# The Effects of Macronutrient and Micronutrient Diet Manipulation on Human Insulin Sensitivity

**Martin de Bock**

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# Abstract

Insulin resistance leading to diabetes is currently at pandemic levels. The associated costs to society and burden on health providers are incalculable. Aside from type 2 diabetes and cardiovascular disease, it is now clear that insulin resistance (and resultant hyperinsulinism) is implicit with the pathogenesis of a wide range of other diseases, including cancers, and neurodegenerative and infectious diseases. Dietary manipulation as part of lifestyle modification is recognised as a fundamental way to prevent obesity and type 2 diabetes. However, as yet, there is no consensus on the best dietary interventions to improve glucose homeostasis or the long-term risk of developing type 2 diabetes. The objectives of this thesis are: i) to understand the mechanisms through which insulin resistance causes disease; ii) to review the current knowledge on dietary macro- and micronutrients that can be manipulated to improve insulin sensitivity; and iii) to add to this knowledge base through novel investigations.

Literature review indicates that reducing dietary free sugars and increasing dietary fibre consumption are the most important dietary manipulations to improve insulin sensitivity. With respect to dietary fibre, there is no existing literature examining the impact of supplemented fibre alone on insulin sensitivity in adolescents. Hence, the first aim was to investigate if psyllium (a purified source of fibre) alone could improve insulin sensitivity in adolescents.

To accomplish this, we conducted a participant-blinded, randomized, placebo-controlled, crossover trial in 47 adolescents, supplemented with 6 g/day of psyllium fibre for 6 weeks. 45 subjects completed the study, and compliance was very high: 87% of participants took >80% of prescribed capsules. At baseline, 44% of subjects were overweight or obese but none had impaired glucose tolerance. However, this was a negative study; there was no improvement in insulin sensitivity. Nevertheless, there was a 4% reduction in android to gynoid fat ratio ($p=0.019$), as well as a 0.12 mmol/l (6%) reduction in LDL cholesterol ($p=0.042$).

The effect of dietary micronutrient manipulation on insulin sensitivity is a new and exciting area of research, with considerable commercial interest from a burgeoning nutraceutical industry. However, the existing data are often confusing, conflicting, or of poor quality. Nevertheless, there are several traditional remedies rich in unique micronutrients that hold some promise, many of which are polyphenols. A published commentary contained in this thesis describes the various

difficulties when trying to assess the effects of polyphenols on insulin sensitivity, namely the heterogeneity of the raw product, diverse inter- and intra-individual responses, and lack of robust data. Taking into account these difficulties, a systematic step-wise research program was embarked upon, aiming to investigate the effects of unique olive plant polyphenols (oleuropein and hydroxytyrosol) on insulin sensitivity.

The first step was to establish the bioavailability of olive leaf polyphenols at different doses (lower and higher) and formulations (liquid or capsule) when ingested as olive leaf extract (OLE). Nine volunteers (5 males) aged 42.8 ± 7.4 years were randomized to receive either capsulated or liquid OLE as a single lower (51.1 mg oleuropein, 9.7 mg hydroxytyrosol) or higher (76.6 mg oleuropein, 14.5 mg hydroxytyrosol) dose, and then the opposite strength (but same formulation) a week later. Conjugated metabolites of hydroxytyrosol were the primary metabolites recovered in plasma and urine after OLE ingestion, and we showed plasma concentrations approached those used in in-vitro experiments. However, as predicted, there was wide inter-individual variation, with plasma time-course, peak concentrations, and area under the curve all influenced by preparation, dose, and gender. With the knowledge that olive polyphenols are absorbed when ingested as OLE, we conducted a clinical trial investigating the effects of OLE supplementation on insulin sensitivity.

In a randomized, placebo-controlled, double-blind, crossover trial, 46 participants (aged 46.4 ± 5.5 years and with BMI 28.0 ± 2.0 $kg/m^2$) received capsules with OLE or placebo for 12 weeks. All participants took >96% of prescribed capsules. OLE supplementation was associated with a 15% improvement in insulin sensitivity ($p=0.024$) compared to placebo. There was also a 28% improvement in pancreatic β-cell responsiveness ($p=0.013$).

Translating practical research results such as these to the general population has many obstacles. Nevertheless, the results from our studies provide an important contribution to our understanding of important dietary supplements and their effects on insulin sensitivity and other cardiovascular risk factors, which can be applied at both an individual and population level.

# Acknowledgements

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## List of commonly used abbreviations

| | |
|---|---|
| AD | Alzheimer's disease |
| AGEs | advanced glycation end products |
| α-MSH | melanocyte stimulating hormone α |
| ApoB | apolipoprotein-B |
| APOE-ε4 | apolipoprotein ε4 allele |
| ARC | arcuate nucleus |
| CE | cholesterol ester |
| CHO | carbohydrate |
| CNS | central nervous system |
| CV | co-efficient of variation |
| CVD | cardiovascular disease |
| DEXA | dual-energy x-ray absorptiometry |
| FFA | free fatty acids |
| GIP | gastric inhibitory polypeptide |
| GLP | glucagon-like peptide |
| HDL | high density lipoprotein |
| HOMA-IR | homeostasis model of assessment of insulin resistance |
| IGF | insulin like growth factor |
| IPAQ | international physical activity questionnaire |
| IRS | insulin receptor substrate |
| ISI | insulin sensitivity index |
| LDL | low density lipoprotein |
| MAP | mitogen activated protein |

| | |
|---|---|
| NF κB | nuclear factor kappa B |
| NPY | anorexigenic neuropeptide y |
| PAI-1 | plasminogen activator inhibitor – 1 |
| PAQ-A | physical activity questionnaire for adolescents |
| PI | phosphatidylinositol |
| PIP2 | phosphatidylinositol 3,4-disphosphate |
| POMC | pro-opiomelanocortin |
| PPAR-γ | peroxisome proliferator activated receptor gamma |
| PVN | paraventricular nucleus |
| ROS | reactive oxygen species |
| SFA | saturated fatty acid |
| TF | tissue factor |
| TG | triglycerides |
| TLR4 | toll like receptor 4 |
| TNF-α | tumour necrosis factor alpha |
| VLDL | very low density lipoprotein |

# Chapter 1. Introduction

Disordered glucose homeostasis contributes to human morbidity and mortality across a broad range of pathologies worldwide. It is inextricably linked to obesity, and, as such, the burden of disordered glucose homeostasis on global health (and the subsequent stress this places on health systems) is increasing due to the obesity pandemic[1]. There exists a spectrum of disordered glucose homeostasis as a result of progressive insulin resistance[2]. Even before established clinical end point measures of poor glucose regulation are met, for example type 2 diabetes, there is a continuum of increased disease risk with progressive insulin resistance and subsequent hyperinsulinism. Importantly, insulin resistance and hyperinsulinism is becoming increasingly prevalent in the younger population due to the obesity pandemic, which has major implications for disease risk as this generation ages.

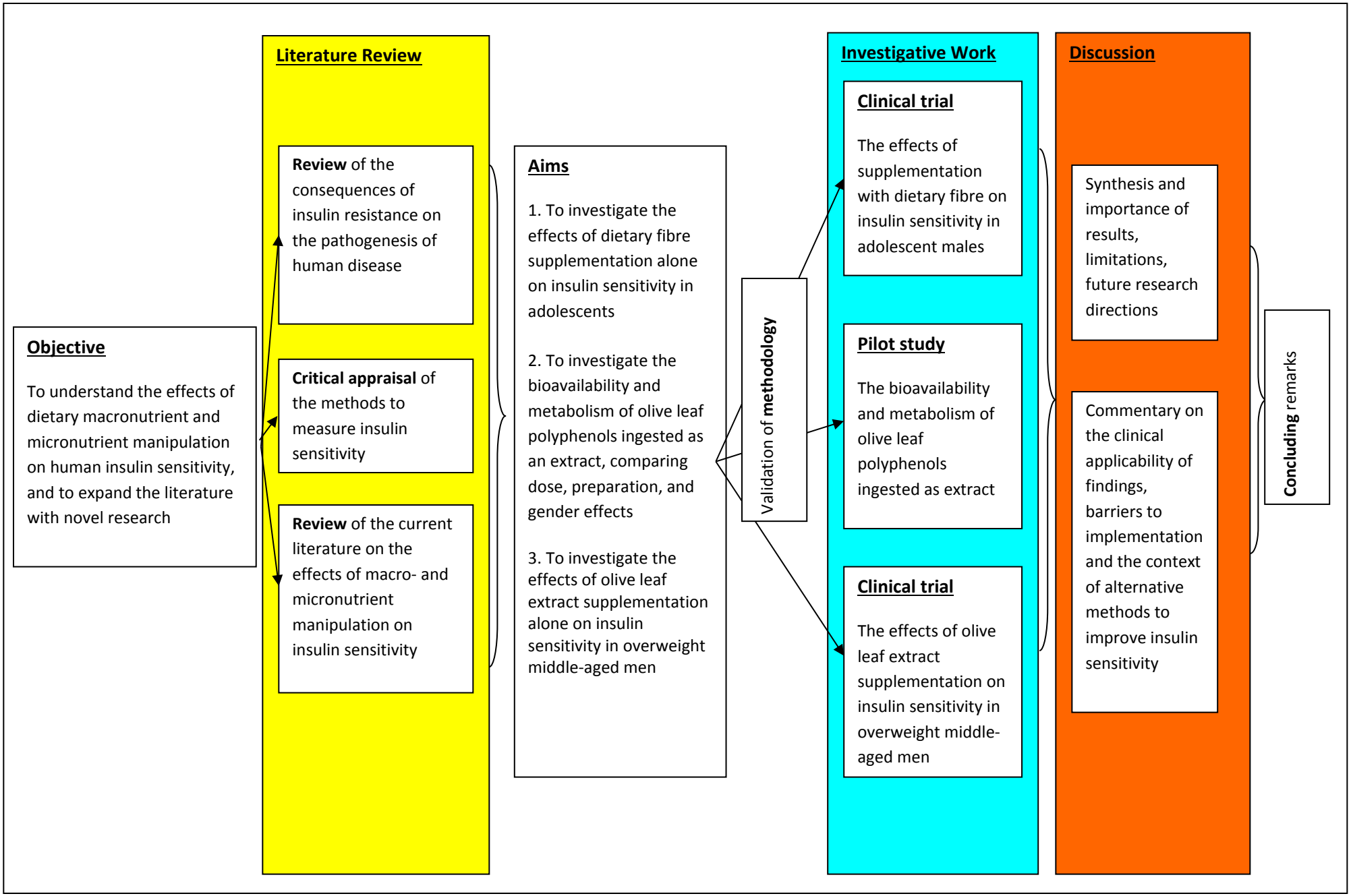
In the following literature review, the various pathogenic mechanisms through which insulin resistance and resultant hyperinsulinism contribute to a wide variety of diseases (cardiovascular, cancerous, neurodegenerative, and infectious) are described. By understanding these pathways, possible mechanisms through which nutritional interventions can improve insulin resistance can be hypothesized and tested.

The relationship between insulin resistance and measures of insulin sensitivity are complex. Insulin resistance relates to the ability of insulin to exert its actions at the insulin receptor and post receptor pathways, and encompasses many tissues and the plethora of physiological properties that insulin has (glucose and non-glucose related). Measures of insulin sensitivity are tools used to define one aspect of insulin action – that is, tissue glucose disposal, which has saturatable kinetics. While those with poor insulin sensitivity are defined as “insulin resistant”, it is important to note that measures of insulin sensitivity, do not measure the wider implications of insulin resistance – in particular the downstream effects of compensatory hyperinsulinism on non-glucose homeostasis insulin action. There are several ways to measure insulin sensitivity that are critically appraised in the literature review.

Dietary intervention is an example of an acceptable, affordable and effective method to improve glucose homeostasis, but there is no consensus on the best approach[3]. Manipulation of dietary macro- and micronutrients can improve insulin sensitivity resistance, and these are also explored in detail in the literature review. Further, the discovery of micronutrients with bioactive properties has opened up a new field of research (and commercial market), resulting in new potential tools to fight the scourge of diseases associated with disordered glucose homeostasis. Dietary polyphenols are one such group that have recently been heralded as beneficial to human health.

Based on the literature review, it became apparent that there are several unanswered questions, even in relation to dietary fibre that arguably has the most and best evidence for improving insulin sensitivity. The following thesis overview demonstrates the aims that were formulated, and the subsequent novel investigative work conducted.

# Thesis overview



## 1.1 Disordered glucose homeostasis and disease

As mentioned, disordered glucose homeostasis affects a number of human organ systems. In the following section, the impact of unfavourable glucose homeostasis on major categories of disease will be reviewed, and where possible, mechanisms discussed.

### 1.1.1 Cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of death in patients with type 2 diabetes, with >50% of this population dying from coronary heart disease, and a further 15% from stroke[4]. The Framingham cohort was one of the first studies to clearly define the risk of CVD in those with type 2 diabetes, where after adjustment for other factors, type 2 diabetes doubled the risk of any CVD[5]. Subsequent epidemiological studies have shown similar results, with one particular study demonstrating a two to four fold increase in risk for coronary artery disease alone in individuals with type 2 diabetes[6]. While the risk is great when type 2 diabetes is established, recent literature has identified that increased morbidity and mortality risk due to CVD is present even before this threshold (on the spectrum of disordered glucose homeostasis) has been reached [4]. Physiologically, the hallmark of "pre diabetes" is insulin resistance and consequent hyperinsulinism[7]. Yip *et al*. found that amongst healthy non diabetic, non obese volunteers, those in the highest tertile of insulin resistance had a one in five chance of having a serious CVD event within five years, compared with no events occurring in those in the lowest tertile[8]. Insulin resistance and hyperinsulinism have subsequently been identified as the central mediator to underlying pathophysiological processes that ultimately lead to CVD; dyslipidemia, hypertension, clotting, atherogenesis, and artery wall dysfunction[9].

The process whereby insulin resistance leads to dyslipidemia is outlined in Figure 1. The initializing step is at the level of the adipocyte, where insulin resitance results in the release of excess free fatty acids (FFA)[1]. Hepatocytes exposed to increased FFAs release increased triglyceride (TG), apolipoprotein-B (ApoB) and very low density lipoprotein (VLDL)[10]. In addition, increased VLDL stimulates the exchange of cholesterol esters (CE) from high density lipoprotein (HDL) and low density lipoprotein (LDL) for VLDL. TG enriched LDL can be

oxidised to become smaller and more dense 'small dense LDL' which may then more easily penetrate and stick to the artery wall. Reverse cholesterol transport by HDL is further compromoised by apoliprotein-A-I (ApoA-I) dissociation and renal clearance. Low levels of HDL and the presence of small dense LDL are each independent risk factors for CVD. There are changes in gene expression that drive this process and have been reviewed elsewhere[9].

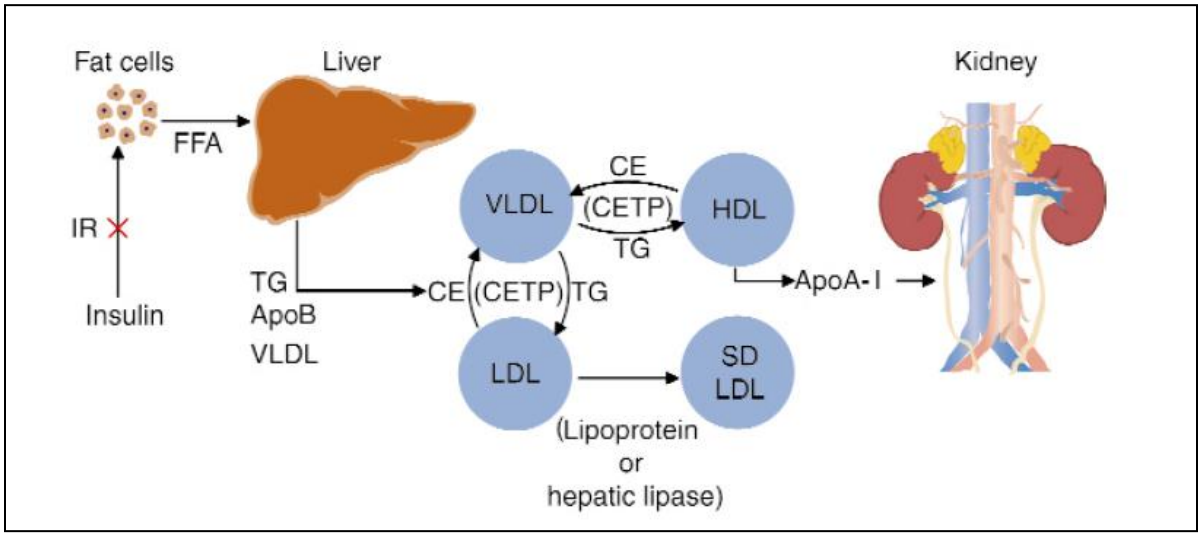



Figure 1: Insulin resistance leading to dyslipidemia***[9]***.

*Reproduced from [9] with permission from the Journal of Clinical Investigation. Additional abbreviations: IR insulin resistance, CETP cholesterol ester transport protein.*

The association between insulin resistance (and resultant hyperinsulinism) and hypertension is firmly established [11-16]. While obesity is also closely related to the development of hypertension, the independent effect of hyperinsulinism on blood pressure has been verified[17, 18], even in the non-obese[19]. Moreover, mechanisms through which hyperinsulinism causes hypertension have been elucidated, and these are i) increased renal Na+/water reabsorption, ii) sympathetic nervous system activation, iii) decreased Na+-K+-ATPase activity, iv) increased Na+-H+ pump activity, v) increased cellular Ca2+ accumulation, and vi) stimulation of growth factors[2].

Many aspects of clotting are disordered in the setting of diabetes, contributing to an increased risk of stroke and atherothrombosis[20, 21]. Table 1 shows there is considerable overlap between clotting factors that are known to be abnormal in diabetes[20], and those that are known to increase atherothrombotic risk[21].

**Table 1: Clotting abnormalities in diabetes.**

| **Clotting abnormalities in diabetes** | **Atherothrombotic Risk Factors** |
|---|---|
| ↑ Plasminogen Activator Inhibitor-1 | ↑ Plasminogen Activator Inhibitor-1 |
| ↑ von Willebrand factor | ↑ von Willebrand factor |
| ↑ Fibrinogen | ↑ Fibrinogen |
| ↑ Platelet activation and aggregation | ↑Prothrombin fragment F1 + 2 |
| ↑ Factor VII | ↑ Factor VII |
| ↑ Factor VIII | ↑ Factor VIII |
| ↑ Tissue Factor | ↑ Factor IX |
| ↑ Antithrombin III | ↑ Factor X |
| ↑ Protein C | ↑ Factor XIII A- and B-subunit |
| ↑ Protein S | |

Some of these clotting abnormalities, for example increased Plasminogen Activator Inhibitor-1 (PAI-1) which leads to poorer fibrinolysis, are also present in the "pre diabetic" insulin resistant individual[22]. Increased PAI-1 levels can be used as an example to demonstrate the complex relationship between hyperinsulinism and gene expression and also to illustrate how a multitude of other atherothrombotic risk sequelae (eg platelet aggregation) can result from increased plasma insulin levels (Figure 2).

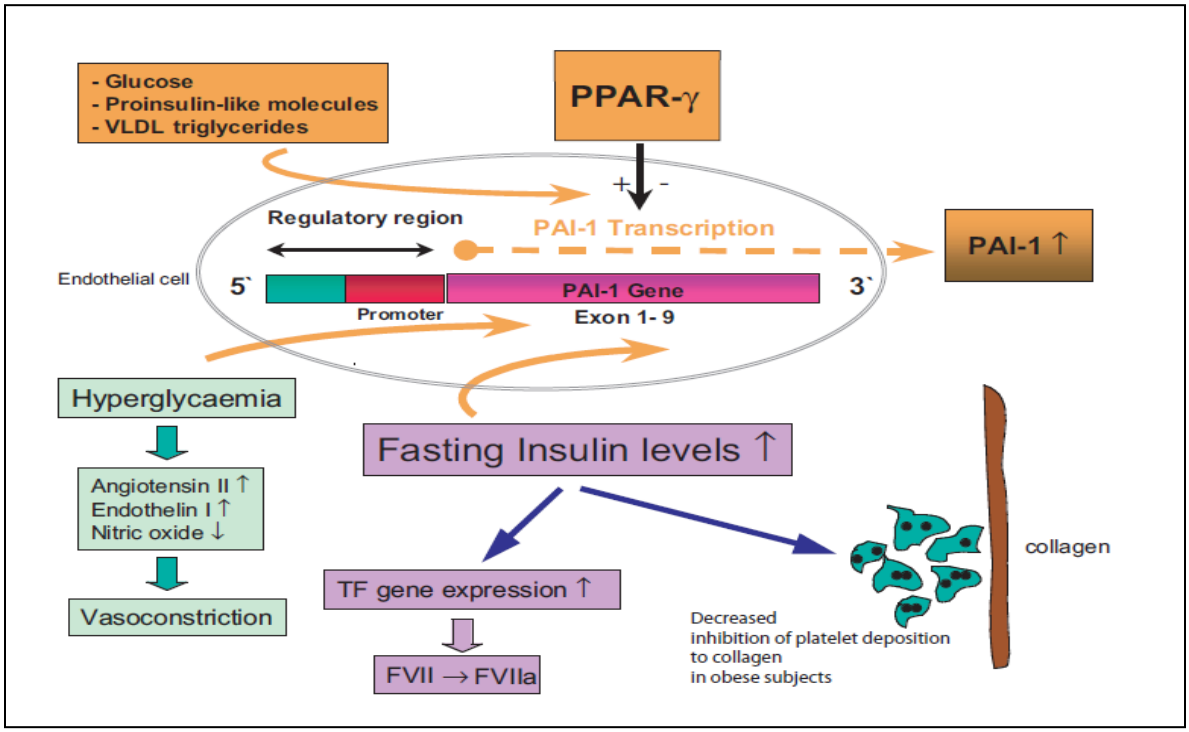



Figure 2: Insulin resistance and disordered clotting*[21]*.

*Insulin, glucose, proinsulin-like molecules, and VLDL induce PAI-1 gene expression. Further, hyperglycaemia leads to increased angiotensin II, endothelin 1, and decreased nitric oxide to induce vasoconstriction. Insulin also induces Tissue Factor (TF) expression which activates the extrinsic clotting pathway. Reproduced from [21] with permission from Swiss Medical Weekly. Additional abbreviation: PPAR-g peroxisome proliferator-activated receptor gamma.*

Once sustained hyperglycaemia in the setting of type 2 diabetes is established, the formation of advanced glycation endproducts (AGEs) adds to the toxic milieu. For example, AGEs act as a molecular glue causing cells to stick to the vessel wall, leading to platelet activation and coagulation initiation[23]. AGEs also have important pathological effects on artery walls, and these will be discussed below.

The atherosclerotic plaque consists of excessive lipid and collagen, foam macrophages and smooth muscle proliferation[24], all of which are affected by plasma insulin concentrations[2]. The central role of insulin in atherogenesis is aptly demonstrated in the classic experiment by

WritCruz *et al*., where a chronic insulin infusion directly into the femoral artery of a dog resulted in pathological changes in the ipsi-, but not on the contra lateral side[25]. In humans, insulin resistance is important, as shown by Laakso *et al*., where the only metabolic difference between a group with atherosclerosis and controls without disease was insulin sensitivity as measured by the euglycacemic clamp (there were no differences in blood pressure or lipid profile)[26]. Further, results of the "Insulin Resistance Atherosclerosis Study" (IRAS) study showed a direct correlation between insulin sensitivity and carotid intima media thickness[27]. In-vitro evidence for the role that insulin has in atherogenesis also exists, with proliferation of smooth muscle cells, raised LDL levels, increased formation and decreased degradation of atherosclerotic plaques, stimulation of connective tissue synthesis, and stimulation of growth factors all being documented[2]. The stimulation of growth factors by insulin through mitogen-activated protein (MAP) kinase despite insulin resistance is an important pathological process, and is expanded upon in subsequent sections.

Endothelial dysfunction mediated by insulin resistance and subsequent hyperinsulinism is common to hypertension, CVD and atherosclerosis[28]. The molecular pathways by which hyperinsulinism results in endothelial dysfunction are shown in Figure 3. Nitric oxide (vasodilator) synthesis and glucose transporter type 4 (GLUT4) uptake are selectively inhibited during insulin resistance. However preservation of the MAP kinase pathway results in increased endothelin-1 (vasoconstrictor) synthesis due to hyperinsulinism[28].

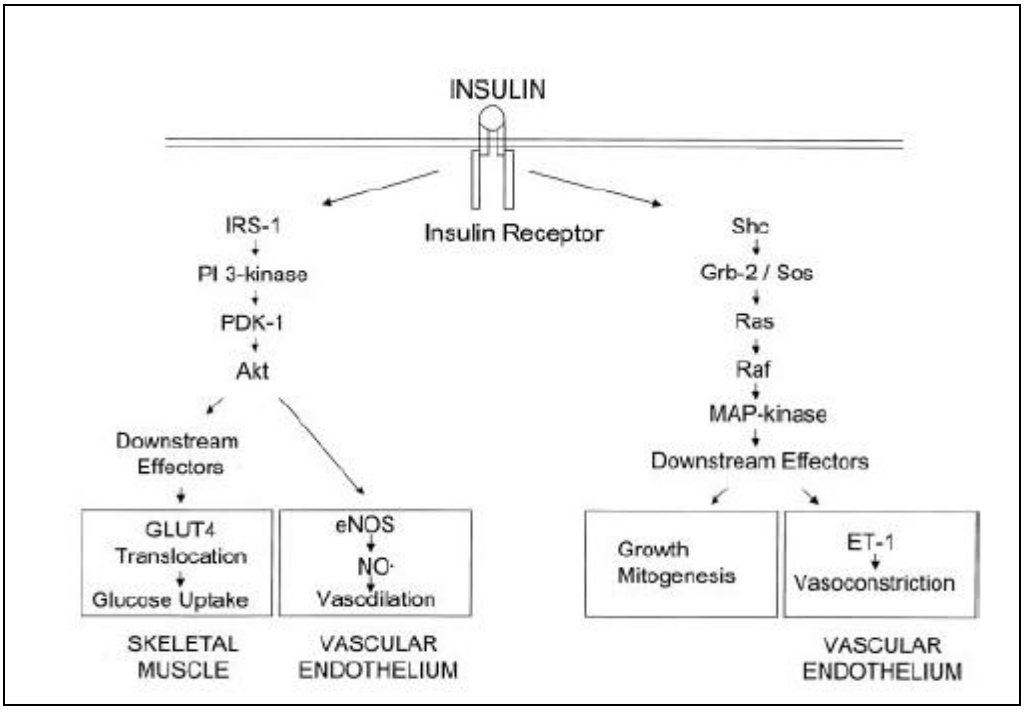



Figure 3: Hyperinsulinism and endothelial dysfunction*[28]*.

*Under normal physiology insulin signalling follows the phosphoinositide 3 (PI3) kinase pathway (left side of diagram), favouring glucose uptake and vasodilation. In the setting of insulin resistance, there is preservation of the MAP-kinase pathway (right side of the diagram) promoting vasoconstriction. Reproduced from [28] with permission from Wolters Kluwer Health.*

Apart from insulin, a host of other factors commonly seen in the metabolic syndrome deleteriously affect endothelial function. A detailed description of these can be found in the review by Kim [28], and are summarised in Figure 4. A vicious reciprocal relationship is produced, accelerating the development of CVD, and importantly, insulin resistance is central to this process.

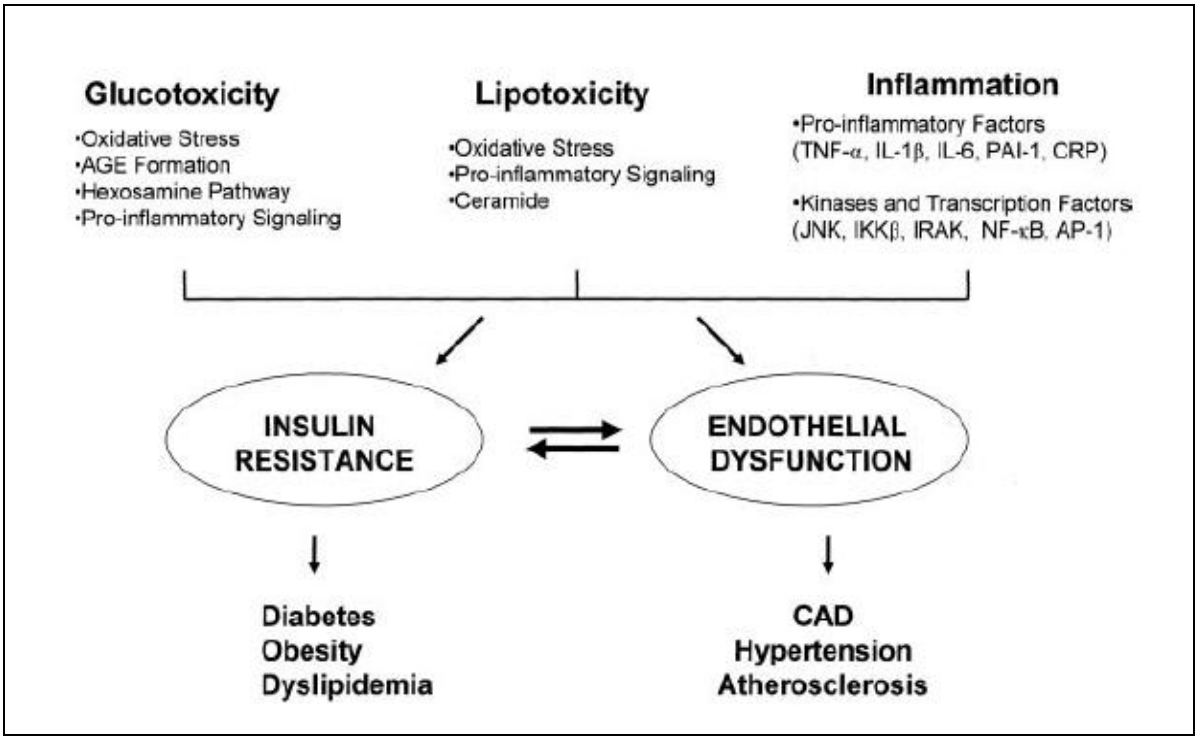



Figure 4: Insulin resistance and cardiovascular disease*[28]*.

*Shared and interacting effects of glucotoxicity and inflammation on insulin resistance and endothelial dysfunction result in a vicious cycle of reinforcement accelerating the pathogenesis of CVD. Reproduced from [28] with permission from Wolters Kluwer Health. Additional abbreviations: CAD coronary artery disease.*

### 1.1.2 Cancer

Disordered glucose homeostasis and cancer share a complex relationship, but clear evidence exists to show that insulin resistance (and resultant hyperinsulinism) is an independent risk factor for the development of many types of cancer. Recent studies in patients with type 2 diabetes, and a well conducted meta-analysis confirm an increased risk for cancer affecting the liver, pancreas, kidney, endometrium, and colon-rectum, as well as an increased risk for non-Hodgkins lymphoma in patients with type 2 diabetes[29]. The relative risk ranges from 1.2 (breast), to 2.5 (liver and pancreas)[29]. Not only is the relative risk of developing increased, but cancer mortality is also exaggerated[29]. Several pathophysiological mechanisms which are likely to

mediate, or at least contribute to, the increase in cancer risk exist. These include; i) hyperinsulinism causing increased mitogenesis, ii) chronic inflammation, iii) hyperglycaemia, and iv) increased free fatty acids.

