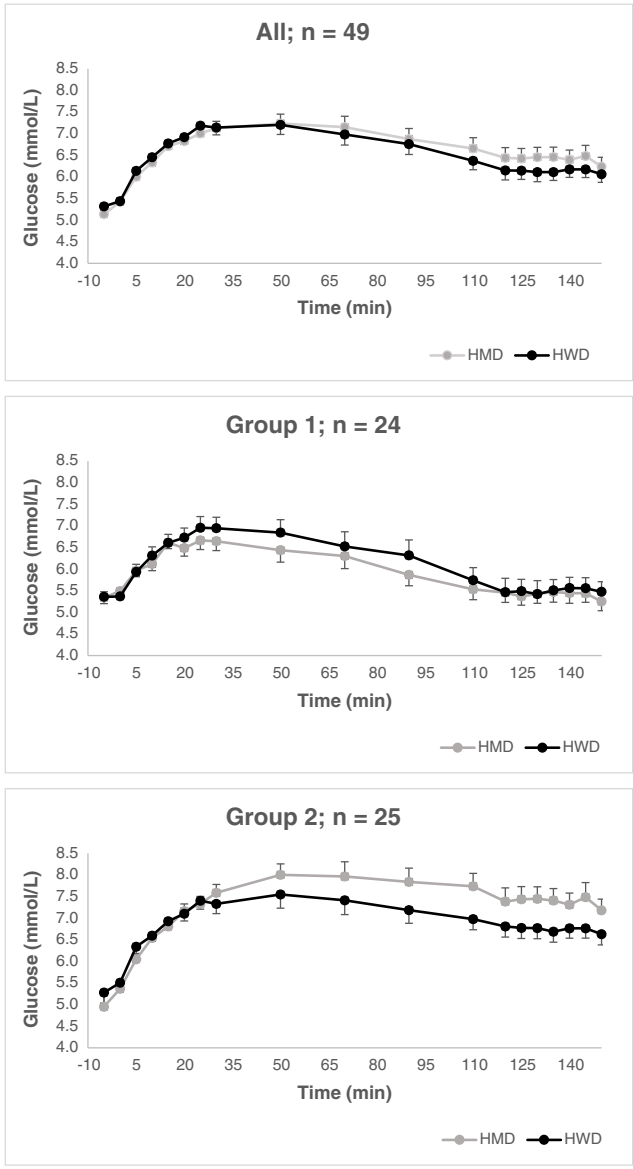


Fig. 2 – Mean ± SE changes in glucose concentrations over the course of the 2.5-h LDIGIT assessed at the end of each diet period. When group was added as a between subject factor diet by group ($P = 0.008$), time by group ($P < 0.001$) and diet by time by group ($P < 0.001$) were all significant. Therefore, repeated measures ANOVA was performed separately for each group. In group 2, statistically significant differences were observed between the two diets with a mean difference of 0.46 mmol/L ($P = 0.012$), whereas in group 1, no statistical significance was observed. n = 49; n = 24 per group 1; n = 25 per group 2. Groups 1 and 2 are based on the insulin values of LDIGIT $_{(120–150min)}$, not randomized groups. HMD, a high red and processed meat and refined grains diet; HWD, a high whole grain, nuts, dairy and legumes diet.

higher BMI, fat mass and % fat compared to group1. Body weight remained very stable throughout the study period. The average weights were 77.9, 77.8 and 77.9 kg during the HMD period (baseline, 2 weeks and 4 weeks) and 77.8, 77.7 and 77.8 kg during the HWD period.

3.2. *Dietary Compliances and Intakes*

The composition of the study diets consumed during two diets periods is presented in Table 3. There were no significant differences in total energy, protein and saturated fat between the two diets. Total fat, polyunsaturated fat, monounsaturated fat, sugars, dietary fiber, thiamine, riboflavin, potassium, magnesium, calcium and folate were significantly higher on HWD, while cholesterol, starch, sodium, alcohol, vitamin C and zinc were significantly higher on HMD. Carbohydrate intake was significantly higher on HMD ($P < 0.001$) which is largely explained by potato and indulgence foods. Volunteers chose more potatoes, bread or rice than biscuits, cake and soft drink. Sugar intake was significantly higher on HWD ($P < 0.001$) due to lactose in dairy products. Significantly high levels of unsaturated fat ($P < 0.001$) can be ascribed to the 60–90 g/d of nuts. Milk, cheese, legumes and nuts contributed to higher thiamine and

Table 2 – Baseline characteristics of participants. [a]

	Group 1 (n = 24)	Group 2 (n = 25)	P value
Sex (M/F)	9/15	6/19	0.3 [**]
Age (y)	35.5 ± 15.6	35.6 ± 16	1
NGT (n)	10	6	0.2 [**]
IFG/IGT (n)	14	19	
Baseline fasting glucose (mmol/L)	5.3 ± 0.8	5.7 ± 0.6	0.07
Baseline 2 h glucose (mmol/L)	6.96 ± 1.5	7.55 ± 1.7	0.2
HOMA-IR (HWD)	0.29, 0.47	0.39, 0.5	0.07 [***]
HOMA-IR (HMD)	0.21, 0.54	0.65, 0.8	0.01 [***]
LDIGIT $_{(120–150min)}$ insulin (HWD pmol/L)	33.4, 27.8	122.7, 149	< 0.001 [****]
LDIGIT $_{(120–150min)}$ insulin (HMD pmol/L)	29.6, 20.3	153, 180	< 0.001[****]
Baseline weight (kg)	69.7 ± 15.1	85.7 ± 21.7	0.005
BMI (kg/m^2)	24.4 ± 4.6	29.4 ± 5.9	0.002
Baseline SBP (mmHg)	110.4 ± 9.2 [b]	114.3 ± 12.4 [c]	0.3
Baseline DBP (mmHg)	69.1 ± 9.9 [b]	72.5 ± 9.5 [c]	0.3
Total Fat Mass (kg)	20.6 ± 11.4	34.3 ± 13.3	< 0.001
Total Lean Mass (kg)	45.4 ± 11.4	47.7 ± 12.1	0.5
Total Fat Mass (%)	30.3 ± 12.8	41.0 ± 8.6	0.001

Groups were contrasted with an unpaired t-test. Values are means ± SDs except for HOMA-IR, LDIGIT $_{(120–150min)}$ insulin, which is median and interquartile range. Groups 1 and 2 are defined a posteriori based on the insulin values of LDIGIT $_{120–150min}$, not randomized groups. M, male; F, female; NGT, normal glucose tolerance; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; HMD, a high red and processed meat and refined grains diet; HWD, a high whole grain, nuts, dairy and legumes diet; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

[a] n = 49.

[b] n = 22.

[c] n = 19.

[**] P values were obtained by chi-square test.

[***] P values were obtained by Mann–Whitney nonparametric test.

[****] P values were obtained by unpaired t tests after log transformation.

Table 3 – Reported dietary intake assessed by weighed food records.

	HMD	HWD	P value
Energy kJ	8443 ± 1180	8421 ± 1079	0.8
Protein g	97.39 ± 12.3	97.57 ± 11.9	0.6
Total fat g	73 ± 19	88 ± 16	P < 0.001
Saturated fat g	26.2, 9.6	26.1, 9.6	0.9
Polyunsaturated fat g	8.7, 4.5	17.2, 6.5	P < 0.001
Monounsaturated fat g	31.5 ± 9	37 ± 7.6	P < 0.001
Cholesterol mg	219.4, 57	158.6, 67	P < 0.001
Carbohydrate g	218.5, 43	182, 42	P < 0.001
Sugars g	58.4, 26	89, 27	P < 0.001
Starch g	153.7, 47.8	93.5, 44.8	P < 0.001
Alcohol g	0, 8.7	0, 2	P < 0.05
Dietary fiber g	18 ± 4	29 ± 6	P < 0.001
Thiamine mg	1.5, 0.6	1.8, 0.5	P < 0.05
Riboflavin mg	1, 0.4	3, 0.7	P < 0.001
Niacin mg	46.2, 11.6	45.2, 9.4	0.2
Vitamin C mg	90.5, 51	53, 40	P < 0.001
Total folate μg	236, 140	386, 122	P < 0.001
Sodium mg	2084, 928	1586, 511	P < 0.001
Potassium mg	2895 ± 541	3456 ± 452	P < 0.001
Magnesium mg	229, 53	519, 132	P < 0.001
Calcium mg	245, 119	1418, 269	P < 0.001
Iron mg	11.5, 3.1	11.5, 4	0.7
Zinc mg	13.8, 4.2	12.1, 2.6	P < 0.001
Protein % energy	19.2, 3.1	19.7, 3.1	0.5
Fat % energy	32 ± 6	38 ± 5	P < 0.001
Carbohydrate % energy	44 ± 5.9	37 ± 5.2	P < 0.001
Fiber % energy	1.7, 0.5	2.7, 0.7	P < 0.001

These values are based on an average of two weighed food diaries per dietary period. HMD, a high red and processed and refined grains diet; HWD, a high whole grain, nuts, dairy and legumes diet (n = 49). Normally distributed values are presented as means ± SDs and P values were determined by paired t tests. Non-normally distributed variables (shown as medians and interquartile ranges) were log transformed and P values were determined by paired t tests for saturated fat, polyunsaturated fat, cholesterol, carbohydrate, sugars, starch, folate, sodium, calcium, zinc, and % energy from fiber, and Wilcoxon signed rank nonparametric tests for alcohol, thiamin, riboflavin, niacin, vitamin C, magnesium, iron and % energy from protein. The approximate glycemic index and glycemic load amounted to 65 and 180 respectively for HMD and 45 and 100, respectively for HWD.

riboflavin, while beans, whole grains and nuts resulted in higher dietary fiber and magnesium on the HWD.

3.3. *Insulin, Glucose and Insulin Sensitivity Index (ISI) During the Last 30 Min of the LDIGIT*

Insulin, glucose and ISI during the last 30 min of the LDIGIT are shown in Table 4. On repeated measures ANOVA diet was not significant ($P = 0.16$) but there was a diet by group interaction ($P < 0.05$) for ISI before and after adjustment for BMI so each group was subsequently analyzed separately. Adding activity levels as a covariate in the initial ANOVA improved the diet effect ($P = 0.005$) and activity itself was a predictor ($P = 0.012$) of the change in ISI. Age, gender, BMI, body composition and diet order did not influence the results nor did presence of impaired fasting glucose/impaired glucose tolerance (IFG/IGT). Activity levels were positively related to age ($r = 0.33$; $P = 0.017$), ISI on HMD ($r = 0.28$; $P = 0.05$) and inversely related to % fat ($r = -0.3$; $P = 0.03$). Activity levels were not different between insulin sensitivity groups ($P = 0.3$).

In group 1, there were no differences between diets in insulin and glucose concentrations or log ISI before or after adjustment for activity. By contrast, in group 2, insulin concentrations were significantly higher on the HMD compared to the HWD [(median and interquartile range) 153, 180 compared to 123, 149 pmol/L, respectively; $P = 0.019$]. Glucose concentrations at 120–150 min were significantly higher on HMD compared to HWD (7.4 ± 1.3 vs. 6.7 ± 1.2 mmol/L respectively; $P = 0.05$). ISI was significantly lower after HMD compared to HWD [(median and interquartile range) 21.1, 34.2 for HMD vs.31.6, 39.4 for HWD; $P = 0.014$]. Adding activity level as a covariate ($P = 0.054$) did not attenuate the diet effect ($P = 0.010$). In this group, log ISI after HMD was significantly correlated with BMI ($r = -0.51$; $P = 0.009$), fat mass ($r = -0.55$; $P = 0.004$) and self-reported activity levels ($r = 0.45$; $P = 0.024$) and the r values for HWD were very similar.

Table 4 – Effects of diets on insulin, glucose, ISI and cholesterol measured at the end of each diet period.

	All (n = 49)				Group 1 (n = 24)			Group 2 (n = 25)		
	HMD	HWD	P value	P Group by time	HMD	HWD	P value	HMD	HWD	P value
Insulin $_{(120–150min)}$ (pmol/L)	56.9, 123.2	52.1, 94.7	0.2	0.07	29.6, 20.26	33.4, 27.78	0.7	153, 180	123, 149	0.02
Glucose $_{(120–150min)}$ (mmol/L)	6.4 ± 1.5	6.1 ± 1.3	0.1	0.05	5.4 ± 1.0	5.5 ± 1.2	0.6	7.4 ± 1.3	6.7 ± 1.2	0.05
ISI_{LDIGIT} ml/kg/min/pmol/L × 10^{-3}	58.8, 119.5	76.4, 103	0.2	0.04	139.8, 108.9	132.8, 163.3	0.7	21.1, 34.2	31.6, 39.4	0.01
Glucose AUC $_{(0–150min)}$ (mmol/L)	16.9 ± 3.3	16.6 ± 3.1	0.45	0.03	14.99 ± 2.55	15.56 ± 3.16	0.3	18.78 ± 2.79	17.66 ± 2.82	0.04
Total cholesterol (mmol/L)	4.82 ± 1.01	4.6 ± 0.97	0.01	0.07	4.63 ± 1.2	4.26 ± 0.98	0.001	5.0 ± 0.83	4.9 ± 0.85	0.6

P values for insulin $_{(120–150min)}$ and glucose $_{(120–150min)}$, ISI_{LDIGIT}, total cholesterol and glucose AUC $_{(0–150min)}$ were determined by paired t tests. Insulin $_{(120–150min)}$ and ISI_{LDIGIT} were log transformed. Values are expressed as medians and interquartile ranges except for glucose $_{(120–150min)}$, total cholesterol and glucose AUC $_{(0–150min)}$ which are presented as means ± SDs. Groups 1 and 2 are defined *a posteriori* based on the insulin values of LDIGIT $_{(120–150min)}$, not randomized groups. HMD, a high red and processed meat and refined grains diet; HWD, a high whole grain, nuts, dairy and legumes diet; AUC, area under the curve; ISI, insulin sensitivity index.

There was no relationship on linear regression between log ISI HWD with nuts, yoghurt, total dairy, total red meat or processed meat or fat type after adjustment for log ISI HMD in the whole population or either group (data no shown).

The log ISI HWD was positively correlated with the amount of carbohydrates in the HWD (Beta-coefficient 0.27; t = 3.5; P = 0.001) after adjustment for log ISI HMD (beta-coefficient 0.79; t = 10.3; P < 0.001). Carbohydrates contributed to 7.4% regression of the variance in log ISI HWD. Fiber and magnesium were not related to log ISI HWD.

3.4. *Lipid Profiles*

There was a borderline significant group by time interaction for total cholesterol. Total cholesterol (P = 0.01) and triglyceride (P = 0.05) concentrations were significantly higher after HMD than after HWD while HDL cholesterol was unchanged (Table 4).

3.5. *Fasting Glucose, Fasting Insulin and HOMA-IR*

Fasting glucose, fasting insulin and HOMA-IR were examined by nonparametric tests, as normality was not achieved by log transformation. In neither group did diet have an effect on these variables. There was no interaction between diet and group (Table 5).

3.6. *C-peptide During the First 30 Min of the LDIGIT*

There were no significant differences in fasting C-peptide, average C-peptide $_{(5–30min)}$ and iAUC for C-peptide $_{(0–30min)}$ and

Table 5 – Effects of diets on fasting glucose, fasting insulin and C-peptide measured at the end of each diet period.

	All (n = 49)				Group 1 (n = 24)			Group 2 (n = 25)		
	HMD	HWD	P value	P Group by time	HMD	HWD	P value	HMD	HWD	P value
Fasting glucose (mmol/L)	5.29, 0.61	5.28, 0.61	0.7	0.2	5.26, 1.03	5.25, 0.8	0.7	5.37, 0.5	5.34, 0.7	0.3
Fasting insulin (pmol/L)	11.4, 15.02	8.67, 11.24	0.5	0.1	5.5, 14.6	7.1, 13	0.9	16.4, 19.4	9.2, 13.4	0.3
HOMA-IR	0.49, 0.63	0.36, 0.44	0.4	0.2	0.21, 0.54	0.29, 0.47	0.98	0.65, 0.8	0.39, 0.5	0.3
Fasting C-peptide (nmol/L)	0.64, 0.45	0.63, 0.45	0.4	0.6	0.47, 0.27	0.54, 0.3	0.9	0.76, 0.5	0.88, 0.5	0.2
Average C-peptide $_{(5–30min)}$ (nmol/L)	1.11, 0.62	1.24, 0.78	0.64	0.5	0.87, 0.46	0.79, 0.61	0.9	1.37, 0.92	1.49, 0.78	0.3
iAUC for C-peptide $_{(0–30min)}$ (nmol/L/min)	27.7, 19.8	32.8, 20.9	0.37	0.7	23.5, 16.6	21.6, 18.3	0.86	34.6, 27.2	40.5, 25.3	0.2
Triglyceride (mmol/L)	0.95 ± 0.47	0.87 ± 0.43	0.05	0.9	0.8 ± 0.46	0.72 ± 0.35	0.19	1.1 ± 0.44	1.0 ± 0.46	0.2
HDL-C (mmol/L)	1.4 ± 0.43	1.39 ± 0.42	0.46	0.9	1.63 ± 0.38	1.6 ± 0.38	0.6	1.19 ± 0.35	1.17 ± 0.34	0.6
hsCRP(mg/L)	0.79, 2.77	0.58, 1.86	0.73	0.2	0.35, 1.31	0.15, 1.18	0.44	1.9, 3.7	1.3, 3.1	0.8

P values for fasting glucose, fasting insulin, HOMA-IR, iAUC for C-peptide $_{(0–30min)}$ and hsCRP were obtained from nonparametric tests. P values for fasting C-peptide, average C-peptide $_{(5–30min)}$, triglyceride and HDL-C were determined by paired samples t tests. Fasting C-peptide and average C-peptide $_{(5–30min)}$ were log transformed. Values for triglyceride and HDL are means ± SDs. Fasting glucose, fasting insulin, HOMA-IR, fasting C-peptide, average C-peptide $_{(5–30min)}$, iAUC for C-peptide $_{(0–30min)}$ and hsCRP are presented as medians and interquartile ranges. HMD, a high red and processed meat and refined grains diet; HWD, a high whole grain, nuts, dairy and legumes diet; iAUC, incremental AUC, area under the curve; HDL-C, high density lipoprotein cholesterol; hsCRP, high sensitivity C-reactive protein.

hs CRP between the two diets. There was no interaction between diet and group (Table 5).

3.7. Glucose During the Whole LDIGIT

Overall there was a strong effect of time ($P < 0.001$) and a weak, but significant, time by diet interaction ($P = 0.024$). When group was added as a between subject factor diet by group ($P = 0.008$), time by group ($P < 0.001$) and diet by time by group ($P < 0.001$) were all significant so the groups were analyzed separately. Group 1 showed no significant time by diet interaction. Group 2 showed a significant diet by time interaction with a mean difference between diets of 0.46 mmol/L over the 150 min ($P = 0.012$). Fig. 2 presents glucose concentrations over the course of the LDIGIT.

There was a significant group by diet interaction for the AUC for glucose $_{(0–150min)}$ ($P = 0.025$). Group 2 showed significant differences between two diets (18.78 ± 2.79 for HMD vs. 17.66 ± 2.82 for HWD; $P = 0.042$; $n = 25$), while no differences between the two diets were seen overall or in group 1 (Table 4).

4. Discussion

The main findings of this weight-stable randomized crossover study are that in the *a posteriori* defined group with a normal to high insulin responses during the LDIGIT, and which contained predominantly overweight and obese participants with IFG/IGT, the daily consumption of a high red and processed meat and a low fiber, medium glycemic index/glycemic load diet for 4 weeks significantly increased insulin and glucose concentrations during the last 30 min of the LDIGIT, compared to the consumption of a high dairy, whole grains, nuts and legumes and low GI/low glycemic load diet.

It would appear that a dramatic improvement in diet quality could increase insulin sensitivity by nearly 50% in only four weeks in people at risk of developing T2DM. No effect was seen in people with a lower risk of developing T2DM as assessed by low LDIGIT insulin levels. It would appear that the source of protein and the type of fat was not related to the change in insulin sensitivity but an increase in the carbohydrate containing foods in the HWD was related to an increase in insulin sensitivity. Fiber was not related to insulin sensitivity suggesting that it was other components in whole grains such as resistant starch.

Resistant starches have been shown a positive effect on insulin sensitivity [25–27]. Resistant starch is not digested in the small intestine and is fermented by the gut flora in the large intestine with an increase in the production of short chain fatty acids (SCFAs — acetate, butyrate and propionate) leading to improved insulin sensitivity through several potential mechanisms [28]. In a randomized cross-over study of 20 healthy middle-aged subjects, the consumption of barley kernel-based bread high in resistant starch and NSP (37.6 g/d of total dietary fiber) for 3 consecutive days showed increases in fasting glucagon like peptide-1 (GLP-1), postprandial peptide tyrosine-tyrosine (PYY) and GLP-2, breath H_2 excretion (a marker of gut microbiota fermentation), fasting SCFAs, as well as an improvement in insulin sensitivity along with lower glucose and insulin responses to the standardized breakfast, compared to white wheat bread (9.1 g/d of total dietary fiber) [29]. The consumption of muffins containing 2.3 g soluble fiber and 5 g of resistant starch per 100 g decreased postprandial glucose and insulin concentrations with a greater effect when both were combined producing a 59% reduction in the AUC for insulin [30]. A multiethnic cohort of 75,512 participants with a 14-year follow-up has shown that grain fiber was associated with a lower risk of T2DM [31]. A prospective cohort study of 35,988 older women with 6-year follow-up also found inverse associations between intakes of whole grains and dietary fiber and risk of T2DM [32]. Resistant starch would have also been a significant component of whole grains. A very high fiber diet (25 g of soluble fiber and 25 g of insoluble fiber) has reduced circulating glucose and insulin concentrations in patients with T2DM compared to a moderate fiber diet (8 g soluble fiber and 16 g of insoluble fiber) [33] and 70–110 g/d of fiber improved insulin sensitivity in healthy young and old adults compared to a usual diet of 10–13 g/d [34]. In contrast to these findings, whole grains (mainly based on milled wheat) for 6 weeks did not alter insulin sensitivity in overweight adults compared to refined grains [35] suggesting that lower amounts of fiber, as in our study, have little effect on insulin sensitivity.

A high magnesium intake has been inversely associated with the risk of T2DM in a meta-analysis of prospective studies [36] and cross-sectional studies [37]. A good source of magnesium in the HWD diet was fermented dairy which has been associated with protection from T2DM despite the high saturated fat content [38]. However, in our study although magnesium was related to carbohydrate intake it was not related to insulin sensitivity.

Nuts were a major source of unsaturated fat in the HWD and displaced a significant amount of starch from the diet. The Predimed study has shown that the consumption of a Mediterranean diet supplemented with polyphenol-rich olive oil or mixed nuts reduced the incidence of T2DM by 51%–52% when assessed by annual OGTT, compared to a control diet over 4 years in the absence of changes in body weight in the Reus substudy [18]. Almond consumption decreased 24-h insulin secretion in hyperlipidemic adults without diabetes [39] and enhanced glycemic control in subjects with T2DM [40]. Unsaturated fatty acids, phytosterols, polyphenols, arginine, fiber, potassium, calcium, and magnesium from nuts may together exert a protective effect on T2DM [41]. Even though increased polyunsaturated fatty acids from walnut consumption decreased fasting insulin [42], the effect of polyunsaturated fatty acids on insulin sensitivity is uncertain with very limited human interventions [43].

Our findings are also consistent with meta-analyses of prospective studies indicating a positive correlation between red and processed meat and an increased risk of T2DM with risk estimates of 1.13 to 1.19 per 100 g per day of total red meat and 1.19 to 1.51 per 50 g processed meat per day [4,5,22]. If the removal of 200 g of red meat and 50 g of processed meat per day from the diet was responsible for the benefit seen with the HWD diet then one might expect a 38%–50% improvement in insulin sensitivity assuming pancreatic function was not influenced by the diet. Saturated fat intake was the same in both diets but red and processed meats may

be a richer source of advanced glycation end products (AGEs) than chicken, fish and dairy products [22]. High glycemic index (GI) carbohydrates such as potato and white bread in the HMD have been linked to an increased risk of T2DM [44,45] and are a significant source of AGEs [46]. A very recent updated meta-analysis of 74,248 women from the Nurses' Health Study, 90,411 women from the Nurses' Health Study II and 40,498 men from the Health Professionals Follow-Up Study has shown a 33% higher risk of T2DM in those in the highest quintile of energy-adjusted GI and a 10% higher risk of T2DM in the highest quintile of energy-adjusted glycemic load (GL), compared to those in the lowest quintile. Those with a combination of a high GI or GL and low fiber diet had a 50% higher risk of T2DM compared to a low GI or GL and a high fiber diet [44]. Our HMD diet had an 80% higher glycemic load, which could contribute to the reduction seen in insulin sensitivity. However, a recent meta-analysis of 8 European countries showed no association between GI or GL and risk of T2DM [47] and another observational study found no association between GI and insulin resistance as assessed by HOMA but an inverse association between GL and HOMA [48]. Sodium was significantly higher on the HMD. It may be explained by high sodium present in processed meat [49] as well as in bread and other starchy foods. Evidence from an epidemiological study suggests that a high sodium intake from processed meat was more strongly associated with the increased risk of T2DM than the saturated fat, heme Fe and nitrate in processed meat [50]. However, data on salt and insulin sensitivity are conflicting [22]. Red meat is a source of heme iron. Increased dietary intake of heme iron is associated with an increased risk of T2DM in some studies via several mechanisms [22,51].

In line with our findings that insulin-sensitive subjects showed no statistically significant beneficial effects of the HWD on insulin sensitivity compared to the HMD, high dairy consumption did not alter glucose and insulin in normal weight subjects [52] but improved HOMA in overweight and obese subjects [53]. However, we have previously shown that a high dairy diet alone appeared to have adverse effects on insulin sensitivity in overweight and obese subjects [6].

To our knowledge this is first weight-stable intervention study of the effect of a combination of red and processed meat and refined grains on insulin sensitivity. We used a dynamic measure of insulin sensitivity that has been validated against the gold standard clamp although it has not been used prognostically. The crossover, isocaloric design, with tight control of weight and exercise patterns enabled us to detect the effects of diet which have not been seen in larger studies [54]. The main limitation was potentially insufficient power to analyze the data separately by insulin sensitivity group although significance was actually achieved in our post hoc analysis. Another limitation was that the effect of diets on measures of insulin sensitivity was assessed in groups categorized a posteriori on insulin concentrations during the LDIGIT. Moreover, meal preparation and consumption outside of a clinical research center could be a potential limitation. A further limitation was that dietary intake was not measured before the intervention at baseline.

In conclusion, our study suggests that a diet high in red and processed meat and refined grains reduces insulin sensitivity compared to a diet in high in whole grains, nuts, dairy products and legumes only in relatively insulin-resistant individuals and not in sensitive individuals but further work needs to be done with a prospectively enrolled insulin-resistant group.

Author Contributions

PMC designed research; YK conducted research; YK and PMC analyzed data; YK, JBK and PMC wrote the paper; PMC had primary responsibility for final content. All authors read and approved the final manuscript.

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Conflict of Interest

The authors report no conflicts of interest.

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10 DIFFERENTIAL EFFECTS OF RED MEAT/REFINED GRAIN DIET AND DAIRY/CHICKEN/NUTS/WHOLE GRAIN DIET ON GLUCOSE, INSULIN AND TRIGLYCERIDE IN A RANDOMIZED CROSSOVER STUDY

10.1 Preface

We conducted an acute study in order to investigate the effects of a meal consisting of white bread, leg ham and orange juice on postprandial glucose, insulin, C-peptide and triglyceride compared with a meal consisting of whole grain bread, chicken, milk, yoghurt and nuts matched for total energy.

This meal tolerance test was carried out after a 4-week weight-stable intervention. The two meal types are representative of the two different chronic intervention diets; a diet high in red and processed meat and refined grains and a diet high in whole grains, nuts and dairy.

10.2 Published Article

Kim Y, Keogh JB, Clifton PM. Differential Effects of Red Meat/Refined Grain Diet and Dairy/Chicken/Nuts/Whole Grain Diet on Glucose, Insulin and Triglyceride in a Randomized Crossover Study. Nutrients. 2016;

I would like to correct the word meat misspelled (meet) in the second paragraph in the introduction of this published article.

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Article

Differential Effects of Red Meat/Refined Grain Diet and Dairy/Chicken/Nuts/Whole Grain Diet on Glucose, Insulin and Triglyceride in a Randomized Crossover Study

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Abstract: Epidemiological studies suggest that a diet high in processed meat, with a high glycemic index is associated with an increased risk of type 2 diabetes. It is not clear if this is due to altered insulin sensitivity or an enhanced postprandial glucose. We aimed to compare the acute metabolic response of two different types of meals after ingestion of the matching diet for four weeks. The study was a randomized, crossover acute meal study. Volunteers consumed either a red meat/refined grain meal or a dairy/chicken/nuts/wholegrain meal after four weeks of the matching diet. After a three-week washout period and four weeks of the alternate diet, they consumed the matching meal. The diets differed with respect to both protein and carbohydrate sources. Blood samples were taken for 180 min for the measurement of glucose, insulin, C-peptide and triglyceride. Fifty-one participants (age: 35.1 ± 15.6 years; body mass index: 27.7 ± 6.9 kg/m^2, 17 with normal and 34 with impaired glucose tolerance) completed two meal tests. The area under the curve ($p < 0.001$) and incremental area under the curve ($p = 0.001$) for insulin was significantly higher after the red meat/refined grain diet than after the dairy/chicken/nuts/whole grain diet. There was an interaction between meal and glucose tolerance group ($p < 0.05$) in the area under the curve (AUC) and the incremental area under the curve (iAUC) of glucose; the red meat/refined grain diet increased glucose relative to the dairy/chicken/nuts/whole grain diet only in the normal group (+2.5 mmol/L/3 h). The red meat/refined grain diet increased glucose and insulin responses compared with the dairy/chicken/nuts/whole grain diet. This meal pattern would increase pancreatic stress long term and may account for the increased risk of type 2 diabetes with this diet.

Keywords: red meat/refined grain diet; dairy/chicken/nuts/whole grain diet; meal tolerance test; postprandial glucose; postprandial insulin

1. Introduction

The prevalence of diabetes worldwide is rapidly rising from 382 million people in 2013 to an estimated 592 million by 2035 [1]. Diabetes prevention strategies are vital to reducing the health expenditure on diabetes and its complications [2,3]. Insulin resistance plays a key role in incidence of type 2 diabetes (T2DM) [4]. Weight loss, along with regular exercise, can reduce insulin resistance [5,6]. Changing dietary patterns without caloric restriction has been little investigated despite the positive epidemiology [7,8].

Prospective studies showed that a Mediterranean dietary pattern, rich in whole grains, nuts, fresh vegetables, fruit, moderate in dairy products and fish or poultry, and low in red and processed meet, refined grains and sugar-sweetened foods and beverages, was strongly associated with a decreased

risk for T2DM ranging from 12% to 85% [9]. Fruits and vegetables alone have not been strongly linked to diabetes and there are no positive intervention studies [10,11].

Meta-analyses of prospective studies showed that processed meat intake of 50 g/day was associated with an increased risk of T2DM, ranging from 19% to 51% [12–14]. A Western dietary pattern high in red and processed meat and refined grains showed a positive association with the risk of T2DM in men [15]. There are no intervention studies on the effects of processed meat on insulin resistance or glucose homeostasis and this study directly addresses this question. The PREDIMED trial showed that a Mediterranean diet supplemented with mixed nuts reduced the risk of T2DM by 18% in the absence of weight change compared with a control diet after 4.1 years of follow-up [16].

Although the association between a diet high in red and processed meat and refined grains and an increased risk of T2DM is clear, it unknown if this is attributable to decreased insulin sensitivity or enhanced postprandial glucose responses or due entirely to confounding. These questions cannot be addressed without an intervention study. Therefore, this study aimed to determine the acute effects of two different types of meals on postprandial glucose, insulin, C-peptide and triglyceride after consumption of a matching diet for four weeks in order to elucidate mechanisms for changes in insulin sensitivity during the dairy/chicken/nuts/wholegrain diet.

We hypothesized that a meal consisting of white bread, leg ham and orange juice would increase postprandial glycemia and insulinemia compared with a meal consisting of whole grain bread, chicken, milk, yogurt and nuts matched for total energy.

2. Methods

2.1. Participants

Fifty-one participants were enrolled in this acute meal study. Eligible participants were adults aged over 18 years (body mass index (BMI), 18–45 kg/m^2) without type 2 diabetes. Exclusion criteria were medication or supplements that could affect glucose metabolism, a history of metabolic illness including liver or kidney disease and allergy or intolerance to dairy, nuts or other components of th diet. Women who were pregnant or breastfeeding or likely to be pregnant and people who experienced weight gain or weight loss over the last three months were also excluded. A recruiting agency and public advertisement were used for the recruitment. An initial screening questionnaire was conducted via email or telephone to identify suitable participants. People who passed the screening criteria visited the Sansom Institute for Health Research Clinical Trial facility at the University of South Australia, in the morning after an overnight fast. A 75 g oral glucose tolerance test (OGTT) was undertaken to exclude people with frank diabetes. This study was approved by the University of South Australia Human Research Ethics committee and registered with the Australian New Zealand Clinical Trials Registry (ACTRN12614000519651). Written, informed consent was obtained. Recruitment started in June 2014 and ended in September 2015. Participant recruitment and completion is shown in Figure 1. All participants who completed both chronic dietary phases had meal tolerance tests (MTTs) analyzed. Those with only one MTT were not analyzed.