Hyperinsulinism acting via the MAP-kinase pathway causes post receptor mitogenic effects, and is the fundamental pathological mechanism explaining the increased risk of oncogenesis. Figure 5 shows the detailed post receptor signalling cascade in the setting of hyperinsulinemia. As illustrated insulin resistance and hyperinsulinsim leads to abnormal insulin receptor substrate 1 (IRS-1) phosphorylation, due to mammalian target of rapamycin (mTOR) overactivation resulting in attenuation of metabolic effects. Conversely insulin receptor substrate 2 (IRS-2) is over expressed and leads to downstream mitogenetic effects. Cancer cells have increased insulin receptor expression, forming a "double jeopardy"[30]. Further, mitogenic effects are also mediated directly through the insulin like growth factor (IGF) -1 receptor (which is 80% homologous with the insulin receptor)[31].

Insulin resistance is often associated with a low grade inflammatory response[32]. This low grade inflammation results in increased DNA damaging reactive oxidative species (ROS)[33]. For example, raised adipocyte derived tumour necrosis factor alpha (TNF-$\alpha$) is often seen in patients with insulin resistance[34], and known to induce many types of cancer by strongly activating nuclear-factor kappa B (NF-$\kappa\beta$)[35]. It is difficult to assess the independent contribution of hyperglycaemia to oncogenesis (as it is nearly always accompanied by hyperinsulinism), but there are mechanistic possibilities including impairment of the immune system, and formation of ROS[36]. Finally, fatty acid synthase expression is increased in insulin resistance[37], which is linked to hepatocarcinoma[38], and tumour progression in general[39].

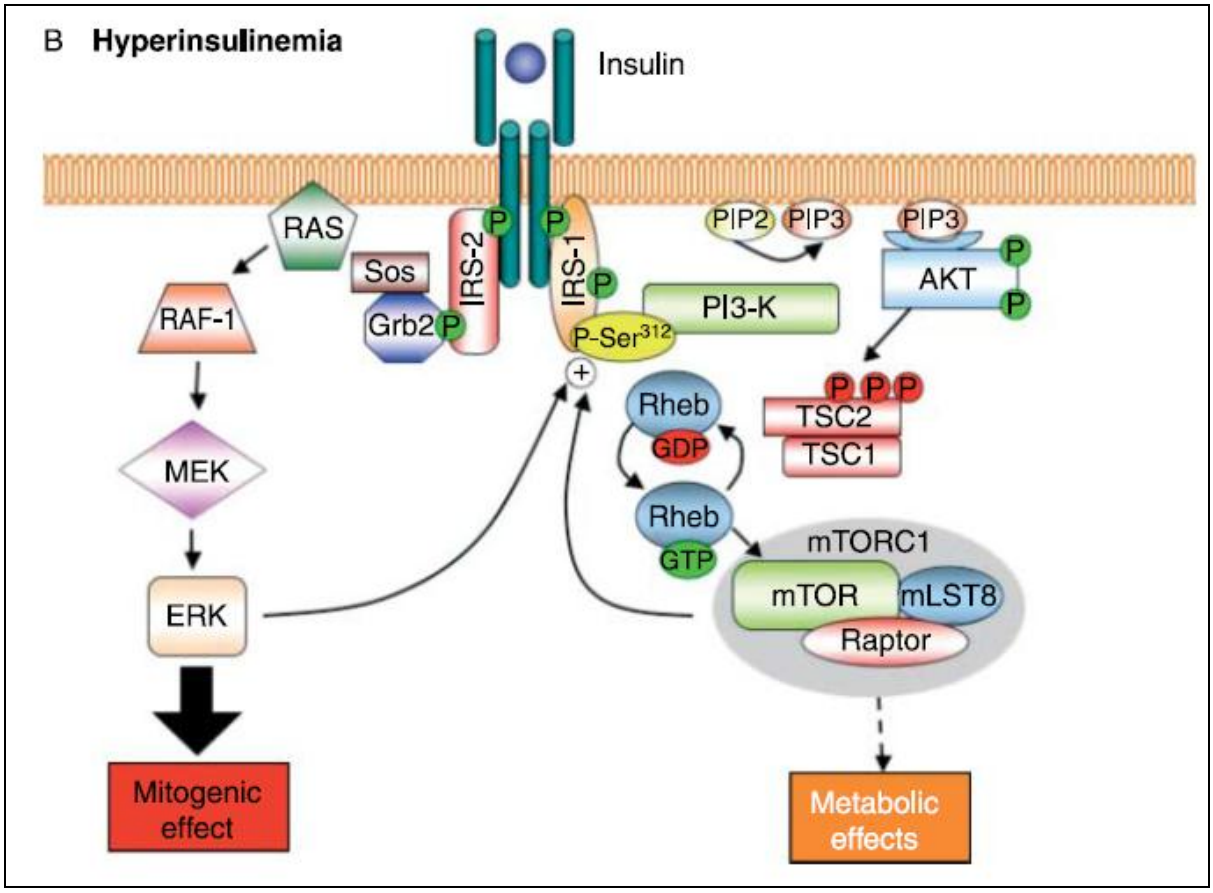



Figure 5: Post receptor insulin signalling during insulin resistance favouring mitogenic outcomes*[29]*.

*Abnormal serine phosphorylation (rather than tyrosine) of the insulin receptor substrate 1 (IRS-1) inhibits PI3-kinase due to mammalian target of rapamycin (mTOR) over expression resulting in attenuation of metabolic effects. Paradoxically, IRS-2 expression is conserved, and mitogenic effects are stimulated by extracellular regulated kinase (ERK) via MEK (which is MAP/ERK kinase). Reproduced from with [29] permission from the Society for Endocrinology.*

### 1.1.3 Neurodegenerative disease

Insulin is an important hormone for normal brain physiology and function. Insulin is transported across the blood brain barrier by an insulin-receptor mediated process[40]. Acutely increased peripheral insulin levels result in higher central nervous system (CNS) insulin, but prolonged hyperinsulinism down regulates blood-brain barrier insulin receptors and insulin transport into the brain. Insulin receptors are abundant in the brain, and selectively distributed[40]. For memory function there is a paradoxical relationship where acute increases in insulin improves memory, but the inverse is true in the setting of insulin resistance[41]. At a molecular level, insulin has a multitude of actions which affect memory[41].

Dysregulation of normal glucose homeostasis is common in neurodegenerative disease, for example Alzeimer's disease (AD), vascular dementia, Parkinson's disease, and Huntington's disease. Pathophysiological mechanisms related to disordered glucose homeostasis shared by these disorders include decreased cerebral glucose metabolism, increased inflammation, increased oxidative stress, increased AGEs, increased vascular dysfunction, decreased neurogenesis, and decreased neuronal repair[40].

The link between insulin resistance and AD originated from the observation that many patients with AD suffered from diabetes[40]. At a population level, increased risk for developing AD, and subsequent memory decline, has been shown in those with hyperinsulinism[42]. Hyperinsulinism is prominent in patients with early AD, despite having similar physical activity and body composition to similarly aged adults without AD[43]. Further, those patients without the apolipoprotein ε4 allele (APOE-ε4), a well established genetic risk factor for AD, are characterised by insulin resistance and hyperinsulinism (emphasising that in those without the common genetic risk factor, insulin resistance plays an important independent role in AD pathogenesis). Two pathological hallmarks of AD – intracellular neurofibrillary tangles containing an abnormally hyperphosphorylated form of tau protein, and extracellular senile plaques composed of aggregated b-amyloid, are promoted by insulin resistance and hyperinsulinism[44]. The chronic inflammatory often associated with insulin resistance, insulin action via the IGF-1 receptor, and genetic variations in insulin signalling may also contribute to AD pathogenesis[40].

Compared to AD, insulin related mechanisms specific to vascular dementia, Parksinson's disease, and Huntington's disease are not as well characterised. Vascular dementia as a result of micro-infarcts is an end result of endothelial dysfunction (insulin resistance causes endothelial dysfunction, see section 1.1.1). Loss of dopaminergic neurons in the substantia nigra is the pathological hallmark of Parkinsons's disease. Loss of insulin-receptor immune reactivity and mRNA in the substantia nigra have been shown in patients with Parkinson's disease[40]. For Huntington's disease insulin related abnormalities include a polyglutamine expansion, which may be associated with reduced insulin gene expression, and modulation of post insulin receptor signalling[40].

While the focus above has been on age-related degenerative disease, cognitive dysfunction has been seen in adolescents with type 2 diabetes[45]. Several studies in children and adolescents with the metabolic syndrome (and who are therefore likely to be insulin resistant) have shown poor cognitive outcomes[46]. This data highlights the potential devastating long term sequelae of obesity and insulin resistance that is observed in progressively younger population cohorts.

### 1.1.4 Infectious disease

Despite a general belief amongst clinicians that diabetes leads to an increased susceptibility to infection, it was not until recently that this was clearly defined[47]. There are now several studies confirming this suspicion [47-52]. To date there is no meta-analysis of this data, but odds ratios and relative risks (depending on the study design) range from 1.2 to over 3 – with all common infectious diseases accounted for[47-52]. There is evidence that poorer diabetic control exacerbates the risk for contracting infectious diseases[48]. The studies referenced above also show increased mortality rates once the infectious disease is contracted in patients with diabetes. From a public health perspective this explains the rationale for patients with diabetes in New Zealand being eligible for the free annual influenza vaccine[53].

While it is logical that hyperglycaemia (and thereby an environment with excess fuel) would more easily incubate opportunistic infection (e.g. blood and urinary tract infections) [54], currently there is a lack of explicit data showing that individuals who are "pre-diabetic" are also more susceptible to infection. We can extrapolate from the obese population who are likely to be insulin resistant, as there is evidence of heightened risk of acquiring infection and poor wound

healing in this population[55-58]. Whatever the underlying mechanisms are, infectious agents would still need to evade the immune system, implying that the immune system must be impaired.

Disordered glucose regulation compromises then human immune system in many ways (Table 2)[54]. Hyperglycaemia appears to be a core reason for immune dysfunction[54]. An exception is seen in polymorphonuclear cells (PMN) where insulin resistance at the cellular level may impede energy access (glucose), and therefore PMN function[59]. More research is required to determine the effects of insulin resistance on the immune system before we can confidently assume that this is the pathogenic mechanism causing increased infections in obese individuals.

**Table 2: Summary of immune dysfunction in diabetics.**

| | Humoral | Cellular |
|---|---|---|
| Innate | Complement ↓<br>Cytokines without stimulation ↑<br>Cytokines with stimulation ↓= | Polymorphonucleocytes ↓=<br>Monocytes / macrophages ↓ |
| Adaptive | Immunoglobulins = | T lymphocytes ↓ |
| Adherence | ↑ | |

### 1.1.5 Perspective

Disordered glucose homeostasis results in a broad range of morbidity and mortality. Many of the changes in physiology which accompany insulin resistance and compensatory hyperinsulinism have been shown to contribute to the mechanisms leading to this increase in morbidity and mortality and hyperglycaemia (in end stage disease) further exacerbates disease risk. But how does this transfer to the management of disordered glucose homeostasis and diabetes in general? At least two points come to mind;

a) Firstly, is there evidence that improving insulin resistance and hyperinsulinism (through medication or lifestyle modification) decreases that burden of disease?
b) Secondly, given administrating exogenous insulin in supraphysiological concentrations is the cornerstone of management for type 1 diabetes and end stage type 2 diabetes, what are the ramifications of creating a physiological environment so high in insulin?

With regard to the first question, there are numerous examples in the literature showing that disease risk can be ameliorated by improving insulin sensitivity (by medication or lifestyle intervention) for CVD[60-62], cancer [63-67], neurodegenerative disease[67-70], and infectious disease[48]. This provides strong rationale for research in the area of improving insulin sensitivity (and hence this thesis).

With regard to the second question, the long acting insulin (glargine) and cancer link scare that emerged in 2009 but was subsequently deemed unwarranted[71, 72] captured the attention of clinicians and researchers globally as it suggested that insulin could have adverse outcomes.. Controversy still exists with respect to CVD, where previous studies have shown intensive management with insulin and a sulfonylurea (which increases insulin secretion) improves mortality in type 2 diabetes [73], but more recent literature has shown the use of a sufonylurea alone caused higher mortality from CVD in type 2 diabetic patients compared to metformin alone, or combination therapy[62]. Ultimately, treating the underlying problem (insulin resistance), rather than the disease (with insulin), appears to be the safest approach (although admittedly, this is not always possible when end stage disease is present). Of course, prevention of insulin resistance from a young age would be better still[74].

## 1.2 Physiology of insulin resistance

The pathophysiology of insulin resistance is complex, and is affected by a host of factors including genetics, age, acute exercise, physical fitness, dietary nutrients, medications, obesity, and body fat composition. Only by understanding the pathophysiology of insulin resistance will effective methods targeting to improve insulin sensitivity be developed. The following section will focus on the origins of insulin resistance, and while it will detail perturbations of normal insulin signalling at a molecular level, an organ system approach has been used overall discussing insulin resistance in skeletal muscle, adipose tissue, liver, brain, and the gut.

### 1.2.1 Insulin resistance in skeletal muscle

As skeletal muscle is the predominant site of post prandial glucose disposal (accounting for 80 – 90% of glucose uptake during euglycaemic hyperinsulinaemic clamp), it stands to reason that the earliest stages of insulin resistance should be traced here[75]. Many studies have shown that normal glucose tolerant offspring of diabetic parents (who are highly likely to develop type 2 diabetes) show impaired skeletal muscle glucose uptake before the appearance of obesity related insulin resistance biomarkers such as increased FFA, or inflammatory cytokines[75]. It follows then, that examination of the molecular machinery of insulin signalling at a skeletal muscle level should yield insight into mechanisms of insulin resistance.

In skeletal muscle, insulin binding to the cell surface insulin receptor causes translocation of the GLUT4 channel to the plasma membrane, which facilitates glucose entry into the cell. Glucose is rapidly phosphorylated and directed primarily to the non-oxidative pathway (glycogen production) and to a lesser degree the oxidative pathway (creating carbon dioxide and water)[75]. Referring back to Figure 5, defects in IRS-1 tyrosine phosphorylation and PI3 kinase activity are evident in the skeletal muscle of normal glucose tolerant offspring of diabetic parents[76]. The consequence is defective glucose entry and phosphorylation (predetermining glycogen synthesis)[77]. The endpoint is reduced glycogen synthesis in skeletal muscle[78]. Serine phophorylation of the IRS complex is pivotal in the disruption of insulin signalling, and is

seen in insulin resistant normal glucose tolerant offspring of diabetic parents[79]. Increased serine phosphorylation of the IRS complex in skeletal muscle is contributed to by high circulating levels of FFAs which are seen in the prediabetic state[80]. Increased intramyocellular lipid content is seen in pre-diabetics and leads to increased diacylcgylcerol, long chain fatty acyl coAs and ceramides, all of which induce serine phosphorylation of IRS-1[81]. Usually, mitochondria oxidise FFAs, but normal glucose tolerant offspring of diabetic parents have reduced expression of key mitochondrial genes involved with fatty acid oxidation[82]. However, it is unclear whether disordered mitochondrial function results in intramyocellular lipid accumulation, or if increased FFA levels lead to mitochondrial dysfunction[75].

### 1.2.2 Insulin resistance in adipose tissue

Adipose tissue is a network consisting of adipocytes, macrophages, nerves, fibroblasts, and vascular cells, all of which are vulnerable to dysfunction that leads to insulin resistance. Adipose tissue specific insulin resistance appears to be an early and irreversible defect which has genetic or embryonic programming origins[83]. Adipose tissue produces many factors (see Table 3) that are implicated with insulin resistance, highlighting the importance of adipose tissue in the pathophysiology of insulin resistance[83]. Broadly, dysfunction of adipose tissue falls into three categories which will be discussed further; a) adipocyte size and buffering ability, b) adipose tissue metabolism, perfusion and inflammation, and c) adipose tissue distribution.

**Table 3: Adipose tissue derived factors implicated with insulin resistance.**

| Factor | Effects |
|---|---|
| FFAs<br>- Elevated in obesity and diabetes<br>- Primarily release by visceral fat[84] | Decreased whole body, hepatic and muscle insulin sensitivity (impaired signalling)<br>Decreased β-cell function<br>Decreased insulin clearance<br>Increased liver triglyceride synthesis<br>Increased organ fat content and oxidative stress |
| Leptin<br>- Elevated in obesity<br>- Primarily released by subcutaneous fat[85] | Increased whole body, hepatic and muscle insulin sensitivity<br>Increased metabolic rate<br>Decreased appetite<br>Decreased organ fat content<br>? Increased blood pressure<br>Decreased endothelial function |
| Adiponectin<br>- Reduced in insulin resistance and obesity<br>- Low levels are predictive of diabetes<br>- Decreased capacity by large subcutaneous adipocytes seen in obesity[86] | Infusion improves insulin resistance<br>Stimulates fatty acid oxidation |
| Resistin<br>- Elevated in obesity and diabetes | Decreased whole body, and hepatic insulin sensitivity |

| | |
|---|---|
| - Primarily secreted by visceral fat [87] | Increased liver TG synthesis |
| Corticoids<br>- Increased 11β-Hydroxy-steroid dehydrogenase-1 in obesity.<br>- Higher expression in visceral fat[88] | Increased fat cell size<br>Increased insulin resistance<br>Increased glucose<br>Increased blood pressure<br>Increased lipids |
| Endocannabinoids<br>- Increased in insulin resistance<br>- Primarily secreted by visceral fat[89] | Decreased adiponectin expression<br>Decreased adipose tissue energy expenditure and fat oxidation |
| TNF-α, IL-6<br>- Primarily secreted by visceral fat[90] | Stimulates lipolysis<br>Increased VLDL secretion<br>Decreased whole body, hepatic and muscle insulin sensitivity (impaired signalling)<br>Decreased adiponectin expression |
| Visfatin, Omentin, Vaspin<br>- Primarily secreted by visceral fat[91] | Increased whole body insulin sensitivity |
| Retinol binding protein-4<br>- Primarily secreted by visceral fat[92] | Decreased muscle insulin sensitivity (insulin signalling)<br>Enhanced liver gluconeogenesis |

Increased circulating FFA is seen early in the development of insulin resistance[93]. Under normal circumstances adipose tissue is plastic and able to buffer postprandial fatty acid influx[94]. Aside from disrupting insulin signalling by causing abnormal IRS-1 serine phosphorylation, FFAs also cause inflammation in adipocytes through toll like receptor 4 (TLR4)[95]. Large insulin resistant adipocytes are less able to buffer fatty acid influx, and are seen in normal glucose tolerant offspring of diabetic parents before the development of metabolic abnormalities[96], and independently predict for the development of type 2 diabetes[97]. In contrast, smaller adipocytes are better equipped to buffer fatty acid influx[97]. Lifestyle or pharmacological interventions that reduce adipocyte size improve insulin resistance[83].

Blood supply to adipose tissue is decreased in patients who are obese and have type 2 diabetes [94]. This leads to decreased TG clearance and higher FFA levels. Further, fat hypoxia results in decreased adiponectin levels (predictive for type 2 diabetes), the production of ROS and local inflammation (by recruited macrophages), exacerbating the insulin resistant process[83].

Fat distribution contributes to insulin resistance, where central fat (abdomen and chest) is more pathogenic than peripheral fat[98]. Intra-abdominal adipocytes express more genes for secretory proteins, and release more proteins per adipocyte compared to those in peripheral fat[98]. Furthermore, central fat is more lipolytic and therefore releases more FFA[99], and due to proximity to the liver, the resultant hepatic insulin resistance compounds the pathogenic state[98].

While increased adiposity is generally attributed to lifestyle factors, there is considerable evidence that genetic and early environmental effects are also contributory[83]. For example, low birth weight newborns have more fat, and are at higher risk of developing metabolic disease in adulthood[100]. Twin studies also confirm a genetic aspect to body fat distribution and metabolic disease[101]. Alarmingly, it has been suggested by one study that insulin resistance in the adipose tissue is not improved by weight loss, highlighting the need for preventative measures[102].

### 1.2.3 Insulin resistance and the liver

Non-alcoholic fatty liver disease is an established feature of the metabolic syndrome[103]. Individuals with fatty liver disease have impaired insulin mediated inhibition of hepatic glucose output, decreased insulin stimulated glucose uptake in skeletal muscle, and decreased inhibition of lipolysis[103]. The hypothesis that hepatic insulin resistance plays an early and central role in the development of whole body insulin resistance is illustrated by three observations, each of which will be discussed in more detail; a) intrahepatic fat has a stronger predictive value for whole body insulin resistance than other fat depots (muscle, visceral, peripheral), b) many inflammatory cytokines implicated with insulin resistance originate in the liver, and c) severe insulin resistance is seen in the setting of liver cirrhosis.

The primary role of intrahepatic fat as a strong correlate for insulin resistance and a predictor for the development of type 2 diabetes is shown by several models. Firstly, lower intrahepatic fat is strongly associated with retained insulin sensitivity, more so than peripheral adiposity or intramyocellular lipid content, in metabolically healthy obese individuals[104] as well as lean individuals[105]. Secondly, in obese adolescents whom are laying the foundation for future metabolic syndrome, it is intrahepatic fat rather than intramyocellular lipid or visceral fat that correlates most strongly with insulin resistance[106]. Finally, in those with established metabolic syndrome, a hypo-caloric low-fat diet intervention results in an 80% reduction of intrahepatic fat, improvement in basal and insulin-suppressed hepatic glucose production, and improved fasting glucose values, but does not produce any significant improvement in insulin stimulated peripheral glucose uptake[107].

Inflammatory cytokines are an early marker of insulin resistance, and as already discussed, originate from adipose tissue, but the liver is also involved. Biopsies from patients with fatty liver disease have increased expression of genes involved with monocyte and marchophage recruitment and inflammation[108]. The link to peripheral insulin resistance is suggested to be mediated by hepatic production of NF-□κβ. Increased FFA metabolites (diacylcgylcerol, long chain fatty acyl coAs, ceramides) induce liver inflammation and NF-□ κβ production which subsequently results in peripheral insulin resistance and the release of other pro-inflammatory cytokines like interleukin-6[103]. Inflammatory cytokines disrupt normal tyrosine

phosphorylation of the IRS complex by suppression of cytokine signalling (SOCS) proteins[109].

Liver cirrhosis (whatever the etiology) is commonly associated with diabetes, and interestingly, induces significant peripheral insulin resistance (shown by decreased insulin mediated peripheral glucose uptake and glycogen synthesis)[103]. If patients undergo liver transplant, once immune-suppression has been weaned, glucose metabolism normalises[103].

Just as there is selective disruption in post receptor signalling in insulin resistance favouring mitogenesis, insulin mediated hepatic function is also selectively affected. For example, glucose related liver function (glycogen synthesis and insulin mediated suppression of hepatic gluconeogenesis) is impaired in the metabolic syndrome, but insulin mediated lipogenesis (suggested to work through the transcription factor forkhead box-1), is maintained and exacerbates the pathophysiological process[103].

Overall, it is clear that the liver is an important player in the development of insulin resistance, which is not totally surprising given that it is responsible for endogenous glucose production, fatty acid oxidation, and insulin degradation.

### 1.2.4 Insulin resistance and the brain

The CNS, particularly the hypothalamus, is sensitive to insulin, adipokines, and FFAs, and responds via the production of neuropeptides and electrical signals to regulate energy intake as well as whole body glucose metabolism[110]. It is thus plausible that insulin resistance at the level of the brain could lead to weight gain through excess energy intake and disordered whole body glucose metabolism.

In the brain, insulin and leptin signalling converge[111], which is important because both of these hormones are raised in the setting of obesity and insulin resistance. The molecular mechanisms through which insulin and leptin modulate energy intake by inducing anorexigenic peptides in hypothalamic neurons are summarised in Figure 6[111]. Activation of the insulin receptor within the arcuate nucleus (ARC) results in PI3K activation. PI3K phosphorlylates phosphatidylinositol 3,4-disphosphate ($PIP_2$) to phosphatidylinositol 3,4,5-triphosphate ($PIP_3$), which induces hypopolarization and reduced activity of the neuron via the $K_{ATP}$ channel. Leptin,

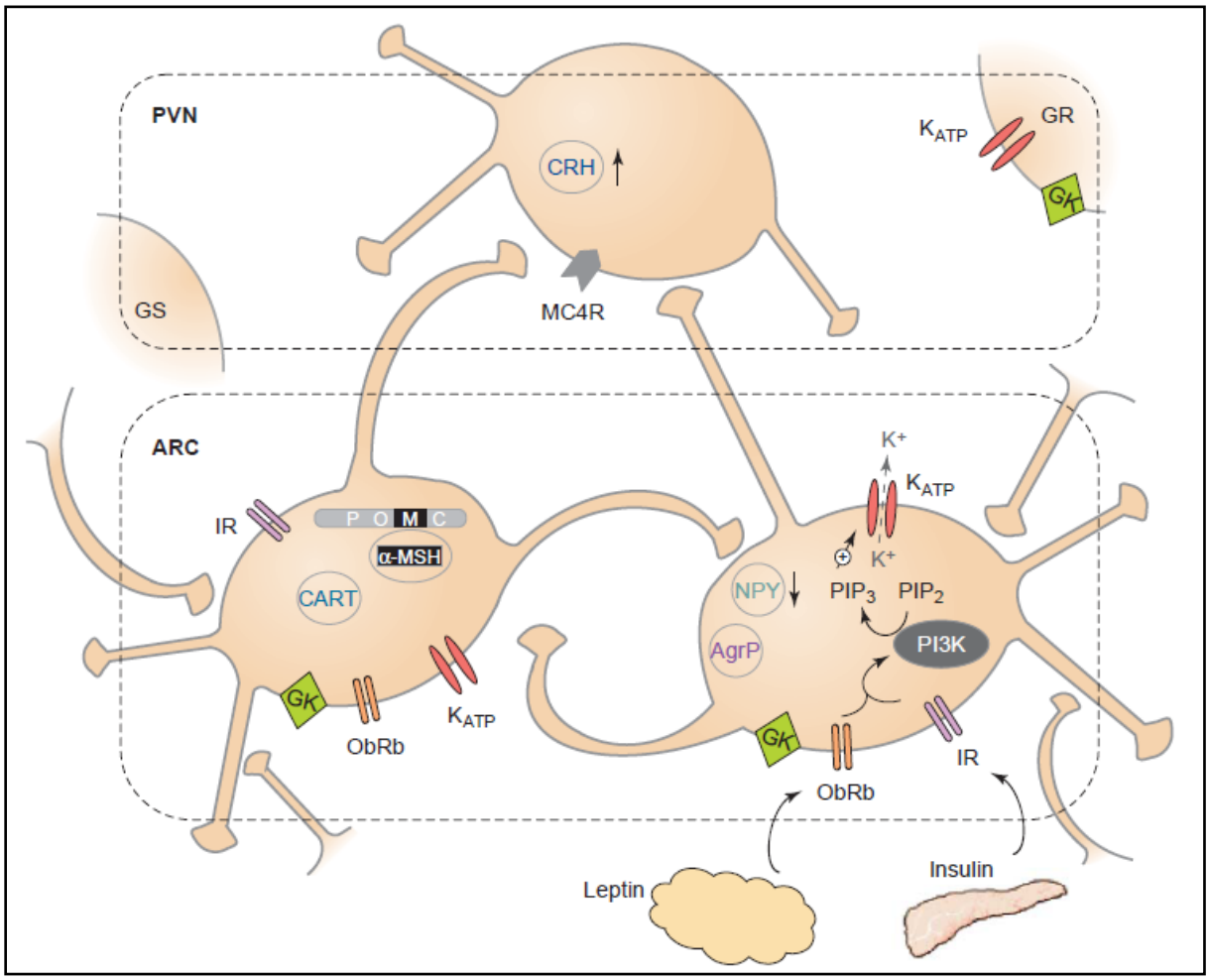



Figure 6: Insulin and appetite regulation*[111]*.

*Molecular mechanisms through which insulin and leptin regulate energy balance in the hypothalamus. Reproduced from [111] with permission from Elselvier. Abbreviations: AgrP, agouti-related peptide; α-MSH, melanocyte stimulating hormone α; ARC, arcuate nucleus; CART, cocaine and amphatamine regulated transcript; CRH, corticotropin releasing hormone; GK, glucokinase; GR, glucose responsive neuron; GS, glucose sensitive neuron; IR, insulin receptor; MC4R, melanocortin receptor 4; NPY, neuropeptide Y; ObRb, leptin receptor; PI3K, phosphotidyl inositol 3 kinase; PIP2, phosphotidyl inositol 3,4 diphosphate; PIP3, phosphotidyl inositol 3,4,5 triphosphate; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus.*

acting on the same neurones, down regulates anorexigenic neuropeptide y (NPY), which in turn increases expression of the anorexigenic corticotrophin-releasing hormone in second order

neurones of the paraventricular nucleus (PVN). Insulin also activates the melanocortin system, containing anorexigenic hormone α-MSH[111].

Central regulation of glucose metabolism occurs primarily through influencing hepatic glucose output. As shown in Figure 6, insulin signalling in the hypothalamus decreases the expression of agouti-related peptide (AgrP), which in turn decreases hepatic glucose production by reducing glucose phosphatase expression[112]. It follows that if insulin signalling is impaired (insulin resistance), it is possible that failures of appetite suppression and suppression of hepatic glucose output may ensue. Indeed, there are animal models that support this[110]. Further, the incretin response relies on central messages, and is discussed below.

### 1.2.5 Insulin resistance and the gut

The gut secretes a variety of peptides in response to nutrition that influence energy homeostasis [113]. While most of these (for example ghrelin, petide YY, cholecystokinin, pancreatic polypeptide, oxyntomodulin) are related to appetite regulation, the incretins (glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1)), directly influence glucose metabolism[113]. The incretin effect augments insulin secretion by three to four times[114] and is severely blunted in the setting of type 2 diabetes[115].

The role of GIP in the pathogenesis of insulin resistance is mainly due to resistance to its effect at a receptor level[115]. GIP is secreted in response to nutrients by K cells, which are most concentrated in the duodenum[115]. Insulin secretion is augmented when GIP binds to its receptor on the pancreatic β-cell, initiating a cascade that increases cAMP[115]. In type 2 diabetes, GIP levels are normal[116], but when GIP levels are elevated via intravenous infusion, the incretin response remains poor[117]. This would seem to indicate a problem with GIP receptor expression, which could have a genetic basis. In support of this hypothesis, it has been observed that 50% of 1$^{st}$ degree relatives (with normal glucose homeostasis) of patients with type 2 diabetes had a reduced incretin response to infused GIP compared to controls with no family history of type 2 diabetes[118]. Despite this, several polymorphisms of the GIP receptor have been described, none of which are associated with diabetes[119], arguing against a genetic basis. While the aetiology of GIP resistance is unclear, patients with type 2 diabetes have normal GIP levels, but have a poor GIP induced incretin response[120]. Because type 2 diabetes is a GIP

resistant state, anti-diabetic medication mimicking GIP has not been pursued, while the opposite is true for GLP-1 (as sensitivity is retained).

GLP-1 acts as an acute incretin in the same way as GIP, but also stimulates all steps of insulin biosynthesis and gene expression, and has trophic affects on pancreatic β-cells[121]. GLP-1 is secreted by L cells which are distributed throughout the gut[115]. In type 2 diabetes, levels of GLP-1 are very low in response to an oral glucose load, and those with impaired glucose tolerance have an intermediate response[116]. The reasons why the GLP-1 response is poor in diabetes remains unclear, however, similar to GIP it appears to be a result of diabetes, rather than a cause[115]. Nevertheless, as patients with diabetes still respond to GLP-1 given exogenously, GLP-1 agonists and dipeptidyl peptidase IV antagonists (which decrease the rate of GLP-1 breakdown), are now well established therapeutics in the treatment of type 2 diabetes.