2.2. Dietary Intervention

This study was part of a randomized crossover trial with two different dietary patterns which will be published separately. The effect of the chronic diets on insulin sensitivity was assessed with a low dose infusion of glucose and insulin. Participants followed either a diet high in red meat and processed meat and refined grains or a diet high in whole grains, nuts, and dairy products and no red meat for four weeks and then were crossed-over to the other dietary pattern. Food was not given to participants but an AU$ 50 and an AU$ 80 voucher were provided for the purchase of nuts and red meat, respectively, at the end of each diet period. Participants were specifically instructed regarding what to eat for each four-week period by providing eight different dietary guidelines in accordance with BMI and gender. The dietary guidelines included daily food selection goals, samples of a meal

plan and recipes, cooking methods and a standard serving size guide. A kitchen scale was provided to participants. Participants were free-living but were asked not to change their lifestyle and to maintain body weight. Participants were encouraged to contact investigators whenever they had inquiries via telephone or email. The two diets were intended to be isocaloric. Daily checklists and three-day weighed food diaries recorded fortnightly including cooking technique, food brand names, quantity and leftovers, were collected to ensure dietary compliance. Food-intake data (2 × 3-day weighed food diaries) were analyzed with FoodWorks Professional Edition 8.0 (Xyris Software, Brisbane, Australia). The recommended menu for the two different diets is shown in Table 1.

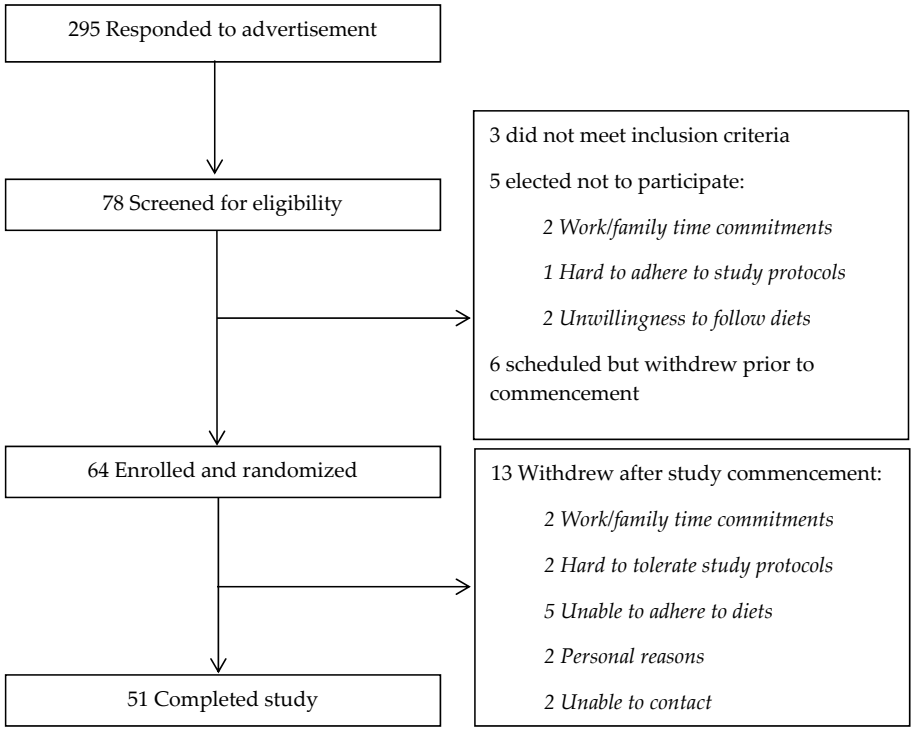


Figure 1. A participant flow chart.

Table 1. The recommended menus for each of the diets.

Red Meat/Refined Grain Diet		**Dairy/Chicken/Nuts/Whole Grain Diet**	
Food	Daily Goal	Food	Daily Goal
Red meat	200–300 g	Chicken Fish Cooked legumes	80–150 g 70–150 g 150–225 g (1 cup–1.5 cups)
Processed meat	≥50 g	Nuts	60–90 g
Refined grains	4–6 serves	Whole grains	3–4 serves
Dairy products	minimal	Dairy products	4 serves
Potato	200–300 g		
Vegetables	1–2 serves	Vegetables	1–2 serves
Fruits	1–2 serves	Fruits	1–2 serves
Oil/spread	3–9 serves	Oil/spread	2–7 serves
Jam or marmalade	1 serve	Jam or marmalade	≤1 serve
Wine/Alcohol	Optional	Wine/Alcohol	Optional
Indulgence food	3–4 serves To replace refined grains or potato if desired		

Serving sizes were based on the guidelines of the Australian National Health and Medical Research Council.

There was on average a three-week washout period on their usual diet in between the two four-week weight-stable diet periods. Randomization was undertaken using an online random number generator (www.randomization.com) by a researcher not involved in the study. The investigator who carried out data analysis and staff who measured blood samples were unaware of the diet order of participants. The MTT was conducted in the morning after an overnight fast at the end of each four-week diet period. The meal order in MTT was based on the diet that volunteers had followed for four weeks, which was randomized. Two different kinds of sandwiches were created in accordance with the key food profiles of two different diets. Food was not provided in the four-week diet intervention. The ham sandwich, representing a diet high in red meat and processed meat and refined grains was comprised of: 80 g white bread, 105 g leg ham, 20 g iceberg lettuce, 10 g tomato slices, 3 g onions, 3 g pickles and 250 mL 25% orange juice. The chicken sandwich, representing a diet high in whole grains, nuts and dairy products and no red and processed meat consisted of: 80 g wholegrain bread, 50 g chicken breast, 20 g iceberg lettuce, 10 g tomato slices, 100 g natural Greek yogurt, 200 g fat reduced milk, 7 g cashew nuts and 7 g peanuts. Participants were advised to consume the meal within 10 min. The food composition of each meal is summarized in Table 2. The two test meals were designed to be enriched in red meat or enriched in dairy but contained the same energy. Other macronutrients differed. The glycemic load of the dairy/chicken/nuts/wholegrain meal was 58% of the red meat/refined grain meal.

2.3. Clinical Measurements

An intravenous catheter (BD Nexiva catheters; 20GA 1.25IN 1.1 × 32 mm, Becton Dickinson and Co., Franklin Lakes, NJ, USA) was placed in a large antecubital vein. Blood samples were taken at −5, 0, 10, 20, 30, 40, 50, 60, 75, 90, 120, 150, and 180 min into both a tube with a sodium fluoride EDTA (ethylenediaminetetracetic acid) and a tube with no additives. The tube with no additives for serum insulin, C-peptide and triglyceride (TG) was stood upright at room temperature for 30 min, and then it was placed on ice, whereas the sodium fluoride EDTA tube for plasma glucose was placed straight on ice until centrifugation and processing. Blood samples were centrifuged at 4000 rpm at 4 °C for 10 min (Universal 32R, Hettich Zentrifugen, Tuttlingen, Germany). Plasma and serum aliquots were stored at −80 °C until analysis. The measurements of plasma glucose and serum TG were made using an automated spectrophotometric analyzer (Konelab 20XTi, ThermoFisher Scientific, Waltham, MA, USA). Serum insulin and C-peptide analyses were conducted by commercial ELISA kits (Alpha Diagnostic, San Antonio, TX, USA, Kit # 0030N for insulin, Kit # 0040 for C-peptide).

2.4. Analysis

Statistical analysis was performed using SPSS V22 (IBM, Chicago, IL, USA).

A sample size of 50 overweight and obese participants without diabetes was required to have 80% power to detect a 20% change (alpha level = 0.05) in insulin sensitivity with 80% power as assessed by LDIGIT (low dose insulin and glucose infusion test) [17]. No specific calculation was made for the MTT.

The area under the curve (AUC) and incremental AUC (iAUC) for glucose, insulin, C-peptide and TG were calculated using the trapezoidal method. The Shapiro-Wilk test, Q-Q plots, and histograms were used to test for the normality of distribution. Non-normally distributed data such as fasting glucose, insulin AUC, insulin iAUC, C-peptide iAUC and TG AUC were log transformed. Differences between two meals were assessed by repeated-measures ANOVA. A Wilcoxon signed rank nonparametric test was used as fasting insulin was still skewed after log transformation. TG iAUC was also analyzed by a Wilcoxon signed rank nonparametric test. Data for glucose AUC, glucose iAUC and C-peptide AUC are presented as the mean ± standard deviation (SD). Data for skewed variables such as fasting glucose, fasting insulin, insulin AUC, insulin iAUC, C-peptide iAUC, TG AUC and TG iAUC are expressed as medians and interquartile ranges. Chi-square tests and unpaired *t* tests were performed to compare participants' baseline characteristics by randomization order. Statistical significance was defined as $p < 0.05$.

Table 2. Nutrient composition of the test meals.

	Amount (g)	Energy (kJ)	CHO (g)	Food GI	Protein (g)	Fat (g)	Sat Fat (g)	Cholesterol (mg)	Sugar (g)	Dietary Fibre (g)	Sodium (mg)
Red meat/refined grain meal											
Bread, white,	80	848	39.2	70	6.4	1.52	0.24	0	2.32	2.16	320
25% orange juice	250	481	26.8	52	0.3	0	0	0	25.7	0.2	1
Lettuce, iceberg, raw	20	8	0.08		0.2	0.02	0	0	0.08	0.3	5.2
Tomato, raw	10	7.4	0.24		0.1	0.01	0	0	0.23	0.12	0.8
Ham, leg, lean & fat	105	804	1.5		20.7	11.6	4.7	73.5	0.1	0	1614
Onion, raw	3	3.81	0.14		0.05	0	0	0	0.14	0.06	0.3
Pickle	3	10.44	0.5		0.02	0.02	0	0	0.44	0.04	18.6
Total		**2162.65**	**68.46**	**60.4**	**27.77**	**13.17**	**4.94**	**73.5**	**29.01**	**2.88**	**1959.9**
Dairy/chicken/nuts/wholegrain meal											
Bread, wholegrain,	80	840	34.6	53	7.6	2.56	0.32	0	2.8	4.16	320
Nuts, cashews, raw	7	170.59	1.18	22	1.19	3.44	0.59	0	0.39	0.41	0.77
Nuts, peanuts, raw	7	166	1.12	14	1.82	3.43	0.49	0	0.28	0.63	1.26
Yoghurt, greek, natural	100	557	6.9	27	4.7	9.7	6.4	-	6.9	0	62
Chicken, breast, lean, raw	50	219	0		11.15	0.8	0.25	29.5	0	0	20.5
Milk, reduced fat	200	372	9.6	37	6.64	2.6	1.8	-	9.6	0	86
Lettuce, iceberg, raw	20	8	0.08		0.2	0.02	0		0.08	0.3	5.2
Tomato, raw	10	7.4	0.24		0.1	0.01	0		0.23	0.12	0.8
Total		**2339.99**	**53.72**	**44.9**	**33.4**	**22.56**	**9.85**	**29.5**	**20.28**	**5.62**	**496.53**

CHO, carbohydrate; GI, glycemic index.

3. Results

Fifty-one participants (15 men and 36 women) completed the study (Figure 1). A total of 13 participants withdrew from the study after study commencement. Two participants withdrew on the day of the blood collection after first four-week diet period due to the fear of blood collection. Five participants withdrew from the diet within five days of the first diet commencement: three could not adhere to red meat/refined grain diet and two could not comply with dairy/chicken/nuts/wholegrain diet. Four participants were enrolled but withdrew due to work and family time commitments, and for personal reasons. Two participants could not be contacted. Baseline characteristics of all participants and baseline characteristics of participants by sequence order is shown in Table 3. This was a small difference in 2 h glucose between randomization order groups.

Table 3. Baseline characteristics of a total of participants and participants by sequence order [1].

	All Participants	Participants Randomized to Red Meat/Refined Grain Diet First and Then Dairy/Chicken/Nuts/Whole Grain Diet (n = 27)	Participants Randomized to Dairy/Chicken/Nuts/Whole Grain Diet First and Then Red Meat/Refined Grain Diet (n = 24)	p Value
Sex (Male/Female)	15/36	9/18	6/18	0.5
Age (year)	35.1 ± 15.6	33 ± 12.7	37.4 ± 18.3	0.3
NGT (n)	17	10	7	0.6
IFG/IGT (n)	34	17	17	
Baseline fasting glucose (mmol/L)	5.5 ± 0.7	5.6 ± 0.7	5.4 ± 0.7	0.4
Baseline 2-h glucose (mmol/L)	7.3 ± 1.6	6.9 ± 1.3	7.8 ± 1.8	0.04 *
Baseline weight (kg)	79.4 ± 21.36	82.6 ± 21.4	75.8 ± 21.2	0.3
BMI (kg/m^2)	27.7 ± 6.9	28.6 ± 7.0	26.7 ± 6.9	0.3
Baseline SBP (mmHg)	112.2 ± 10.7 [2]	112.2 ± 12.4 [3]	112.2 ± 8.4 [4]	1
Baseline DBP (mmHg)	70.7 ± 9.7 [2]	70.4 ± 9.7 [3]	71.3 ± 9.9 [4]	0.8
Total Fat Mass (kg)	29 ± 15.7	30.4 ± 15.6	27.4 ± 16.0	0.5
Total Lean Mass (kg)	46.6 ± 11.5	48.4 ± 12.3	44.6 ± 10.4	0.2
Total Fat Mass (%)	36.6 ± 12.6	37.3 ± 12.2	35.9 ± 13.3	0.7

p values were obtained by chi-square tests and unpaired t tests to compare participants' characteristics by randomization order. Values are means ± SDs. NGT, normal glucose tolerance; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. [1] n = 51; [2] n = 42; [3] n = 24; [4] n = 18.

Analyses of six-day weighed food diaries showed that compliance with the protocol was satisfactory. Total energy intake did not differ between two diets. Reported dietary intake is shown in Tables 4 and 5. Overall, intakes of key foods assessed by food diaries were similar to the recommended quantities of key foods. Body weight was measured at baseline, week two and week four. Mean body weight was almost unchanged throughout the intervention period.

Table 4. Reported dietary intake of key foods assessed by weighed food records [1].

Red Meat/Refined Grain Diet		Dairy/Chicken/Nuts/Wholegrain Diet	
Food	Average intake	Food	Average intake
Red meat	241.3 g	Chicken Fish Cooked legumes	105.5 g 110.5 g 154.3 g
Processed meat	55.8 g	Nuts	70 g
Refined grains	5.2 serves (320.4 g) *	Whole grains Dairy products	3.9 serves (235.8 g) * 3.8 serves (687.4 g)

These values are based on an average of two weighed food diaries per dietary period. [1] n = 51. * Weight (g) was calculated based on cooked for rice and pasta, and raw for cereal, oats and muesli.

Table 5. Reported dietary intake of nutrients assessed by weighed food records [1].

	Red Meat/Refined Grain Diet	Dairy/Chicken/Nuts/Whole Grain Diet	*p* Value
Energy (kJ)	8406 ± 1172	8382 ± 1078	0.76
Protein (g)	95, 13.7	97, 14	0.41
Total fat (g)	73 ± 18	87 ± 16	$p < 0.001$
Saturated fat (g)	26.2, 8.5	26, 9.3	0.93
Polyunsaturated fat (g)	8.7, 4.4	17.2, 6.6	$p < 0.001$
Monounsaturated fat (g)	31 ± 9	37 ± 7	$p < 0.001$
Cholesterol (mg)	219.4, 52	158, 67	$p < 0.001$
Carbohydrate (g)	218.3, 43	181,40	$p < 0.001$
Sugars (g)	61, 26	89, 26	$p < 0.001$
Starch (g)	151.3, 46	93, 43	$p < 0.001$
Alcohol (g)	0, 8.7	0, 1.8	$p < 0.05$
Dietary fibre (g)	17, 6.4	27,8	$p < 0.001$
Thiamine (mg)	1.5, 0.6	1.9, 0.5	$p < 0.05$
Riboflavin (mg)	1, 0.5	2.8, 0.7	$p < 0.001$
Niacin (mg)	46.1, 12	44, 9.4	0.12
Vitamin C (mg)	90.5, 50	54, 40	$p < 0.001$
Total folate (μg)	236, 157	386, 131	$p < 0.001$
Sodium (mg)	2092, 918	1588, 523	$p < 0.001$
Potassium (mg)	2886 ± 531	3441 ± 450	$p < 0.001$
Magnesium (mg)	229, 51	518, 138	$p < 0.001$
Calcium (mg)	245, 116	1418, 253	$p < 0.001$
Iron (mg)	11.5, 3	11.5, 4	0.7
Zinc (mg)	13.6, 3.8	12, 2.6	$p < 0.001$
Protein (% energy)	19.2, 3	19.6, 3	0.4
Fat (% energy)	32 ± 5	39 ± 5	$p < 0.001$
Carbohydrate (% energy)	44 ± 6	37 ± 5	$p < 0.001$
Fibre (% energy)	1.7, 0.4	2.7, 0.7	$p < 0.001$

These values are based on an average of two weighed food diaries per dietary period. [1] $n = 51$. Normally distributed values are presented as means ± SDs and *p* values were determined by paired *t* tests. Non–normally distributed variables (shown as medians and interquartile ranges) were log transformed and *p* values were determined by paired *t* tests for protein, saturated fat, polyunsaturated fat, cholesterol, carbohydrate, sugars, starch, dietary fiber, sodium, calcium, zinc and % energy from fiber, and Wilcoxon signed rank nonparametric tests were performed for alcohol, thiamin, riboflavin, niacin, vitamin C, folate, magnesium, iron and % energy from protein.