### 1.2.6 Perspective

The literature on the mechanisms of insulin resistance is ever expanding, with the drive coming from a desire to generate new therapeutic agents or approaches to combat the scourge of type 2 diabetes. To some degree new therapeutics are becoming available – for example the GLP-1 agonists, but as each new agent becomes available and added to the existing arsenal, each subsequent clinical benefit for the individual is smaller. Hence, while the search continues, the discovery of the "magic bullet" appears to be increasingly elusive and unlikely to be found.

An alternative approach would be to look at the food we eat. If we interrogate the known pathogenic mechanisms that lead to insulin resistance in response to the food we eat, we may be able to dissect out what aspects of nutrition influence these mechanisms. To approach this, we can start by looking at the macronutrients in our diet, but then delve deeper into micronutrients, and this is the subject of section 1.4, as well as the clinical trials contained in this thesis.

## 1.3 Measuring insulin sensitivity

Insulin sensitivity (or insulin resistance) is frequently measured in research because of its association with common diseases such as obesity, type 2 diabetes, CVD, and polycystic ovarian syndrome[122]. Broadly, insulin sensitivity can be quantified by: a) the measurement of glucose utilization and/or glucose production rates during steady-state hyperinsulinemia (clamp method) or b) measurement of insulin-mediated changes in glucose disappearance rates after intravenous glucose challenge (minimal model)[122]. Only the clamp method is accepted as the gold standard technique. However, both are costly, labour intensive, and technically challenging, several "proxy" methods have been developed to serve as surrogates for the clamp and minimal model.

A detailed description of the clamp method was first described in 1979 by DeFronzo and colleagues[123]. When devising this method, DeFronzo aimed to uncouple the in-vivo feedback relationship between plasma glucose and insulin, so that the β-cell response to glucose and tissue sensitivity to insulin could be assessed individually. β-cell response is measured by the hyperglycaemic clamp where plasma glucose is kept constant by infusing glucose to increase the plasma level to 125 mg/dL. Under these conditions, insulin secretion is biphasic – with an early burst within six minutes, followed by a slow increase in plasma insulin concentrations. The initial response and subsequent slower increase can be compared across subjects. Tissue sensitivity is measured by the hyperinsulinemic euglycaemic clamp, where this time plasma insulin is kept constant (100μU/mL) by an intravenous infusion. Glucose levels are kept constant at basal levels using a glucose infusion. Under this euglycaemic condition, the glucose infusion rate determines glucose uptake by all the tissues and is therefore a measure of tissue sensitivity to insulin.

The minimal model was first described by Bergman *et al*. in 1979 (in dogs) [124] and in 1981 (in humans)[125]. It was promoted as an alternative to the technically difficult and laborious nature of DeFronzo's clamp method. Similar to the principles behind the clamp method, the minimal model uses mathematical techniques to separate the glucose/insulin feedback into a) the effect of insulin to accelerate glucose uptake and b) the effect of glucose to enhance insulin secretion, thereby giving measures of β-cell response and insulin sensitivity. The minimal model is derived

from the frequently sampled intravenous glucose tolerance test[125]. Comparisons between the minimal model and clamp technique conclude that there is a satisfactory correlation between the two techniques across the spectrum of insulin resistant states [126-128]. Nevertheless, the need for simpler, less invasive, and cheaper methods has spurred the development of several alternative proxy methods to assess insulin sensitivity.

The performance of such proxy methods compared to the gold standard methods has recently been examined by Lorenzo *et al.*[129]. Table 4 briefly summaries the multitude of proxy methods compared by Lorenzo *et al.* (the original references and explanation of abbreviations can be found in[129]).

**Table 4: Proxy methods to measure insulin sensitivity.**

| Name | Formula |
|---|---|
| *Based on fasting measurements* | |
| Fasting glucose | $G_0$ |
| Fasting insulin | $I_0$ |
| Raynaud | $40/I_0$ |
| HOMA IR | $(I_0 \times G_0) / 22.5$ |
| HOMA2 | Computer modelling available at www.ocdem.ox.ac.uk. Uses fasting glucose and insulin values only. |
| FIRI | $(I_0 \times G_0) / 25$ |
| IGR | $I_0 / G_0$ |
| ISI $_{basal}$ | $10^4 /(I_0 \times G_0)$ |
| QUICKI | $1/(\log I_0 + \log G_0)$ |
| Bennett's $S_I$ | $1/(\log I_0 \times \log G_0)$ |

| | |
|---|---|
| Belfiore's ISI(gly) $_{basal}$ | $2/ [(I_{O/N} \times G_{O/N}) + 1]$ |
| McAuley | $e^x$, where $x=2.63 – 0.28In (I_o) – 0.31ln(Tg_o)$ |
| *Based on oral glucose tolerance test* | |
| 2-hr glucose | $G_{120}$ |
| 2-hr insulin | $I_{120}$ |
| $IGR_{2h}$ | $I_{120}/ G_{120}$ |
| $ISI_{2h}$ | $10^4 /(I_{120} \times G_{120})$ |
| Gutt's ISI $_{0, 120}$ | $(m/[G_0 + G_{120})/2]/log [I_0 + I_{120})/2]$ |
| Avignon's SiM | [(w x Sib) + Si2h]/2 |
| Stumvoll $_{(0,120)}$ | $0.156 – 0.0000459 \times I_{120} – 0.0000321 \times I_0 – 0.00541 \times G_{120}$ |
| Stumvoll with demographics | $0.222 – 0.00333 \times BMI – 0.0000779 \times I_{120} – 0.000422 \times age$ |
| Stumvoll MCR $_{OGTT}$ | $18.8 – 2.71 \times BMI – 0.0000645 \times I_{120} – 0.00375 \times G_{90}$ |
| Stumvoll ISI $_{OGTT}$ | $0.226 – 0.0032 \times BMI – 0.0000645 \times I_{120} – 0.00375 \times G_{90}$ |
| Belfiore's ISI(gly) $_{area}$ | $2[I_a meanI_a \times G_a/meanG_a) + 1]$ |
| $SI_{IS}$OGTT | $1/[log(g_0 + G_{30} + G_{90} + G_{120}) + log (I_0 + I_{30} + I_{90} + I_{120})]$ |
| Matsuda | $10^4/(G_0 \times I_0 \times mean\ G_{OGTT} \times mean\ I_{OGTT})^{0.5}$ |

Lorenzo *et al.* found that the best correlate with the gold standard clamp was the Matsuda method, with a Spearman correlation coefficient of 0.77[129]. However, methods based on an oral glucose tolerance only improved the correlation to the clamp minimally compared to those based on fasting samples only. For example the often used Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) had a Spearman correlation coefficient of 0.69[129]. Although all the proxy measures are considerably cheaper and easier to perform than the gold standard methods, there is also a degree of cost versus additional benefit factors *within* the proxy measures that an investigator must weigh up. The choice of method will depend on the research budget, study design and the primary question the research is attempting to address.

Buchanan, in an editorial based on the Lorenzo *et al.* study, argued that while proxy measures are useful tools in cross-sectional and epidemiological studies, they are flawed in their use for genetic and longitudinal studies[122]. For genetic studies, two studies have demonstrated variable and poor associations between proxy measures and genetic contributions to insulin resistance [130, 131]. With respect to longitudinal data, Buchanan cited unpublished data where the Matsuda correlated to the intravenous glucose tolerance test well at two time points, but the correlation coefficients for the change in insulin resistance values were poor (between 0.3 and 0.4)[122]. However, the reliability of the Matsuda method over repeated measures has been demonstrated by others[132]. Further, the choice of method may be influenced by the study participant characteristics. For example the Matsuda method appears to be more sensitive at detecting differences in those with normal glucose tolerance and impaired glucose tolerance compared to the clamp[133]. When assessing insulin sensitivity in children, highly variable fasting insulin levels render proxy models that are dependent on this value (e.g. HOMA-IR) unreliable and are not recommended[134]. Despite the technical difficulties of applying the clamp in children it is still recognised as safe and the gold standard method[134].

For impaired glucose tolerance to progress into type 2 diabetes, defects in insulin sensitivity and pancreatic β-cell function are required[135, 136]. Both of these factors are interdependent, where in healthy patients, decreased insulin sensitivity is compensated for by increase changes in β-cell responsiveness, so that the product of insulin sensitivity and insulin secretion is constant [137]. This inverse relationship creates a hyperbolic curve and is termed the "disposition index". Accordingly, when a patient becomes insulin resistant, and can no longer compensate by

increasing insulin release, diabetes ensues. There are many ways to assess pancreatic β-cell function with the gold standard derived from an intravenous glucose tolerance test and hyperglycaemic clamp, but there also proxy surrogates which use fasting values only[137].

There is some debate as to the clinical validity of the gold standards tests for insulin sensitivity, as intravenous glucose challenges bypass the incretin response on β-cell function that are only stimulated by oral glucose ingestion[138]. Indeed, the same hyperbolic relationship produced by the intravenous glucose tolerance test can be recreated in response to the oral glucose tolerance test (the oral disposition index) [139, 140], and independently predicts for the progression of impaired glucose tolerance to type 2 diabetes[140].

To summarise, the clamp is regarded as the gold standard to assess insulin sensitivity and β-cell function. However, due to technical difficulty and cost, a number of proxy methods are available. The choice of method will depend on budget, technical skills, size and design of study, and the population studied. The acceptance of proxy measures derived from the oral glucose tolerance test is highlighted by recent changes in what the European Food Safety Authority considers as satisfactory evidence for health claims on foods relating to glucose homeostasis[141].

# 1.4 Nutrition and glucose homeostasis

Several lifestyle intervention programs have been shown to slow the progression of impaired glucose tolerance to type 2 diabetes[142-145]. The beneficial effects are sustained even many years after the active intervention is discontinued[146, 147]. In these studies lifestyle interventions included a variety of dietary counselling interventions along with advice to increase physical activity, with the goal of inducing weight loss. While a hypocaloric diet will induce weight loss and therefore improve insulin sensitivity, long term compliance with such a program inevitably fails. Diets that target weight loss through drastic changes in macronutrient contributions to total energy intake (for example the Atkins very low carbohydrate, or the standard low saturated fat diet) go in and out of favour like fashion trends. Similarly, new wonder super foods full of healthy micronutrients are constantly thrust upon the consumer with a promise of improved health. It is therefore little wonder that clinicians have a lack of consensus over the best nutritional strategy to combat obesity[3].

Obesity is currently in epidemic proportions and is largely blamed for increasing CVD mortality. As a result most of the literature on nutrition in diabetes focuses on weight loss. While weight loss is important, the following section will specifically look at the macronutrient and micronutrient *direct* contribution to insulin sensitivity (and where possible) independent of weight loss.

## 1.4.1 Macronutrients

### 1.4.1.1 Carbohydrate

Carbohydrate (CHO) is the principal macronutrient in the diet for the vast majority of civilisation. CHO can be classified on a chemical basis (Table 5)[148]. The way in which CHO in food affects blood glucose and insulin sensitivity can be approached on three levels 1) the overall proportion of CHO as an energy source in food, 2) the blood glucose increase after the ingestion of a specific CHO food source as compared to white bread – termed the ‘glycaemic index’, and 3) the effect of specific CHO sub types (both as a proportion of total CHO, or simple

ingestion of). Dietary fibre is technically defined as a CHO, but due to specific properties relating to insulin sensitivity, will be discussed separately.

**Table 5: Classification of carbohydrate.**

| Class (degree of polymerisation) | Subgroup | Examples |
|---|---|---|
| Sugars (1 – 2) | Monosaccharide<br>Disaccharides<br>Polyols | Glucose, galactose, fructose, sucrose, lactose, trehalose, sorbitol, mannitol |
| Oligosaccharides (3 – 9) | Malto-oligosaccharides<br>Other oligosaccharides | Maltodextrins, raffinose, stachyose, fructo-oligosaccharides |
| Polysaccharides (>0) | Starch<br>Non-starch polysaccharides | Amylose, amylopectin, modified starches, cellulose, hemicelluloses, pectins, hydrocolloids |

Guidelines as to the amount of CHO in one's diet that is considered 'healthy' exist. The Acceptable Macronutrient Distribution Range (AMDR) for CHO depends on the reference document, but generally the theme is to have most energy come from CHO, because the alternative would be high consumption of (health undesirable) fat. For example, the New Zealand recommendation is that 45 – 65% of energy comes from CHO in those aged 14 and over[149]. The World Health Organisation (WHO) / Food and Agriculture Organisation (FAO) expert committee recommends 55 – 75% of total energy intake should come from CHO, but goes further to say that 400g per day should come from fruit and vegetables (excluding tubers)[150]. By including a guideline on food sources of CHO, the WHO/FAO guidelines aimed to provide

not only an appropriate amount of CHO, but also enough dietary fibre[150]. Further, the intention here is to avoid greater than 10% of energy intake originating from “free sugars”, which also fall under the CHO umbrella, the rationale for which will be discussed later. While the New Zealand guidelines specifically avoid an acceptable range for children, the 2010 Dietary Guideline for Americans maintain the 45 – 65% range for all ages[151]. Most reference documents relating to CHO intake put strong emphasis on recommending wholegrain breads, cereals and vegetables as sources for CHO – again with the aim of providing enough dietary fibre, and shifting CHO intake away from “free sugars”. By doing so, recommended CHO food sources tend to have a low glycaemic index.

The glycaemic index ranks food based on the rise of blood sugar compared to that of a reference food – usually plain white bread or glucose[152]. The glycaemic load takes into account the amount of food eaten, and is the product of glycaemic index and the amount of available CHO in that food, and is more predictive of postprandial glycaemia and insulinaemia then either glycaemic index or available CHO alone[153]. Since the original observation that diets with high glycaemic loads led to an increased risk of type 2 diabetes[154], dozens of other studies following tens of thousands of people have shown a similar pattern[155]. In type 2 diabetes, a Cochrane review indicates the benefit of following a low glycaemic index or glycaemic load diet[3].

There are well established physiological mechanisms which explain why diets with a high glycaemic index and glycaemic load are diabetogenic. The acute metabolic response comparing a low and high glycaemic load meal is summarised in Table 6[156].

**Table 6: Metabolic response based on glycaemic load of meal.**

| | Low glycaemic load meal | High glycaemic load meal |
|---|---|---|
| Early postprandial response<br><br>(0 – 2 hours) | Glucose increases<br><br>Incretin response<br><br>Insulin secretion<br><br>Glucagon suppression | Major glucose increase<br><br>Exaggerated incretin response<br><br>Exaggerated insulin secretion<br><br>Exaggerated glucagon suppression<br><br>➔ Results in exaggerated glycogenesis, lipogenesis, and suppression of gluconeogenesis and lipolysis |
| Mid postprandial response<br><br>(2 – 4 hours) | Glucose decreases<br><br>Suppression of Free Fatty Acids | Rapid fall of glucose often to hypoglycaemic range due to persisting high insulin and low glucagon<br><br>Exaggerated suppression of Free Fatty Acids |
| Late postprandial response<br><br>(4 – 6 hours) | No counter regulatory response<br><br>Compared to baseline, no change in levels of:<br><br>- Free Fatty Acids<br>- Glycogenolysis<br>- Gluconeogenesis | Exaggerated counter-regulatory hormone response due to hypoglycaemia leading to:<br><br>- Elevated Free Fatty Acids<br>- Decreased glycogenolysis<br>- Increased gluconeogenesis |

Referring to the mechanisms discussed in section 1.2, it can be shown how a high glycaemic index and glycaemic load diet can predispose to insulin resistance and diabetes. Firstly, hyperinsulinism *per se* induces insulin resistance in skeletal muscle, but sensitivity is maintained in adipose tissue, favouring adiposity (which has pathogenic consequences – see section 1.2.2)[157]. Hyperglycaemia, excess counter-regulatory hormone secretion, and excess FFAs are all contributory to insulin resistance[156]. When you consider that many factors present in the metabolic response to a high glycaemic load diet inhibit pancreatic β-cell function (hyperglycaemia, FFAs, oxidative stress) a damaging two hit model emerges (impaired insulin sensitivity in the face of increased insulin secretion)[156]. Further, given that a host of disease processes are accelerated by hyperinsulinism as outlined in section 1.1, it is not surprising that high glycaemic index and glycaemic load diets are pathogenic for obesity, cancer and CVD[156]. It also appears that individuals with a genetic predisposition to developing type 2 diabetes have a poor metabolic response to high glycaemic load meals[156]. This individual response based on genetics is further explored in the context of sucrose and very recent literature later.

While the glycaemic index is an excellent and easily understood way for a food to be "scored" from a consumer perspective, there are some criticisms of using it as the solitary way to define whether a food is "good" or "bad". For example low glycaemic index foods may be energy dense – although this can partially be taken into account by factoring in glycaemic load. Also, low glycaemic index foods may contain substantial amounts of sugar, fat, or fatty acids which do not contribute to good health outcomes[150]. A further limitation is the large inter-individual variation in glycaemic response to food[150]. While health improvements have been demonstrated when following a low glycaemic index diet as cited earlier, in general they are not as impressive as diet and lifestyle interventions that target weight loss[158]. Further, many studies assessing low glycaemic index diets utilise nutrition plans rich in cereals, fruit and vegetables, which amongst other micronutrients, contain high levels of polyphenols. Polyphenols have a distinct bioactivity which will be discussed in depth in section 1.4.1.2. However as the food plans used when assessing low glycaemic index diets often contain high levels of micronutrients (such as polyphenols) shown to have health benefits, it is difficult to ascertain whether the health benefits are conferred solely through the glycaemic index of the diet or via the effect of certain micronutrients. For all these reasons there is some criticism of food

manufacturers using glycaemic index scores as a marketing tactic, as glycaemic index can be artificially lowered, and distracts for the nutritional impact of the whole food[158].

There is mounting evidence that "free sugars" are diabetogenic. They are defined as all monosaccharides and disaccharides added to foods by the manufacturer, cook, or consumer, plus sugars naturally present in honey, syrup and fruit juices, and are recommended to contribute less than 10% of energy intake[159]. Sugar sweetened beverages are exceptionally high in, and can be a major dietary source of free sugars. Furthermore the increased consumption of sugar sweetened beverages parallels (and is possibly contributory) to the obesity epidemic, an observation contributing to the recommendation that free sugars should make up less that 10% of total energy intake. There is growing evidence linking "free sugar" intake and chronic diseases such as type 2 diabetes, coronary artery disease, and hypertension[160]. Despite guidelines, in America, sugar intake from sweetened beverages (which are the largest single caloric food source in the United States) is well over the 10% threshold in several population groups, particularly those from ethnic minorities at high risk of developing type 2 diabetes [161]. Sugar-sweetened beverages primarily use one of two types of sugar source to sweeten; high-fructose corn syrup (45% - 58% glucose, 42-55% fructose), or sucrose (50% glucose, 50% fructose).

Consumption of fructose can be considered a cardiovascular poison, by virtue of its properties to promote dyslipidemia, insulin resistance, hepatic de novo lipogenesis, and visceral adiposity[162]. These findings are the result of study by Stanhope *et al*., where obese middle aged participants were given either pure glucose sweetened beverages, or fructose sweetened beverages (this energy source contributed 15 – 20% of total energy intake per day for 10 weeks), and the metabolic response compared[162]. They concluded that the mechanism through which fructose induced insulin resistance is mediated is through hepatic de novo lipogenesis, and subsequent higher hepatic diacylglycerol levels (see section 1.2.3) [162]. There was no increase in FFA in this study to explain the observed increase in insulin resistance, however given the subjects also developed increased visceral adiposity and weight gain, as well as raised TG levels[162], the failure to observe raised FFA may be due to the short length of intervention. Recently, a gene-environment interaction between obesity predisposition genes and the intake of sugar sweetened beverages has been shown by Qi *et al.*[163]. In this study, two large longitudinal cohorts and one replicate cohort were examined, and it was found that those with a

greater genetic predisposition to obesity were more susceptible to weight gain when they had higher rates of sugar sweetened beverage intake[163].

To further endorse the need for reduced sweetened beverage intake in order to prevent obesity and type 2 diabetes, it has been observed that the detrimental effects of this environmental pathogen can be reversed. De Ruyter *et al.* showed that by replacing a sugar containing beverage with a sugar free beverage significantly reduced weight gain and fat accumulation in normal weight children[164]. Ebbeling *et al*. intervened by decreasing sugar containing beverages for one year in obese adolescents and found an improved BMI, especially in Hispanics who have a higher risk of obesity and type 2 diabetes, but by a year after the intervention, these improvements were lost[165]. A limit to this study is that no other CVD risk factors were measured, such as insulin sensitivity, lipid profile, anthropometry, or blood pressure. Interestingly, intake of "diet" drinks is also associated with type 2 diabetes, but this probably due to other associated lifestyle factors[166]. Overall, so strong is the evidence for the adverse effects of sweetened beverages, proposals aimed at limiting intakes through taxes, or size of containers are being controversially touted.

#### 1.4.1.2 Fibre

In the human diet the main sources of dietary fibre are fruit, vegetables, legumes, and grains[167]. It was early studies observing the diet of primitive African tribes and the apparent lack of obesity and type 2 diabetes in these populations which lead to the coining of the term that diabetes was a "fibre deficient" disease[168]. Since then, an enormous body of literature has emerged regarding dietary fibre, insulin sensitivity, and obesity, and the call continues for ongoing research with particular attention to the mechanisms through which dietary fibre confers health benefits[167]. Despite such a large body of literature, it was not until very recently, after the 2009 Codex meeting, that an internationally accepted definition of fibre was agreed upon. Codex was established by FAO and WHO in 1963, and is responsible for developing harmonized international food standards, guidelines and codes of practice to protect the health of the consumers. The definition of fibre is as follows[169]:

Dietary fibre means carbohydrate polymers with ten or more monomeric units which are not hydrolysed by the endogenous enzymes in the small intestine of humans and belong to the following categories:

- Edible carbohydrate polymers naturally occurring in the food as consumed,
- carbohydrate polymers, which have been obtained from food raw material by physiological, enzymic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities,
- synthetic carbohydrate polymers which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities.

This inclusive definition is illuminating, both in that it is the only food definition that includes reference to physiological activity that benefits health, and that it covers a diverse range of sources (and therefore types) of fibre. Early attempts to classify fibre based on solubility (where soluble fibre dissolves in the digestive system eg guar gum, and insoluble fibre does not eg cereal fibre from oats) are obsolete as this chemical property of fibre is not exclusively responsible for physiological effects[170]. The complexity of fibre is nicely illustrated when you consider its constituents (Table 7)[171]. Considering dietary fibre as a single entity is flawed, as different food sources of fibre, made up of different constituents have very different physiological properties.

**Table 7: Constituents of dietary fibre.**

| | |
|---|---|
| **Non-Starch Polysaccharides and Resistant Oligosaccharides**<br>Cellulose<br>Hemicellulose<br>Arabinoxylans<br>Arabinogalactans<br>Polyfructoses<br>Inulin<br>Oligofructans<br>Galactooligosaccharides<br>Gums<br>Mucilages<br>Pectins | **Analogous Carbohydrates**<br>Indigestible dextrins<br>Resistant maltodextrins (from corn and other sources)<br>Resistant potato dextrins<br>Synthesized carbohydrate compounds<br>Polydextrose<br>Methyl cellulose<br>Hydroxypropylmethyl cellulose<br>Indigestible ("resistant") starches |
| **Lignin** | **Substances Associated with the Non-Starch Polysaccharide and Lignan Complex in Plants**<br>Waxes<br>Phytate<br>Cutin<br>Saponins<br>Suberin<br>Tannins |

There is an abundance of epidemiological evidence that demonstrates that populations with higher consumption of dietary fibre is associated with lower rates of obesity and the development of type 2 diabetes in children and adults.. The following studies these are well controlled epidemiological studies and the majority take into account confounding factors such as ethnicity, activity, and obesity. While the statistics are impressive, it is important to note that epidemiological studies are not designed to establish causality. Nevertheless, prospective cohorts following over 427,000 adults show that those with high consumptions of whole grains or cereal fibre demonstrate a 29% reduction in the development of type 2 diabetes [172]. The effect is even more pronounced in those with impaired glucose tolerance where a 62% reduction in the conversion to type 2 diabetes was observed in those with the highest fibre intake over 4.1 years[173]. In adolescents Steffan *et al.* investigated the effect of whole grain consumption on insulin sensitivity (measured by euglycaemic clamp test) in 285 adolescents in Minnesota[174]. When split into 3 tertiles there was a statistically significant trend of improved insulin sensitivity with increased whole grain intake. Increased whole grain intake mirrored an increase in fibre intake which was statistically significant. Further evidence in adolescents has recently been published, showing an inverse relationship between prevalence of the metabolic syndrome and dietary fibre intake[175]. In type 1 diabetes (paediatric and adult), patients with higher fibre intake have better glycaemic control [176-178], however these cohort studies did not take into account physical activity. Another weakness of these epidemiological studies is that it is not possible to separate fibre intake specifically from whole grain consumption (which is important as whole grains also contain numerous other micronutrients that can improve insulin sensitivity).

Many interventional studies have examined whether improving the intake of dietary fibre can improve glycaemic control in diabetes, and other important parameters of the metabolic syndrome – both in adults and children. In adults with type 2 diabetes, a meta-analysis of moderate CHO/high fibre diet compared to moderate CHO/low fibre diet interventions showed improved fasting and postprandial glucose, improved HbA1c, and improvements in other associated CVD risk factors such as lipid profile, blood pressure, and endothelial function for the moderate CHO/high fibre diets[179]. Until the study that is included in this thesis[180], there have been no prospective randomized controlled studies investigating the effect of increasing dietary fibre *alone* on insulin sensitivity in children and adolescents. Nevertheless, some information can be gained from the other studies that have been done in adolescents and are

summarised in Table 8. These studies appear to suggest benefit, but as they have used crude measures of insulin sensitivity, and often included exercise interventions, it is not possible to confidently say the observations are solely due to increased fibre intake.

**Table 8: Fibre intervention trials in children and adolescents.**

| Author | Study Participants | Intervention | Outcomes and comments |
|---|---|---|---|
| Ventura *et al*., 2009[181] | 54 overweight Latinos aged 15.5 years (SD = 1) | Dietary counselling to consume a high fibre, low sugar diet<br><br>Duration of study 16 weeks | Those that increased fibre by 5g/d had improved BMI and visceral adiposity but not insulin sensitivity |
| Ebbeling *et al*. 2003[182] | 16 obese predominantly European individuals aged 13 - 21 years | Dietary counselling; low glycaemic index diet versus traditional low fat diet<br><br>Duration of study 12 months | Both groups increased fibre content by 2g/1000kcal (less than 4g total)<br><br>Insulin resistance (measured by HOMA) did not increase in the low glycaemic index group but increased as would be expected with puberty in the low fat group<br><br>Activity levels not examined |
| Chen *et al*. 2006[183] | 16 obese individuals aged 10 – 17 years | Exercise, high fibre (>40g/day) and low fat diet<br><br>Duration of study | Significant improvement in insulin sensitivity (measured by HOMA)<br><br>Can't separate effect of fibre from the rest of the intervention |

| | | | |
|---|---|---|---|
| | Ethnicity not stated | two weeks | |
| Ludwig *et al.* 1999[184] | 12 obese adolescents aged 15.7 years (SD = 1.4)<br><br>Ethnicity not stated | Acute insulin and glucose response to test meals, low glycaemic index versus high glycaemic index | Low glycaemic index meals (containing 2 – 3 times more fibre) produced a lower insulin response |
| Roberts *et al.*<br><br>2007[185] | 19 overweight children and adolescents aged 8 – 17 years<br><br>Ethnicity not stated | Residential program, prepared high fibre, low fat meals<br><br>2.5 hours per day exercise<br><br>Duration of study 2 weeks | Improved oxidative stress biomarkers<br><br>Improved lipid profile<br><br>Can't separate effect of fibre from the rest of the intervention |
| Rovner *et al.* 2009[186] | 23 type 1 diabetics aged 13.1 years<br><br>Ethnicity not stated | Low glycaemic index meals for one day versus normal diet | Continuous glucose monitoring showed improved glucose profile with the low glycaemic meals despite no difference in total energy intake<br><br>Physical activity not recorded |

There are numerous proposed mechanisms through which dietary fibre can improve glucose homeostasis, including altering gastric emptying, sequestration of ingested CHO, influencing the incretin response, and via physiological properties of absorbed products produced from fibre fermentation. From a broad perspective, soluble fibre reduces the acute postprandial insulin and glucose surge and contributes most to colonic fermentation, making it the best candidate for bioactivity[187]. It would then be expected that soluble fibre should protect against diabetes due to repeated consumption causing lower circulating insulin levels, and subsequent up regulation of insulin receptors and secondary insulin signalling molecules[188]. However, this is not the case, as demonstrated by a recent meta-analysis of 328,212 subjects, that found no association between type 2 diabetes risk and either fruit (relative risk for extreme quintiles (RR) 0.96; 95% CI 0.88–1.04) or vegetable (RR 1.04; 95% CI 0.94–1.15) dietary fibre intake. In contrast, high intake of cereal dietary fibre is consistently associated with a markedly reduced type 2 diabetes risk (RR 0.67; 95% CI 0.62–0.72)[189]. While insoluble and soluble fibre share some physiological mechanisms (weight loss, low energy density, increased satiety, effects on gut hormones and inflammatory markers) there must be mechanisms unique to insoluble fibres that protect against the development of type 2 diabetes that are not established[190]. From a practical perspective, it is important to note that most high fibre foods contain both insoluble and soluble fibre.

Delayed gastric emptying slows CHO delivery to the small bowel and therefore ameliorates postprandial glucose and insulin excursion[191]. Many studies show viscous polysaccharides such as pectin and guar gum (when part of liquid meal) delay gastric emptying and improve glucose tolerance[192]. But not all viscous fibres have this property, for example psyllium does not delay gastric emptying[193]. The effect in solid meals is controversial given that a delayed, no effect, or accelerated gastric emptying has been observed when purified fibre is added to a solid meal[192]. The story is even murkier when the literature on whole food is considered, and it may be that disruption of the food form (by processing etc.) is more important than the fibre content[192]. Therefore, some sources of fibre delay gastric emptying and improve glucose homeostasis, but this cannot be applied to all dietary fibre.

Fibre and whole grains have been shown to reduce the availability of CHO to be absorbed across the small intestine epithelium[187]. The viscosity of fibre is important in terms of creating a barrier along the intestinal epithelium[187]. Psyllium is a good example of a predominantly

soluble fibre that improves postprandial glycaemia, even to a second meal, which is at least partly due to its gel forming properties[194]. Intact whole grains also contribute to the sequestration of CHO from absorption, and this effect is probably due to the kernel rather than the endosperm[195].

The gut-neural-endocrine system is complicated, and studies assessing the relationship that fibre has with it have yielded inconsistent results[190]. Acute changes to satiety hormones such as cholecystokinin, ghrelin, and anorexigenic peptide YY have been measured, but with no end effect on actual satiety ratings[190]. GLP-1 can potentiate the action of insulin, but most studies have shown no change in GLP-1 in response to fibre[190]. However, favourable results have been shown with adiponectin[190] which can improve insulin sensitivity as previously reviewed.