Fasting glucose concentrations between two meals did not differ (median, interquartile range 5.3, 0.9 mmol/L for red meat/refined grain diet vs. 5.3, 0.6 mmol/L for dairy/chicken/nuts/whole grain diet). Total glucose AUC was not different between two meals (16.8 ± 2.8 mmol/L/3 h for red meat/refined grain diet vs. 16.2 ± 2.5 mmol/L/3 h for dairy/chicken/nuts/whole grain diet). The iAUC for glucose significantly differed between two meals (0.9 ± 2.3 mmol/L/3 h for red meat/refined grain diet vs. 0.3 ± 1.6 mmol/L/3 h for dairy/chicken/nuts/wholegrain diet, $p < 0.05$). There was a group by meal effect ($p < 0.05$ for total glucose AUC and $p = 0.016$ for total glucose iAUC) so glycemic control groups were analyzed separately. The group with normoglycemia ($n = 17$) had a significant diet effect for total glucose AUC ($p = 0.017$) and total glucose iAUC ($p = 0.024$) while in the group with impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) diet ($n = 34$) was not insignificantly different. Postprandial glucose concentrations after two meals are shown in Figure 2. Individual data for glucose AUC and iAUC is shown in Figure 3.

There was a small difference in fasting insulin, which was higher after the dairy/chicken /nuts/wholegrain diet vs. the red meat/refined grain diet. However, insulin AUC after red meat/refined grain diet was significantly higher than for the dairy/chicken/nuts/whole grain diet (94.4, 117 mU/L/3 h for red meat/refined grain diet vs. 65.9, 74.3 mU/L/3 h for dairy/chicken/nuts/wholegrain diet, $p < 0.001$). The insulin iAUC was significantly higher after the red meat/refined grain diet (79.7, 119.2 mU/L/3 h for red meat/refined grain diet vs. 54.3, 71.9 mU/L/3 h for dairy/chicken/nuts/whole grain diet, $p = 0.001$) than after dairy/chicken/nuts/wholegrain diet. Postprandial insulin concentrations after the two diets are shown in Figure 4. Individual data for insulin AUC and iAUC is shown in Figure 5. Both the C-peptide

AUC (12.5 ± 5.8 nmol/L/3 h for red meat/refined grain diet vs. 10.2 ± 4.1 nmol/L/3 h for dairy/chicken/nuts/wholegrain diet, $p = 0.006$) and iAUC (8.6, 6.5 nmol/L/3 h for red meat/refined grain diet vs. 6.4, 4.1 nmol/L/3 h for dairy/chicken/nuts/wholegrain diet, $p = 0.002$) were higher after the red meat/refined grain diet compared to the dairy/chicken/nuts/wholegrain diet. There was no interaction with glycemic group. Individual data for C-peptide AUC and iAUC is shown in Figure 6.

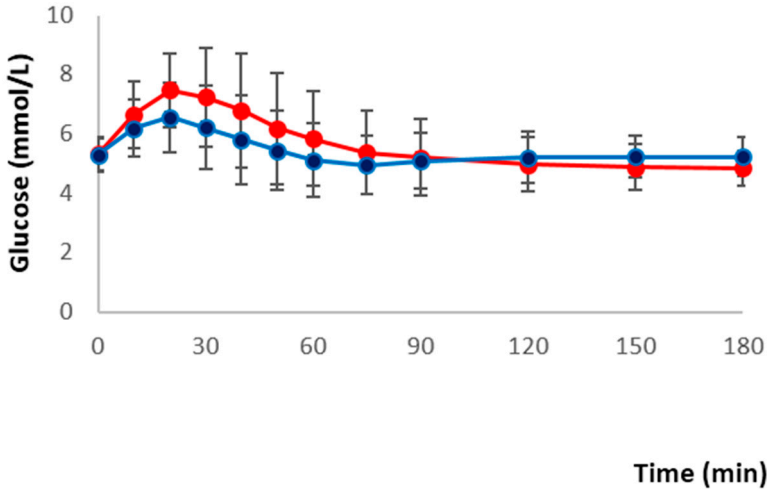


Figure 2. Mean ± standard deviation (SD) changes in postprandial glucose concentrations after two diets ($n = 51$). A repeated-measures ANOVA showed significance for the treatment ($p = 0.003$) and time-by treatment interaction ($p < 0.001$; $n = 51$). red meat/refined grain diet; dairy/chicken/nuts/whole grain diet.

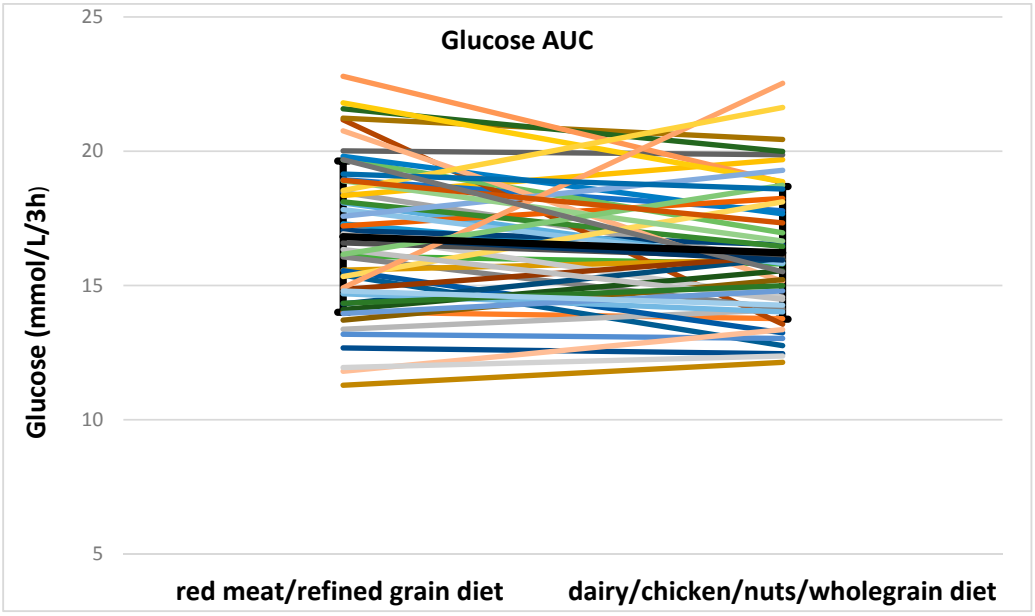


Figure 3. *Cont.*

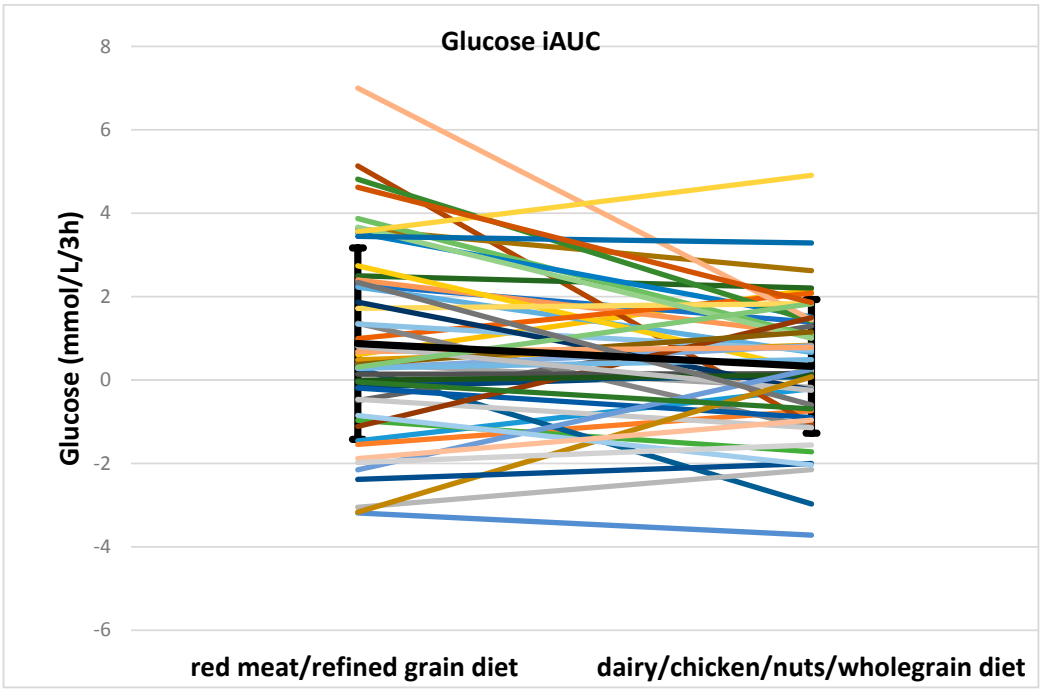


Figure 3. Individual data for glucose AUC and iAUC. The bold black line shows the mean and SD. AUC, area under the curve; iAUC, incremental area under the curve.

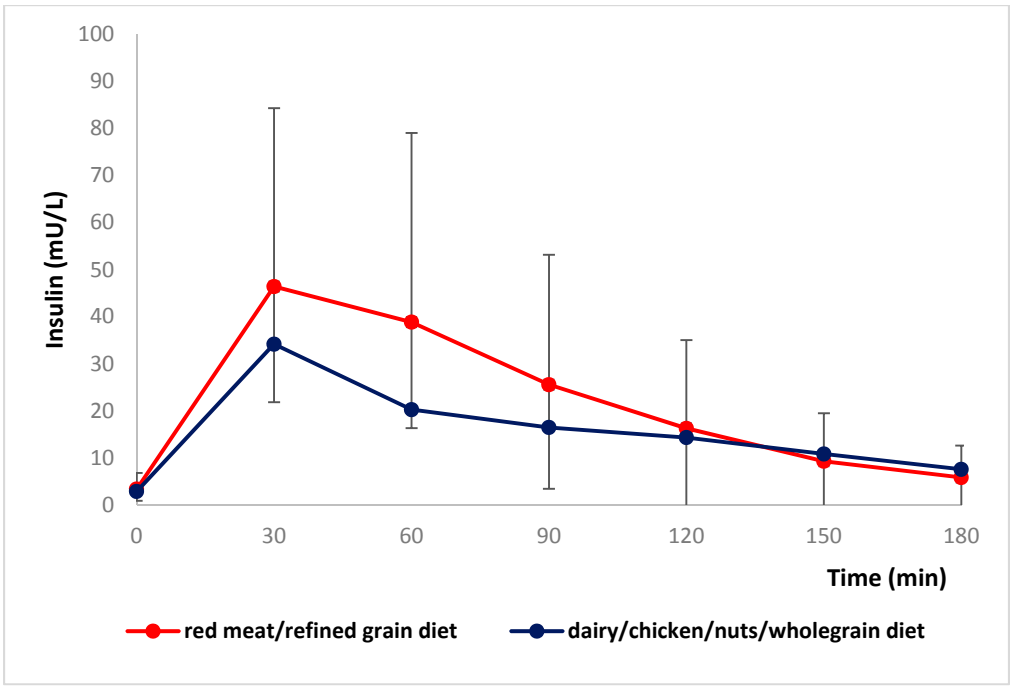


Figure 4. Mean ± SD changes in postprandial insulin concentrations after two diets (n = 51). A repeated-measures ANOVA of log transformed data showed significance for the treatment ($p = 0.004$) and time-by-treatment interaction ($p < 0.001$; $n = 51$).

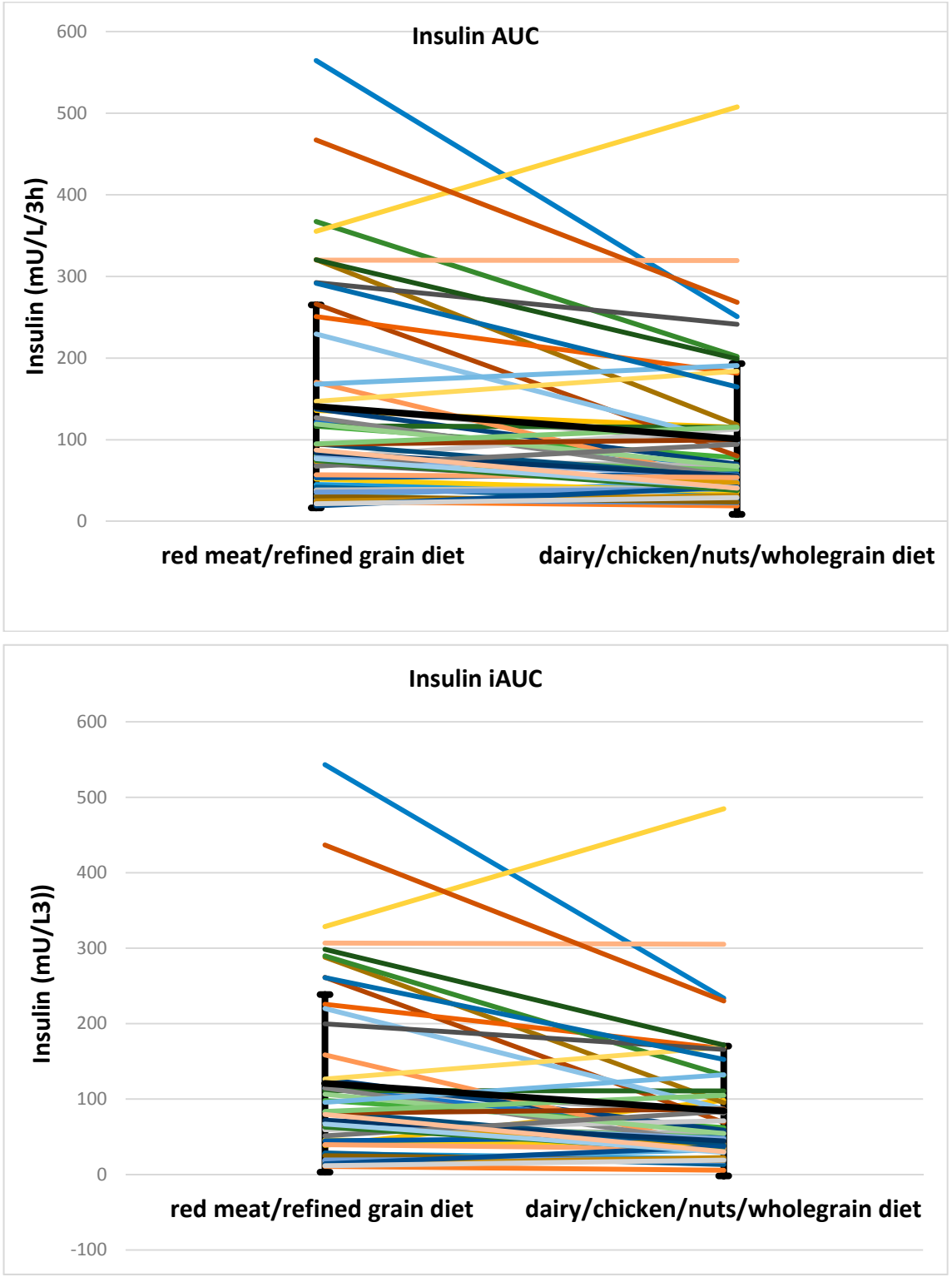


Figure 5. Individual data for insulin AUC and iAUC. The bold black line shows the mean and SD. AUC, area under the curve; iAUC, incremental area under the curve.

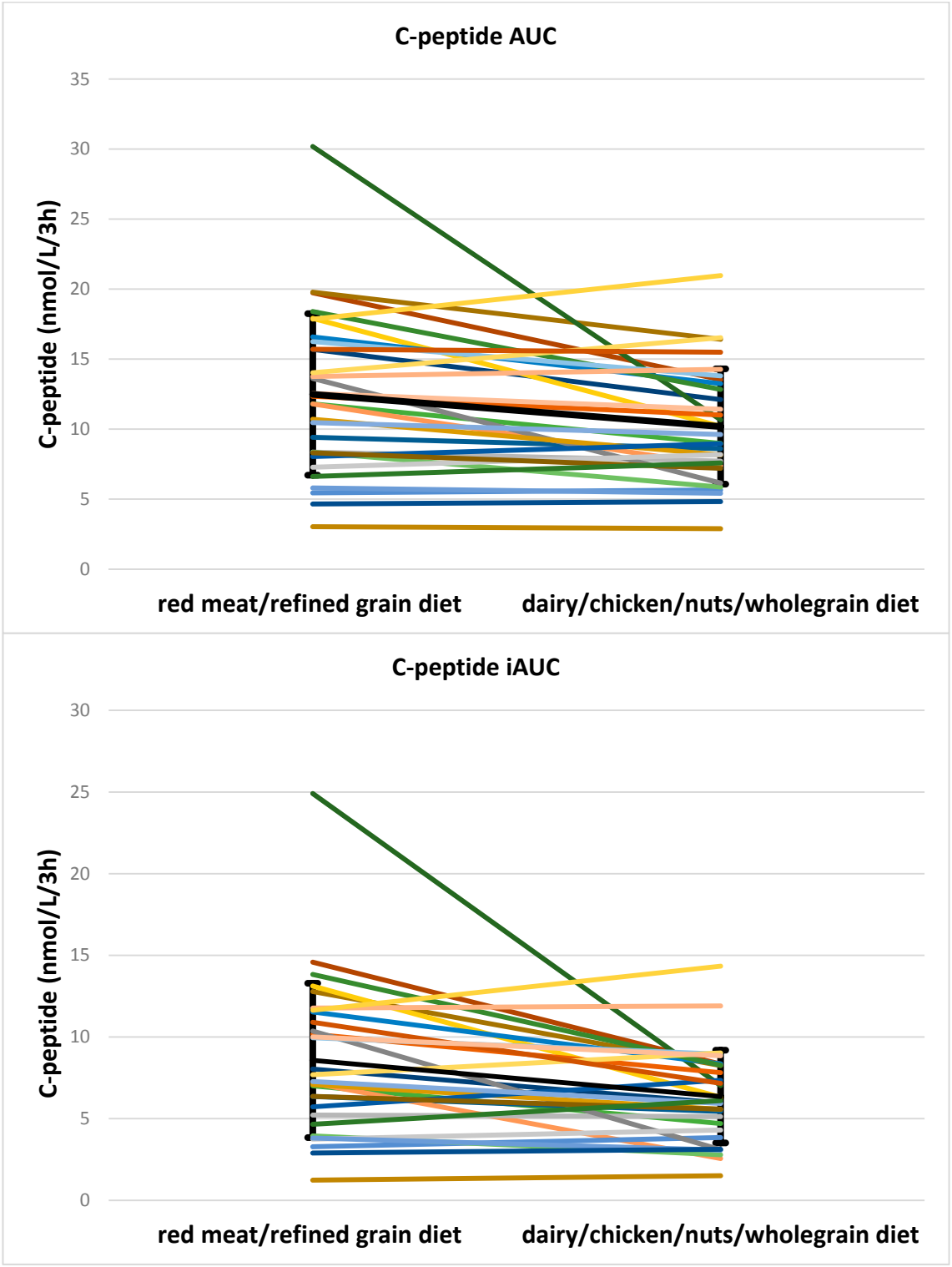


Figure 6. Individual data for C-peptide AUC and iAUC. The bold black line shows the mean and SD. AUC, area under the curve; iAUC, incremental area under the curve.

TG AUC did not differ between two meals (2.3, 2.4 mU/L/3 h for red meat/refined grain diet vs. 2.6, 1.8 mU/L/3 h for dairy/chicken/nuts/wholegrain diet, $p = 0.172$) but there was a small significant difference in TG iAUC (−0.2, 0.5 mU/L/3 h for red meat/refined grain diet vs. 0.0, 0.4 mU/L/3 h for

dairy/chicken/nuts/wholegrain diet, $p < 0.001$). Individual data for triglyceride AUC and iAUC is shown in Figure 7. Differences in glucose, insulin, C-peptide and TG are presented in Table 6.

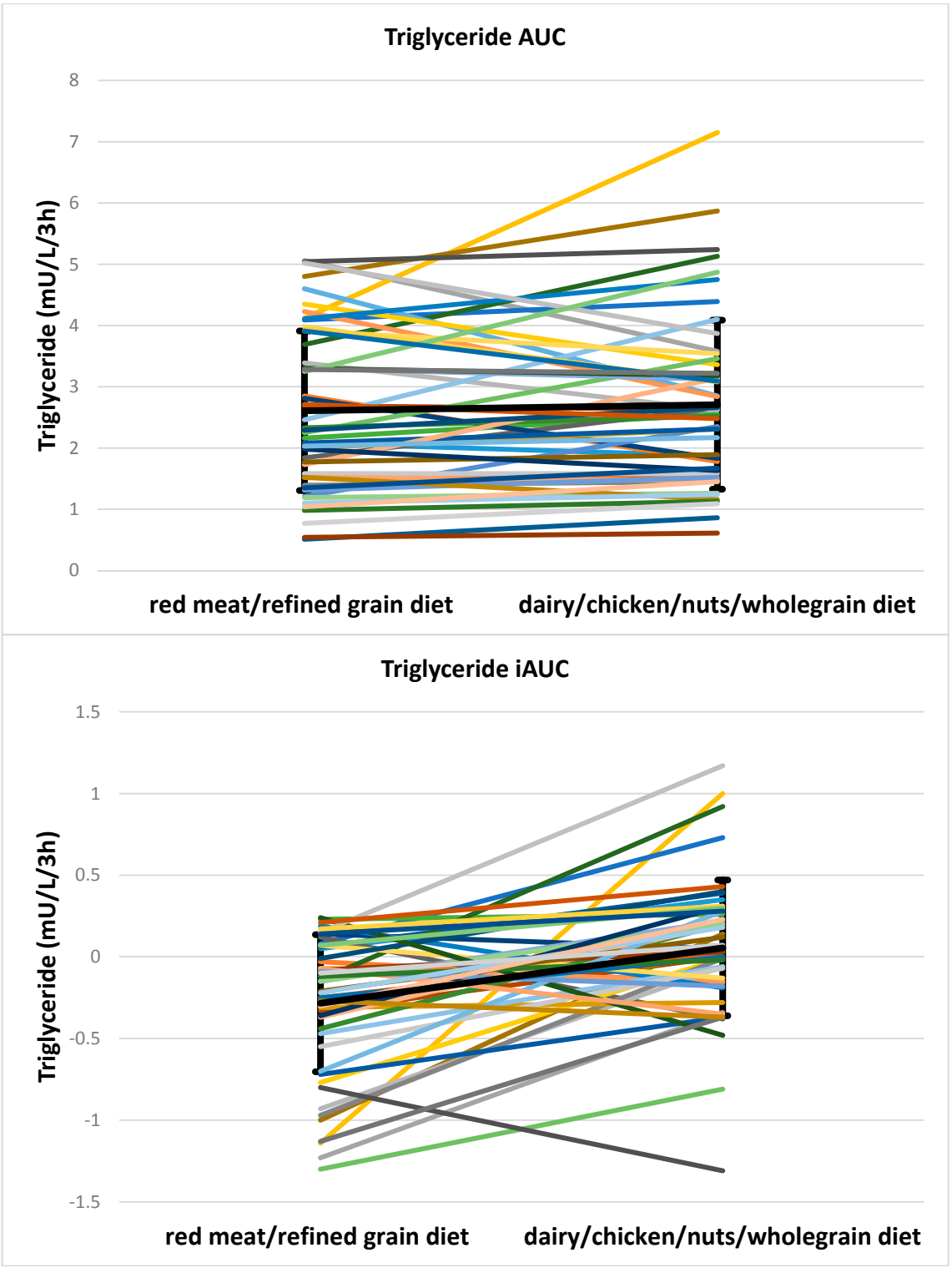


Figure 7. Individual data for triglyceride AUC and iAUC. The bold black line shows the mean and SD. AUC, area under the curve; iAUC, incremental area under the curve.

Table 6. Effects of meals on glucose, insulin and TG measured at the end of each diet period.

	All (n = 51)				Group with NGT (n = 17)			Group with IFG or IGT (n = 34)		
	Red Meat/Refined Grain Diet	Dairy/Chicken/Nuts/ Wholegrain Diet	p Value	p Group by Meal	Red Meat/Refined Grain Diet	Dairy/Chicken/Nuts/ Wholegrain Diet	p Value	Red Meat/Refined Grain Diet	Dairy/Chicken/Nuts/ Wholegrain Diet	p Value
Fasting glucose (mmol/L)	5.3, 0.9	5.3, 0.6	0.828	NS	5.2, 0.6	5.2, 0.8	0.59	5.4, 0.9	5.4, 0.6	0.99
Glucose AUC (mmol/L/3 h)	16.8 ± 2.8	16.2 ± 2.5	0.077	0.047	16.5 ± 2.9	14.9 ± 2.0	0.017	17 ± 2.8	16.9 ± 2.4	0.7
Glucose iAUC (mmol/L/3 h)	0.9 ± 2.3	0.3 ± 1.6	0.046	0.016	1.1 ± 2.9	−0.3 ± 1.7	0.024	0.8 ± 1.9	0.7 ± 1.5	0.7
Fasting insulin (mU/L)	2.2, 1.9	2.2, 1.2	0.023	NS	2.1, 1.1	2.1, 0.88	0.78	2.5, 2.1	2.3, 1.4	0.012
Insulin AUC (mU/L/3 h)	94.4, 117	65.9, 74.3	<0.001	NS	116.5, 169.5	65.9, 116.6	0.012	85.8, 129.4	62.1, 74.1	0.001
Insulin iAUC (mU/L/3 h)	79.7, 119.2	54.3, 71.9	0.001	NS	95.7, 120.7	54.3, 92.7	0.033	70.2, 134.7	50.8, 67.9	0.008
C-peptide AUC (nmol/L/3 h)	12.5 ± 5.8	10.2 ± 4.1	0.006	NS	10.9 ± 5.0	9.0 ± 4.2	0.075	13.3 ± 6.1	10.8 ± 4.1	0.03
C-peptide iAUC (nmol/L/3 h)	8.6, 6.5	6.4, 4.1	0.002	NS	6.7, 7.3	5.1, 4.6	0.112	7.9, 6.1	6.2, 3.1	0.014
TG AUC (mU/L/3 h)	2.3, 2.4	2.6, 1.8	0.172	0.013	2.0, 1.5	2.4, 2.1	0.008	2.8, 2.3	2.7, 1.7	0.78
TG iAUC (mU/L/3 h)	−0.2, 0.5	0.0, 0.4	<0.001	NS	−0.2, 0.8	0.2, 0.5	0.004	−0.2, 0.5	0.0, 0.4	0.001

Fasting glucose, insulin AUC, insulin iAUC, C-peptide iAUC and TG AUC were log transformed. p values for fasting insulin and TG iAUC were obtained from Wilcoxon signed rank nonparametric tests as fasting insulin and TG iAUC were not still normally distributed after log transformation. p values for fasting glucose, glucose AUC, glucose iAUC, insulin AUC, insulin iAUC, C-peptide AUC, C-peptide iAUC and TG AUC were examined by paired t tests. Values are expressed as medians and interquartile ranges except for glucose AUC, glucose iAUC and C-peptide AUC, which are presented as means ± SDs. Group 1 was comprised of participants with normal glucose tolerance (NGT) and group 2 was comprised of participants with impaired fasting glucose (IFG) and impaired glucose tolerance (IGT). AUC, area under the curve; iAUC, incremental area under the curve; TG, triglyceride. For AUC C-peptide n = 10 with NGT and 20 with IFG/IGT.

4. Discussion

In this study, a red meat/refined grain meal resulted in significantly higher postprandial insulin secretion than a dairy/chicken/nuts/wholegrain meal after a similar chronic dietary pattern but did not alter postprandial glucose concentrations. The 27% increase in glycemic load in the red meat/refined grain meal only partially explains the 40%–50% rise in insulin. Therefore, the significantly increased insulin concentrations could be also partly attributable to the four-week red meat/refined grain diet high in refined grains, red and processed meat promoting insulin resistance. The population tested was very diverse from lean insulin sensitive to obese insulin resistant subjects, some of whom had impaired glucose tolerance, so we believe the findings apply to the whole population. Our finding suggests that meals varying moderately in carbohydrate content and glycemic index (GI) do not influence total blood glucose to any major degree.

This finding is consistent with the small study of 25 overweight subjects where there was no difference in mean 24-h or daytime glucose concentrations on diets differing in carbohydrate and glycemic index (GI) [18]. It is interesting in this data set that an increase in glucose was seen only in the normoglycemic group who would be expected to mount a good insulin response but insulin differences between meals were not related to glycemic control group.

C-peptide only increased by 23% for total AUC on the red meat/refined grain diet while insulin increased by 46% compared with the diary/chicken/nuts/wholegrain diet, suggesting that the large difference in insulin levels was due to lowered insulin clearance rather than increased insulin release with the red meat/refined grain diet. This suggests the red meat/refined grain diet for four weeks had reduced insulin sensitivity and one of the early responses with changes in insulin sensitivity is alteration in hepatic insulin clearance rates. Therefore, only half of the increase in insulin was attributable to increased glycemic load and the other half was due to insulin resistance. Several studies showed that decreased insulin sensitivity was associated with decreased insulin clearance [19–21], and reduced insulin clearance correlated with risk of incidence of T2DM [22]. The Insulin Resistance Atherosclerosis Study 5-year follow-up showed a negative correlation between plasma plasminogen activator inhibitor (PAI)-1 concentrations and insulin clearance [23]. Dietary factors such as low GI foods might reduce PAI-1 activity [24,25].

The dairy/chicken/nuts/wholegrain diet contained a reasonable amount of nuts. Dietary fiber in nuts slows gastric emptying and subsequent glucose absorption [26]. Acute nut ingestion usually blunts the postprandial glucose response to carbohydrate foods due to their high fat content as well [27,28]. A pooled analysis of 25 intervention studies showed that the nut consumption of 67 g/day reduced fasting triglyceride concentrations by 10.2% only in people with hypertriglyceridemia but no overall triglyceride-lowering effect of nuts was seen [29]. The dairy/chicken/nuts/wholegrain meal in this study contained 9 g more fat and this was reflected in slightly higher postprandial triglyceride levels.

Studies investigating effects of isoenergetic diets differing in dietary protein and cereal fiber content on insulin sensitivity indicated that a high protein diet was associated with increased insulin resistance compared with a high cereal fiber diet [30,31]. This emphasizes the importance of high quality carbohydrate rich in fiber as observed in our diet and meal of dairy/chicken/nuts/wholegrain.

In line with our finding, Leinonen et al. [32] found that whole kernel rye bread lowered the postprandial insulin response compared with wheat bread, but there was no difference in glucose response between the two breads in healthy subjects. Whey protein has been shown to have an insulinotropic effect compared with non-dairy proteins [33] in some studies but not all [34]. Whey protein consumed in amounts of 20 g/day before or after a carbohydrate meal stimulated insulin, glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) and delayed gastric emptying resulting in decreased postprandial glycaemia in people with diet-controlled T2DM [35,36]. It appears that 2.8 g whey in milk and yogurt of our dairy/chicken/nuts/wholegrain meal has no effect on postprandial glycemia and insulin secretion and that meat and dairy protein probably have equivalent effects on insulin release. As there are inconsistent effects of dairy consumption on insulin

sensitivity [37,38] and few dairy interventions examining acute effects on glucose and insulin [34] exist, the possible explanation for overall effects observed in the dairy/chicken/nuts/wholegrain meal of this present study are not due to dairy but other meal components. Alteration in gut microbiota may play a role in the beneficial effects seen with the dairy/chicken/nuts/wholegrain diet [39].

A limitation of this study was that the macronutrient compositions were not matched and total energy content was slightly different between two meals leading to very slight differences in triglyceride incremental area. A further limitation was that we have no baseline measurements to show that there was no carry-over of the previous diet. However, diet order had no effect on the results, suggesting there was no significant carry-over and the results observed are valid. The study design also assumed that all prognostic factors other than the treatment remained constant across treatment conditions. Randomization might not work to equalize the populations as the sample size was small and there was a small difference in 2-h glucose between the two groups. Even though the compliance was good, self-reported food intake [40] and meal preparation outside the clinical research facility during the intervention periods also could be a potential limitation.

5. Conclusions

In conclusion, a red meat/refined grain meal following four weeks of a similar red meat/refined grain diet elevated insulin and incremental glucose responses in comparison with a dairy/chicken/nuts/whole grain meal, which is consistent with the increased insulin resistance which we observed in relatively insulin-resistant adults in the main study as well as the carbohydrate quality and quantity differences between the meals. The adverse insulin response to the red meat/refined grain meal relative to the dairy/chicken/nuts/wholegrain meal indicates possible mechanisms through which these diets might affect risk of type 2 diabetes. Further work needs to be done on individual components of the meal to define which is the most important factor. The population in this study consisted of 63% Caucasian, 28% Asian and 9% Latin American subjects with over 50% aged <40 years. Future studies should attempt to replicate the results in a population including middle-aged people from the Middle East and the Pacific area.

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Conflicts of Interest: The authors declare no conflict of interest.

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10.7 Table: Effects of the two diets on postprandial glucose, insulin and triglyceride responses divided into the two insulin sensitivity subgroups

We added Data in the following table as the main study was divided along post-hoc subgroup lines of insulin resistance, which is linked to the other measures subsequently reported in this chapter 10. Data are presented as being divided into the two subgroups for the subsequent tests of stimulated blood glucose, insulin and triglyceride measures. There was no significant group by time interaction for any measures. These results are discussed in chapter 12.