The role of low grade inflammation in the pathogenesis of insulin resistance has already been discussed, and there is good evidence that a high fibre diet is anti-inflammatory. Population studies confirm a high fibre diet is associated with lower circulating inflammatory markers such as CRP, IL-6 and TNF-α[196, 197]. There are complex mechanisms through which dietary fibre is anti-inflammatory and these are reviewed in more depth elsewhere[198]. To summarise the proposed mechanisms, they are through: weight reduction, decreased lipid oxidation, decreased lipids, improved bowel microflora and products of fermentation (see later), lower glucose levels, and modulating adiponectin[198]. Again, it is important to note that many studies used whole grains which contain micronutrients other than fibre, and are anti-inflammatory in their own right, once again making it difficult to ascertain whether the results seen were due to fibre alone[195].

Independent of their viscosity or solubility, fibre is partially or fully fermented in the large bowel, producing a range of substances with the potential to improve insulin sensitivity. Short chain fatty acids such as acetate, butyrate, and propionate are produced from fermentation[187]. These short chain fatty acids decrease hepatic glucose output, and improve lipid homeostasis[199]. There are also specific g-protein coupled receptors (GPR40, GPR41 and GPR43) for short chain fatty acids, that modulate β-cell pancreatic insulin secretion, leptin release, and adipogenesis[200]. In animal studies, supplemented butyrate can prevent and treat diet induced insulin resistance[201]. This is an exciting and expanding field with new chemical messengers, for example fibroblast growth factor 19[202], emerging as important modulators of insulin sensitivity in response to nutrients. Further research into new chemical messengers is

essential, particularly with new research showing that although cereal fibre improves whole body insulin sensitivity, it was not due to increased short chain fatty acids, or indeed the composition of the gut flora[203].

With the evidence outlined above, it is clear why all nutrition guidelines promote a high fibre diet. The need to promote such a diet is due to the fact that western diets are fibre poor. The guidelines in New Zealand recommend an adequate fibre intake dependent on age and gender: 2-3 years, M=14g, F=14g; 4–8 years, M=18g, F=18g; 9-13 years, M=24g, F=20g; 14-18 years, M=28g F=22g[148], and 25 – 30g in adults[204]. New Zealand nutrition surveys show that these intakes are often not reached[148, 204]. It can be assumed that a balanced diet, with greater than the 25 – 30g of fibre that is deemed adequate, would contain a variety of dietary fibre, and the individual would therefore benefit from the different physiological actions on insulin sensitivity that each has.

#### 1.4.1.3 Fat

Fat intake in the modern western diet has changed considerably as compared to our ancestors, both as a proportion of total calories, but more significantly, as a change in fat quality. Each gram of fat consumed yields more than double the energy input compared to CHO and protein. It should follow that high fat diets result in excess energy input, and hence lead to obesity and therefore type 2 diabetes. Despite this common sense scenario, there is no epidemiological evidence linking higher total dietary fat and type 2 diabetes [205]. The lack of such an observation has spurred fad diets such as the Atkins diet, where the blame for CVD is linked to dietary CHO (especially refined CHO), and hence the intake of fat is not restricted on these diet regimes[206]. Indeed, the lack of epidemiological evidence linking dietary fat with diabetes combined with data showing ("fad") high fat diets can achieve significant and sustained weight loss[207], has challenged the entrenched dogma that a low fat diet is best for prevention of CVD. In fact, a new perception is that the "low fat" goal actually propelled obesity and type 2 diabetes because calories are replaced with refined CHO, and consumers succumb to food manufacturer advertising endorsing products as fat free, whilst ignoring sugar content[206]. Clearly, a "low fat is best" philosophy is over simplistic. Therefore, research on dietary fat and diabetes has focused on the quality of fat consumed and there is now considerable literature ranging from epidemiological evidence down to in-vitro pathogenic mechanisms.

Dietary fat intake can be categorised by the fatty acid content, and broadly falls into three categories dependent on their chemical structure; saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) (Table 9). MUFAs and PUFAs have double bonds in their carbon chain (one for MUFAs, and more than one for PUFAs). The carbon chains are arranged around their double bond to be either on the same side (cis) or opposite side (trans), with the latter describing trans fats. Trans fats are rare in nature, and usually commercially produced. The essential fatty acids α-linolenic acid and linoleic acid are termed as such as humans are unable to synthesize them and are reliant on acquiring them in the diet. In reality, foods have a mixture of fatty acids, however the proportions can vary considerably[208]. Over the last two centuries, there has been a shift in fatty acid consumption patterns in the Western diet; with an increase in total fat and SFA, and a decrease in the omega-3 (ω-3) to omega-6(ω-6) PUFA ratio[209].

**Table 9: Classification of dietary fat.**

| Type of fatty acid | Chemical structure | Common examples and major source |
|---|---|---|
| Saturated fatty acids (SFA) | $C_nH_{(2n+1)}CO_2H$ | Palmitic acid, Stearic acid } meat, eggs, nuts<br>Lauric acid, Myristic acid } tropical oils |
| Mono-unsaturated fatty acids (MUFA) | $C_nH_{(2n-1)}CO_2H$ | Oleic acid } animal and vegetable oils<br>Palmitoleic acid } animal, vegetable and marine oil |

| | | |
|---|---|---|
| Poly-unsaturated fatty acids | $C_nH_{(2n-3)}CO_2H$ | ω-3: Eicosapentaenoic acid<br>docohexaenoic acid } Fish and plant oil |
| | $C_nH_{(2n-5)}CO_2H$ | α-linolenic acid* |
| | | ω-6: Linoleic acid* } palm and sunflower oils |
| *=essential fatty acids | | |

Most epidemiological evidence indicates that increased SFA consumption is directly associated with insulin resistance and the incidence of type 2 diabetes[210]. However, contrasting evidence can be found. For example in a high quality study that controlled for most confounders, used gold standard measures of insulin sensitivity, and carefully looked at fatty acid quality, there was no association between insulin resistance and SFA intake[211]. This well designed study polarises it from the other epidemiological data which has many short comings; sub-optimal tools to measure insulin sensitivity, poor dietary assessment tools that fail to sub-type SFA or account for MUFA or PUFAs, and failure to control for important covariates – even as fundamental as weight or BMI[208]. Several large cohort studies also failed to demonstrate an association between SFA intake and type 2 diabetes [210].

In an attempt to improve the quality of evidence, biomarkers (plasma fatty acid composition) of dietary SFA intake have been used, which generally show a direct association between SFA intake and insulin resistance[210]. But again, there are problems with this approach as dietary changes have acute effects on biomarkers (weeks) and therefore limit longer term risk profiling, genetic differences can influence the biomarkers, and a deterioration in glucose metabolism itself can affect fatty acid composition[212].

Intervention studies examining the effect of SFA alone are also conflicting, with early studies having methodological flaws, and the only study showing increased insulin resistance used a non-"real world" 50% energy from fat intervention[210]. On the other hand, after three months on a high (18% of energy) SFA diet, there was reduced insulin sensitivity compared to a diet

high in MUFAs (21% of energy)[213]. This latest study introduces the idea that while reducing SFA may not have clear evidence behind it (perhaps because the calories are substituted by refined CHO), substituting SFA with "healthy" fats may offer a better strategy.

Epidemiological studies suggest that unsaturated fat intake is inversely associated with type 2 diabetes and insulin resistance. For example, in a prospective cohort of 84204 women followed for 14 years, Salmeron *et al.* showed no increased risk attributable to total fat or MUFA intake, but for a 5% increase in energy from PUFAs, the relative risk for type 2 diabetes was 0.63[205]. The same caveats as discussed above apply to the epidemiological evidence. Using biomarkers to quantify specific fatty acid intake shows that an increased SFA:linoleic acid ratio in serum phospholipids was inversely associated with insulin mediated glucose disposal in healthy adults, and accounted for about 1/3 of variance in insulin sensitivity[214]. Galgani *et al*. critically reviewed interventional studies, and after applying stringent inclusion criteria, found 15 trials of suitable quality[208]. Of these 15, one study showed impaired insulin sensitivity with SFA enriched diet[213], one study showed improved insulin sensitivity with PUFA enriched versus SFA enriched[215], and in contrast, one study showed impaired insulin sensitivity after fish oil supplementation in patients with type 2 diabetes[216]. The other studies showed no effect. It is notable, that in the same study that showed impaired insulin sensitivity with SFA enrichment, no effect was found for MUFA enrichment, olive oil supplementation (rich in MUFA) or fish oil supplementation[213].

Fish oil, a rich source of ω-3 fatty acids is of particular interest due to several epidemiological studies showing the protective effect of high intakes of fish oil for CVD[217]. However, there is a less clear relationship between ω-3 fatty acid intake and insulin sensitivity and/or type 2 diabetes. For example, while several epidemiological studies report high fish intakes protect against type 2 diabetes[218-221], others have shown either no such relationship[222], or a negative relationship[223-225]. Further, several meta-analyses of dozens of interventional studies show no improvement in glucose homeostasis after fish oil supplementation in patients with type 2 diabetes[226]. One could argue that studying the effects of ω-3 fatty acids in diabetics is the problem, in that insulin resistance is so entrenched at this stage that it is difficult to improve. One very small study in healthy elderly participants (n = 12, aged >60) showed improved insulin sensitivity after 8 weeks of fish oil supplementation[227]. On balance however,

it would need to be concluded that ω-3 fatty acids protect against CVD through other mechanisms (inflammation, lipid profile, endothelial function) rather than glucose homeostasis.

Trans fats have a clear association with decreased insulin sensitivity. For example in the large prospective cohort by Salmeron *et al*. previously mentioned, for a 2% increase in energy derived from trans fat the relative risk for developing type 2 diabetes was 1.39[205]. There are few interventional trials, and most are small, but the theme is consistent, showing reductions in the intake of trans fats decreases insulin resistance, particularly in those who are at higher risk of developing type 2 diabetes. The effect is exaggerated when trans fats are replaced with PUFAs – where the risk of developing type 2 diabetes is further reduced[228]. Longer term interventional studies in humans are not available, but in a 6 year follow up in monkeys fed with a high trans fat diet, fructosamine (a measure of medium term levels of glycaemia) levels were increased compared to those on a higher MUFA diet[229].

While the data concerning fatty acid quality is not completely convincing in observation and clinical studies, there are proposed mechanisms through which fatty acids could modulate insulin sensitivity. Consuming SFAs increases intramyocellular lipid levels[208], and as explained in section 1.2.1 this interrupts normal insulin signalling. While it has been observed that fatty acids can alter cell membrane function, any link to insulin sensitivity is weakly based on cross-sectional data which has major limitations[208]. Inflammatory pathways are also implicated, where SFAs are pro-inflammatory, while this was not observed with ω-3 fatty acid supplementation in a mouse model[95]. Trans fats are also pro-inflammatory[228]. From a protective point of view, in a detailed review by Adkins and Kelley, the mechanisms through which ω-3 fatty acid could protect against CVD are outlined, for which several (anti-inflammatory, down regulating FFAs, up-regulating FFA oxidation) are implicated with improved insulin sensitivity[230].

Overall, while the benefits of a diet low in saturated fat and trans fats for preventing CVD (through a variety of mechanisms including plasma lipid profiles, endothelial function, blood pressure) are not disputed, evidence that this protection is also conferred through improved insulin sensitivity is lacking. There are potential mechanisms, but the translational literature linking the bench-top to epidemiological and clinical studies are methodologically difficult, and the results are often conflicting. There is a clear need for well designed, long term studies, using

gold standard methods examining fatty acid ingestion and insulin sensitivity covering the full spectrum of healthy to diseased (type 2 diabetes) glucose homeostasis. These studies, particularly regarding ω-3 fatty acid, should continue to explore possible physiological mechanisms.

#### 1.4.1.4 Protein

Protein consumption in the Western diet has increased considerably over the past 50 years[231]. Fad diets like the Atkins diet promote high protein nutrition, and have been shown to effectively achieve significant and sustained weight loss[207]. However, cross-sectional[232] and prospective[233-236] epidemiological studies suggest an increased type 2 diabetes risk with high protein intakes, independent of fat intake. This association is dependent on the source of protein, with animal protein being diabatogenic, and vegetable derived protein having no observed effect[237]. To complicate matters, specific protein hydrolysates, bioactive peptides, and amino acids can either improve or inhibit insulin sensitivity and have well defined physiological mechanisms to account for these properties.

High protein diets have been shown to induce insulin resistance in humans, but the association is temporal. For example, short term studies confirm higher secretion of insulin and resultant lower blood glucose levels[207]. In contrast, in another study healthy patients consuming a high protein diet for 6 months had enhanced gluconeogenesis, reduced insulin suppression of hepatic glucose production, and enhanced fasting glucose, despite increased insulin secretion[232]. Similarly, in a type 1 diabetic population, insulin sensitivity was reduced after consuming a high protein diet for a year[238]. The cross sectional and prospective epidemiological data linking high protein intake and diabetes risk appears counter-intuitive given that a high protein, low carbohydrate diet is associated with weight loss[207]. This implies that dietary protein induces insulin resistance independently and more potently than the effects on weight and energy balance.

While the epidemiological studies mentioned above are in adults, infants receiving formula rather than breast milk, provide an insight into the metabolic effects of a comparatively high protein diet. Formula fed babies are noted to have accelerated weight gain compared to breastfed babies[239, 240], and rapid infant weight gain has been shown by numerous studies to be obesogenic[241-243]. This increased weight gain has been shown to be reduced by lowering the protein content in infant formula, however, while the effect was significant at 12 months the

differences were reducing by 24 months[244]. There are many other aspects to breast milk (like satiety hormones in breast milk) which may influence nutrient intake and weight gain. However, as non breast feeding remains prevalent in Western nations, longer term studies investigating the physiological effects of low protein formula will be required to truly assess the impact of infant milk protein on metabolic risk.

The dietary source of protein is important as animal (principally red meat) derived protein preferentially reduces insulin sensitivity as compared to vegetable or fish derived protein[207]. Results from the European Prospective Investigation into Cancer and Nutrition (EPIC)-Netherlands (NL) study demonstrated that after 10 years the hazard ratio for developing type 2 diabetes was 2.15 for total protein and 2.18 for animal protein when the highest quartile intake was compared to the lowest[237]. There was no association found for vegetable protein in this study, which had previously been shown by others[235]. Soy protein is a typical plant derived protein, and has been shown to improve glucose homeostasis in human and animal studies[207]. It is proposed that this property is mediated through suppression of glucagon, and improved insulin signalling in adipose tissue and the liver. Protein from fish also improves glucose homeostasis (independent of omega 3 fatty acids). Experiments using cod protein in rat models have shown improved insulin sensitivity in skeletal muscle through the improvement of the phosphoinositide (PI) 3-kinase/Akt pathway and selectively improved GLUT4 translocation to the T-tubules - a cell-surface domain thought to mediate the bulk of glucose transport in response to insulin[207]. Human studies show improved insulin under the curve following cod protein ingestion compared to milk protein[245]. The contrasting physiological response depending on the protein source is probably due to the different amino acids contained in each source, which are known to have differing effects on glucose homeostasis.

The mechanisms through which certain amino acids induce insulin resistance are well established. The role of mTOR in insulin resistance was introduced in section 1.1.2. The pathway in which such amino acids stimulate mTOR and induce insulin resistance (ultimately by blocking PI3 kinase signalling) is depicted in Figure 7[207]. mTOR activation also results in adipogenesis[207]. Impairment of insulin signalling by amino acids can also occur through the hexosamine pathway[207]. In addition, some amino acids induce hepatic gluconeogenesis, further impairing healthy glucose homeostasis[207].

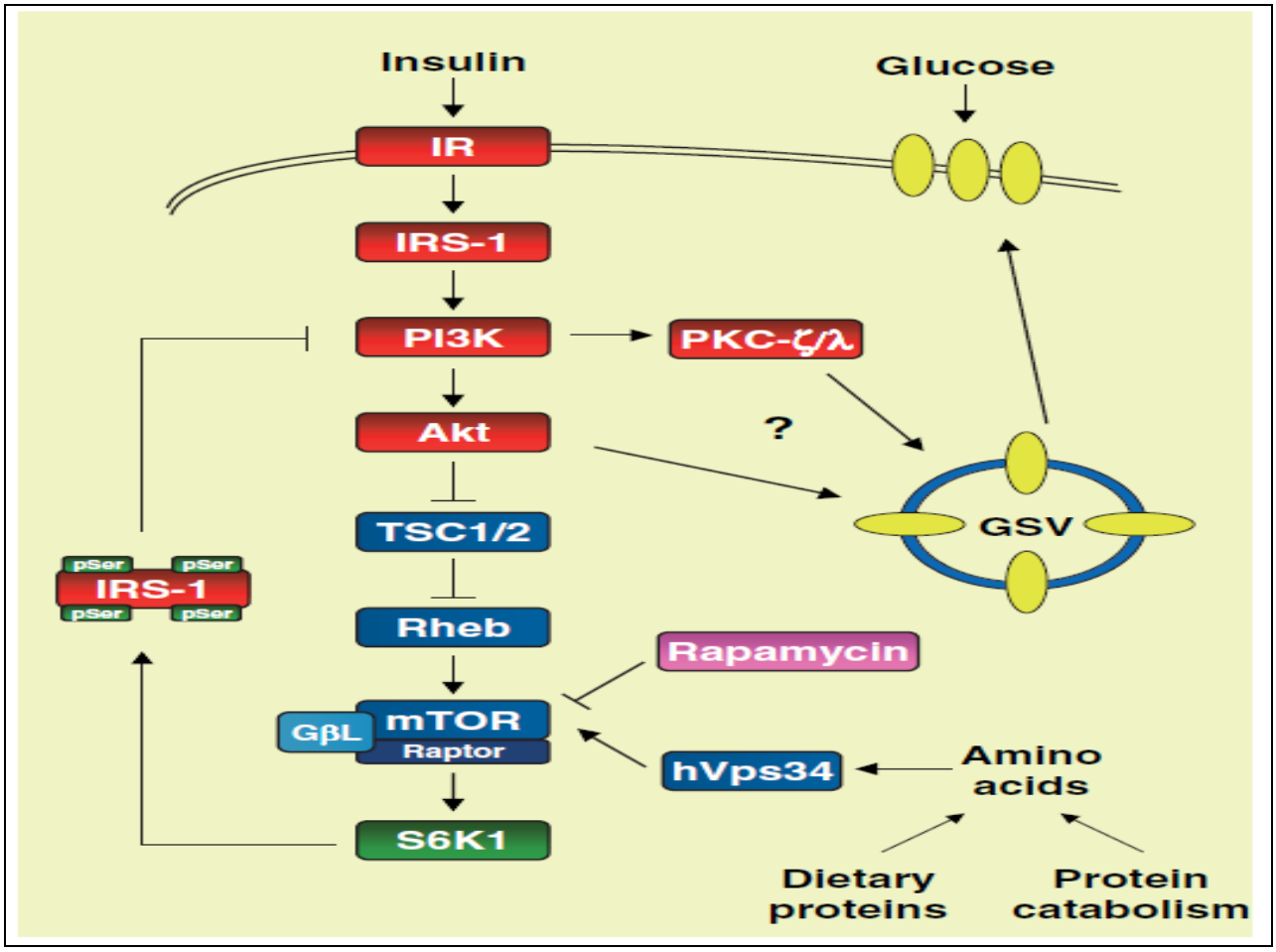



Figure 7: Amino acid induced insulin resistance*[207]*.

*Reproduced from [207] with permission from Annual Reviews Inc. In normal physiology insulin binds and activates the insulin receptor (IR), which phosphorylates insulin receptor substrate-1 (IRS-1). Upon binding to tyrosyl-phosphorylated IRS-1, the activated phosphatidylinositol 3-kinase (PI3K) relays the signal to both Akt and protein kinase C (PKC), which are involved in glucose transporter 4 translocation (GSV) and glucose transport. Akt can also phosphorylate and destabilize tuberous sclerosis complex (TSC)1/2, enabling Rheb-mediated mTOR activation. Amino acids activate mTOR through class III phosphotidylinositol 3 kinase (hVps34). The rapamycin-sensitive mTORC1 (composed of mTOR, raptor, and G protein α-subunit-like protein (GβL)) requires both insulin and amino acids for mediating full activation of S6K1 and promoting inhibitory phosphorylation of IRS-1 on serine residues. Serine-phosphorylated IRS-1 causes insulin resistance by blocking PI3K signalling as previously mentioned.*

In contrast, certain other amino acids and protein hydrosylates appear to have favourable effects on glucose homeostasis. It was first thought that only CHOs invoke an insulin response, but in early pioneering work using intravenous infusions of amino acids, insulin responses to amino acids were demonstrated. Each amino acid had a different insulin secreting potention; In decreasing order of insulin response arginine > lysine > leucine > phenylalanine > methionine[246]. Further studies confirmed synergistic effects when combinations of amino acids were infused, where arginine-leucine had the strongest insulinotropic effect. Oral ingestion of amino acids also increases insulin secretion, and this effect appears to be synergistic when combined with CHO. The proposed mechanisms through with amino acids stimulate insulin secretion and are shown in Figure 8. It is of note that the mechanism of action (closing of the $K_{ATP}$ channel) is the same as that of commonly prescribed insulin secretagogues from the sulfonylurea class of pharmaceuticals. Aside from improving insulin secretion, protein hydrosylates also improve incretin secretion (GIP, but not GLP-1)[247]. However the application of this is guarded given that patients with type 2 diabetes lose sensitivity to GIP[117]

Protein hydrolysates, often with 2 – 10 amino acids are finding their way into nutraceuticals. The drive for this comes from evidence in a series of experiments which show improved insulin secretion and then improved glycaemia in type 2 diabetes with the use of protein hydrosylates. Proof of concept that protein hydrosylates improves insulin secretion three fold in type 2 diabetes was shown by van Loon *et al*.[248]. In a more practical experiment, Manders *et al*. showed that post prandial glucose levels in type 2 diabetes patients were improved when meals were co-ingested with casein hydrosylate (with or without leucine)[249]. Following on from this experiment, the investigators applied the same intervention in a community setting, with 24 hour glucose monitoring showing postprandial hyperglycaemia was reduced by 25% and mean glucose levels were 10% lower[250].

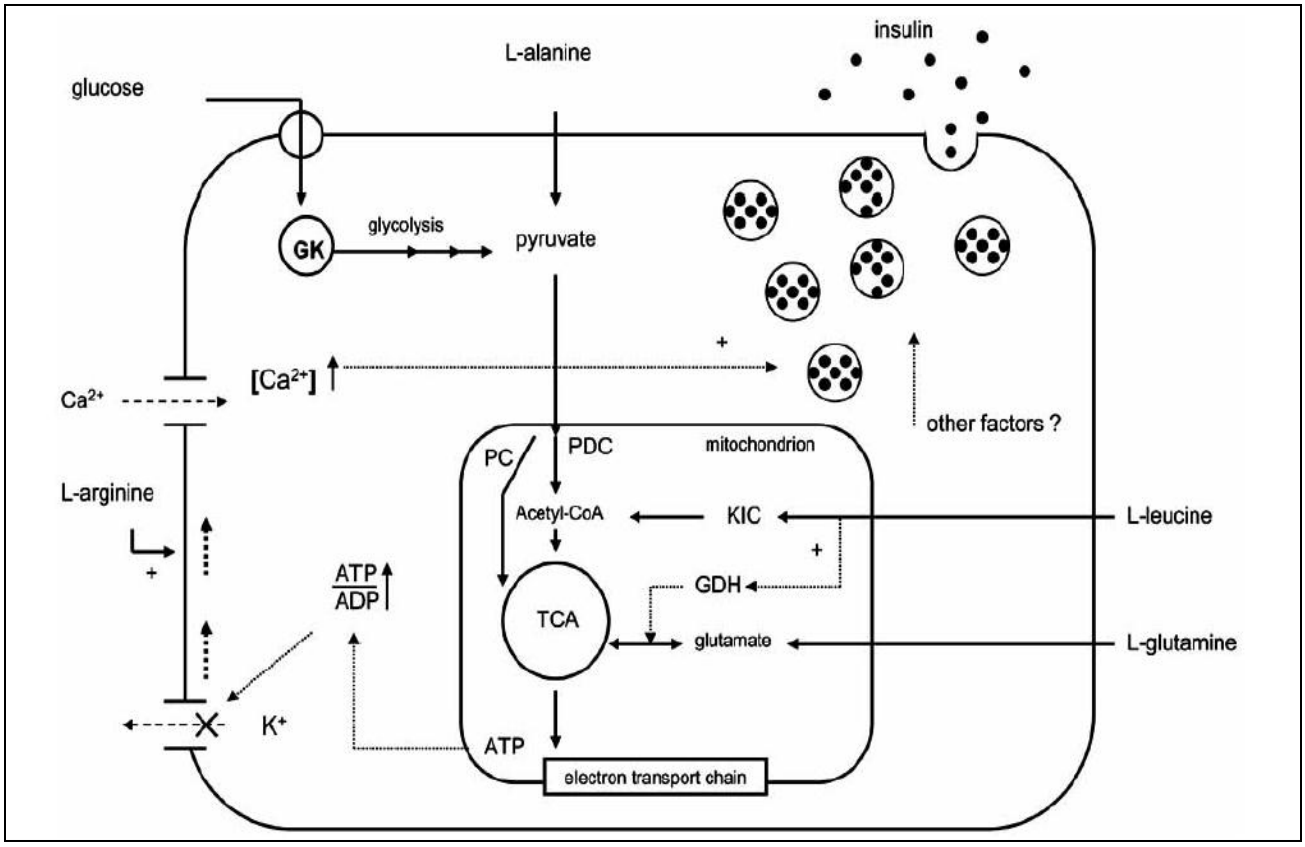



Figure 8: Proposed mechanisms that amino acids improve insulin secretion *[251].*

*Reproduced from [251] with permission from Bentham Science Publishers LTD. The normal sequence for insulin release begins with glucose entering the cell and being phoshorylated by glucokinase (GK). Pyruvate is formed by glycolysis. In the mitochondria pyruvate becomes a substrate for pyruvate dehydrogenase complex (PDC) or pyruvate carboxylase (PC) before entering the citric acid (TCA) cycle. Increased TCA cycle activity results in an increased ATP/ADP ratio, which will lead to the closing of ATP-sensitive K+ channels. The plasma membrane then depolarises, allowing opening of voltage activated Ca2+ channels, resulting in Ca2+ activated insulin exocytosis. Arginine has been reported to be able to directly depolarize the plasma membrane. Metabolizable amino acids are catabolised to generate ATP and, as such, to increase intracellular ATP/ADP ratios and activate insulin exocytosis. Numerous interactions and co-factors are evident in the complex regulation of amino acid induced insulin secretion. For example, leucine-induced insulin secretion is mediated by its oxidative decarboxylation as well as by allosterically activating glutamate dehydrogenase (GDH). Additional abbreviations: KIC;-ketoisocaproate.*

In summary, dietary protein has contrasting effects on insulin sensitivity. On one hand epidemiological evidence, particularly for animal derived protein, seems to show that protein intake is associated with impaired glucose homeostasis, for which there are established mechanisms. On the other, certain amino acids and protein hydrosylates promote insulin secretion and can improve glycaemia in type 2 diabetes, prompting nutraceutical manufacturers to incorporate certain amino acids into commercial products.

#### 1.4.1.5 Perspective

The provision of a single recommendation outlining the optimal proportion of each different macronutrient in the diet that can be applied universally to healthy and diseased populations is not possible. This is because individual responses are likely to be different based on genetic, epigenetic, and environmental factors. Further, geographic, cultural, and socio-economic factors might have considerable influence on dietary patterns, often away from what is considered optimal for cardiovascular health. Application of nutritional guidelines at a population level is fraught with difficulties and will be expanded upon further in Chapter 6. Nevertheless, nutritional messages that promote high fibre intake and avoidance of "free sugars" are likely to positively influence insulin sensitivity based on the literature reviewed, while messages around fat and protein have weaker evidence. While the evidence for high fibre diets is strong, there remain important unanswered questions; i) In a young population who have poor fibre intake and are predisposed to developing type 2 diabetes by virtue of their socio-economic status, ethnicity, and anthropometry, can fibre supplementation improve their glucose homeostasis? ii) What physiological mechanisms translate the intake of fibre to improved insulin sensitivity? Recently the call for such research has come from a leading nutrition journal[167]. In Chapter 3 (our paper investigating the use of supplemented psyllium in adolescent males) I have attempted to answer these questions.

In addition to the literature surrounding macronutrients, there is a growing body of literature examining micronutrients, and traditional (folk) plant based approaches to managing type 2 diabetes. One of the most commonly used medications in diabetes – Metformin – had its origins from the French Lilac plant, which had been used as folk medicine to treat diabetes for centuries

before pharmaceuticals were available[252]. The following section will critically appraise the literature available on micronutrients and plant based folk medicine, and will explore possible mechanisms through which they may work that could ultimately lead to the development of new pharma- and nutraceuticals.

### 1.4.2 Micronutrients

One third of patients with type 2 diabetes in the United States actively use complementary and alternative medicine to treat the disease[253]. The popularity of alternative treatment attests to its acceptability at a population level, and in some settings it is a cheaper alternative to normal prescriptions and medical consultations. Some herbal and plant based therapies have been used for centuries to treat diabetes. With advances in modern technology, the identification and concentration of candidate bio-actives from folk medicine has resulted in a booming global nutraceutical industry. However, physicians remain sceptical and hesitant about endorsing such therapies because of a lack of convincing evidence from well designed trials. Indeed, this scepticism is usually well founded, as a common theme will resonate in the next section – human trials are hampered by flawed methodology.

The following section will firstly discuss commonly used folk remedies, and critically examine the evidence pertaining to glucose homeostasis. Specifically, the use of cinnamon, ginseng, traditional Chinese medicine, traditional Indian medicine, and medicine from Mexican plants will be discussed. Polyphenols are a common class of active ingredient found in many plant based treatments, and are now emerging as being linked to improved glucose homeostasis. There are thousands of natural polyphenols in the plant kingdom (and in derived foods), and these molecules share the basic structure of an aromatic ring with attached hydroxyl groups. Variations in this structure lead to individual classifications of polyphenols, with at least 10 separate classes identified, [254] four of which are important in the human diet: phenolic acids, flavonoids, stillbenes, and lignans. A published commentary on dietary polyphenols is included in this section. Finally, specific minerals and vitamins that have been subjected to human trials will be discussed.

#### 1.4.2.1 Cinnamon

Cinnamon is one of the most commonly consumed spices. The main constituent of cinnamon bark is cinnamon oil, rich in cinnamic acid, cinnamaldehyde and cinnamic alcohol[255]. Of these, the former two have shown the ability to improve glucose homeostasis in animal models, and mechanisms of action have been proposed, including increasing GLUT4 translocation, acting as a peroxisome proliferator activated receptor (PPAR) agonist, and via their anti-inflammatory properties[255]. The insulin potentiating activity appears to derive from the aqueous fraction. In this aqueous fraction, a class of water soluble cinnamon polyphenols have been isolated and shown to having insulin-potentiating activity in-vitro[255]. Several animal models have validated the in-vitro work, and have found that activity is due to enhancement of the insulin signalling pathway (via stimulation of insulin receptor-b and IRS-1 tyrosine phosphorylation)[255]. Further, mechanisms by which cinnamon extract could improve glucose homeostasis other than through insulin signalling have been shown in-vitro, for example increasing both glycogen synthase activity[256], and anti-inflammatory proteins[257].

Several human studies showing improved glucose homeostasis have been conducted using aqueous cinnamon extract, but often have methodological limitations. Khan *et al.* demonstrated improved fasting glucose in adults with type 2 diabetes following supplementation with cinnamon extract for 40 days[258]. However, although there were placebo control groups, the design was not cross-over. Fasting bloods were drawn, but no insulin levels were described in the paper, which makes it difficult to attribute any change in fasting glucose to insulin sensitivity. There was no attempt to control for confounders, for example exercise, diet, and anthropometrical changes. Similarly, a study by Zeigenfuss *et al*. showed improved fasting glucose in patients with metabolic syndrome, but did not measure insulin levels or use a cross over design[259]. Furthermore, in this study there was a decrease in body fat in the intervention group which may have been be responsible for the improved fasting glucose. Wang *et al*. did establish improved insulin sensitivity in women with polycystic ovarian syndrome using a good proxy method (the Matsuda method), but the study was small, and again failed to control for important confounders[260]. On the other hand Vanschoonbeek found no improvement in insulin sensitivity after 6 weeks of cinnamon extract supplementation, but could be criticized as the intervention was only half the length of the previous studies[261]. This theme, where in-vitro

data are promising, yet clinical data are flawed will be repeated for several of therapies appraised in this section.

#### 1.4.2.2 Ginseng

After a systematically reviewing 108 trials examining 36 herbs and 9 vitamins and minerals in patients with type 2 diabetes Yeh *et al*. singled out two supplements with the best evidence for positive effects on glucose homeostasis, one of which is American ginseng[253]. This statement was based on a series of experiments by Vuksan *et al*. as part of an American Ginseng Clinical Testing Program[253]. This collection of experiments used a carefully planned, methodical and step wise approach that covered diabetic and non diabetic patients. Improved glycaemic responses to a 25g OGTT following preprandial ginseng supplementation, as well as improved long term measures of glycaemia were consistently observed.[255] Unfortunately measures of insulin sensitivity were not undertaken, except for a non significant increase in insulin levels suggesting improvement in pancreatic β-cell function[255]. The real strength of this series is the consistent use of the same ginseng preparation, as there can be a huge discrepancy between products due to type of ginseng tree, which part of the tree is used, the age of the tree, the geographical location of the tree, and processing techniques[255]. Interestingly, Jenkins *et al*. combined ginseng with a soluble fibre in a long term well designed study in type 2 diabetics and concluded that it improved glycaemic control comparable to a commonly used class of medication – alpha-glucoside inhibitors[255].

The active components in ginseng are commonly identified as the triterpenoid saponin glycosides (ginsenosides or panaxosides)[255]. Animal and in-vitro studies have shown that ginseng extract or ginsenosides can improve glucose homeostasis by delaying the absorption of glucose, increasing insulin secretion and binding, and improving glucose uptake[255]. Further mechanisms include modulating intracellular signalling systems for example PPAR-γ (the same target as thiazolidinediones), protein kinase c activity, and decreasing TNF-α in macrophages[255]. It is notable that American ginseng has the highest total ginsenoside content

compared to other species of ginseng[262], which may be why it has the best evidence for its use.

#### 1.4.2.3 Herbs in Traditional Chinese Medicine

Modern Chinese medical practice has incorporated western medicine with ancient techniques that have been used for thousands of years. While traditional Chinese medicine (TCM) has many facets, this section will briefly look at the herbs and foods used for the treatment of diabetic symptoms.

Of the many herbs used, bitter melon (Mormordica charantia) has the most evidence for positive effects on glucose homeostasis. In the review by Yeh *et al*., it was identified as having positive preliminary results[253]. This was based on two small non randomized trials demonstrating improved post prandial [263] and fasting [264] plasma glucose levels. The active component is likely to be "vegetable insulin" – a component of bitter melon which has structural similarities to insulin. In fact, one of the aforementioned studies used purified "vegetable insulin" from bitter melon, injected in the same fashion as regular insulin (having a peak action at 6 hours) to obtain a hypoglycaemic effect[264]. There are numerous other herbs used in TCM, however none have been tested human clinical trials. Nevertheless, many have been trialled in animal models, and have suggested mechanisms of action relating to glucose homeostasis. These are summarized in Table 10[255]. Some others herbs are used for the treatment of diabetes symptoms but have no evidence for any properties pertaining to glucose homeostasis[255].

## Table 10: Herbs used in traditional Chinese medicine without human clinical data.

| Herb or supplement | Proposed active components | Proposed mechanisms of action |
|---|---|---|
| *Fructus Corni* (dogwood fruit) | Ursolic acid, oleanolic acid | Upregulation of GLUT4 mRNA expression<br>Antioxidant |
| *Fructus Ligustri Lucidi* (glossy privet fruit) | Oleanolic acid | Antioxidant |
| *Fructus Lycii* (goji) | *Lycium barbarum* polysaccharide | Upregulation of GLUT4 in skeletal muscle |
| *Family Moraceae* (white mulberry) | Moran A (glycoprotein) | Inhibition of intestinal disaccharidases |
| *Radix Astragali* | Primary polysaccharides and flavonoids | Unknown |
| *Radix Ophiopogonis* (dwarf lilyturf tuber) | Homoisoflavones | Unknown |
| *Radix Puerariae* | Flavonoids | Increasing GLUT4 expression<br>Antioxidant |
| *Radix rehmanniae* (Chinese floxglove root) | Polysaccharide extract | Increased insulin secretion<br>Antioxidant<br>Increased hepatic glucose storage |
| *Radix Trichosanthis* | Trichosans | Unknown |
| *Rhizoma Coptidis* | Berberine | Simulates insulin secretion<br>Decreases glucose absorption<br>Increases hepatic glucose uptake |
| *Rhizoma Polygonati* | Spirostanol glycoside | Decreased GLUT2 expression in liver |

| | | |
|---|---|---|
| | | Improved peripheral insulin sensitivity |
| *Rhizoma Anemarrhenae* | Mangiferin | Increased insulin sensitivity |
| *Rhizoma Atractylodis* | Atractans | Unknown |

#### 1.4.2.4 Herbs originating from India

There are many plants and herbs used in traditional Indian medicine to treat type 2 diabetes. Cinnamon and bitter melon have already been covered above. In the same fashion as taken in the previous section regarding TCM, herbs with human data (fenugreek, ivy gourd, Indian holy basil, *Gymnema sylvestre* and *Aloe vera* ) will be focused on, while those with animal data only are presented in Table 11.

Fenugreek (*Trigonella foesnum-graecum*) is a legume commonly used in Indian cooking, and in Ayuverda medicine. There are three randomized controlled trials from a single investigator showing improved glucose homeostasis, covering type 1 and type 2 diabetes, however they are of poor quality and conducted on small numbers (n = 5 to 15) of participants[253]. There are several other non-controlled trails, and open-label cohort studies that lend support to the randomized data[253, 255]. There have been extensive studies in animals showing improved glucose homeostasis[255]. These studies have helped to identify the high dietary fibre composition of fenugreek which is believed to improve glucose homeostasis through the mechanisms covered in section 1.4.1.2. However, it must be noted that the limited mass of fenugreek consumed make it likely to be a small contributor to total daily fibre intake, and hence other, as yet unknown, compounds may be responsible for any physiological action. Yeh *et al*. concluded that there is preliminary evidence for the use of fenugreek in diabetes, but further well conducted studies are required[253].

*Coccinia indica*, (ivy gourd) was identified by Yeh *et al.* as having the best evidence for use in diabetes along with American ginseng[253]. This is based on a well designed double blind randomized controlled study in 32 patients with type 2 diabetes[265], and a non randomized

three armed trial in 70 type 2 diabetic patients[266] showing improved glucose homeostasis. Again, supporting evidence is provided from open label prospective studies[267, 268] and numerous animal studies[255]. The active component is not in the fruit, but in the leaves, root and whole plant[255]. A specific active compound has not yet been identified, but the mechanism of action is proposed to be as an insulin mimetic[253].

While there are a plethora of animal studies demonstrating positive glycaemic outcomes with *Ocimum sanctum* (Indian holy basil), human data are limited by the fact there is only one study[269]. The active component is thought to be eugenol[255], proposed to act by enhancing pancreatic β-cell function and insulin secretion[253].

*Gymenma sylvestre* is a common herb used in Ayuverda, and has a popular Hindi name "gurmar", meaning "destroyer of sugar". The two published studies in humans showing many facets of improved glucose homeostasis[270, 271] are limited by the fact they are both from the same investigative group and are non randomized and open labelled. The active constituents have been identified as gymnemosides and gymnemic acid[255], which are proposed to increase glucose utilization and improve pancreatic β-cell function[253]. At best, the data on *Gymenma sylvestre* could be described as suggestive, with considerable more research required.

Evidence for the use of *Aloe vera* in the treatment of type 2 diabetes in humans is positive, but only limited data exists to draw upon. Again, the two published prospective studies are non-randomized and performed by the same investigative group, but found decreased fasting plasma glucose in patients with established and newly diagnosed type 2 diabetes after oral Aloe vera supplementation[272, 273]. Animal data suggest the mechanism of action is by improving pancreatic β-cell function, and as an anti-inflammatory agent[255]. The active component has not been identified, and clearly more research is required to validate its use in diabetes.

## Table 11: Herbs used for diabetes from Indian origin without human clinical data.

| Herb or supplement | Proposed active components | Proposed mechanisms of action |
|---|---|---|
| *Allium sativum* (garlic) | Allicin<br>S-allyl cysteine sulfoxide | Stimulates insulin secretion<br>Prolongs insulin half life<br>Increased hexokinase activity<br>Decreased hepatic gluconeogenesis |
| *Murraya koenigii* (curry leaf) | Unknown | Increased insulin secretion<br>Increased hexokinase activity<br>Decreased hepatic gluconeogenesis<br>Antioxidant |
| *Scoparia dulcis* (sweet Broom Weed) | Unknown | Increased insulin secretion<br>Antioxidant |
| *Nigella sativa* (black cumin) | Unknown | Increased insulin secretion<br>Decreased hepatic gluconeogenesis<br>Antioxidant |
| *Curcuma longa* (tumeric) | Unknown | Antioxidant |
| *Cuminum cyminum* (cumin) | Flavonoids | Unknown |
| *Caesalpinia bonducella* (Leguminosae) | Unknown | Increased insulin secretion |
| Swertia chirayita (indian gentian) | Ophelic acid<br>Chiratin | Increased insulin secretion<br>Improved glucose uptake<br>Increased glycogen synthesis |

| | | |
|---|---|---|
| *Cassia auriculata (*avaram) | Unknown | Increased insulin secretion<br>Decreased hepatic gluconeogenesis<br>Antioxidant |
| *Phllanthus amarus* | Unknown | Enhanced peripheral uptake of glucose |
| *Tinospora cordifolio* | Unknown | Decreased hepatic gluconeogenesis<br>Increased hexokinase activity |
| *Baeohaavia diffusa* (punarnava) | Unknown | Decreased hepatic gluconeogenesis<br>Increased hexokinase activity<br>Antioxidant |
| *Musa sapientum* (banana flower) | Unknown | Antioxidant |
| *Piper betle* | Unknown | Decreased hepatic gluconeogenesis<br>Increased hexokinase activity |
| *Nelumbo nucifera* (lotus) | Unknown | Unknown |
| *Citrullus colocynthis* (bitter apple) | Saponin fraction | Increased insulin secretion |
| *Brassia juncea* (mustard) | Unknown | Decreased hepatic gluconeogenesis<br>Increased glycogen synthesis |

#### 1.4.2.5 Traditional Mexican plants

While ancient medical practice in Mexico has identified approximately 306 plant species from 235 genera and 93 families for the use in type 2 diabetes, only a few have been formally

investigated, and even fewer in human trials[274] *Opuntia sp.* (Nopal) has the most literature, but there are several other species that potentially have a greater effect. Normally, patients drink a cup of beverage made from the plant with their meals, and by doing so obtain a hypoglycaemic effect for 5-6 hours[255]. Plants with supportive animal, but no human, data are presented in Table 12.

In a recurrent theme as we have seen earlier, a single investigative unit has been responsible for establishing the human clinical data for Nopal, which limits the applicability of the data in a general way. In the series by Frati-Munari *et al*., various preparations of Nopal were tested in patients with type 2 diabetes, demonstrating attenuated acute glucose and insulin responses[275], and decreased fasting plasma glucose and insulin levels[276]. However negative results were also reported when a dehydrated extract was used[277]. All these studies were limited in design, as none were randomized or blinded. Numerous animal studies provide supportive evidence for the use of Nopal in type 2 diabetes, and have proposed increased insulin secretion, increased insulin sensitivity, improved glucose uptake, decreased intestinal glucose absorption, and decreased gluconeogenesis a possible mechanisms of action[255]. Nopal contains several potential bioactive components, namely the fibre content, but also many constituents with antioxidant properties[255]. Yeh *et al*. identified Nopal as having positive preliminary results that require further evaluation[253].

Guarumbo (*Cecropia obtusifolia*) is a tree from which the leaves, bark, and root are boiled in water to treat diabetes. The main active components are isoorientin and chlorogenic acid (a polyphenol)[255]. Human studies were published after the critical review from Yeh *et al.*, but had they been available at the time Yeh *et al.*'s review was written, it is likely the evidence would have been assessed as suggestive. For example in a small (n=12) open label non-randomized, but long term (34 weeks) trial there was sustained improvement in HbA1c but no effect on insulin levels[278]. In a larger study that was double blinded and randomized, patients with poorly controlled type 2 diabetes were supplemented with Guarumbo extract and significantly improved fasting plasma glucose levels were noted after three weeks, however, insulin levels were unfortunately not described in this study[279].

**Table 12: Plants used in traditional Mexican medicine for diabetes without human clinical data.**

| Herb or supplement | Proposed active components | Proposed mechanisms of action |
|---|---|---|
| *Psacalium peltatum* (Matarique) | Peltalose (a CHO) | Increased insulin secretion<br>Improved glucose uptake |
| *Cucubita ficifolia* (Chilacayote) | Dichiroinositol (a CHO) | Increased insulin secretion<br>Decreased hepatic gluconeogenesis |
| *Equisetum myriochaetum* (Cola de caballo) | Flavonol glycosides and other polyphenols | Increased insulin secretion |
| *Bidens pilosa* (Aceitilla) | Polyacetylene glucosides | Immunomodulation |

### 1.4.2.6 Polyphenols

**Preface to publication**:

In sections 1.4.2.1 to 1.4.2.5 whole foods with potential benefits for glucose homeostasis were discussed, and where possible the bioactive chemicals within them identified. Many of these bioactive compounds fall into the class of compound known as 'polyphenols'. There are thousands of natural polyphenols in the plant kingdom (and in derived foods), and these molecules share the basic structure of an aromatic ring with attached hydroxyl groups. Variations in this structure lead to individual classifications of polyphenols, with at least 10 separate classes identified, [254] four of which are important in the human diet: phenolic acids, flavonoids, stillbenes, and lignans (Figure 9). In food, some polyphenols are linked with sugar moieties (glycosylated) or exist as esters, which may alter their metabolism [280]

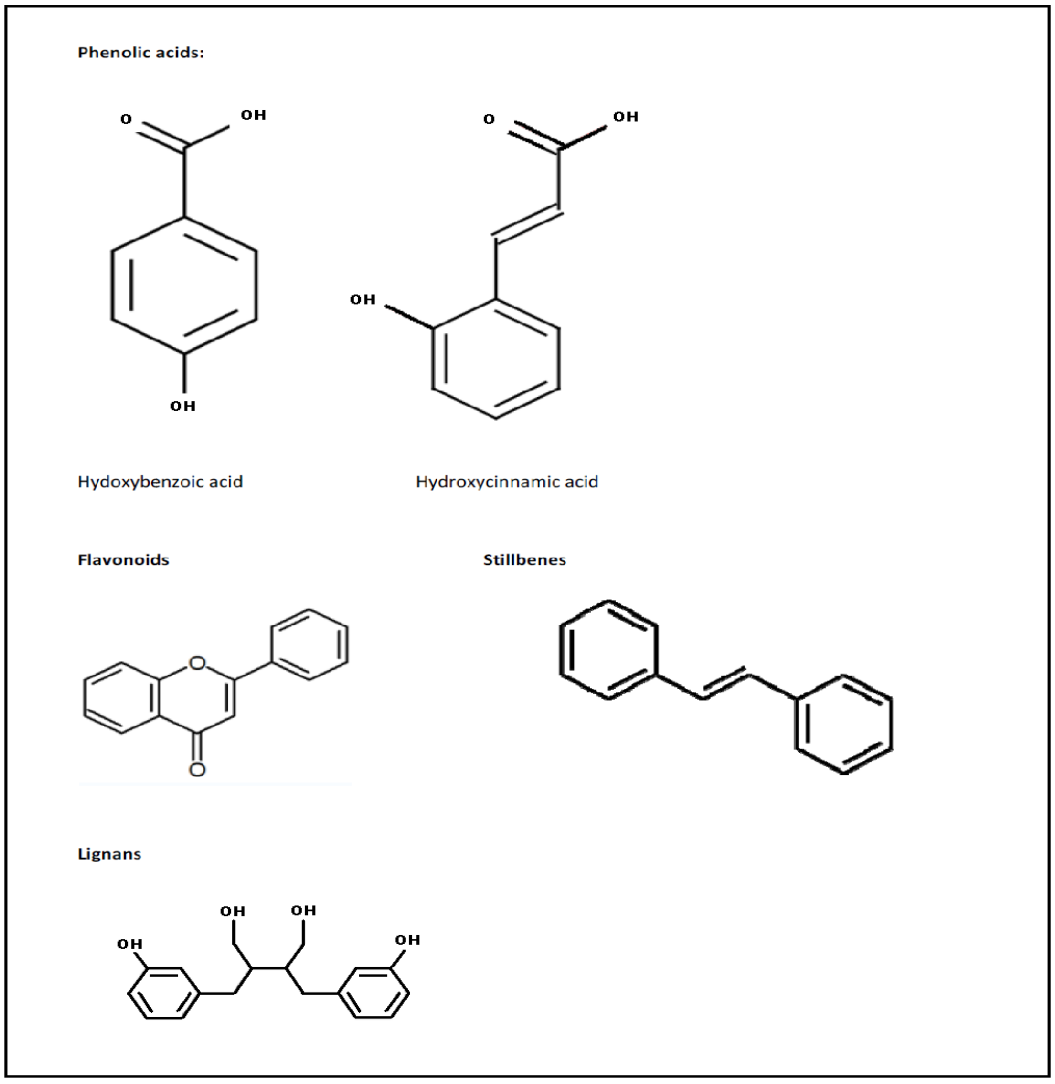



Figure 9: Four classes of polyphenol abundant in the human diet.

The following published commentary on polyphenols and human glucose metabolism was written with the intention to:

a) highlight the current imbalance between the enormous global nutraceutical market and the sparsity of robust clinical data supporting its use

b) to draw attention to the often flawed published data surrounding polyphenols to date

c) to promote that polyphenols are an exciting new area of food research which may lead to the development of nutraceuticals that can be used as adjunctive therapy in type 2 diabetes, or even used in a preventative setting, and

d) to suggest a methodical approach to research in this area which will provide robust and reproducible data that may eventually lead to clinical credibility.

Indeed, it is this methodical approach that I adopted when investigating Olive Leaf Extract (a potent source of the polyphenol oleuropein), the publications for which are later included in this thesis.

The following section contains an unaltered reproduction of the manuscript "Polyphenols and Glucose Homeostasis in Humans" published in the *Journal of the Academy of Nutrition and Dietetics*, 2012. **112**(6): p. 808.

# Polyphenols and Glucose Homeostasis in Humans

Martin de Bock[1], José G B Derraik[1], Wayne S Cutfield[1]

[1] Liggins Institute, University of Auckland, Auckland, New Zealand

**Keywords**: Diet, Glucose Homeostasis, Nutraceuticals, Polyphenols

**ABSTRACT**

There is mounting *in vitro* and animal evidence that polyphenols can improve glucose homeostasis, but human data are still limited. Human epidemiological studies investigating polyphenols have indicated favorable metabolic outcomes, but the few clinical trials extant have yielded conflicting results. Translation from *in vitro* and animal evidence into human clinical trials is hindered by the variable bioavailability and rapid metabolism of polyphenols. Normal dietary intakes of polyphenols rarely achieve serum concentrations reported to be necessary to exert the metabolic effects seen in *in vitro* and animal studies. In an attempt to overcome this issue, nutraceutical products are produced with high concentrations of polyphenols, well-above the normal dietary intake, which may have adverse effects. Currently, the nutraceutical industry is worth billions of dollars, despite a lack of published clinical studies showing clear benefits to human health of supplemental polyphenols. Well-conducted clinical studies are needed to clarify the potential effects of polyphenols on glucose homeostasis before these products are used in diabetes treatment and/or prevention.

## Introduction

The global pandemic of type 2 diabetes mellitus places an incalculable burden on health care systems. In the United States alone, it is estimated that 52% of the population will have diabetes or prediabetes by 2020, conditions that already cost that country US$194 billion a year in healthcare spending [281]. Amelioration of type 2 diabetes risk usually targets lifestyle and diet, primarily with the aim of reducing obesity – the foremost risk factor in the development of insulin resistance and ultimately type 2 diabetes . However, particular dietary components, such as polyphenols, may assist in type 2 diabetes prevention in ways other than weight control.

Dietary polyphenols are chemicals of plant origin that are abundant in fruit, vegetables, chocolate and nuts, as well as in beverages such as tea, coffee, wine and soy milk [282, 283]. In tea leaves for example, polyphenols can account for up to 30% of their dry weight [284]. As such, polyphenols are the most abundant anti-oxidants in the human diet [285]. Dietary polyphenol consumption is of interest to human health because it is associated with lower rates of diabetes and cardiovascular disease [286-289]. There are thousands of natural polyphenols in the plant kingdom (and in derived foods), all of which share the basic structure of an aromatic ring with attached hydroxyl groups. Variations in this structure lead to individual classifications of polyphenols, with at least 10 separate classes identified [254], four of which are important in the human diet: phenolic acids, flavonoids, stilbenes, and lignans [290].

Historically it has been difficult to determine polyphenol concentrations in food items, or to identify foods that contain specific polyphenols. However, a collaborative project between nutritionists, food scientists, epidemiologists and bioinformaticians has recently produced a database encompassing 500 different polyphenols known to be present in over 400 food items [291]. The amount and type of polyphenols consumed is largely dependent on the dietary patterns of individuals and populations. Nonetheless, it previously has been estimated that total polyphenol intake is approximately 1 g/day [292], which is likely to be a conservative figure [280]. Using the aforementioned database, the historical 1 g/day figure has been shown to underestimate intake by approximately 20% [293].

In wealthier nations, such as the USA where the prevalence of type 2 diabetes is an ever growing issue [281], there is an enormous market for polyphenol formulations. The global nutraceutical market was estimated to be worth US$ 117.3 billion in 2007[294]. Currently, there is a major imbalance between the published clinical studies on the benefits of polyphenols to human health and the marketing of these products. There is mounting evidence that polyphenols can reduce insulin resistance in *in vitro* and animal studies (which have been reviewed elsewhere [295]), but data from human studies remains limited.

Thus, we address three primary questions regarding polyphenol action in humans that remain unclear. 1) Do polyphenols improve human glucose homeostasis? 2) What limits the effectiveness of dietary polyphenols? 3) What role does the nutraceutical industry have in research related to the health benefits of polyphenols?

**Do polyphenols improve glucose homeostasis in humans?**

In the attempt to answer this question, we have focused on specific food sources, rich in dietary polyphenols, that highlight important issues in the literature: i) tea – arguably the most studied and single largest source of human polyphenol consumption; these studies are of heterogeneous design and have yielded conflicting results; ii) chocolate – as the variability of results from the studies illustrate the importance of adopting appropriate methods to assess insulin sensitivity; iii) red wine – a well-studied beverage with a polyphenol content that provides a new potential mechanism to improve health but also has potential adverse side effects; and iv) soy – these studies demonstrate that cardioprotection can occur independently from improvement in glucose homeostasis.

The literature on tea epitomizes the challenges in interpreting the data from studies on polyphenols due to conflicting results and heterogeneous design. Tea is rich in catechin (particularly epigallocatechin gallate) from the flavonoid class of polyphenols. Globally, tea is second only to water as a consumed beverage, so that it represents a significant source of polyphenol for a vast proportion of the world's population [284]. Epidemiological studies are contradictory with four studies showing no effect of tea intake on type 2 diabetes incidence [296-299], two showing some type 2 diabetes protection in high consumption groups [300, 301], and one demonstrating type 2 diabetes protection only in those individuals aged less than 60 years [302]. One study showed a cup per day of black tea was protective while green tea consumption was not [303], in contrast to another study showing that only green tea was protective [304]. A meta-analysis of these studies concluded that intake of approximately four cups of tea per day may prevent the development of type 2 diabetes [305].

Subsequent intervention studies (summarized in Table 13) compound the difficulty in interpreting findings due to major variations in study design (including assessment of insulin sensitivity), study populations, adopted dosage of tea polyphenols, and conflicting results [306-316]. Focusing on the study populations alone, all studies differed according to sample size, age ranges studied, gender distribution, health status of participants (from healthy to those with type 2 diabetes ), and ethnicity. This heterogeneity in populations may account for some of these conflicting results, possibly due to background dietary differences and/or variable human enzymatic activity and gut flora (which alter polyphenol metabolism) [317]. Of the eleven

## Table 13: Summary of intervention trials examining the effects of tea consumption on glucose metabolism. Data are mean±SD.

| Reference | Study Design | Intervention | Participants | Follow Up | Measurements | Results | Adverse Events |
|---|---|---|---|---|---|---|---|
| Josic *et al.* 2010 [306] | Randomized, crossover, non-blind | 300 ml green tea, total catechin dose 202 mg, given with test meal | n=14 (50% male)<br>Age 27±3 yrs<br>BMI 22.3±3.4 kg/m$^2$<br>Healthy patients<br>Ethnicity not reported | 2 hours | Glucose<br>Insulin<br>Area under the curve | No difference in area under the curve<br>Higher BGC at 2 hr (p=0.019) (absolute difference <1 mmol/L) | Not reported |
| Brown *et al.* 2009 [308] | Randomized, placebo controlled, double blind | EGCG 800 mg daily, over 8 weeks | n=88 (100% male)<br>Healthy males 40–65 yrs<br>BMI placebo 31.0±2.5 kg/m$^2$<br>BMI intervention 31.2± 2.8 kg/m$^2$<br>Ethnicity not reported | 8 weeks | HOMA(IR) | No difference | Not reported |
| Venables *et al.* 2008 [309] | Counter-balanced, crossover, placebo controlled, non-blind | Green tea extract with 136 mg EGCG, total polyphenol 340 mg | n=12 (100% male)<br>Healthy males<br>23±2 yrs<br>BMI 24.1±1.1 kg/m$^2$ | 2 hours | Matsuda index (ISI) | 13±4% greater (more sensitive) (p<0.05) | Not reported |
| Nagao *et al.* 2008 [307] | Randomized, double blind | Green tea with either 583 mg catechins (intervention) or 96 mg catechins (control) for 12 weeks | n=50 (38% male)<br>Type 2 diabetes not on insulin<br>Intervention age 65±2 yrs<br>Control age 63±2 yrs<br>BMI intervention 25.6±1.8 kg/m$^2$<br>BMI control 24.0±0.9 kg/m$^2$<br>Japanese | 12 weeks<br>Sustained effect checked at 16 weeks | Fasting glucose<br>Fasting insulin<br>$HbA_{1c}$ | Only patients on insulinotropic medications increased insulin production and significantly decreased their $HbA_{1c}$ | None observed |
| Fukino *et al.* 2007 [310] | Randomized, crossover, non-blind | Green tea extract, daily dose 456 mg catechins and 102 mg caffeine | n=60 (85% male)<br>32–73 yrs<br>Fasting glucose >6.1 or non fasting >7.8 mmol/L (borderline diabetes)<br>Japanese | 2 months | Fasting glucose<br>Fasting insulin<br>$HbA_{1c}$<br>HOMA(IR) | Small improvement in $HbA_{1c}$ (p=0.03)<br>No difference in other parameters | Not reported |
| MacKenzie *et al.* 2007 [311] | Randomized, placebo controlled, blind | Teaflavin extract daily<br>Arm 1: 150 mg green tea catechins, 75 mg black tea theaflavins, 150mg other tea | n=49 (53 % male)<br>Type 2 diabetes >6 months, not on insulin<br>Age 65 (49–86) yrs | 3 months | $HbA_{1c}$ | No difference | 1 profuse sweating<br>1 rash |

| Reference | Study Design | Intervention | Participants | Follow Up | Measurements | Results | Adverse Events |
|---|---|---|---|---|---|---|---|
| | | polyphenols<br>Arm 2: 300 mg green tea catechins, 150 mg black tea theaflavins, 300 mg other tea polyphenols | BMI (self reported) 30.7–34.8 kg/m$^2$ over the three arms.<br>47/49 Caucasian | | | | |
| Bryans *et al.* 2007 [312] | Four-way randomized, crossover | Black tea extract<br>1 g arm: 350 mg polyphenols (39 mg flavan-3-ols, 21 mg theaflavins)<br>3 g arm: 1050 mg polyphenols (127 mg flavan-3-ols, 63 mg theaflavins). | n=16 (25% male)<br>36±2 yrs<br>BMI 23.8±0.7 kg/m$^2$<br>Healthy<br>Ethnicity not recorded | 2 .5 hours | Oral glucose tolerance test | Glucose significant lower at 2 hr in tea vs control<br>Insulin levels increased at 90 min in tea vs control | Not reported |
| Ryu *et al.* 2006 [313] | Randomized, crossover, non-blind | 9 g green tea, dose of catechin not stated | n=55 (56% male)<br>Type 2 diabetes<br>53±8 yrs<br>BMI 25.0±2.2 kg/m$^2$<br>Korean | 4 weeks | HOMA(IR) | No difference | Not reported |
| Fukino *et al.* 2005 [314] | Randomized, non-blind | Green tea extract with 456 mg catechins, total 544 mg polyphenols | n=66 (80% male)<br>32–73 yrs with impaired glucose tolerance or diabetes<br>Japanese | 2 months | Fasting glucose<br>Fasting insulin<br>$HbA_{1c}$<br>HOMA(IR) | No improvement compared to control | Not reported |
| Tsuneki *et al.* 2004 [315] | Non-randomized, intervention, non-blind | Green tea powder 1.5 g. Dose of catechins not stated | 22 healthy volunteers<br>No demographic data<br>Presumably Japanese | 2 hours | Oral glucose tolerance test | Lower glucose at 30 and 120 min ($p<0.05$) | Not reported |
| Hosoda *et al.* 2003 [316] | Randomized, crossover, non-blind | 1.5 l oolong tea daily for 30 days, containing 242 mg EGCG, 1,490 mg total polyphenols | n=20 (50% male)<br>61±8 yrs<br>Type 2 diabetes not on insulin<br>BMI 22.6±1.1 kg/m$^2$ | 30 days | Fasting glucose<br>Fructosamine | Fasting glucose decreased 30% ($p<0.001$)<br>Fructosamine improved 21% ($p<0.01$) | None observed |

BGC, blood glucose concentration; BMI, body mass index; EGCG, epigallocatechin gallate; yrs, years of age.

intervention studies, five were negative [306, 308, 311, 313, 314] and the three positive studies only yielded minor findings of questionable clinical significance [310, 312, 315]. Hosoda *et al.* showed clinically significant improvements in fasting glucose and fructosamine concentrations but with a required intake of 6 cups of oolong tea per day; this raises questions regarding the routine application of naturally occurring polyphenols to improve glucose homeostasis. The study by Venables *et al.* showed that with acute consumption of tea glucose homeostasis improved using oral glucose tolerance tests, but the number of participants was small [309]. Only Nagao *et al.* had a robust study design (double-blind randomized trial with 12-week follow up) showing a clinically important result, as those on insulinotropic medications had higher insulin production and lower glycosylated hemoglobin ($HbA_{1c}$) after green tea supplementation [307]. Overall, while tea polyphenols may potentially improve glucose homeostasis and prevent the development of type 2 diabetes , the evidence is inconsistent.