	LDIGIT group 1 and 2 (n = 49)				LDIGIT group 1 (n = 24)			LDIGIT group 2 (n = 25)		
	HMD	**HWD**	***P***	***P* Group by Time**	**HMD**	**HWD**	***P***	**HMD**	**HWD**	***P***
Glucose AUC (mmol/L)	16.8 ± 2.9	16.2 ± 2.5	0.098	0.9	16.1 ± 3	15.6 ± 2.6	0.3	17.5 ± 2.7	16.9 ± 2.3	0.2
Glucose iAUC (mmol/L)	0.9 ± 2.3	0.3 ± 1.6	0.035	0.9	0.36 ± 2.5	-0.26 ± 1.5	0.1	1.49 ± 2	0.9 ± 1.6	0.14
Insulin AUC (mU/L)	94.4, 114	65.9, 73.7	< 0.001	0.097	69.3, 47.3	45.8, 32.2	0.026	147.1, 200	115, 124.8	0.001
Insulin iAUC (mU/L)	79.7, 100.5	54.3, 68.3	0.001	0.2	56.1, 48.5	38.3, 32.1	0.07	126.4, 191.3	95.1, 110	0.006
TG AUC (mU/L)	2.3, 2.3	2.5, 1.7	0.33	0.9	1.8, 2	2.1, 1.9	0.4	2.8, 2	2.8, 1.8	0.6
TG iAUC (mU/L)	-0.2, 0.5	0.0, 0.4	< 0.001	0.6	-0.15, 0.3	0.047, 0.5	< 0.001	-0.25, 0.6	0.3, 0.4	< 0.001

P values (n = 49) for glucose AUC and glucose iAUC were obtained from paired samples t tests as they were normally distributed. P values (n = 49) for insulin AUC and insulin iAUC were examined by paired samples t tests after log transformation. P values (n = 49) for TG AUC and TG iAUC were obtained from nonparametric tests. Values (n = 49) for glucose AUC and glucose iAUC are presented as means ± SDs. Values (n = 49) for insulin AUC, insulin iAUC, TG AUC and TG iAUC, are expressed as medians and interquartile ranges. The groups 1 and 2 are defined *a posteriori* based on the insulin values of LDIGIT (120-150min), not randomized groups. HMD, a high red and processed meat and refined grains diet; HWD, a high whole grain, nuts, dairy and legumes diet; AUC, area under the curve; iAUC, incremental area under the curve; TG, triglyceride.

11 EFFECTS OF TWO DIFFERENT DIETARY PATTERNS ON INFLAMMATORY MARKERS, ADVANCED GLYCATION END PRODUCTS AND LIPIDS IN SUBJECTS WITHOUT TYPE 2 DIABETES: A RANDOMISED CROSSOVER STUDY

11.1 Preface

This chapter outlines analyses of inflammatory and fibrinolytic markers, advanced glycation end products and lipids on samples from our crossover dietary intervention trial.
The objective was to determine if a diet high in red and processed meat and refined grains elevated concentrations of inflammatory and fibrinolytic markers, advanced glycation end products compared with a diet high in dairy, whole grains, nuts and legumes, as well as to examine to the association between these markers and the changes in insulin sensitivity found in the previous study.

11.2 Published Article

Kim Y, Keogh JB, Clifton PM. Effects of Two Different Dietary Patterns on Inflammatory Markers, Advanced Glycation End Products and Lipids in Subjects without Type 2 Diabetes: A Randomised Crossover Study. Nutrients. 2017; 9.

I would like to correct the word ‘are’ misspelled (is) in paragraph 6 in section 2.6 and paragraph 16 in section 4 of this published article.

nutrients

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Article

Effects of Two Different Dietary Patterns on Inflammatory Markers, Advanced Glycation End Products and Lipids in Subjects without Type 2 Diabetes: A Randomised Crossover Study

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Abstract: Epidemiological studies suggest that consumption of red and processed meat and refined grains are associated with type 2 diabetes and metabolic syndrome and increased inflammatory and fibrinolytic markers. We hypothesised that a diet high in red and processed meat and refined grains (HMD) would increase inflammatory markers and advanced glycation end products (AGEs) compared with a diet high in dairy, whole grains, nuts and legumes (HWD). We performed a randomised crossover study of two four-week interventions in 51 participants without type 2 diabetes (15 men and 36 women aged 35.1 ± 15.6 years; body mass index: 27.7 ± 6.9 kg/m^2). No baseline measurements were performed. Plasma fluorescent AGEs, carboxymethyllysine, glucose, insulin, lipids, hs-CRP, interleukin 6 (IL-6) and plasminogen activator inhibitor-1 (PAI-1) were analysed after four weeks on each diet. IL-6, hs-CRP, AGEs and carboxymethyllysine were not different between diets but PAI-1 was higher after the HMD than after HWD ((median and interquartile range) 158, 81 vs. 121, 53 ng/mL $p < 0.001$). PAI-1 on the HWD diet was inversely correlated with whole grains intake ($p = 0.007$). PAI-1 was inversely correlated with insulin sensitivity index (r = −0.45; $p = 0.001$) and positively correlated with serum total cholesterol (r = 0.35; $p = 0.012$) and serum triglyceride (r = 0.32; $p = 0.021$) on HMD. This trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12614000519651).

Keywords: dietary pattern; plasminogen activator inhibitor type 1; advanced glycation products; insulin sensitivity

1. Introduction

Inflammation is thought to play a role in the pathogenesis of type 2 diabetes mellitus (T2DM) [1,2]. Increased concentrations of interleukin 6 (IL-6) and high-sensitivity C-reactive protein (hs-CRP) have been associated with an increased risk of T2DM [3,4]. Elevated plasminogen activator inhibitor type 1 (PAI-1) has also been found to be a predictor of the development of T2DM [5–8].

Dietary intake may affect inflammatory and fibrinolytic markers. In cross-sectional data obtained from the Insulin Resistance Atherosclerosis Study (IRAS), whole grain consumption was inversely associated with plasma PAI-1 and hs-CRP concentrations and refined grain consumption was positively associated with plasma PAI-1 concentrations [9]. However, a diet high in whole grains for six weeks showed no differences in either hs-CRP or IL-6 or PAI-1 concentrations compared with a diet high in refined grains [10], although reducing the glycemic index of a test food appears to lower PAI-1 [11]. Cross-sectional studies have shown that greater consumption of red and processed meat was associated with higher circulating hs-CRP concentrations [12–15], while in dietary interventions, red meat consumption did not change hs-CRP concentrations [16,17].

Advanced glycation end products (AGEs) are endogenously formed when the carbonyl groups of reducing sugars and reactive aldehydes non-enzymatically react with the free amino groups in proteins, lipids and nucleic acids through a series of reactions forming Schiff base and Amadori products [18–20]. The process of AGEs formation is also known as the Maillard reaction [21]. Higher levels of AGEs are produced in foods processed at high temperatures in dry conditions such as grilling, broiling, frying and roasting, compared with foods processed slowly at lower temperatures or in water [22]. The stable, relatively inert and non-fluorescent carboxymethyllysine (CML) is a frequently measured AGE [20]. High levels of AGEs have been implicated in insulin resistance in non-obese, non-diabetic subjects [23,24] and in pancreatic beta cell dysfunction in vitro and in vivo [25,26]. Dietary AGEs-restricted diets are associated with a lower risk of T2DM [27] and a reduction in inflammation and insulin resistance [28,29].

To date, few interventions have investigated the associations between dietary patterns and inflammatory and fibrinolytic markers and advanced glycation end products in subjects without type 2 diabetes.

We reported on the changes in insulin sensitivity by continuous low-dose insulin and glucose infusion tests (LDIGIT) in our previous publication [30], where we found that the participants fell into an insulin-sensitive group (insulin < 56 pmol/L, n = 24), and a relatively insulin-resistant group (insulin > 56 pmol/L, n = 25) in response to the LDIGIT $_{(120–150\ min)}$ on a diet high in red and processed meat and refined grains (HMD). The insulin-resistant group had significantly higher concentrations of insulin and glucose during the LDIGIT $_{(120–150\ min)}$ on the HMD and thus a significantly decreased insulin sensitivity index (ISI), compared with a diet high in dairy, whole grains, nuts and legumes (HWD). In this study, we examined inflammatory and advanced glycation end products that may mediate the insulin resistance, as well as fibrinolytic markers which may be altered by increased insulin resistance. In the previous publication [30], we did not report on any potential mediators of insulin resistance. The specific aim was to determine the effect of a weight-stable diet high in red and processed meat and refined grains compared with a diet high in dairy, whole grains, nuts, legumes without red and processed meat on inflammatory and fibrinolytic markers and advanced glycation end products in individuals without T2DM. It also aimed to examine the association between these markers and the changes in insulin sensitivity found in our previous study [30]. Our hypothesis was that a diet high in red and processed meat and refined grains would elevate concentrations of inflammatory markers (hs-CRP, IL-6), PAI-1 and AGEs (fluorescent AGEs, CML) compared with a diet high in dairy, whole grains, nuts and legumes.

2. Methods

2.1. Ethical Approval and Registration

This study was approved by the University of South Australia Human Research Ethics committee, and all study participants gave their written informed consent prior to participating. The trial was registered with the Australian New Zealand Clinical Trials Registry www.anzctr.org.au/ (ACTRN12614000519651). AUD$240 was offered to the participants on completion of the two diets. Recruitment began in June 2014 and ended in September 2015.

2.2. Study Participants

A total of 51 participants (body mass index (BMI) 18–45 kg/m^2), aged over 18 years, were recruited by public advertisement and a recruiting agency. An initial screening questionnaire was undertaken via email or telephone. Details of all inclusion, exclusion criteria and the change in entry criteria have been reported previously [30]. Respondents who passed the screening criteria visited the Sansom Institute for Health Research Clinical Trial facility at the University of South Australia, in the morning after an overnight fast. Written informed consent was obtained. Blood pressure was measured four times after sitting quietly for 10 min (Omron Corporation, Kyoto, Japan). The interval between blood

pressure measurements was at least 1 min. Height was measured on a wall-mounted stadiometer (Seca, Hamburg, Germany) without shoes and body weight was measured on electronic digital scales (Tanita Corporation, Tokyo, Japan) in light clothing and without shoes. Body composition was measured by whole-body dual-energy X-ray absorptiometry (DXA) (Luna Prodigy, Lunar Radiation Corp., Madison, WI, USA) to calculate lean mass. A 75 g oral glucose tolerance test (OGTT) was performed to exclude people with diabetes. A simple survey on activity levels was carried out at baseline (sedentary or moderately active). We did not collect blood samples at baseline.

A detailed description of participant recruitment and completion has been previously published [30,31].

2.3. Dietary Intervention

Eligible participants were randomly allocated to either a diet high in red and processed meat and refined grains (HMD) or a diet high in whole grains, nuts, legumes, dairy, and devoid of red and processed meat (HWD) for four weeks, and then were crossed over to the alternative diet for four weeks. Randomisation was performed via an online random number generator (www.randomization.com) by a researcher not involved in this study.

The investigator who performed data analysis and staff who measured blood samples were blinded to the diet order. There was a minimum of a two-week washout period (on average three weeks) between the two diets during which participants returned to their usual diet. On HMD daily consumption of 200–300 g red meat, ≥50 g processed meat (ham, bacon and sausage) was recommended as this reflected the amount consumed in the highest quintile of published cohort studies [28,32,33]. In order to maximise the difference between the two diets, 4–6 serves of refined grains (white bread, rice, noodles and pasta) based on volunteers' weights, 1–2 serves of vegetables, 1–2 serves of fruits and a minimal amount of dairy products (e.g., 1–2 teaspoons of milk) were allowed during the HMD. Two hundred to three hundred grams of potatoes, 1 serve of jam or marmalade, 3–9 serves of oil (e.g., olive or canola) or spread (e.g., butter) or 3–4 serves of indulgence food were recommended for additional energy in accordance with volunteers' weights. During the HWD, recommendations, based on the volunteers' weights, were daily consumption of 4 serves of low-fat dairy products including 2 serves of yoghurt, 3–4 serves of whole grain breads, rice and pasta, 60–90 g unsalted nuts and either 70–150 g chicken or fish (or other seafood) or 150–225 g cooked legumes. Serve sizes of cereal foods were as follows: 1 slice (40 g) wholegrain or wholemeal bread, ½ cup (75–120 g) cooked brown rice, ½ cup (75–120 g) cooked wholemeal pasta, ¼ cup (30 g) muesli, ½ cup oats, ⅔ cup (30 g) wheat cereal flakes, ½ cup (120 g) cooked wholegrain porridge, 3 (35 g) wholegrain crisp-breads. Two to seven serves of oil or spread and one serve of jam or marmalade were also advised for additional energy intake in accordance with volunteers' weights. The recommended menus for each of the diets have been published previously [31].

Total energy was matched in the two diets. Vegetables and fruits were limited to 1–2 serves in both diets as epidemiology does not suggest that these components are related to the risk of type 2 diabetes [34,35]. Serving sizes were defined according to the guidelines of the Australian National Health and Medical Research Council [36]. Food was not provided to participants but they were given an AUD$80 voucher for red meat and an AUD$50 voucher for the nuts at the end of each diet period. Participants received specific dietary instructions for each diet along with eight different energy levels based on BMI and gender. For the HWD, healthy cooking methods included boiling, steaming, stewing and poaching rather than deep-frying, grilling, and roasting. Samples of recipes and daily meal plans for the two diets were given. A kitchen scale (Homemaker Slimline Electronic Scale; Kmart, Melbourne, Australia) was provided to measure the quantity of recommended food at home. Participants were encouraged to check their weight every day and maintain usual physical activity. Weight was measured at each visit and in the event of weight change, the diet was adjusted accordingly. A daily checklist during each dietary period and a three-day weighed food diary specifying serving weights and the types of food they consumed within each two-week period were used in order to

monitor dietary compliance. Participants attended the clinic fortnightly and the three-day weighed food diary and daily checklists were checked. All food consumed over these three-day weighed periods were analysed by Food Works Professional Edition 8.0 (Xyris, Kenmore Hills, Qld, Australia). Dietary intake was not measured before the intervention, at baseline or during the washout period.

2.4. Assessments of Insulin Sensitivity

At the end of each diet period, participants attended the Sansom Institute for Health Research Clinical Trial facility from 8 a.m. to 9 a.m. in the morning after an overnight fast for a continuous low-dose insulin and glucose infusion test ((LDIGIT); $n = 49$). All fasting blood samples were taken at this time and individuals had the same appointment time for each visit. Fifty-one participants completed the study but only 49 subjects could be included due to failure of blood sample collections or cannulations for the low-dose insulin and glucose infusion test (LDIGIT). If volunteers were sick prior to the day of the test, we deferred the test till they were well again. The dietary intervention continued until all blood sampling was completed. All medications changes were recorded.

Descriptions of the LDIGIT administration and calculation of ISI and homeostasis model assessment of insulin resistance (HOMA-IR) have been previously reported [30].

During the tests, blood for serum was collected in the tube with no additives and the tube was kept upright in a tube rack at room temperature for 30 min to ensure complete clot formation and then placed on ice. Blood for plasma was collected in the sodium fluoride EDTA tube and the tube was placed immediately on ice until centrifugation and processing. Blood samples were centrifuged at 4000 RPM at 4 °C for 10 min (Universal 32R, Hettich Zentrifugen, Tuttlingen, Germany).

2.5. Biomarkers Analysis

Plasma glucose, fasting serum total cholesterol, triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and hs-CRP were measured using an automated spectrophotometric analyzer (Konelab 20XTi, Thermo Electron, Massachusetts, MA, USA). Serum insulin was assayed by using a commercial ELISA kit (Alpha Diagnostic, San Antonio, TX, USA, Kit # 0030N) with an intra-assay coefficient of variation (CV) of 6.29%–10.40% and inter-assay CV of 5.8%–11.8%. IL-6 was assayed by using a commercial ELISA kit (Elisakit.com, Victoria, Australia, #: 0012 Human IL-6 ELISA Kit) with an intra-assay CV of <10% and inter-assay CV of <10%. PAI-1 was assayed by using a commercial ELISA kit (Affymetrix eBioscience, San Diego, CA, USA, BMS2033/BMS2033TEN Human PAI-1 Platinum ELISA kit) with an intra-assay CV of 4.7% and inter-assay CV of 5.0%. Serum CML was measured by using a competitive ELISA kit (CircuLex, Nagano, Japan, CML/Nε (Carboxymethyl) lysine ELISA Kit, Cat# CY-8066) with an intra-assay CV of 5.2%–7.4% and inter-assay CV of 4.7%–15.2%.

Serum was diluted 50-fold with phosphate-buffered saline and 250 µL of the diluted serum was aliquoted into the black 96-well plate in triplicate. Total fluorescent AGEs were measured in a multi-mode microplate reader (EnSpire® Multimode Plate Reader, PerkinElmer, Waltham, MA, USA) at room temperature to estimate the levels of fluorescent AGEs. Fluorescence was read at excitation and emission wavelengths of 370 nm and 440 nm, respectively.