Dark chocolate is another food item particularly rich in polyphenols, including flavonols. The associated literature demonstrates the importance of which method is chosen to assess insulin sensitivity. Population studies have shown dark chocolate to be cardioprotective [318], and the majority of clinical trials showed favorable effects on glucose homeostasis [319-323] when a proxy measure of insulin sensitivity was used (HOMA-IR). However, when the accepted gold standard method to measure insulin sensitivity (the hyperinsulinemic euglycemic clamp technic) was adopted, Muniyappa *et al.* showed no improvement in insulin sensitivity after 14 days on flavanol rich cocoa supplement, raising concerns on the reliability of proxy measures of insulin sensitivity [324]. Further investigation is warranted, including more participants, long-term follow up, and gold standard technics to measure insulin sensitivity.

The polyphenol content in red wine may explain the “French Paradox”, where there is a low incidence of cardiovascular disease in France despite a relatively high intake of saturated fat [325]. Since Renaud & de Lorgeril’s original article [325], the subsequent literature has focused on the anti-oxidant effect and lipid lowering properties of red wine polyphenols, while some trials have investigated the effect of grape and wine products on glucose homeostasis [326-330]. Yet, these studies have yielded contradictory results: two studies showed no improvement [327, 329], one showed lowered fructosamine but no change in insulin sensitivity [326], another demonstrated acute amelioration of glucose excursion when wine was taken with a meal [330], but only Banini *et al.* found a clinically significant reduction in $HbA_{1c}$ (7.4 to 6.8%) in type 2

diabetes patients after 28 days of wine supplementation [328]. The focus on red wine polyphenols has since shifted to resveratrol, a potent anti-oxidant found in red grapes and their products. Resveratrol has been shown to modulate the ageing gene Sirt1 [331], and Brasnyó *et al.* demonstrated recently that it can improve insulin sensitivity in type 2 diabetes patients [332].

The focus of soy research has been its weak estrogenic influence on the cardiovascular system in post-menopausal women [333-337]. Cardioprotective effects have been shown in epidemiological studies [338, 339], but they seem to be independent of improved glucose homeostasis. Two studies failed to show an improvement in glucose homeostasis [333, 334], while two others yielded minimal changes of questionable clinical significance [336, 337]. The study by Ho *et al.* only found improvement in fasting glucose concentrations in those individuals with the highest baseline results [335], and did not investigate insulin sensitivity. All the studies were performed exclusively on post-menopausal women, putting into question the broader applicability of the findings. The balance of clinical results suggests that the cardioprotective effects seen in epidemiological studies are not mediated through improvement in glucose metabolism.

Another consideration is whether total dietary polyphenol intake is more important in regulating glucose homeostasis, in comparison to the intake of a single polyphenol in large quantities. A systematic review of 35 clinical trials confirmed improvement in insulin sensitivity on the Mediterranean diet [340], but an attempt to narrow improved insulin sensitivity down to olive oil polyphenols yielded a null result [341]. Although it is an enticing prospect to identify a single polyphenol that can improve glucose homeostasis, population data examining total dietary polyphenol load more consistently showed positive associations [289, 300, 301, 305, 342, 343]. However, a common problem with intervention trials using whole foods is the lack of specific information regarding which polyphenols are present and in what quantities.

*In vitro* and animal data covering a wide range of polyphenols have comprehensively shown favorable results in most physiological processes involved in glucose homeostasis [295]. Unfortunately, there is no conclusive evidence that individual polyphenols can favorably influence glucose homeostasis in humans. Reproducing promising non-human data in clinical studies is challenging.

**What limits the effectiveness of dietary polyphenols?**

The metabolism of polyphenols is likely to be an important factor determining whether a normal diet can deliver effective concentrations of polyphenols to target tissues. The bioavailability of differing polyphenols is highly variable, involving complex and diverse physiological processes. There are also between-subject variations, which are partially explained by heterogeneity in human enzymatic activity and gut flora [317].

In addition, the physiological activity of polyphenol metabolites is yet to be fully understood. In human investigations; polyphenol metabolite concentrations after normal dietary exposure rarely rise above 1 μmol/L, with most polyphenols achieving concentrations significantly below this mark [282]. *In vitro* studies use media usually in excess of 1 μmol/L for sustained periods of time [295]. Blood concentrations may provide information on polyphenol absorption, but they do not necessarily correspond to target tissue levels. Thus, measuring polyphenol concentrations in plasma may not accurately assess exposure [282]. Indeed, *in vitro* and animal studies showed that polyphenols affect glucose homeostasis in many tissues beyond the blood stream, including carbohydrate digestion and glucose absorption in the intestine, stimulation of pancreatic β-cell insulin secretion, liver glucose metabolism, activation of insulin receptors, and glucose uptake in insulin-sensitive tissues [295].

There are variations in the way ingested polyphenols are metabolized and absorbed [280], which may explain differences in their bioavailability. Initially, polyphenols are likely to be metabolized via gut enzymes, but if this does not occur, they are variably processed by gut microflora into metabolites that may be absorbed. For example, the metabolism of many polyphenols requires hydrolysis via lactase [344]. As a result, there is wide variation in this process among different ethnic groups, as 95% of adults of Asian descent, 70% of African, and 10% of European descent are lactase deficient [345]. In contrast, gut microflora are required for the metabolism of polyphenols that are esterified or glycosylated with rhamnose [346]. Since gut microflora vary considerably among individuals [347], this will also affect polyphenol bioavailability. As an exception, flavonols do not require hydrolysis before absorption [280].

Once absorbed, polyphenols undergo rapid reconjugation and excretion through similar pathways as pharmaceutical drugs [280]. However, while pharmaceutical drugs manage to saturate these pathways, polyphenols in normal dietary concentrations do not [280]. The rate of reconjugation of polyphenols is also associated with genetic polymorphism (e.g. catechol-0-

methyltransferase) and environmental factors (e.g. smoking) [348-354]. In addition, the half-life ($EC_{50}$) and activity of metabolites remain poorly understood, and they may have more potent properties than polyphenols in their core state [291, 355].

Nevertheless, the net effect of diverse bioavailability, failure to saturate metabolic pathways, and rapid clearance explains why plasma concentrations of polyphenols in most human studies do not reach the required levels as per *in vitro* and animal studies. Although intestinal absorption can be high, measurement of the parent polyphenol in blood rarely exceeds 1% of the raw product [280]. The $EC_{50}$ values for anti-oxidant, anti-inflammatory, and anti-cancer polyphenol properties obtained *in vitro* are 5–100 μmol/l [356], a target rarely achieved in human studies. For example, eating five apples led to no detectable anti-oxidant effects, even though just 1% polyphenol bioavailability would likely be necessary to yield the observed *in vitro* effects [357]. Catechin given in large supplemented doses leads to detectable blood concentrations, which would only be achieved by drinking many litres of wine [358, 359]. Flavanones (found only in citrus fruit and soy) are an exception, and have been shown to reach serum concentrations of 5 μmol/l [282].

To overcome the limitations associated with variable bioavailability and rapid metabolism, several studies of the effects of polyphenols on glucose homeostasis have used large doses with some success. For example, 20 g/day of decaffeinated coffee solids (equivalent to ten cups of coffee) lowered fasting glycemia in healthy volunteers after two weeks [360]. Similarly, supplementation with 6 cups of green tea per day decreased fasting glucose concentrations by 30% [316].

Thus, to mimic an *in vitro* environment, several intakes a day of polyphenol-rich food would be required to ingest the amount and mix of polyphenols necessary to exert the observed beneficial effects on glucose homeostasis. Although this may not be feasible in practice in many cases, it is possible that subtle long-term dietary intake of polyphenols may yield health benefits.

**What role does the nutraceutical industry have in research related to the health benefits of polyphenols?**

The size of the nutraceutical market indicates that consumers are willing to take polyphenol products despite the paucity of robust scientific data on health benefits, as previously discussed in relation to glucose homeostasis. If concentrated polyphenol formulations were shown to

improve glucose homeostasis, the health benefits would be enormous, as the global incidence of type 2 diabetes is estimated to reach 360 million by the year 2030 [361]. However, robust data from clinical trials using polyphenols are still lacking, and positive health outcomes following their application in individuals with type 2 diabetes are elusive.

Most clinical trials have attempted to deliver large quantities of individual polyphenols to overcome issues of rapid clearance, in the hope of achieving measurable health effects (see Table 1). Typically, nutraceuticals contain polyphenol doses 100–1000x greater than those obtained from normal dietary intake. For example, a 150mL glass of red wine contains on average 0.5 mg of resveratrol [362], but commercial formulations use up to 500 mg [363]. However, even though human trials have used these commercially available products, these results have been inconsistent.

Although nutraceuticals are not subject to the same level of regulation as pharmaceuticals, there is some governance over their health claims and most countries have an associated regulatory framework. While regulation is important for quality assurance, prohibition of claims on health benefits or disease prevention associated with food items limits consumers' ability to make healthy food choices [364]. Nutraceutical companies are regulated against making health claims until there is robust scientific evidence. However, this is not unreasonable due to safety concerns associated with the intake of high concentrations of individual polyphenols, which are powerful anti-oxidants. Several polyphenols have been shown to promote oxidative damage to DNA, lipids, and deoxyribose under certain conditions *in vitro* [365-369]. Safety is rarely reported in clinical studies. As long-term effects of supraphysiological doses of polyphenols are unknown, detailed safety profiles should be reported in future clinical trials in humans.

Nonetheless, supraphysiological doses of polyphenols appear to be the only method adopted to achieve measurable concentrations approaching those seen *in vitro* and in animal studies. It is possible that nutraceuticals containing concentrated polyphenols could potentially exert effects on human glucose homeostasis. Pharmaceutical companies have altered chemical structures to improve metabolism, and this may be a valid pathway for the nutraceutical industry. Whether nutraceuticals should deliver a single polyphenol or multiple polyphenols is yet to be determined, but it is still unknown whether nutraceuticals can actually deliver greater protective benefit against type 2 diabetes than a normal dietary intake of polyphenol-rich foods.

To clarify the role of polyphenols in glucose homeostasis, it is important that robust experimental designs are employed. Thus, future clinical trials should be consistent in design and outcome measurements to help reproducibility. Therefore, we suggest the following framework:

1. Pilot studies should precede clinical trials, and assess bioavailability and metabolism of the polyphenol being investigated to establish dose response and plasma concentrations.
2. Robust trial design, such as double-blind randomized placebo-controlled trial with crossover and washout periods.
3. Accurate or gold standard methods to assess insulin sensitivity should be used, e.g. hyperinsulinemic euglycemic clamp, minimal model, Matsuda method, etc.
4. When assessing the effects of polyphenols on glucose homeostasis, both the acute (e.g. mixed meal response) and chronic responses should be examined.
5. When assessing the effects of polyphenols on glucose homeostasis, consider measuring a range of peptides involved in glucose regulation beyond just insulin, such as IL-6, adipocytokines, and incretins.

**Conclusions**

Despite promising data from *in vitro* and animal studies, the influence of polyphenols on human glucose homeostasis has not been consistently shown. Further research in humans should adopt robust randomized placebo-controlled study design. Gold standard technics to assess insulin sensitivity should be used where possible. Researchers should investigate molecular pathways involved in glucose homeostasis that may translate into long-term health benefits that are not observed in short-term studies. However, a limitation in clinical studies is the heterogeneous bioavailability and rapid metabolism of polyphenols. In an attempt to overcome this issue, nutraceutical products are produced with high concentrations of polyphenols well-above the normal dietary intake, which may lead to adverse effects. Despite the paucity of robust data showing beneficial health outcomes associated with polyphenols in humans, these compounds already have a large commercial value. Further research in this area is urgently needed, as prescribable polyphenols to treat and preferably offset the type 2 diabetes pandemic are an exciting prospect.

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#### 1.4.2.6 Minerals

Minerals are important co-factors for many enzymes in the body, including those involved with insulin signalling and glucose homeostasis. Chromium and magnesium and have received the most attention, while others such as vanadium have only come to interest recently. Research into minerals and diabetes has stemmed from the realisation that mineral deficiencies are common in at population groups at risk of diabetes – such as the elderly[370].

Chromium has been an obvious candidate for research into glucose homeostasis, as it is a cofactor for insulin. Chromium increases insulin binding, the number of insulin receptors, and insulin receptor phophorylation. Further, chromium inhibits phosphotyrosine phosphates (which reduce insulin sensitivity at the insulin receptor level). In the normal diet, rich sources of chromium include broccoli, beer, whole grains, mushrooms, cheese, oysters, and liver. However, modern food technology (i.e. the use of refined grains) has dramatically lowered chromium intake[255]. A proviso here is that most individuals with type 2 diabetes are not chromium deficient[253].

Chromium picolinate has been the preferred preparation of chromium used for supplementation as it is well established as having the best bioavailability[255]. There are 12 published randomized controlled trials investigating chromium picolinate supplements in type 2 diabetes, and all but two suggested positive effects[255]. With regard to the negative studies, one was performed in poorly controlled patients, and another was of very short duration[255]. Three trials specifically examined insulin sensitivity. Martin *et al*. used a double blind randomized controlled trial study design, and the gold standard hyperinsulinemic euglycaemic clamp to measure insulin sensitivity to demonstrate substantial improvement[371], however there was a significant improvement in fat mass while on chromium supplementation which would have contributed to this result. Morris *et al*. showed improved glucose uptake using an insulin tolerance test, and provided further evidence using HOMA-IR[372]. Finally, Ravina *et al*.[373] performed a study investigating insulin sensitivity following chromium supplementation, but unfortunately this study could not be accessed for critique into the methodology. Turning our attention to healthy individuals, a meta-analysis including 15 randomized controlled trials found chromium had no effect on glucose or insulin concentrations, but this review did not allow for differences in chromium form (this is important because some forms have poor bioavailability)[374]. Based on positive results in a well designed long term study in obese non diabetic individuals, which measured insulin with the gold standard minimal model technique[375], the US Food and Drug Administration (FDA) issued a qualified health claim

for chromium picolinate in diabetes prevention, but pointed out the relationship was uncertain[376]. While the collective results in type 2 diabetes management are encouraging, it should be noted that much of the data comes from China which may have regional nutritional effects, and that the dose ranges of chromium used are variable. As such, replicating these studies in Western populations is pertinent. Reassuringly, there have been no concerns to date regarding toxicity[376].

Magnesium has been identified as a potential beneficial supplement for patients with diabetes due to i) its action as a cofactor for many enzymes involved with glucose homeostasis[253], ii) the fact that magnesium depletion is common in patients with severe type 2 diabetes[253], and iii) epidemiological evidence suggesting those with the highest magnesium intake have lower rates of type 2 diabetes [377]. Despite this, the results of clinical trials have been disappointing because inconsistent results have been demonstrated[253].

Vanadium is a trace element found in very small quantities in food, and has insulin-mimetic properties[378]. Small non-randomized clinical trials suggest a positive effect on glucose homeostasis[253], however gastro-intestinal side effects are common and will likely dampen further efforts into researching this element.

#### 1.4.2.7 Vitamins

Vitamins A, C and E are strong anti-oxidants, and have therefore been investigated as potential agents in the treatment of type 2 diabetes. Vitamin E (the tocopherols) is the most studied due to its direct free radical scavenging properties[379]. While animal and observational studies initially suggested positive results[379], a large (n = 38,716) long term (median 10 year follow up) prospective study showed vitamin E supplementation did not prevent type 2 diabetes [380]. With respect to trials in patients with type 2 diabetes, in four out of six published studies the direction was favourable, but the largest of the studies showed no improvement[253].

Vitamin C is also a strong anti-oxidant, and while population data shows a strong inverse relationship with diabetes risk, clinical trials investigating Vitamin C supplementation in diabetic patients have mainly been negative[379]. There is also some concern with regard to Vitamin C, as when it is consumed in very high doses, there may be an elevated risk of CVD mortality in post-menopausal women with type 2 diabetes[381].

The role of Vitamin A and carotenoids is still exploratory, but is based on anti-oxidant properties and the fact that its carrier protein (retinol binding protein) induces insulin resistance in animal models and in humans[379]. There are no randomized controlled trials investigating Vitamin A and carotenoids alone.

Literature on Vitamin D and diabetes is expanding exponentially, and while the normal way to obtain Vitamin D is through sun exposure, as it is also supplemented orally, the recent literature is worth highlighting. Large long term epidemiological cohort studies show a strong association between vitamin D deficiency and the incidence of type 2 diabetes [382, 383]. However, it is broadly accepted that population data are confounded as Vitamin D is a good marker of excellent health. In a recent meta-analysis of 8 observational studies, higher vitamin intake (>500IU per day) decreased the risk of type 2 diabetes by 13% compared to those taking less than 200IU per day[384]. Pancreatic β cells have Vitamin D receptors, and mechanisms of action have been proposed to improve glucose homeostasis: improved insulin secretion, increased expression of the insulin receptor, enhanced insulin responsiveness, and improved systemic inflammation[385]. In pooled analysis from clinical trials, eight studies in patients with normal glucose tolerance, and in three small trials studying patients with type 2 diabetes, there was no demonstrated improvement in parameters of glucose homeostasis[384]. Hence while there are plausible links between Vitamin D and improved glycaemia, the population data are severely confounded, and clinical trials do not support vitamin D supplementation to improve glucose homeostasis in type 2 diabetes.

#### 1.4.2.7 Summary

Chapter one has established the wide ranging health repercussions of disordered glucose homeostasis, and how insulin resistance and resultant hyperinsulinism is a central pathogenic process. Knowledge of insulin signalling, and the many aspects of insulin resistance that have been discovered over the last few decades have allowed researchers to find possible interventions to improve insulin sensitivity. Ongoing research into possible ways to improve insulin sensitivity such as that contained in this thesis is central to developing novel approaches to improve this burden of disease. However, measuring insulin sensitivity is complex and labour intensive, and hence proxy measures have been developed. A non-pharmacological nutritional approach to improving insulin sensitivity is a well established tool in managing diabetes, and also has an important preventative role, but there are several unanswered

questions. Thefore the following aims were formulated and investigated in the original research contained in this thesis hereafter:

1) To investigate the impact of supplemented fibre alone insulin sensitivity in an adolescent population at high risk of developing type 2 diabetes later in life
2) To investigate the bioavailability of olive polyphenols when ingested as olive leaf extract, containing concentrated levels of olive polyphenols (particularly oleuropein and hydroxytyrosol), and the effects of dose and preparation (liquid or capsule) on absorption and metabolism
3) To investigate the impact of supplemented olive leaf polyphenols alone on insulin sensitivity in middle aged overweight mean, who by virtue of their body habitus, are at risk of developing type 2 diabetes.

# Chapter 2. Methods

In the upcoming chapters (Chapter 3, Chapter 4, and Chapter 5) a series of manuscripts are included. While these manuscripts include methods sections, on the whole they are brief. The purpose of this chapter is to expand on and provide rationale for the scientific methods chosen in the experimental trials where it is not provided in the manuscripts and deemed relevant. Chapter 3 and Chapter 5 are both randomized placebo controlled clinical trials investigating the effect of different nutritional supplements on insulin sensitivity in two very different population settings. The two trials share some methodology, and when this occurs it shall be highlighted. Chapter 4 describes an experiment investigating the bioavailability of the nutritional supplement tested in Chapter 5. As the manuscript included in Chapter 4 has a detailed methods supplement describing the chemical analysis, it is not included in the current chapter.

Other methods that are considered aptly described in the manuscripts and will not be expanded upon. Specifically these are: height and weight protocols, biochemical assays (glucose, insulin, lipids, cytokines, inflammatory markers, liver function tests), and assessment of wellbeing.

## 2.1 Clinical trial design

Chapter 3 and Chapter 5 share the same trial design; they are randomized placebo controlled, blinded (participant-blinded in Chapter 3, double-blind in Chapter 5) cross-over trials. The basic framework of such a study is shown in Figure 9.. Between treatment phases a defined washout period was used. The primary advantage of the cross-over design is that a comparison of two different treatments on the same individual is obtained and is considered more precise than the assessment of two different treatments on two different subjects (for example when using a parallel design). This is because intra-individual variability is assumed to be less than inter-individual variability[386]. From a practical perspective, a significant advantage when using a cross-over design is that only half the amount of recruits are required compared to a parallel design[387], although each participant needs to be studied more than twice as long.

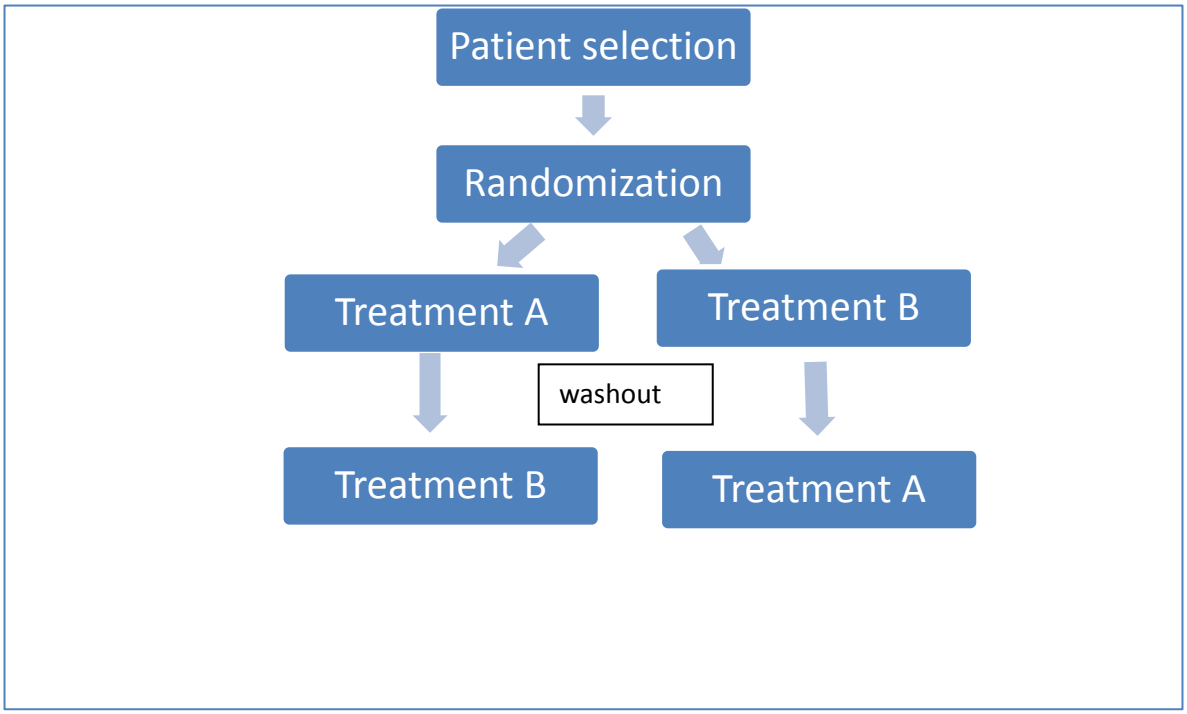


Figure 10: Crossover trial design.

There were important potential deficiencies of using a cross-over design that were considered before making this choice. Firstly, because each participant is required to be studied for longer (compared to other possible study designs), patient drop out can become problematic and compromise results. Secondly, possible sequence effects can arise. Thirdly, there is the potential for residual effects (carry-over from first sequence) to impact on findings, and finally changes that occur irrespective of the intervention may further confound results[387]. In the cross-over experiments contained in this thesis, attempts to minimise for these potential short comings of the crossover trial were made by including long washout periods between phases, by controlling for factors that are known to affect the primary outcome (which in our case is insulin sensitivity) by collecting data on diet and exercise, and also by including only male participants in order to avoid the effects of the menstrual cycle on insulin sensitivity. Further, the statistical method used (as described in the manuscripts) checked for any sequence effect. While the length of time each participant was in the study was considerable, there was minimal participant drop out in the studies.

# 2.2 Measurement of insulin sensitivity

In both clinical trials, the Matsuda method was used to assess insulin sensitivity. As described in section 1.3 the Matsuda method is the best performing of the proxy methods designed to assess insulin sensitivity[129]. The Matsuda method is based on a 75g oral glucose tolerance test with plasma glucose and insulin values taken at 0, 30, 60, 90 and 120 minutes. It provides a composite measure of whole body insulin sensitivity (hepatic and peripheral tissue) and considers insulin sensitivity both in the basal state and after the ingestion of a glucose load. The following formula was derived to give an insulin sensitivity index (ISI), as originally described by Matsuda and DeFronzo[388]:

$$ISI = 10000 \div \sqrt{[(G_{fasting} \times I_{fasting}) \times (G_{OGTT\ mean} \times I_{OGTT\ mean})]}$$

In this formula the square root is to correct for the nonlinear distribution of insulin and the 10,000 is a scaling factor.

There are good scientific reasons why the Matsuda method was chosen over the gold standard hyperinsulinaemic-euglycaemic clamp. For example, when using the hyperinsulinaemic-euglycaemic clamp, steady state is attempted, which is less representative of the normal highly dynamic relationship between glucose and insulin, so clamping may not accurately reflect insulin sensitivity or insulin resistance under 'normal' physiological conditions. A key reason for this may be the secondary pathways involved in glucose and insulin metabolism which have their own control systems and may respond to prolonged infusion of insulin in an unrepresentative manner. Such pathways can operate at the level of the gut as well as peripheral tissues providing further justification for the use of oral-based dynamic challenges to insulin secretion and glucose uptake such as the Matsuda method.

There were also numerous practical reasons for why the Matsuda method was chosen. The clamp method is time consuming, labour intensive, and requires medical supervision. Further, for studies in young people, the clamp method is not recommended due to its invasive nature and safety concerns[134] (particularly important as one of our studies was in adolescents).

The Matsuda index has been validated against the hyperinsulinaemic-euglycaemic clamp in 153 subjects (66 men and 87 women, aged 18–71 years, BMI 20–65 kg/m$^2$) with varying degrees of glucose tolerance (62 subjects with normal glucose tolerance, 31 subjects with

impaired glucose tolerance, and 60 subjects with type 2 diabetes)[388]. Whole-body insulin sensitivity measured with the hyperinsulinaemic-euglycaemic clamp correlated closely with the Mastuda index in subjects with normal glucose tolerance ($r$ = 0.73, $P$ <0.0001), with impaired glucose tolerance ($r$ = 0.66, $P$<0.0001) and with type 2 diabetes ($r$ = 0.54, $P$<0.0001). Mastuda's index gave better correlations than the ISI(HOMA) ($r$ = 0.69, $P$<0.0001), ISI(Ceder) ($r$ = 0.62, $P$<0 .0001) and ISI(Bel) ($r$ = 0.54, $P$<0.0001) estimates of whole-body insulin sensitivity compared with the hyperinsulinaemic-euglycaemic clamp. The correlation coefficients derived from the Cederholm and Belfiore estimates were significantly lower and there was no correlation between the G/I ratio during the OGTT and the hyperinsulinaemic-euglycaemic clamp [388]. The Matsuda method has also been shown to be reproducible for moderately sized clinical trials requiring repeated measurements and also appropriately ranked categories of fasting glucose tolerance[132].

## 2.3 Measurement of body composition

Dual-energy x-ray absorptiometry (DEXA) (Lunar Prodigy 2000, General Electric, Madison, WI, USA in conjunction with the software package Encore 2007 v.11.40.004,). was the method chosen to analyse body composition in the two randomized clinical trials. Dual-energy x-ray absorptiometry derives an image from photons that are attenuated differently dependent upon the impedance of different body tissues[389]. It is necessary to measure body composition, as total body fat and fat distribution are important modulators of insulin resistance and other parameters of the metabolic syndrome[390]. Accurate assessment of total and body fat are required in order to ensure subtle changes are not responsible for any observed changes in insulin sensitivity.

There are several ways in which composition can be measured as a predictor for type 2 diabetes risk, but DEXA is the one of the most accurate non-invasive methods. For example DEXA has a co-efficient of variation of 0.8% for body fat, compared to up to 10% in magnetic resonance imaging (MRI) techniques[389]. In terms of predicting for future type 2 diabetes, simple waist circumference has been shown to perform best compared to skin fold thickness, BMI, and waist-hip ratio[391] which highlights the importance of central fat distribution. It follows then, that DEXA which calculates an android (central) to gynoid (hip) fat ratio with precision serves an excellent predictor for future metabolic disease[390]. The details of the DEXA equipment and protocols used are included in the accompanying manuscripts.

## 2.4 Measurement of blood pressure

Raised blood pressure is an important predictor of CVD. Mechanisms through which insulin resistance can lead to hypertension have been discussed previously in section 1.1.1. Standard bedside blood pressure measurement provides a poor estimate of risk in individual patients due to poor technique of the observer, the "white coat" effect, and the inherent variability of blood pressure[392]. Ambulatory blood pressure on the other hand is a sensitive tool that is able to determine a mean blood pressure, but also detect diurnal variation, as well as providing a crude insight to variation within the day[392]. Abnormal diurnal variation, for example loss of nocturnal dipping, and nocturnal hypertension are stronger predictors for CVD mortality than mean blood pressure (especially in normotensive patients)[393, 394]. For these reasons, ambulatory blood pressure monitoring is considered the gold standard for blood pressure monitoring[392]. Hence, ambulatory blood pressuring monitoring was used in both clinical trials, and details of the protocol used are included in the manuscripts.

## 2.5 Collection of nutrition information – food diaries

Short term changes in dietary patterns (macronutrient proportion and total energy) can acutely influence insulin sensitivity; hence an accurate assessment of diet whilst participating in an interventional trial like ours is required. The prospective food diary is recognised as the most accurate "gold standard" assessment of dietary intake (apart from supplying all food), provided the participants are motivated and able to complete the diary[395]. However, there are potential pitfalls with using a prospective food diary – inaccurate records, diets that change depending on the day of the week, and participant fatigue can all affect the quality of the data collected[396]. We elected not to use a food frequency questionnaire such that is commonly used in epidemiological studies, as this method is not sensitive for acute dietary changes.

In both clinical trials a three day food diary was collected prospectively in the week prior to assessment, the template and instructions for which are shown in Appendix I: Food diary. Participants were instructed to fill out two week days, and one weekend day to account for

changes in dietary patterns that often occur in the weekend (in order to minimize the impact of diet changes according to the day of the week). Participants were encouraged to fill the diary out each time they ate any food, rather than rely on recall at the end of the day, or three days. All participants were instructed how to fill out the diary, and shown common household measures (metric cup, teaspoon etc.) to refer to, so that volumes were accurately recorded. Further, when participants arrived for assessment, the diaries were collected and checked for missing items and any ambiguous foods or volumes were clarified. As a single investigator was responsible for training and checking, this helped to minimize any potential for inaccuracy or inconsistency. The data from each food diary was then entered into the Foodworks software program (v6.0, Xyris Software, Brisbane, Australia) which allowed for total energy and macronutrient ratios to be calculated utilizing the New Zealand FOODfiles 2010 database (see http://www.foodcomposition.co.nz/). The same single investigator responsible for food diary training and checking was also responsible for entering data into the Foodworks software, further reducing error by ensuring that any substitutions used when entering data were consistent. As a cross-check, 3 food diaries and their analysis were independently reviewed by a qualified nutritionist, and all results were within a 10% error margin.

## 2.6 Collection of exercise data

As exercise acutely influences insulin sensitivity[397], validated ways of assessing the exercise levels of participants during a trial like ours are required to reduce the impact exercise levels may have on study outcome. A common flaw in many of the clinical studies that were discussed in Chapter 1 is that no attempt was made to assess exercise level. Assessment of physical activity is possible in at least three different ways. The most accurate of these methods is using criterion methods (for example double labelled water or indirect calorimetry), however these are labour intensive and invasive. Next are objective measures, for example the use of pedometers and accelerometers, and finally the least accurate are subjective methods, which require validation against objective measures[398]. Criterion methods were not appropriate for the clinical trials in this thesis as they are overly invasive and burdensome, particularly when used for a measure that was not a primary outcome of the study. Objective measures also have draw backs – for example pedometers and accelerometers are not very accurate at assessing energy expenditure[398]. A further consideration is cost, and inconvenience to the study participant. Given participants were already burdened with ambulatory blood pressure

monitoring and several visits to the assessment unit, it was opted to use subjective measures, choosing validated methods.

In Chapter 3, the trial enrolled teenagers, and hence the Physical Activity Questionnaire for Adolescents (PAQ-A) (University of Saskatchewan, Saskatoon, Canada) was chosen. The PAQ-A is a retrospective 7 day activity and exercise recall. The PAQ-A has established convergent validity – that is, it correlates well with other exercise questionnaires, and objective measures[399]. As the PAQ-A was initially designed for a Canadian population, for use in this trial, it was modified slightly to reflect activities common to New Zealand youth (for example ice skating was replaced by skateboarding). The modified PAQ-A used is included in Appendix II: Physical activity questionnaire for adolescents.

In chapter 5, the trial enrolled middle aged men, and therefore the International Physical Activity Questionnaire (IPAQ) was used. The IPAQ has been used extensively, across a diverse range of populations. It covers activity across four domains – transport, home, work and leisure. Validity has been shown against accelerometer data[400]. The IPAQ was used unmodified, and is included in Appendix III: Physical activity questionnaire for adults.

## 2.7 Measurement of carotid intima media thickness

Atherosclerosis causing coronary artery obstruction or leading to embolic cerebrovascular events is a common CVD endpoint. As such, investigators have identified tools that predict atherosclerosis – namely endothelial function. Typically, this involves methods such as the Brachial Index, which examines elasticity of arteries through measurements of the pulse wave. However, as technology has advanced, it is now possible to directly measure early atherosclerosis by radiological imaging. Imaging techniques include MRI, computed tomography (CT), and angiography but these are either prohibitively expensive or overly invasive for a research setting requiring multiple assessments.

The ultrasound technique of measuring carotid intima-media thickness (cIMT) is non-invasive, rapid, proven to accurately assess treatment effects, and is a validated and reproducible measure that is predictive of cardiovascular and cerebrovascular risks[401]. Hence this method was chosen, and a single investigator was trained to perform the measurement by an experience radiologist. cIMT was measured using an M-Turbo ultrasound system (Sonosite, Bothel, USA),

with images attained based on a standard protocol[402]; the right common carotid artery was scanned with study participants in a supine position and with the neck slightly extended and rotated away from the scanning probe. The image was focused on the far wall of the right common carotid artery wall, focusing on the area proximal to the carotid bulb as shown in Figure 11. Gain settings were used to optimize image quality. The probe angle was adjusted to create an image that showed the greatest distance between the lumen-intima interface and the media-adventitia interface. At least four images were recorded and frozen at the end of diastole. Digitally stored images were analysed by the same single investigator who was trained in and performed the scans using computer software automated callipers (SonoCalctm v.4.1, Sonosite). A maximal cIMT measurement approximately 10 mm proximal to the carotid bulb was used for comparative. To assess reproducibility, triplicate measures were taken of seven healthy volunteers over a 7-day interval, and resulted in an intra-observer co-efficient of variation (CV) of 3.7%.

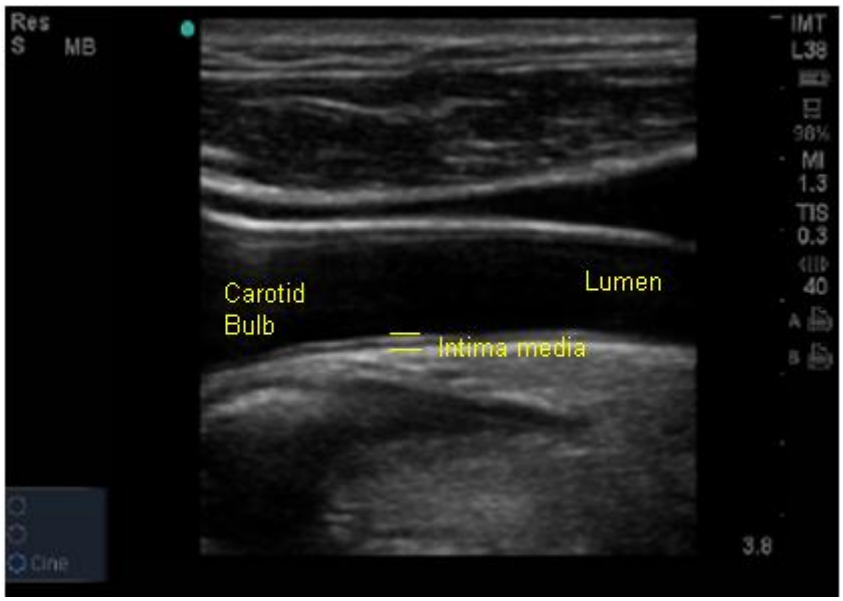


Figure 11: cIMT image.

*An image obtained from ultrasound of the cIMT, identifying important landmarks, for example the carotid artery lumen, the carotid bulb, and the intima media measured approximately 10mm proximal to the carotid bulb*.

# 2.8 Resting energy expenditure (resting metabolic rate)

Body weight and anthropometry is known to influence resting energy expenditure (REE), and REE was thus assessed during the second clinical trial in adult males (Chapter 5)[403]. REE reflects energy consumed at rest and what fuels are oxidised[403]. REE is affected by a number of things including prior physical activity, ambient temperature and food consumption. In order to reduce the impact of physical activity on results, REE measurement was performed as the first investigation after participants arrived for their clinical testing session. REE was measured using open circuit indirect calorimetry using a transparent canopy in the dilution testing mode (TrueOne, Parvomedics). Participants lay supine in a temperature controlled room (22 – 24 degrees Celsius), which was quiet and darkened (Figure 12). Measurements were taken continuously for 30 minutes, and averaged to 1 minute intervals. The first 10 minutes were discarded so steady state was ensured. The REE was calculated using an abbreviated Weir equation (without urinary nitrogen): REE (kilocalories per day) = (3.9 VO2 + 1.1 VCO2) * 1.44. The REE result was expressed in the unit ‘kilocalories per 24 hours’. The TrueOne dilution method is considered the best performing method to measure REE from available machines[404].

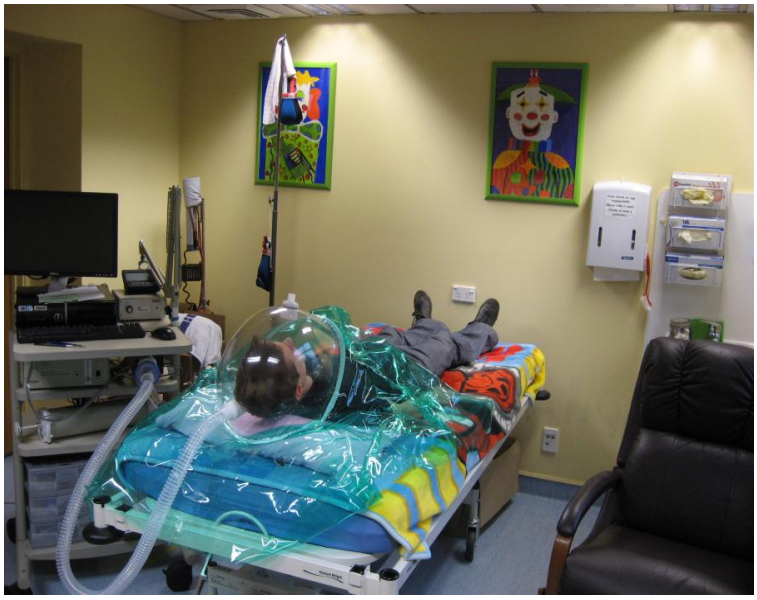

Figure 12: Resting energy expenditure measurement using the Parvo system.

## 2.9 Additional biochemical assays

In chapter 3, active GLP-1 was measured and is not mentioned in the published manuscript, the results for which are included as supplementary data after the included publication. Active GLP-1 was measured by ELISA (Linco Research, Missouri, USA) and glucagon by Radioimmunoassay (Millipore, Missouri, USA). The assays were performed by an experienced technician offsite.

Particular care was required to stabilize the drawn blood sample at the point of collection, as GLP-1 is quickly broken down under normal circumstances. 2mL of whole blood was placed immediately into EDTA tubes on ice, which contained added aprotonin (400KIU/mL of blood), and DPPIV inhibitor (10μl/mL of blood). As soon as practically possible the samples were spun down and plasma was frozen at -80 degrees Celsius until time of analysis.

# Chapter 3. Can supplemented psyllium improve insulin sensitivity in an adolescent population at high risk of developing metabolic syndrome?

**Preface to publication:**

In section 1.4.1.2 it was recognized that no prospective studies investigating the effects of supplemented dietary fibre alone on insulin sensitivity had been published. The following manuscript details the first such investigation. The rationale for doing the trial is on the basis that early interventions are likely to have longer lasting (and possibly preventative) effects, and that adolescents living in poor socioeconomic areas have higher risk of developing metabolic disease later in life.

Psyllium (the husks of the seeds from the plant Plantago *ovata*) was the selected source of dietary fibre used as a supplement for our adolescent participants. Psyllium husks encompass a mixture of neutral and acid polysaccharides containing galacturonic acid, with a 70/30 ratio of soluble/insoluble fibre. This dietary fibre was chosen as it has an established safety record in children (managing familial hypercholesterolemia), is well tolerated with a minimal side effect profile, and because psyllium can be (and already is) added to foodstuffs easily without seriously affecting taste or texture, meaning it could also have a possible role in public health initiatives.

The following section contains an unaltered reproduction of the manuscript "Psyllium supplementation in adolescents improves fat distribution & lipid profile: a randomized participant-blinded placebo controlled crossover trial" published in the *PLOS one* 2012. **7**(7): p. e41735.

Following the manuscript, supplementary results are presented. Although the manuscript contains its own discussion, a broader discussion of the results of this and our other trials is presented in Chapter 6 to help place this data in perspective with current thoughts regarding nutrition intervention and insulin sensitivity.

# Psyllium supplementation in adolescents improves fat distribution & lipid profile: a randomized participant-blinded placebo controlled crossover trial

Martin de Bock[1], José G B Derraik[1], Christine M Brennan[1], Janene B Biggs[1], Greg C Smith[2], David Cameron-Smith[1], Clare R Wall[3], Wayne S Cutfield[1,*]

[1] Liggins Institute, University of Auckland, Auckland, New Zealand

[2] Department of Molecular Genetics, University of Auckland, Auckland, New Zealand

[3] Department of Nutrition, University of Auckland, Auckland, New Zealand

*Author for correspondence: Liggins Institute, University of Auckland, Private Bag 92019, Auckland, New Zealand; Ph: +64.9.923.5118; Fax: +64.9.373.8763; Email: w.cutfield@auckland.ac.nz

**Short title:** Psyllium supplementation trial in adolescents

**Keywords:** cholesterol, clinical trial, fibre, metabolic syndrome, *Plantago*, Psyllium, supplementation.

**Trial registration:** ACTRN12609000888268 (Australian New Zealand Clinical Trials Registry). http://www.anzctr.org.au/trial_view.aspx?id=320688

## ABSTRACT

**Aims:** We aimed to assess the effects of psyllium supplementation on insulin sensitivity and other parameters of the metabolic syndrome in an at risk adolescent population.

**Methods:** This study encompassed a participant-blinded, randomized, placebo controlled, crossover trial. Subjects were 47 healthy adolescent males aged 15–16 years, recruited from secondary schools in lower socio-economic areas with high rates of obesity. Participants received 6 g/day of psyllium or placebo for 6 weeks, with a two-week washout before crossing over. Fasting lipid profiles, ambulatory blood pressure, auxological data, body composition, activity levels, and three-day food records were collected at baseline and after each 6-week intervention. Insulin sensitivity was measured by the Matsuda method using glucose and insulin values from an oral glucose tolerance test.

**Results:** 45 subjects completed the study, and compliance was very high: 87% of participants took >80% of prescribed capsules. At baseline, 44% of subjects were overweight or obese. 28% had decreased insulin sensitivity, but none had impaired glucose tolerance. Fibre supplementation led to a 4% reduction in android to gynoid fat ratio ($p=0.019$), as well as a 0.12 mmol/l (6%) reduction in LDL cholesterol ($p=0.042$). No associated adverse events were recorded.

**Conclusions:** Dietary supplementation with 6 g/day of psyllium over 6 weeks improves fat distribution and lipid profile (parameters of the metabolic syndrome) in an at risk population of adolescent males.

## INTRODUCTION

The metabolic syndrome encompasses a set of biochemical and physical parameters that are associated with a greater risk for the development of type 2 diabetes mellitus and cardiovascular disease, and all cause mortality [405]. These parameters include central adiposity, blood pressure, lipid profile, and insulin sensitivity [406]. The emergence of the metabolic syndrome in the paediatric population is primarily a result of dramatic increases in childhood obesity [407], and tracks from adolescence into adulthood [408]. Therefore, a range of initiatives are frequently employed in the attempt to decrease the incidence of the metabolic syndrome and obesity in childhood. These are mostly community-based interventions, aiming to foster increased physical activity and dietary changes. Nutritional management in particular, varies greatly (from caloric restriction to changes in macronutrient composition and energy ratio), as there is a lack of consensus on the optimal approach [3].

In children, the effects of dietary fibres on parameters of the metabolic syndrome are not well established. Cross sectional data have shown that fibre and whole grain consumption in adolescence is associated with increased insulin sensitivity [409] and a lower incidence of the metabolic syndrome [175]. However, the majority of clinical studies have focused on dietary fibre combined with either exercise and/or other dietary interventions [183, 410]. Thus, to our knowledge, there have been no placebo-controlled clinical trials investigating the effect of supplementation with dietary fibre alone on parameters of the metabolic syndrome in adolescents.

Dietary fibres encompass a broad array of compounds (primarily of plant origin) with known physiological benefits, including laxation, and improvements in glucose homeostasis and cholesterol [171]. The gel-forming water-soluble fibres are those that appear to have the most beneficial effects on post-prandial glycemia [411]. Such fibres include the seed husks of psyllium (*Plantago* spp., in particular *P. ovata*), also known as ispaghula, which is a common dietary source of fibre often used to enrich cereals and other food items. Psyllium husks encompass a mixture of neutral and acid polysaccharides containing galacturonic acid, with a 70/30 ratio of soluble/insoluble fibre. Psyllium has been used safely in children and adolescents, and was shown to improve hypercholesterolemia [412-414]. In this study, we aimed to investigate the effect of psyllium fibre supplementation alone on insulin sensitivity and other parameters of the metabolic syndrome in an at risk adolescent population.

## MATERIALS AND METHODS

### Ethics approval

Ethics approval for this study was provided by the Northern Y Regional Ethics Committee. Written informed consent was obtained from participants and caregivers.

### Subjects

Healthy adolescent males aged 15–16 years were recruited from high schools in Auckland (New Zealand) to participate in the study (Figure 1). We targeted schools in lower socio-economic areas with high rates of obesity, in order to select adolescents at greater risk of developing the metabolic syndrome. Exclusion criteria included those receiving medications that alter glucose metabolism (e.g. steroids, stimulants, and insulin), and smokers. Participants provided written informed consent if they were 16 years and over.

**Study design**

This study was a participant-blinded, randomized, placebo controlled, crossover trial. Randomization and allocation to trial group were done using computer random number generation. All participants were randomized into a 6-week intervention with either 6 g/day of psyllium (*P. ovata*) (equating to 6 g of dietary fibre) or 6 g/day of potato starch placebo (Figure 13). The dose of 6 g/day was adopted based on review of the existing literature [415], as well as on the volume of fibre and placebo each dose would equate to, so as not to affect compliance with study protocol. After a 2-week washout period, participants crossed over to receive the opposite intervention for a further six weeks (Figure 13). Both the psyllium and potato starch were packed as 500 mg capsules (Douglas Pharmaceuticals, Auckland, New Zealand). The capsules were blister-packed to aid compliance, and participants were instructed to consume the 12 capsules per day with large amounts of water. Capsules could be consumed all at once or divided in doses, and with or without food. Adherence to dosing was monitored by counting non-consumed capsules in returned blister packs at the end of each 6-week intervention. Participants were advised to continue their normal eating and exercise patterns during the study period.

**Study parameters**

All clinical assessments were carried out at the Maurice & Agnes Paykel Clinical Research Unit (Liggins Institute, University of Auckland). Subjects were assessed at three time points after an overnight fast: baseline, end of the first 6-week intervention, and end of the second 6-week intervention (Figure 13). Height was measured using a Harpenden stadiometer. Weight and body composition were assessed using both body mass index (BMI) and whole-body dual-energy x-ray absorptiometry (Lunar Prodigy 2000, General Electric, Madison, WI, USA). Body composition data of interest were total percentage body fat and the ratio of android fat to gynoid fat. Note that android and gynoid fat values were determined by the manufacturer's software, based on an automated sectioning of specific areas of the body [416]. BMI data were converted to standard deviation scores (BMI SDS) according to British 1990 standards [417].

A fasting blood sample was obtained to assess metabolic factors. Glucose, triglycerides, cholesterol, HDL, and LDL concentrations were measured on a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) by enzymatic colorimetric assay (Roche, Mannheim, Germany) with an interassay CV of less than 2.5%. Insulin concentrations were measured using an Abbott AxSYM system (Abbott Laboratories, Abbott Park, IL 60064, USA) by microparticle enzyme immunoassay with an interassay CV of 5.4%. Insulin

sensitivity was assessed by a 75 g oral glucose tolerance test (OGTT) using the Matsuda method, with glucose and insulin samples collected at 0, 30, 60, 90, and 120 minutes [388]. The Matsuda method has a strong correlation with the hyperinsulinemic euglycaemic clamp (r=0.77) [129], and excellent reproducibility during multiple measures [132].

24-hour ambulatory blood pressure was assessed prior to each clinical assessment. Participants were fitted with a Spacelabs 90207 or 90217 (Spacelabs Medical Inc., Redmond, Washington, USA), with each subject being assigned the same device model for all assessments. Measurements were performed every 20 minutes from 0700–2200, and every 30 minutes from 2200–0700. Only profiles with a total of at least 40 readings over a 24-hr period were included for analysis [418].

Three dietary records were collected at baseline and at clinical assessment following each 6-week intervention. Each dietary report encompassed an itemized nutritional intake recorded during two school days and one weekend day. Nutritional intake was recorded using standard household measures, as well as the information from food labels where appropriate. Participants were instructed by a nutritionist [CW] on how to fill out the food diary accurately. A trained investigator [MdB] reviewed all food records with each participant to address unclear descriptions, errors, omissions, or doubtful entries. Records were subsequently entered into Foodworks software (v6.0, Xyris Software, Brisbande, QLD, Australia) by the trained investigator [MdB]. Accuracy of food record entry was also externally confirmed by the nutritionist [CW], randomly selecting and verifying 10% of all records.

Physical activity was assessed using the Physical Activity Questionnaire for Adolescents (PAQ-A) (University of Saskatchewan, Saskatoon, SK, Canada). Leisure activities were modified to reflect those engaged by New Zealand youth. This self-administered 7-day recall questionnaire has been validated for use in adolescents [399].

Demographic data were also collected on all subjects. Socio-economic status (SES) was classified using the New Zealand Index of Deprivation 2006 (NZDep2006) [419]. This uses household census data reflecting nine aspects of material and social deprivation to divide New Zealand into tenths (scored 1–10) by residential address, where a higher score reflects lower SES [419].

**Statistical analysis**

Baseline data associations were assessed using simple linear regressions, but the association between SES and insulin sensitivity was examined using non-parametric Spearman's rank correlation. Baseline analyses were carried out in Minitab v.16 (Pennsylvania State University, State College, PA, USA). Crossover trial data were analysed in SAS v.9.2 (SAS Institute, Cary, NC, USA) using a linear mixed model design based on repeated measures, which accounted for treatment sequence (Placebo→Fibre vs Fibre→Placebo), treatment phase (Stage 1 vs Crossover; Figure 13), ethnicity, SES, as well as participant as a random factor. Importantly, models also incorporated the baseline value of the outcome response as a co-variate, to account for the different starting points for each subject at the beginning of the study. The Johnson transformation was adopted when necessary to stabilize the variance. Data are expressed as means ± SEM.

**RESULTS**

A total of 47 healthy adolescent males (aged 15.8 ± 0.1 years at the start of the study) met the inclusion criteria and were enrolled in the study. Randomization order was established prior to recruitment of subjects, and we aimed for a minimum of 42 subjects (i.e. 21 in each group) as required by the power calculation to detect a 25% change in insulin sensitivity [420]. At the point at which we had enrolled and successfully studied 45 subjects it was obvious that study failure rates were far lower than anticipated, thus the study recruitment was stopped. This explains the uneven ratio of subjects randomly allocated between groups 1 and 2 (Figure 13). Subsequently, two participants were lost to follow up, and were excluded (Figure 13).

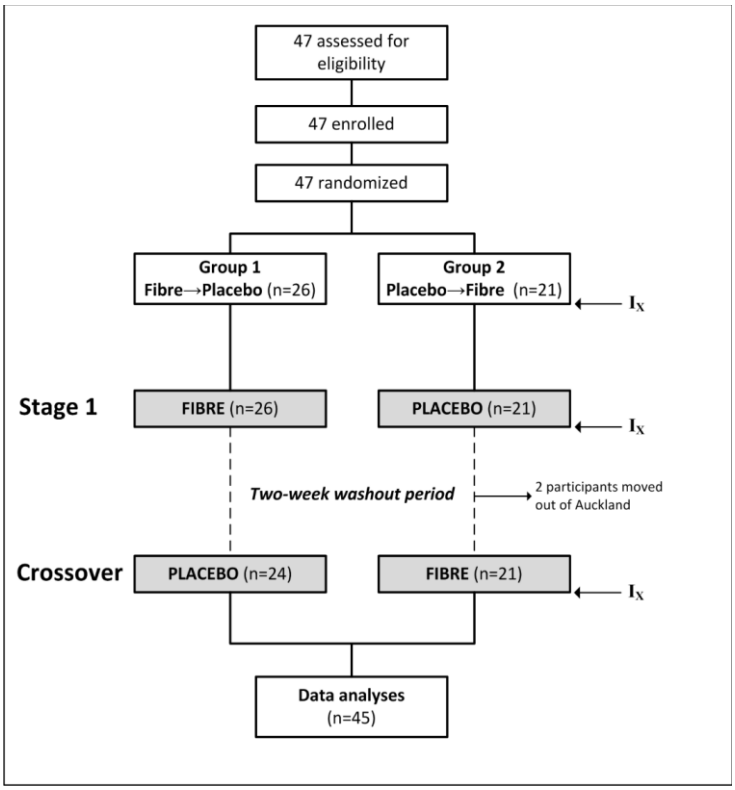


Figure 13: Summary of study's recruitment process and trial execution.

*$I_X$ indicates timing of assessments.*

All participants were from areas of lower SES, with 44% from the lowest quintile of SES in New Zealand. Subjects were of Pacific Island (46%), European (37%), Maori (15%), and Indian (2%) ethnicities. Mean BMI at baseline was 25.8 ± 0.7 kg/m$^2$, with 24% of subjects obese (BMI ≥30 kg/m$^2$) and a further 20% overweight (BMI ≥25 but <30 kg/m$^2$); mean percentage body fat was 23.5 ± 1.7%. Participants' compliance with the study was very high: 87% of participants took more than 80% of prescribed capsules over the 12 weeks of intervention. No associated adverse events, including gastrointestinal, were recorded during this study.

Mean pre-study dietary fibre intake was 23.1 ± 1.7 g/day (Table 14: Baseline daily dietary parameters among study subjects.), with only 33% of subjects meeting the recommended daily intake of 28 g/day for this age group [421]. As a result, the addition of 6 g/day of psyllium during the treatment period equated to a mean individual increase in daily dietary fibre intake of 36.4 ± 4.6%, with an equivalent 50% or more increase recorded in four subjects. Baseline data demonstrate a high intake of energy derived from fat, including saturated fat (Table 14).

**Table 14: Baseline daily dietary parameters among study subjects.**

| Dietary parameter | Mean ± SEM |
|---|---|
| **Total energy (kJ)** | 10673 ± 560 |
| **Fibre (g)** | 23.1 ± 1.7 |
| **Calories from fat (%)** | 35.9 ± 1.2 |
| **Calories from saturated fat (%)** | 15.9 ± 0.7 |
| **Calories from carbohydrates (%)** | 43.5 ± 1.6 |
| **Calories from sugars (%)** | 16.2 ± 1.1 |
| **Calories from protein (%)** | 17.0 ± 1.2 |

**Baseline**

Insulin sensitivity at baseline was positively associated with mean daily intake of dietary fibre ($r^2$=0.20; p<0.01; Figure 14), and inversely associated with BMI SDS ($r^2$=0.38; p<0.001; Figure 15). SES was also correlated with insulin sensitivity (p=0.037), so that the higher the index of deprivation the lower the Matsuda index (ρ=-0.31). 28% of subjects were insulin resistant with a baseline Matsuda score lower than 2.5. BMI SDS was associated with baseline triglycerides ($r^2$=0.24; p<0.001), total cholesterol ($r^2$=0.26; p<0.001), LDL ($r^2$=0.26; p<0.001), HDL:LDL ratio ($r^2$=0.19; p<0.01), but not HDL ($r^2$=0.00; p=0.65) concentrations.

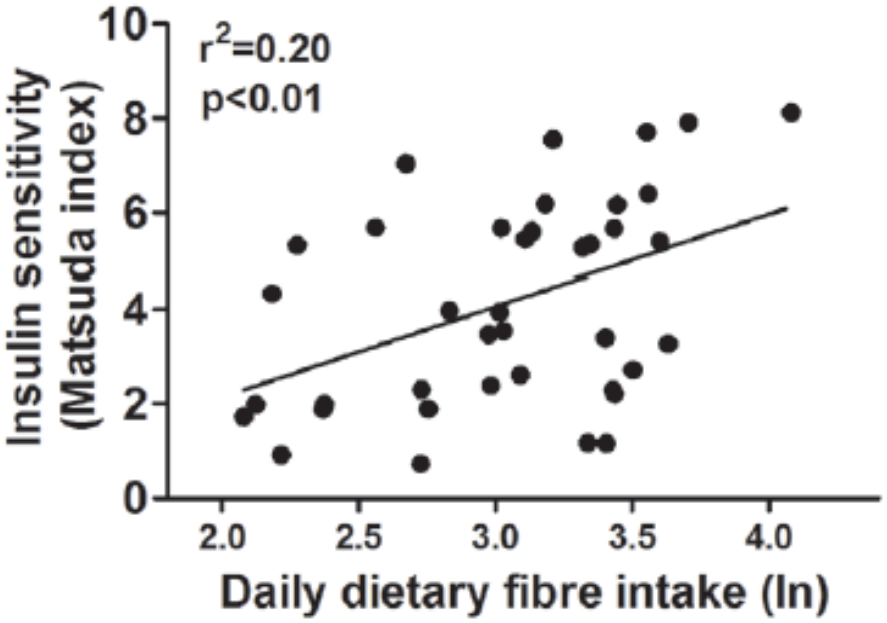


Figure 14: The association between baseline daily dietary fibre intake (log-transformed) and insulin sensitivity (Matsuda index).

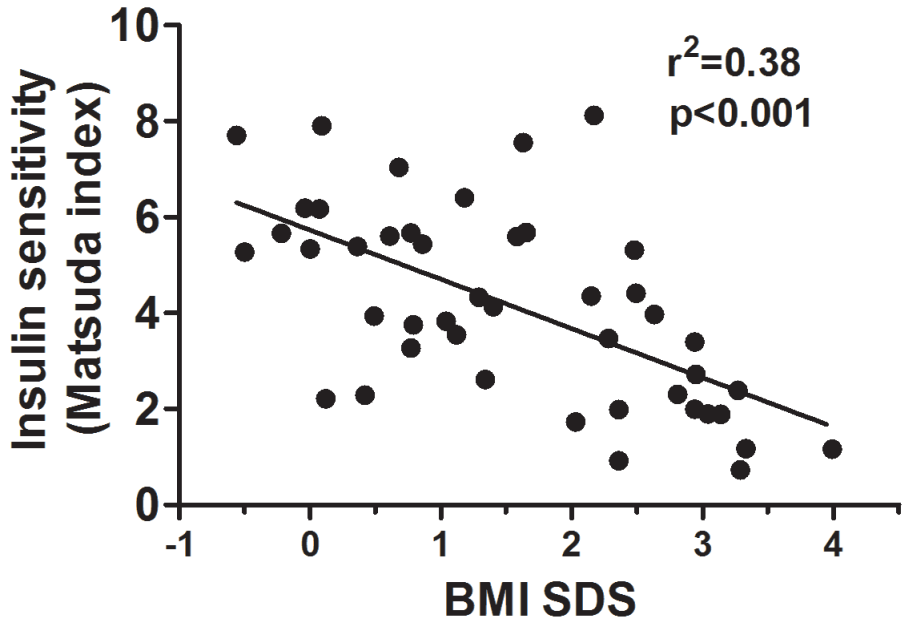


Figure 15: The association between BMI SDS and insulin sensitivity (Matsuda index).

**Crossover trial**

Dietary intake among individual participants did not change significantly throughout the study. Thus, total caloric intake (p=0.43), total fibre intake (p=0.44), and the percentage of total calories from saturated fat (p=0.17) at baseline were not different to the respective intake consumed during placebo and fibre treatment. In addition, there was also no change in physical activity levels among groups throughout the study periods.

Although fibre supplementation did not lead to a reduction in weight, BMI SDS, or body fat percentage, it did lead to a 4% reduction in android to gynoid fat ratio (p=0.019; Table 15). Psyllium supplementation also led to a 0.12 mmol/l (6%) reduction in LDL cholesterol (p=0.042; Table 15). There were no observed effects on insulin sensitivity, fasting plasma insulin, or glycemic status (i.e. fasting plasma glucose), irrespective of ethnicity, baseline fibre intake, or BMI SDS. Ambulatory blood pressure parameters were similar with placebo and fibre intake, except nighttime systolic blood pressure that tended to be on average 3.1 mmHg lower with psyllium supplementation (p=0.073; Table 15).