2.6. Statistical Analyses

Data were analyzed with SPSS V22 (IBM, Chicago, IL, USA). The power calculation was based on our primary outcome of insulin sensitivity with a sample size of 50 overweight and obese subjects without diabetes providing 80% power to detect a 20% change (alpha level = 0.05) in insulin sensitivity as assessed by LDIGIT [37]. The Shapiro–Wilk test, Q–Q plots, and histograms were used to test for the normality of distribution. Non-normally distributed variables were log transformed before analysis but untransformed data is shown. Differences between diets were tested by paired samples *t*-tests. Wilcoxon signed-rank nonparametric tests were also used as some variables (e.g., fasting glucose, fasting insulin, HOMA-IR, hs-CRP, PAI-1 and IL-6) were still skewed in one or both diet periods after

log transformation. Associations were examined using Pearson correlation coefficients for parametric correlations, and Spearman correlation coefficients and Kendall's tau for non-parametric correlations.

An unpaired *t*-test, a chi-square test and a Mann–Whitney nonparametric test were performed to contrast variables between post hoc insulin sensitivity groups. *p* values for group by time interaction were determined by repeated measures of ANOVA. Data are presented as the means ± standard deviations (SDs) except for skewed variables which are expressed as medians and interquartile ranges. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Study Participants

Fifty-one participants completed the two diets and forty-nine participants underwent a successful LDIGIT. Table 1 shows the baseline characteristics of a total of 51 participants divided into 2 LDIGIT groups (a total of 49 participants) defined a posteriori based on insulin values during the $LDIGIT_{120–150\ min}$, not from randomised groups: an insulin-sensitive group <56 pmol/L with a median insulin of 33 pmol/L ($n = 24$) and an insulin-resistant group >56 pmol/L with a median insulin of 122 pmol/L ($n = 25$).

Body weight remained very stable throughout the study period. The average weights were 77.9, 77.8 and 77.9 kg during the HMD period (baseline, 2 w and 4 w) and 77.8, 77.7 and 77.8 kg during the HWD period. In the LDIGIT insulin-resistant group, insulin ($p = 0.019$) and glucose ($p = 0.05$) were higher after HMD than after HWD, resulting in a decreased ISI ($p = 0.014$). Log ISI HMD was correlated with BMI ($p = 0.009$) and fat mass ($p = 0.004$). Log ISI HWD was positively correlated with the amount of carbohydrates in the HWD (Beta-coefficient 0.27; t = 3.5; $p = 0.001$) after adjustment for log ISI HMD (Beta-coefficient 0.79; t = 10.3; $p < 0.001$).

Table 1. Baseline characteristics of participants.

Variable	All Participants [1]	Participants Who Completed LDIGIT ($n = 49$)		
		Insulin-Sensitive Group ($n = 24$)	Insulin-Resistant Group ($n = 25$)	*p*
Sex (M/F)	15/36	9/15	6/19	0.3 †
Age (year)	35.1 ± 15.6	35.5 ± 15.6	35.6 ± 16	1
NGT (*n*)	17	10	6	0.2
IFG/IGT (*n*)	34	14	19	
Baseline fasting glucose (mmol/L)	5.5 ± 0.7	5.3 ± 0.8	5.7 ± 0.6	0.07
Baseline 2 h glucose (mmol/L)	7.28 ± 1.6	6.96 ± 1.5	7.55 ± 1.7	0.2
HOMA-IR (HWD)	0.37, 0.43	0.29, 0.47	0.39, 0.5	0.07 ‡
HOMA-IR (HMD)	0.53, 0.66	0.21, 0.54	0.65, 0.8	0.01 ‡
$LDIGIT_{(120–150\ min)}$ insulin (HWD pmol/L) [2]		33.4, 27.8	122.7, 149	<0.001 #
$LDIGIT_{(120–150\ min)}$ insulin (HMD pmol/L) [2]		29.6, 20.3	153, 180	<0.001 #
Baseline weight (kg)	79.4 ± 21.36	69.7 ± 15.1	85.7 ± 21.7	0.005
BMI (kg/m^2)	27.7 ± 6.9	24.4 ± 4.6	29.4 ± 5.9	0.002
Baseline SBP (mmHg)	112.2 ± 10.7 [3]	110.4 ± 9.2 [4]	114.3 ± 12.4 [5]	0.3
Baseline DBP (mmHg)	70.7 ± 9.7 [3]	69.1 ± 9.9 [4]	72.5 ± 9.5 [5]	0.3
Total Fat Mass (kg)	29 ± 15.7	20.6 ± 11.4	34.3 ± 13.3	<0.001
Total Lean Mass (kg)	46.6 ± 11.5	45.4 ± 11.4	47.7 ± 12.1	0.5
Total Fat Mass (%)	36.6 ± 12.6	30.3 ± 12.8	41.0 ± 8.6	0.001

Groups were contrasted with an unpaired *t*-test. † *p* values were obtained by chi-square test. ‡ *p* values were obtained by Mann–Whitney nonparametric test. # *p* values were obtained by unpaired *t*-tests after log transformation. Values are means ± SDs except for HOMA-IR and $LDIGIT_{(120–150\ min)}$ insulin, which are medians and interquartile ranges. The insulin-sensitive group and insulin-resistant group were defined a posteriori based on the insulin values of $LDIGIT_{120–150\ min}$, not from randomised groups: insulin-sensitive group <56 pmol/L with a median insulin of 33 pmol/L ($n = 24$) and insulin-resistant group >56 pmol/L with a median insulin of 122 pmol/L ($n = 25$). M, male; F, female; NGT, normal glucose tolerance; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDIGIT, low-dose insulin and glucose infusion tests; HOMA-IR, homeostasis model assessment of insulin resistance; HWD, high in dairy, whole grains, nuts and legumes; HMD, high in red and processed meat and refined grains; SD, standard deviation. [1] $n = 51$; [2] $n = 49$, [3] $n = 42$; [4] $n = 22$; [5] $n = 19$.

3.2. Dietary Compliances and Intakes

The reported dietary intake of nutrients and key foods assessed by weighed food records (n = 51) during the two diet periods has been previously published [31]. Analyses of three-day weighed food diaries of every two weeks (a total of six-day weighed food diaries for each four-week dietary period) indicated that dietary compliance was good with similar energy intake in the two diets, with intakes of key foods very similar to the recommended quantities of key foods. We achieved our planned differences in red and processed meat and whole and refined grains. The key foods consumed were 241 g of red meat, 56 g of processed meat (19% protein) and 320 g of refined grains (44% carbohydrate) compared with 70 g of nuts, 236 g of wholegrains and 687 g of dairy foods (19% protein, 37% carbohydrate). We took no biomarkers of food intake as protein intake was planned to be the same on both diets and estimated protein intake was not different at 95 and 97 g/day while total fat and carbohydrate, which were a little different, have no agreed sensitive biomarkers other than HDL cholesterol and triglyceride, which were measured. Fat energy was 6% higher and carbohydrate 7% lower on the HWD.

3.3. Inflammatory Markers

Table 2 summarises the differences in insulin sensitivity, lipids, inflammatory and fibrinolytic markers, and AGEs between the two diets measured at the end of each dietary period in 51 participants. There were no confounding illnesses or medications or smoking at the time of the blood sampling and the one person who felt unwell at the time of their appointment was rescheduled for three days later. No medication was taken. Diet order had no effect in either LDIGIT group. PAI-1 was significantly higher after HMD than after HWD ($p < 0.001$; n = 51). PAI-1 HWD was inversely correlated with the log of whole grains intake (r = 0.29; p = 0.04; n = 51). There was a strong relationship on linear regression between PAI-1 HWD and whole grains intake (Beta-coefficient = −0.35; t = −2.8; p = 0.007) and PAI-1 HMD (p = 0.001).

Table 2. Insulin sensitivity, lipids, inflammatory and fibrinolytic markers, and AGEs measured at the end of each dietary period in all participants (n = 51).

Variable	HMD	HWD	p
Fasting glucose (mmol/L)	5.3, 0.6	5.3, 0.5	0.9
Fasting insulin (pmol/L)	11.8, 15	9, 11	0.3
HOMA-IR	0.53, 0.66	0.37, 0.43	0.25
TG (mmol/L)	0.91, 0.72	0.86, 0.69	0.041
HDL-C (mmol/L)	1.39 ± 0.43	1.37 ± 0.42	0.4
Total cholesterol (mmol/L)	4.8 ± 1.0	4.6 ± 1.0	0.032
hs-CRP (mg/L)	1.01, 2.98	0.61, 2.55	0.7
IL-6 (pg/mL)	8, 12	8, 12	0.65
PAI-1 (ng/mL)	158, 81	121, 53	<0.001
Fluorescent AGEs (absorbance units)	1388, 304	1370, 454	0.16
CML (µg/mL)	1.37, 0.67	1.49, 0.56	0.07

p values (n = 51) for fluorescent AGEs, TG, CML, total cholesterol and HDL-C were determined by paired t-tests. Fluorescent AGEs, TG and CML were log transformed. p values (n = 51) for fasting glucose, fasting insulin, HOMA-IR, hs-CRP, PAI-1 and IL-6 were obtained from nonparametric tests. Values (n = 51) are expressed as medians and interquartile ranges except for HDL-C and total cholesterol, which are presented as means ± SDs. HMD, a high red and processed meat and refined grains diet; HWD, a high whole grain, nuts, dairy and legumes diet; HOMA-IR, homeostasis model assessment of insulin resistance; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; hs-CRP, high sensitivity C-reactive protein; IL-6, interleukin 6; PAI-1, plasminogen activator inhibitor type 1; AGE, advanced glycation end product; CML, carboxymethyllysine.

PAI-1 HMD was inversely correlated with log ISI $_{\text{LDIGIT}}$ after HMD (r = −0.45; p = 0.001, n = 49). When two groups were defined a posteriori based on the insulin values of LDIGIT$_{(120–150\ \text{min})}$, there was a borderline significant group by time interaction for PAI-1 (p = 0.05; n = 49) and the change in PAI-1 was greater in the insulin-resistant group (177, 61 for HMD vs. 128, 52 ng/mL for HWD; $p < 0.001$) than in the insulin-sensitive group (137, 80 for HMD vs. 113, 39 ng/mL for HWD; p = 0.02). There was no effect of diet order on these results nor was there a difference between glycemic control groups [normal compared with impaired fasting glucose (IFG)/impaired glucose tolerance (IGT)].

PAI-1 HMD was positively correlated with serum total cholesterol on the HMD (r = 0.35; $p = 0.01$; $n = 51$) and with log serum TG after the HMD (r = 0.32; $p = 0.02$; $n = 51$).

There were no differences in hs-CRP ($p = 0.7$; $n = 51$) and IL-6 ($p = 0.7$; $n = 51$) between the two diets.

3.4. Advanced Glycation end Products

Total fluorescent AGEs were not different between the diets. Fluorescent AGEs after the HWD were correlated with dietary sugars (r = 0.3; $p = 0.04$; $n = 51$). CML did not differ between the two diets ($p = 0.07$; $n = 51$). Glycemic status (normal compared with IFG/IGT) had no effect.

3.5. Lipids

Total cholesterol was significantly higher after HMD than after HWD ($p = 0.03$; $n = 51$). HDL-C did not differ between the two diets ($p = 0.4$; $n = 51$). Triglyceride was significantly higher after HMD than after HWD ($p = 0.04$; $n = 51$). Glycemic status (normal compared with IFG/IGT) had no effect.

4. Discussion

The present weight-stable randomised crossover study showed that the consumption of a high red and processed meat and a low-fibre, medium glycemic index (GI) and medium glycemic load (GL) diet for four weeks significantly increased PAI-1 concentrations compared with consumption of a high dairy, whole grains, nuts and legumes low GI and low glycemic load diet. In addition, there was a significant LDIGIT group by time interaction for the PAI-1, showing that this effect was more marked in the insulin-resistant group. This finding strongly supports our previous conclusion that an increase in whole grain foods in the HWD rather than the source of protein and the type of fat was related to an increase in insulin sensitivity in only relatively insulin-resistant subjects.

Our finding of PAI-1 being inversely correlated with whole grains intake is consistent with the finding that refined grains intake was positively, and whole grains intake inversely, associated with PAI-1 concentrations in a cross-sectional study in the nondiabetic population [9].

Our finding is consistent with other randomised crossover [11,38,39] and parallel [40] grain interventions. These studies show that an oat bran diet (102 g/day of oat bran added to a low-fiber diet) for two weeks lowered PAI-1 concentrations compared with a low-fiber diet in 24 young healthy subjects [38] and a low-GI diet reduced PAI-1 concentrations in 20 patients with T2DM [11], 12 men with T2DM [39] and 50 overweight women [40]. However, the data is quite mixed and there are studies showing that a whole grain diet in subjects with normal or impaired carbohydrate metabolism [41], obese subjects with metabolic syndrome [42] and healthy, overweight subjects [10] had no effect on PAI-1 compared with a refined grain diet.

PAI-1, a single-chain glycoprotein (47 kilodaltons), is an inhibitor of tissue plasminogen activator (tPA) [43]. PAI-1 plays a role in thrombus formation and cardiovascular disease [43,44]. The Insulin Resistance Atherosclerosis Study suggested that elevated PAI-1 concentrations might be a very early risk marker of the insulin resistance syndrome leading to T2DM [6]. Our finding that PAI-1 was inversely correlated with insulin sensitivity in the HMD strongly supports this suggestion.

Moreover, our current study found that the HMD significantly increased serum total cholesterol and triglyceride compared with the HWD. Positive correlations between PAI-1 and serum cholesterol and triglyceride were observed. These correlations suggest that PAI-1 is closely allied with other risk factors for atherosclerosis [45]. An intervention [38] showing a decrease in both PAI-1 and lipid profiles (total cholesterol, HDL-C and low-density lipoprotein cholesterol (LDL-C)) after an oat bran diet, however, failed to find the association between the changes in PAI-1 and lipid profiles. Two interventions [11,39] which showed favourable effects of a low-GI diet on insulin sensitivity, lipid profiles and PAI-1 in subjects with T2DM compared with a high-GI diet, however, did not find an association between PAI-1 and insulin sensitivity or lipid profiles. Therefore, our study is the first dietary intervention observing correlations between PAI-1, lipid profiles (total cholesterol and triglyceride) and insulin resistance as previously shown in the cross-sectional studies.

In this study, we could not show differences in CML levels between a diet high in red and processed meat and refined grains compared with a diet high in whole grain, legumes, nuts and dairy products. A wide range of foods in a modern diet contains AGEs [46,47]. Uribarri et al. [46] reported that the highest dietary CML levels were observed in beef and cheese, followed by poultry, pork and fish. CML contents in roasted nuts (peanuts, walnuts, almonds and cashews) range from 6447 to 9807 kU/100 g, which are the almost same amount of AGEs in 100 g of beef steak strips, stir-fried with or without oil from 7 min to 15 min [46]. Even though red and processed meat and white bread are rich sources of dietary CML, cooking methods could drive CML formation in the HWD diet. For example, the AGE content of boiled or stewed chicken (1124 kU/100 g) increases when the same amount of chicken is broiled (5828 kU/100 g). The AGE content of broiled chicken is similar to the AGE content of broiled beef (5963 kU/100 g) while the AGE content of boiled or stewed beef is 2230 kU/100 g [46]. Tessier et al. [21] reported that breakfast cereal and dairy products accounted for 12% and 4% of the entire dietary intake of CML [21]. Cheese and grilled fish (e.g., salmon ranging from 3000 to 4300 kU/100 g) also contribute to dietary intake of CML [46].

In light of the suggestion that foods rich in protein and fat are more likely to have a higher AGEs content [48], a similar intake of protein and saturated fat in the HMD and HWD should mean little potential difference in CML contents between the two diets. In addition, there was a significantly higher intake of vitamin C (an antioxidant involved in inhibiting AGE formation [19]) from potato in the HMD which cannot be ignored as CML is known to be a glyoxidation product [19,20]. It has been proposed that dietary AGEs are associated with generation of pro-inflammatory cytokines in healthy subjects [49]. In our study, no differences in the inflammatory molecules hs-CRP and IL-6 between the two diets may be explained by the absence of a difference in CML levels between the two diets. The absence of differences in hs-CRP and IL-6 at the end of the two interventions could be partly attributed to the low inflammatory milieu of the study sample.

Recent evidence has challenged the concept that IL-6 has a negative impact on metabolic homeostasis [50,51]. The further measurements of IL-1beta/IL-1ra or TNF alpha which are associated with insulin resistance [52] may be useful. However, these measurements were not undertaken in this study.

The strength of this study is that, to our knowledge, this is the first randomised crossover intervention in weight stability showing significantly higher PAI-1 concentrations after the consumption of red and processed meat and refined grains than after the consumption of whole grains, nuts, dairy products and legumes. This is also the first dietary intervention showing a correlation between PAI-1, insulin resistance and lipid profile changes. The crossover and isocaloric study design with tightly controlled weight and activity levels is a strength of this study.

There are several limitations to take into account when the results are interpreted. We did not measure faecal and urinary CML. Studies show that only about 10%–30% of dietary AGEs are absorbed in the intestine and one-third of ingested AGEs are excreted in urine and faeces, and two-thirds are retained in tissues [22,53,54]. We did not have baseline measurements for any variable. Meal preparation and consumption outside the clinical research facility could be a potential limitation, although we believe compliance to the diets was excellent. The other potential limitation could be that dietary intake was not measured before the intervention, at baseline or during the washout and we had no control group, nor did we have objective measures of nutrient intake.

In conclusion, our study suggests that a diet high in red and processed meat and refined grains for four weeks significantly elevated PAI-1 concentrations compared with a diet high in whole grains, nuts, dairy products and legumes. Increased PAI-1 may be associated with insulin resistance and elevated lipids. This study provides more evidence in favour of a healthy diet pattern for disease prevention and that higher-quality fibre-rich carbohydrate may be the most important element in this healthy diet.

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11.3 Table: Effects of the two diets on blood IL-6, PAI-1, fluorescent AGEs and CML divided into the two insulin sensitivity subgroups

We added Data in the following table as the main study was divided along post-hoc subgroup lines of insulin resistance, which is linked to the other measures subsequently reported in this chapter 11. Data are presented as being divided into the two subgroups for the blood IL-6, PAI-1, fluorescent AGEs and CML analysis. There was a borderline significant group by time interaction for PAI-1. These results are discussed in chapter 12.

	LDIGIT group 1 and 2 (n = 49)				LDIGIT group 1 (n = 24)			LDIGIT group 2 (n = 25)		
	HMD	**HWD**	***P***	***P* Group by time**	**HMD**	**HWD**	***P***	**HMD**	**HWD**	***P***
IL-6 (pg/ml)	8, 11	8, 11	0.5	0.4	8, 18	8, 17	0.5	8, 10	9, 11	0.8
PAI-1 (ng/ml)	159, 81	121, 50	< 0.001	0.05	137, 80	113, 39	0.02	177, 61	128, 52	< 0.001
Fluorescent AGEs (absorbance units)	1392, 294	1430.6, 438.	0.16	0.5	1448.7, 361.6	1451.7, 413	0.1	1383, 333.1	1367, 486	0.3
CML (µg/ml)[#]	1.37, 0.6	1.49, 0.6	0.074	0.7	1.26, 0.65	1.4, 0.5	0.3	1.49, 0.54	1.59, 0.57	0.1

P values (n = 49) for IL-6, PAI-1 and fluorescent AGEs were obtained from nonparametric tests except for CML determined by paired samples t tests. Values (n = 49) for IL-6, PAI-1, fluorescent AGEs and CML are expressed as medians and interquartile ranges. The groups 1 and 2 are defined *a posteriori* based on the insulin values of LDIGIT $_{(120\text{-}150\text{min}),}$ not randomized groups. HMD, a high red and processed meat and refined grains diet; HWD, a high whole grain, nuts, dairy and legumes diet; IL-6, interleukin 6; PAI-1, plasminogen activator inhibitor type 1; AGE, advanced glycation end product; CML, carboxymethyllysine.

12 SUMMARY AND CONCLUSIONS

12.1 Discussion

Although lifestyle modification including increased physical activity, weight loss, energy restriction and improved diet quality can lower the risk of T2DM, exactly which dietary patterns or components in the absence of energy restriction and increased physical activity are able to influence the risk of T2DM is unclear.

Epidemiological studies have indicated an increased risk of T2DM with high consumption of red meat and processed meat, and a lower risk with high consumption of dairy and whole grains. Nuts are inconsistent in epidemiological studies.

However, the few interventions for dairy, whole grains and legumes have shown mixed findings and very few interventions have investigated the effect of the consumption of red and processed meat and glucose homeostasis.

Therefore, the primary endpoint of this dietary intervention was to investigate the effect of a diet high in red and processed meat and refined grains on insulin sensitivity compared with a diet high in whole grains, dairy, legumes, nuts and devoid of red and processed meat in weight stability.

The secondary endpoint was to determine possible mediators of insulin sensitivity including inflammatory and fibrinolytic markers and advanced glycation end products (AGEs) which have been associated with the risk of T2DM in cross sectional studies.

The key findings from this research:

- A diet high in red and processed meat and refined grains decreased insulin sensitivity compared with a diet in high in whole grains, nuts, dairy and legumes but only in relatively insulin-resistant individuals and not in insulin-sensitive individuals.
- In the insulin-resistant group, insulin sensitivity index (ISI) was correlated with BMI and fat mass.
- Total cholesterol and triglyceride were significantly higher after a diet high in red and processed meat and refined grains than after a diet high in whole grains, nuts, dairy and legumes.

- ISI on the diet high in whole grains, nuts, dairy and legumes was positively correlated with the amount of carbohydrates in the diet high in whole grains, nuts, dairy and legumes after adjustment for ISI on the diet high in red and processed meat and refined grains.
- The fibrinolysis inhibitor, PAI-1, was significantly higher after a diet high in red and processed meat and refined grains than after a diet high in whole grains, nuts, dairy and legumes, although inflammatory markers (hs-CRP and IL-6) and AGEs [fluorescent AGEs and carboxymethyllisine (CML)] were not different between diets.
- PAI-1 on the diet high in whole grains, nuts, dairy and legumes was inversely correlated with whole grain intake after adjustment for PAI-1 on the alternative diet
- PAI-1 was inversely correlated with ISI, only after a diet high in red and processed meat and refined grains.
- PAI-1 was positively correlated with serum total cholesterol and TG after a diet high in red and processed meat and refined grains.

An acute meal tolerance test was also conducted at the end of each 4-week dietary intervention period. The hypothesis of the test was that a red meat/refined grain meal would elevate postprandial glycemia and insulinemia compared with the dairy/chicken/nuts/whole grain meal matched for total energy and that this elevation may be partly responsible for the change in insulin sensitivity during the chronic diet.

The key finding from the meal tolerance test:

- A red meat/refined grain meal induced significantly higher postprandial insulin release than a dairy/chicken/nuts/whole grain meal after a similar chronic dietary pattern.
- A red meat/refined grain meal significantly increased C-peptide total AUC compared with a dairy/chicken/nuts/whole grain meal.
- There was no difference in postprandial glucose concentrations between two meals.
- Elevated postprandial insulin levels after the red meat/refined grain meal could be caused by the 4-week diet high in red and processed meat and refined grains reducing insulin sensitivity or alternatively the meal responses could be the cause of the change in insulin sensitivity.
- Meals varying moderately in carbohydrate content and glycaemic index (GI) did not affect total blood glucose to any major degree.

- The difference between a 23% increase in the C-peptide total AUC and a 46 % increase in the insulin total AUC could be due to decreased hepatic insulin clearance rates with the red meat/refined grain diet.
- The 27% increased glycemic load in the red meat/refined grain meal contributed to the 40%–50% increase in insulin while increased insulin resistance after a 4-week diet high in red and processed meat and refined grains may be responsible for the other half of the increase in insulin.

The main finding in this thesis is that a diet high in red and processed meat and refined grains for 4 weeks had a clear, adverse effect on insulin resistance in a relatively insulin-resistant group but not in an insulin-sensitive group. This finding links to the highly topical clinical and research area of personalised health. However as this is a post hoc observation we need to design a future study to a priori address the issue that the diet studied may have differing effects in different groups of people.

We separately analysed the data for postprandial glucose, insulin and triglyceride responses presented in chapter 10 and for the blood IL-6, PAI-1, fluorescent AGEs and CML concentrations presented in chapter 11 in accordance with two post-hoc subgroups of insulin resistance and found there was no significant group by time interaction for any measures. However, there was a borderline significant group by time interaction for PAI-1 ($P = 0.05$; $n = 49$) and the change in PAI-1 was greater in relatively insulin-resistant individuals ($P < 0.001$) than in insulin-sensitive individuals ($P = 0.02$) after a diet high in red and processed meat and refined grains compared with a diet high in whole grains, nuts, dairy and legumes. As discussed in the published article presented in chapter 10, increased insulin resistance was associated with decreased insulin clearance which correlated with increased PAI-1.

Our findings from the human intervention trial suggest that insulin-resistant people are more susceptible to a dietary pattern high in red and processed meat and refined grains leading to increased insulin resistance. Insulin resistance significantly increases the risk for cardiovascular disease. The most important factor influencing insulin sensitivity appears to be the quality and quantity of carbohydrate.

12.2 Limitations

Initially the aim was to recruit only overweight and obese participants but slow recruitment led to widening the entry criteria to normal weight subjects. A sample size of 50 overweight and obese subjects without diabetes was calculated to provide 80% power to detect a 20%

change (alpha level = 0.05) in insulin sensitivity as assessed by LDIGIT but we could not recruit this number. Insulin resistance was not calculated prior to randomisation.

In our study investigating the effect of two diets on insulin sensitivity reported in chapter 9, groups were categorized *a posteriori* based on higher *vs*. lower insulin levels during the LDIGIT. The need to divide the groups in this way was caused by recruitment of two very diverse groups: lean and insulin sensitive participants and overweight and obese insulin resistance participants. A larger group consisting of the latter alone would have been desirable but this was not possible in the time scale allowed for the PhD. We did not power the study based on two separate smaller groups but fortunately there was adequate power to see a diet by group interaction.

There were no dietary intake and other measurements at baseline and at the end of the wash-out period. We assumed that prognostic factors other than the dietary treatment would remain constant across both dietary treatment.

Participants were not given foods so meal preparation and consumption outside the clinic facility could be a potential limitation even though the compliance appeared to be satisfactory.

In the study reported in chapter 11, we only measured serum AGEs without measurements of AGEs in faecal and urine samples to specifically determine AGE content differences between two diets.

As described in chapter 9, the prescription of such a large quantity of red meat reflects the amount consumed in the highest quintile in the cohort studies [322-324] and was done in order to maximize the difference between two diets. However, it could be viewed as a limitation as the amount of red meat (200 - 300 g/day) prescribed is not representative of a typical intake. Prescription of a more usual amount of red meat could be considered in future research.

12.3 Future Directions

To confirm our findings, a large, multicentre, randomised crossover clinical trial should be performed to correct for the limitations of our study design including a prospectively enrolled insulin-resistant group, a reasonable run-period prior to study commencement, dietary intake and other measurements at baseline and during the washout period, as well as key foods being provided.

The population in this study consisted of 63% Caucasian, 28% Asian and 9% Latin American subjects with over 50% aged <40 years, and future studies should attempt to replicate the key results in a population including middle-aged people from the Middle East and the Pacific area, and older adult populations.

According to the finding in chapter 9, total energy was matched in the two diets but fat and carbohydrate intake differed between the two groups; it would appear that the source of protein and the type of fat was not related to the change in insulin sensitivity but an increase in carbohydrate containing foods in the HWD was related to an increase in insulin sensitivity. Dietary fiber was not related to insulin sensitivity suggesting that it was other components in whole grains such as resistant starch.

As a consistently observed association between whole grain carbohydrate and insulin sensitivity in our studies reported in chapter 9, 10 and 11, further laboratory analyses may be of interest to establish the effect of resistant starch on insulin sensitivity.

As reviewed in chapter 6, resistant starch-derived short chain fatty acids are known to play a key role in glucose metabolism through several potential mechanisms which involve enhanced proliferation of favourable gut bacterial species, decreased intestinal permeability, increased secretions of glucagon-like peptide-1 (GLP-1) and peptide YY (PYY). Therefore, a potential area for future laboratory analyses are postprandial GLP-1 and PYY, serum TLR4 (a maker of gut barrier integrity), faecal analyses for short chain fatty acids and specific bacterial populations.

Additional analyses for AGEs such as serum methylglyoxal (MG: a precursor of AGEs), 24-hour urine and faecal CML and methylglyoxal (MG) may be useful. It also would be useful to analyse other markers of hemostasis especially fibrinogen and factor VII which are associated with increased insulin resistance and increased cardiovascular risk [325, 326].

12.4 Conclusion

In comparison with a diet in high in whole grains, nuts, dairy products, legumes and devoid of red and processed meat, a diet high in red and processed meat and refined grains for 4 weeks increased insulin resistance only in relatively insulin-resistant individuals and not in sensitive individuals. Significantly increased PAI-1 concentrations on this diet were correlated with insulin resistance, as well as with carbohydrate in this diet, while improved insulin sensitivity on the diet high in whole grains, nuts, dairy products and legumes was

favourably correlated with carbohydrate in this diet. In the meal tolerance test a red meat/refined grain meal increased postprandial insulin and glucose responses, compared with a dairy/chicken/nuts/whole grain meal, strongly supporting the findings on carbohydrate quality observed in the chronic intervention.

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14 APPENDICES

14.1 Statement of Authorship: A review of potential metabolic etiologies of the observed association between red meat consumption and development of type 2 diabetes mellitus

STATEMENT OF AUTHORSHIP

Chapter 2

A review of potential metabolic etiologies of the observed association between red meat consumption and development of type 2 diabetes mellitus

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Metabolism 2015; 64 (7):768-79. doi: 10.1016/j.metabol.2015.03.008.

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14.2 Statement of Authorship: Response to the comment by Kuipers and Pruiboom.

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14.3 Statement of Authorship: Polyphenols and Glycemic Control

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Polyphenols and Glycemic Control

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14.4 Statement of Authorship: Consumption of red and processed meat and refined grains for 4 weeks decreases insulin sensitivity in insulin-resistant adults: A randomized crossover study

STATEMENT OF AUTHORSHIP

Chapter 9

Consumption of red and processed meat and refined grains for 4 weeks decreases insulin sensitivity in insulin-resistant adults: A randomized crossover study.

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Metabolism. 2017; 68:173-83.

Kim Y, Keogh JB, Clifton PM. Consumption of red and processed meat and refined grains for 4 weeks decreases insulin sensitivity in insulin-resistant adults: A randomized crossover study. Metabolism. 2017; 68:173-83.

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14.5 Statement of Authorship: Differential Effects of Red Meat/Refined Grain Diet and Dairy/Chicken/Nuts/Whole Grain Diet on Glucose, Insulin and Triglyceride in a Randomized Crossover Study

STATEMENT OF AUTHORSHIP

Chapter 10

Differential Effects of Red Meat/Refined Grain Diet and Dairy/Chicken/Nuts/Whole Grain Diet on Glucose, Insulin and Triglyceride in a Randomized Crossover Study.

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Nutrients. 2016; 8.

Kim Y, Keogh JB, Clifton PM. Differential Effects of Red Meat/Refined Grain Diet and Dairy/Chicken/Nuts/Whole Grain Diet on Glucose, Insulin and Triglyceride in a Randomized Crossover Study. Nutrients. 2016; 8.

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14.6 Statement of Authorship: Effects of Two Different Dietary Patterns on Inflammatory Markers, Advanced Glycation End Products and Lipids in Subjects without Type 2 Diabetes: A Randomised Crossover Study

STATEMENT OF AUTHORSHIP

Chapter 11

Effects of Two Different Dietary Patterns on Inflammatory Markers, Advanced Glycation End Products and Lipids in Subjects without Type 2 Diabetes: A Randomised Crossover Study

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Nutrients. 2017; 9.

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14.7 Examples of Weighed Food Diary and Food Checklists

Weighed Food Diary

Time	Brand name	Food/drink and cooking method	Quantity/ Amount	Leftover/ Wastage

Food Checklist

FOOD CHECKLIST WEEK: DATE:	**DAY 1**	**DAY 2**	**DAY 3**	**DAY 4**	**DAY 5**	**DAY 6**	**DAY 7**
Chicken (80-150 g) or Fish (70-150 g) or Legumes [150-225 g (l cup–1½ cups)]							
Nuts (1 serve =30 g)							
Nuts (1 serve =30 g)							
Nuts (1 serve =30 g)							
Whole grains (1)							
Whole grains (2)							
Whole grains (3)							
Whole grains (4)							
Dairy products (1)							
Dairy products (2)							
Dairy products (3)							
Dairy products (4)							
Vegetables (1)							
Vegetables (2)							
Fruits (1)							
Fruits (2)							
Oil/spread (2-7 serves)							
Jam or marmalade (≤ 1 serve)							
Wine/Alcohol (Optional)							

FOOD CHECKLIST WEEK: DATE:	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Red meat (200-300 g) **e.g.Beef, pork, lamb, veal**							
Processed meat (≥50 g) **e.g. Ham, bacon,sausage**							
Refined grains (1)							
Refined grains (2)							
Refined grains (3)							
Refined grains (4)							
Refined grains (5)							
Refined grains (6)							
Vegetables (1-2 serves)							
Potato (200-300g)							
Fruits **(1-2 serves)**							
Oil / spread (3-9 serves) **Jam or marmalade(1 serve)**							
Indulgence foods (3-4 serves) **To replace refined grains or potato if desired**							
Wine/Alcohol (Optional)							