**Table 15: Outcome measures following a 6-week supplementation with 6 g/day of psyllium fibre or placebo. Data are means ± SEM, and p-values refer to results from multivariate models.**

| Variable | Placebo | Fibre | p-value |
|---|---|---|---|
| **Anthropometry** | | | |
| Weight (kg) | 83.4 ± 3.1 | 83.0 ± 3.0 | 0.65 |
| BMI (kg/m$^2$) | 26.2 ± 1.0 | 26.0 ± 0.9 | 0.92 |
| % body fat | 23.8 ± 1.7 | 23.4 ± 1.7 | 0.95 |
| Android to gynoid ratio | 0.99 ± 0.04 | 0.95 ± 0.04 | 0.019 |
| **Ambulatory blood pressure** | | | |
| Daytime diastolic (mmHg) | 69.8 ± 0.9 | 69.8 ± 0.9 | 0.86 |
| Daytime systolic (mmHg) | 123.9 ± 1.5 | 122.6 ± 1.3 | 0.44 |
| Nighttime diastolic (mmHg) | 56.2 ± 1.1 | 55.1 ± 0.8 | 0.57 |
| Nighttime systolic (mmHg) | 109.4 ± 1.7 | 106.3 ± 1.3 | 0.073 |
| Diastolic dip (mmHg) | 19.2 ± 1.4 | 20.3 ± 1.4 | 0.58 |

| | | | |
|---|---|---|---|
| Systolic dip (mmHg) | 11.4 ± 1.2 | 13.6 ± 1.0 | 0.13 |
| **Plasma** | | | |
| Glucose (mmol/l) | 5.20 ± 0.07 | 5.11 ± 0.06 | 0.19 |
| LDL (mmol/l) | 2.46 ± 0.09 | 2.32 ± 0.09 | 0.042 |
| HDL (mmol/l) | 1.19 ± 0.04 | 1.17 ± 0.04 | 0.40 |
| HDL to LDL ratio | 0.53 ± 0.03 | 0.54 ± 0.03 | 0.59 |
| Triglycerides (mmol/l) | 0.95 ± 0.06 | 0.94 ± 0.06 | 0.99 |
| **Hormones** | | | |
| Insulin (μU/l) | 14.8 ± 1.5 | 15.3 ± 1.5 | 0.51 |
| **Insulin sensitivity** (Matsuda index) | 3.88 ± 0.3 | 3.85 ± 0.3 | 0.90 |

## DISCUSSION

This is the first randomized, participant-blinded, crossover trial investigating the effects of psyllium supplementation on parameters of the metabolic syndrome in adolescents. Our data show that even in the context of a relatively short intervention, psyllium supplementation improves LDL cholesterol and android to gynoid fat ratio. Conversely, there was no improvement in insulin sensitivity, HDL, and blood pressure, which are the other parameters of the metabolic syndrome. These results have public health implications as commercial food manufacturers often use psyllium to fortify products such as cereal and baked goods to boost their fibre content.

Our study corroborates previous data showing that psyllium has lipid lowering properties in children and adolescents. The 6% improvement in LDL cholesterol concentrations we observed is comparable to other studies that have shown improvements of 0–23% using psyllium doses ranging from 5– 25 g/day [412-414, 422]. The lipid lowering action of soluble fibres such as psyllium occurs by binding bile acids and cholesterol, increasing faecal excretion of bile salts, and reducing cholesterol synthesis via production of short-chain fatty acids [423]. Importantly for this study, the reduction of LDL provides evidence that psyllium can be absorbed in the more palatable capsulated form.

We also observed a reduction in the android to gynoid ratio of fat distribution with fibre supplementation, which indicates a decrease in central adiposity. Similarly, a recent large descriptive study in adolescents showed decreased visceral fat among subjects with the highest fibre intake [424]. Thus, although we observed no change in BMI SDS, our findings are important as central obesity is an independent risk factor for the development of the metabolic

syndrome and associated cardiovascular disease risk [425]. Possible explanations for the observed effect in fat distribution include altered dietary fat lipolysis and subsequent absorption [426], or modulation of sex steroids that effect fat distribution [427]. Importantly, the results could not be explained by changes in exercise patterns.

In this study, psyllium supplementation over 6 weeks did not affect insulin sensitivity. However, previous studies in adults with type 2 diabetes showed that food supplementation with psyllium led to improved glucose metabolism, as examined by post-prandial glucose and insulin excursion [428, 429]. This improvement is likely explained by the solubility and viscosity of psyllium, which sequesters carbohydrate absorption [411], and delays gastric emptying and intestinal transit time [430]. In contrast, our study investigated the effects of psyllium on insulin sensitivity in the longer term. Anderson et al. have previously shown that supplementation with 10.2 g/day of psyllium over three days improves post-prandial glucose concentrations, but not insulin sensitivity (measured by euglycaemic hyperinsulinaemic clamp) in adults with type 2 diabetes [429]. Changes in insulin sensitivity would require additional physiological properties of psyllium, such as the production of short-chain fatty acids [190]. Thus, our null result may be explained by the poor fermentation of psyllium to produce short-chain fatty acids as compared to other sources of dietary fibre [431, 432]. However, the effects of short-chain fatty acids on insulin sensitivity are questionable [433], and these may even be deleterious in the long-term as observed in animal models [434]. A further possible explanation (and a weakness of our study) relates to our chosen method to assess insulin sensitivity; i.e. we adopted an oral glucose tolerance test rather than the labour-intensive gold standard euglycaemic hyperinsulinaemic clamp. One trial examining the effect of resistant starch on insulin sensitivity detected an improvement using the clamp technique, but did not demonstrate a difference using the Homeostasis Model Assessment (HOMA) proxy [435].

Commercial food producers have capitalised on the broad benefits of fibre, commonly using psyllium to enrich cereals and other foods. By definition, fibre encompasses a broad range of edible plant compounds, which have physiological health benefits including laxation, lowered cholesterol, and improved glucose metabolism [171]. However, given that dietary fibre encompasses such a diverse range of compounds, there is a wide variation in their physiological effects. The implication is that while psyllium is a highly soluble and palatable fibre that can easily be added to food products, it may not deliver all the health benefits associated with the consumption of different forms of fibre. While we do not dispute the overall benefits of dietary fibre, it is important that consumers and food producers become aware that not all forms of fibre are equal in terms of physiological action.

The adequate intake for dietary fibre for adolescents is 28 g/day in Australia-New Zealand [421]. Dietary fibre intake in our study population was poor, as only 40% of participants consumed 28 g/day or more. Our observation is not unusual, and similar figures have been obtained for other adolescent populations [436]. These findings are reason for concern, as a recent cross-sectional study in adolescents showed that those in the highest quintile of fibre intake had a three-fold reduction in the incidence of the metabolic syndrome compared to those in the lowest quintile [175].

In conclusion, we showed that fibre supplementation using psyllium improves fat distribution and lipid profile, even after a relatively short intervention of six weeks. Conversely, psyllium supplementation did not improve insulin sensitivity. Due to the enormous burden that cardiovascular diseases have on public health, our findings have potentially important public health implications. Continued awareness and promotion of the value of dietary fibre in the adolescent diet is required. It is possible that commercial food manufacturers, through fortification of food with dietary fibre such as psyllium, could play a role in the prevention of cardiovascular diseases. However, further research is warranted to investigate the best types of fibre, delivery method, dose, and length of treatment to determine the appropriate fibre supplementation and associated health benefits.

**Acknowledgement**

We thank Douglas Pharmaceuticals for supplying the capsulated psyllium and placebo. We would like to thank the LENScience Group (Liggins Institute, University of Auckland) for forging the relationship between the Liggins Institute and the secondary schools from which our study population was drawn. We acknowledge the Paykel Trust for long-term funding of the Maurice & Agnes Paykel Clinical Research Unit at the Liggins Institute.

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## Supplementary results

### GLP1 and Glucagon

There was no effect of psyllium supplementation on active GLP1 levels (p=0.43). There was also no baseline association of active GLP1 levels with BMI SDS (p=0.54), body weight SDS (p=0.5) or % body fat (p=0.95).

### Physical activity

The IPAQ-A score at baseline was 2.32 (+/- 0.71), where a level of 1 would is considered very low physical activity, and 5 the highest level. This value would reflect a moderate level of physical activity. There was no statistical difference in physical activity over the three assessment points.

# Chapter 4. How are the unique olive plant polyphenols absorbed and metabolised?

**Preface to publication:**

The medicinal use of the olive plant has occurred for centuries, including for the treatment of diabetes. The olive plant has unique polyphenols, namely oleuropein and hydroxytyrosol which have been shown in-vitro to improve aspects of glucose homeostasis (and is expanded upon in the following manuscript).

In section 1.4.2.6 it was highlighted that the absorption and metabolism of polyphenols in the human diet is highly variable, and dependent on many factors. It was also proposed that a systematic approach is required in order to reliable link clinical effects to laboratory data. The first stage is to verify that possible bioactive constituents of a food product are absorbed in meaningful concentrations, and to assess how rapid the metabolites are cleared from the body. The following manuscript is our approach to investigating the bioavailability and metabolism of oleuropein and hydroxytyrosol when ingested as part of an olive leaf extract complex. The effect of preparation and dose of the olive leaf extract were examined, as any well gender difference in bioavailability and metabolism.

The following manuscript is an unaltered version of that in the second stage of review in the journal: *Molecular Nutrition and Food Research*. The manuscript has been formatted as per the request of the journal into two sections. The first is continuous document highlighting the background, results and discussion. The second section has been prepared as an online supplement and describes the methodology in detail.

# Human absorption and metabolism of oleuropein and hydroxytyrosol ingested as olive (Olea europaea L.)leaf extract

Martin de Bock[1], Eric B. Thorstensen[1], José G B Derraik[1], Harold V Hen*derson*[2], *Wayn*e S Cutfield[1,3*]

[1] Liggins Institute, University of Auckland, Auckland, New Zealand

[2] AgResearch, Ruakura Research Centre, Hamilton, New Zealand

[3] National Centre for Growth and Development (NRCGD), University of Auckland, Auckland, New Zealand

*Author for correspondence: Liggins Institute, University of Auckland, Private Bag 92019, Auckland, New Zealand; Ph: +64.9.923.5118; Fax: +64.9.373.8763; Email: w.cutfield@auckland.ac.nz

**Short title:** Olive polyphenol bioavailability in humans

**Abbreviations:** AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; INR, international normalised ratio; OLE, olive leaf extract.

**Disclosure and conflicts of interest**

This study was supported by a TECHNZ grant (University of Auckland – UniS 30475.001) through the New Zealand Ministry of Science and Innovation (MSI). TECHNZ grants are funded 50% by the MSI, and 50% by a commercial partner following an extensive independent science review process. In this project, the commercial partner was the olive leaf extract manufacturer (Comvita). The first author [MdB] was funded by the Joan Mary Reynolds Trust. All authors are completely independent from the funders (Comvita and MSI), so that neither the MSI nor the capsules supplier (Comvita) had any role in study design, data collection and analysis, decision to publish, or preparation of this manuscript. All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare that (1) all authors have had no relationships with Comvita that might have an interest in the submitted work; (2) the authors' spouses, partners, or children also have no financial relationships that may be relevant to the submitted work; and (3) none of the authors have any non-financial interests that may be relevant to the submitted work.

**ABSTRACT**:

Polyphenols derived from the olive plant (*Olea europaea* L.), particularly hydroxytyrosol and oleuropein, have been shown to have many beneficial effects *in vitro*. Olive leaves are the richest source of olive polyphenols, and olive leaf extract (OLE) is now a popular nutraceutical taken either as liquid or capsules. To quantify the bioavailability and metabolism of oleuropein and hydroxytyrosol when taken as OLE, nine volunteers (5 males) aged 42.8 ± 7.4 years were randomized to receive either capsulated or liquid OLE as a single lower (51.1 mg oleuropein, 9.7 mg hydroxytyrosol) or higher (76.6 mg oleuropein, 14.5 mg hydroxytyrosol) dose, and then the opposite strength (but same formulation) a week later. Plasma and urine samples were frequently collected at fixed intervals for 24 hours post-ingestion. Polyphenol content was analysed by LC-ESI-MS/MS. Conjugated metabolites of hydroxytyrosol were the primary metabolites recovered in plasma and urine after OLE ingestion. There was wide inter-individual variation, with plasma time-course, peak concentrations, and area under the curve all influenced by preparation, dose, and gender. Olive leaf extract is an effective vehicle for delivering oleuropein and hydroxytrosol metabolites to plasma in humans.

**Keywords**: Bioavailability, hydroxytyrosol, oleuropein, olive leaf extract, polyphenols.

The health benefits associated with the Mediterranean diet are well established, including protection from cardiovascular disease, age-related cognitive decline, and cancer [437]. Such benefits were thought to be associated with the consumption of MUFA, but have more recently been attributed to the high intake of polyphenol rich olive oil [438]. Polyphenols derived from the olive plant (*Olea europaea* L.) have a range of beneficial health effects, with antioxidant, anti-inflammatory, anti-atherogenic, and anti-carcinogenic properties [439].

Previous literature on olive polyphenol metabolism has focused on olive oil consumption, as this is the most common dietary source. However, olive plant polyphenols are mostly concentrated in the leaves. These were previously discarded as by-products of fruit harvesting, but have recently emerged as valuable nutraceuticals. Despite the growing market of olive leaf extract (OLE), there is scarce literature examining the consequent absorption and metabolism of the principal polyphenols oleuropein and hydroxytyrosol. In contrast, the literature on olive oil polyphenols is extensive [440, 441].

The unique olive plant polyphenol is oleuropein, which is most abundant in the leaves (up to 264 mg/g of dry leaf, when expressed as tyrosol equivalents) [442]. Oleuropein concentrations are comparatively lower in extra-virgin olive oil [443], and especially so in refined oil due to its hydrolysis into tyrosol and hydroxytyrosol during processing [440]. It is important to investigate the fate of oleuropein and hydroxytyrosol ingested via OLE rather than olive oil, due to different polyphenol concentrations, chemical forms, and modes of delivery (e.g. capsules vs liquid). All of these factors are likely to affect metabolism, and should be explored before any bioactivity can be attributed to OLE consumption (as a nutraceutical).

Thus, we aimed to quantitatively determine the absorption and metabolism of oleuropein and hydroxytyrosol in humans, following acute ingestion of different doses and formulations of olive leaf extract. We used a previously validated technique [444], specifically looking for oleuropein, hydroxytyrosol, and homovanillic acid. Further, we also adopted β-glucuronidase hydrolysis to determine conjugated (sulphated and glucuronidated) metabolites of hydroxytyrosol.

Ethics approval for this study was provided by the Northern Y Regional Ethics Committee, Ministry of Health, New Zealand (NTY/11/02/015). Written informed consent was obtained from participants. Male and female subjects were recruited in equal numbers within the University of Auckland via email advertising. All subjects were healthy, free of chronic illnesses, and non-smokers. Women were excluded if menstruating during the study. Five men and five women participated, aged 42.8 ± 7.4 years (range 31.7–54.0), with BMI 26.9 ± 1.9 $kg/m^2$ (range 24.6–29.8).

Participants were randomized to receive OLE in either capsulated or liquid form (Comvita, Auckland, New Zealand) to assess effects of delivery method. A possible dose affect was also investigated, with each subject receiving two single doses of OLE commonly found in nutraceutical formulations: either a lower (51.1 mg oleuropein,) or higher (76.6 mg oleuropein) dose. Participants received the opposite strength (but same formulation) a week later.

The oleuropein dose was matched between the two different formulations, but hydroxytyrosol concentration was higher in capsules than in liquid preparation (9.7 mg vs 5.4 mg for the lower dose, and 14.5 mg vs 8.1 mg for the higher dose). Polyphenol content in capsules and liquid

preparations was independently verified (Conmac Laboratory Services, Queensland, Australia) (Table 20). Note that minor changes in other polyphenols present at much lower concentrations were not examined.

Concentrations of oleuropein and its metabolites in plasma and urine was quantified by LC-ESI-MS/MS [444] (Supporting information). Data were analysed using a linear mixed model design with repeated measures (SAS Institute, Cary, USA). Models accounted for preparation (capsule vs liquid), dose (high vs low), sex (male vs female), and between-subject differences. Data are expressed as means and 95% confidence intervals, adjusted for confounding factors in the multivariate models.

Nine patients completed both doses (one woman taking liquid formulation did not complete). There were no adverse effects noted, and liver function tests (AST, ALT, ALP, GGT, and INR) were unaffected. Conjugated metabolites of hydroxytyrosol (glucuronidated and sulphated) made up most of OLE polyphenol metabolites detected in plasma (96–99%), with a much smaller amount of oleuropein recorded. We did not detect hydroxytyrosol acetate sulphate, which has recently been identified in plasma [445]. Homovanillic acid was only detected in trace amounts in urine and plasma (data not shown). LC-ESI-MS/MS data on recorded metabolites is provided (Figure 16).

Table 16 summarizes the data on plasma polyphenol metabolites following OLE consumption. Liquid preparation resulted in peak plasma oleuropein concentrations that were 6-fold higher than with capsules ($p=0.004$), as well as an area area-under-the-curve (AUC) four times greater ($p=0.040$). No further oleuropein was recovered following β-glucuronidase hydrolysis. OLE dose did not significantly affect plasma parameters (peak and AUC) for oleuropein or conjugated metabolites of hydroxytyrosol (Table 16), however this could be due to sample size and/or marked inter-individual variation.

Detection of plasma oleuropein and hydroxytyrosol metabolites was rapid after ingestion (range 23–93 minutes), with oleuropein concentrations peaking earlier than conjugates (Table 16; Figure 17). Peak concentrations were reached earlier with liquid preparations compared to capsules (Table 16; Figure 17).

There was considerable variation in peak plasma metabolite concentrations among participants (coefficients of variation (SD/mean): 46–122%), depending also on formulation and dose (data

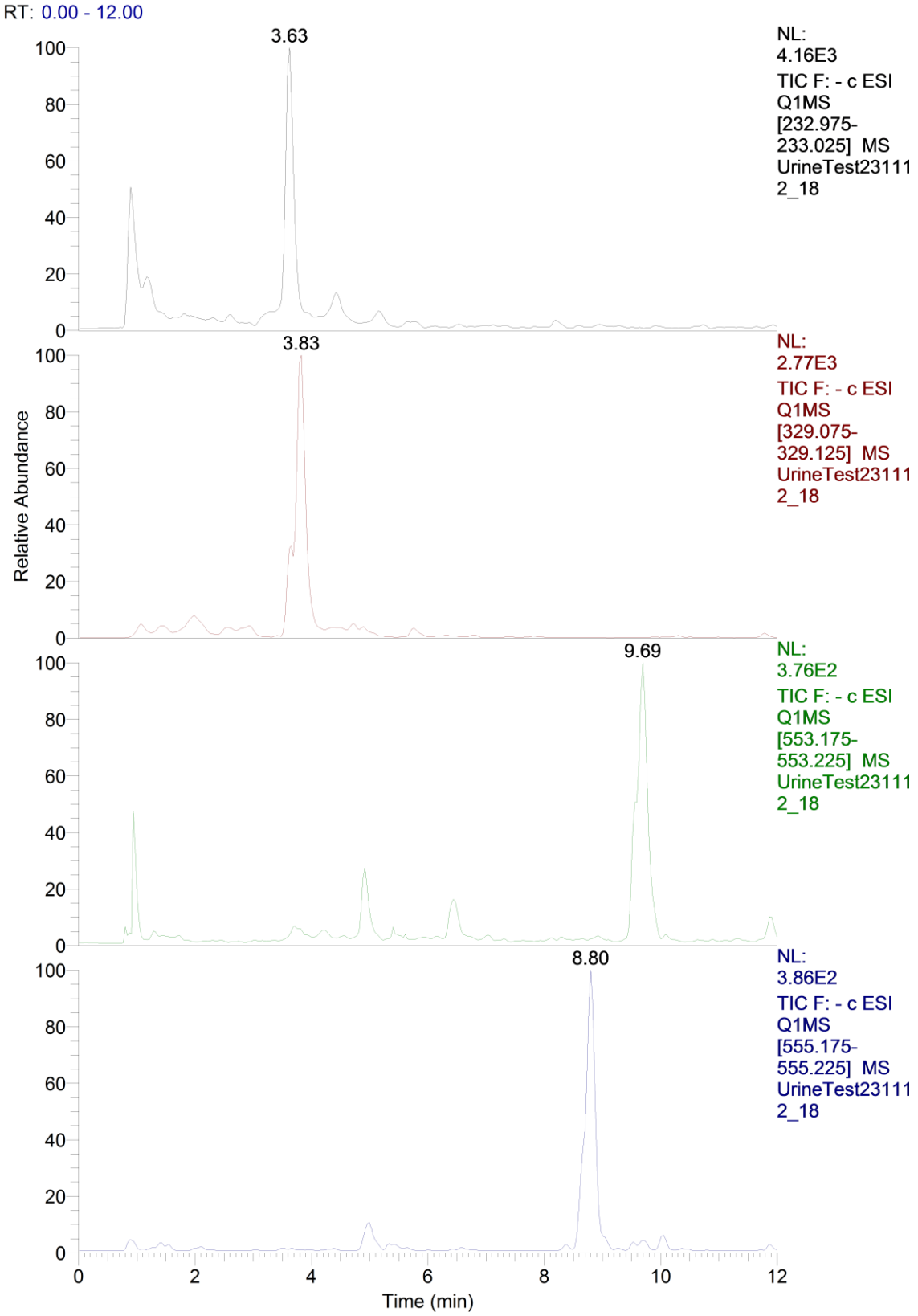


Figure 16: Sample single ion monitoring (SIM) output showing urine metabolites.

*A, sulphated hydroxytyrosol; B, glucuronidated hydroxytyrosol; C and D, open-ring glucuronidated oleuropein metabolites*

**Table 16:The effects of preparation, dose, and gender on the bioavailability of oleuropein and conjugated (glucuronidated and sulphated) metabolites of hydroxytyrosol. Data are the results from the multivariate models, and are expressed as means and 95% confidence intervals adjusted for other confounding factors.**

| | **Preparation** | | | **Dose** | | | **Sex** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Biochemical** | **Capsule** | **Liquid** | **p-value** | **Lower** | **Higher** | **p-value** | **Males** | **Females** | **p-value** |
| n | 10 | 8 | | 9 | 9 | | 5 | 4 | |
| **Oleuropein** | | | | | | | | | |
| peak (ng/ml) | 0.47 (0.26–0.86) | 2.74 (1.34–5.61) | **0.004** | 1.00 (0.62–1.61) | 1.28 (0.80–2.06) | 0.24 | 0.89 (0.48–1.64) | 1.44 (0.70–2.95) | 0.27 |
| AUC (ng/ml) | 58 (26–130) | 224 (88–570) | **0.040** | 126 (66–243) | 104 (54–199) | 0.57 | 67 (30–149) | 194 (76–494) | *0.085* |
| time to peak (min) | 38 (22–54) | 23 (4–42) | *0.089* | 31 (17–45) | 30 (16–44) | 0.90 | 25 (8–41) | 36 (17–55) | 0.31 |
| **Conjugated metabolites of hydroxytyrosol** | | | | | | | | | |
| peak (ng/ml) | 56 (20–156) | 59 (18–196) | 0.94 | 50 (23–108) | 66 (31–143) | *0.089* | 117 (42–325) | 28 (9–94) | *0.076* |
| AUC (ng/ml) | 5700 (2210–14700) | 5180 (1700–15700) | 0.88 | 4820 (2360–9830) | 6120 (3000–12500) | *0.084* | 11600 (44800–29900) | 2550 (840–7750) | **0.048** |
| time to peak (min) | 93 (77–109) | 64 (45–83) | **0.031** | 72 (58–85) | 85 (72–98) | 0.10 | 78 (62–94) | 79 (60–98) | 0.91 |

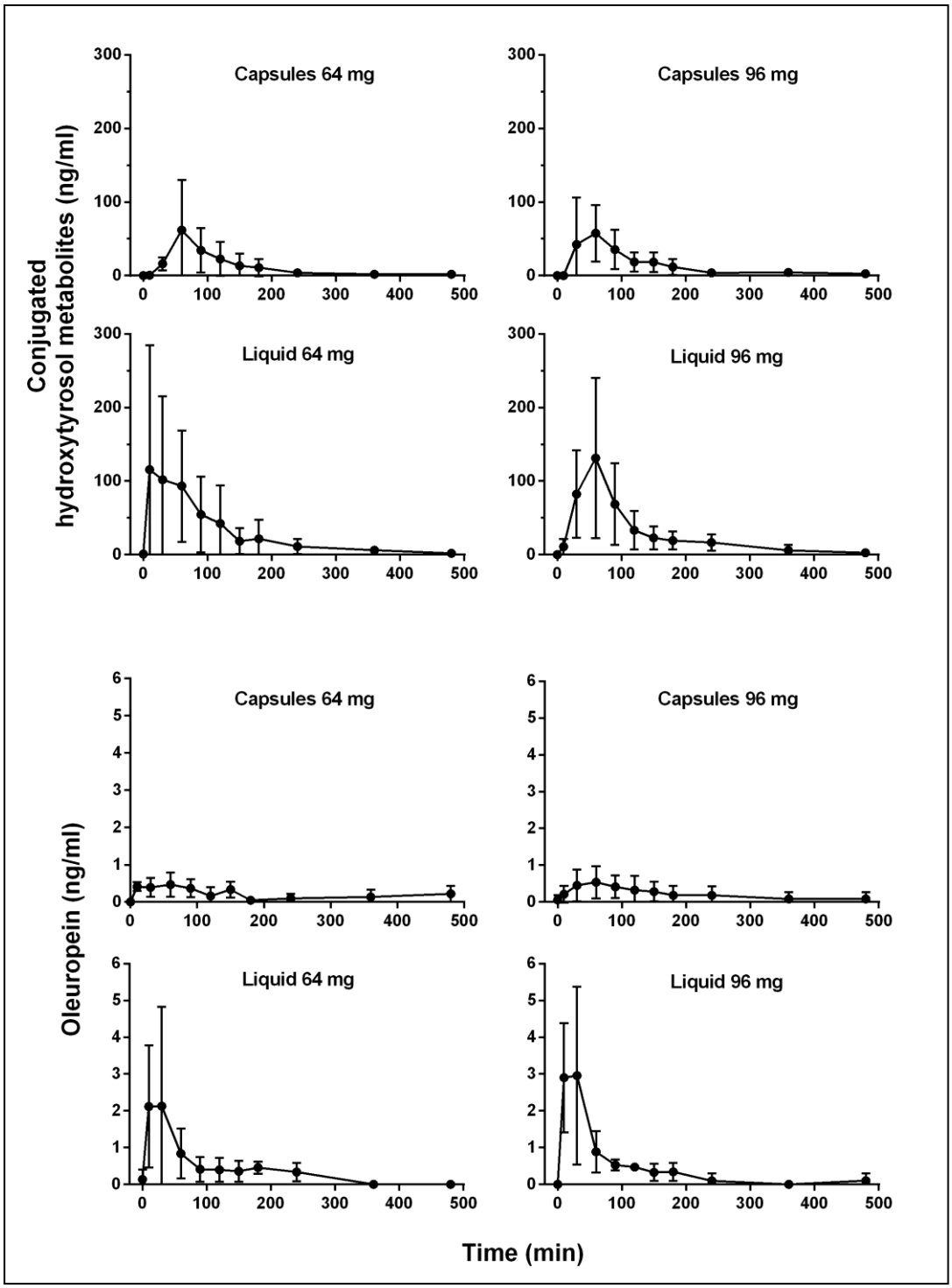


Figure 17: Plasma conjugated (glucuronidated and sulphated) hydroxytyrosol and oleuropein concentrations.

*8 hours following olive leaf extract ingestion in capsule and liquid forms, at lower (64 mg) and higher (96 mg) oleuropein doses.*

not shown).

Further, despite the small sample size, subgroup analysis showed a marked gender effect (Table 16). Males tended to have greater peak conjugated hydroxytyrosol concentrations than females (p=0.076; Table 16). Males also had plasma conjugated hydroxytyrosol AUC 4.5x higher than in females (p=0.048), but plasma oleuropein AUC that tended to be 3x lower (p=0.085; Table 16).

Conjugated (sulphated and glucuronidated) hydroxytyrosol were the primary metabolites detected in urine. Most were recovered in the initial eight hours post-ingestion (Figure 18).

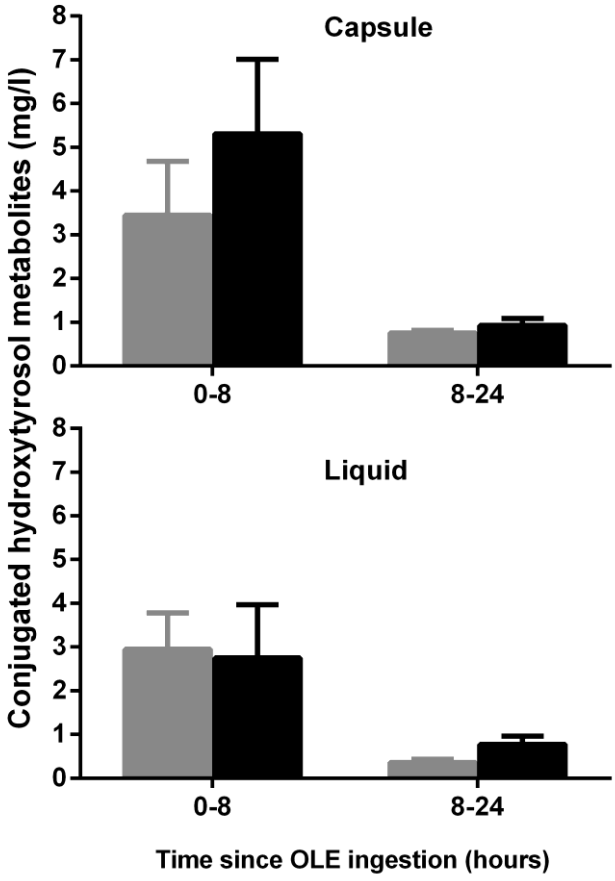


Figure 18: Urine recovery of conjugated (glucuronidated and sulphated) hydroxytyrosol metabolites.

*24 hours following olive leaf extract (OLE) ingestion in capsule and liquid forms, at low (gray bars) and high (black bars) doses.*

This is the first study to assess the absorption and metabolism of oleuropein and hydroxytyrosol in human plasma following OLE ingestion (and to establish its time-course). The predominant olive polyphenol metabolites detected in plasma and urine were conjugated (sulphated and glucuronidated) hydroxytyrosol. Our data are compared to previous studies in Table 17.

Our data contrast to the only other study that examined oleuropein metabolism taken as OLE. After chronic ingestion of OLE, Kendall et al. found almost exclusively phase II metabolites of oleuropein in the urine (glucuronidated at different positions), and no conjugated metabolites of hydroxytyrosol [446]. Phase II metabolites of oleuropein were also detected, but these could not be quantified due to a lack of a standard. Our contrasting findings may be due to subtle differences in polyphenol composition of the extracts consumed, which are known to be altered by agronomic and technological factors [447], methodological issues, as well as individual differences among study participants.

The main metabolites recorded were sulphated and glucuronidated conjugates of hydroxytyrosol, while we observed only trace amounts of homovanillic acid and no hydroxytyrosol acetate sulphate. However, sulphated and glucuronidated conjugates could not be individually quantified due to unavailability of specific standards. Our findings differ slightly to those of previous studies (Table 17), two of which were carried out in rats [448, 449], and two human studies used olive cake to enrich the olive oil (altering the ratio of oleuropein

## Table 17 : Published literature on the metabolic pathways of the olive polyphenols.

### [1] *Animal studies performed in rats.*

| Source of olive polyphenol | Polyphenol content | Phase I metabolism | Phase II metabolism | Plasma | Urine |
|---|---|---|---|---|---|
| Leaf extract (present study) | - mainly oleuropein<br>- some hydroxytyrosol<br>- some tyrosol | oleuropein extensively hydrolysed, liberating more hydroxytyrosol | - glucuronidated and sulphated | - predominantly glucuronidated and sulphated hydroxytyrosol<br>- some oleuropein | - - predominantly glucuronidated and sulphated hydroxytyrosol<br>- some oleuropein |
| Leaf extract [446] | - mainly oleuropein<br>- some hydroxytyrosol | oleuropein glucoside escapes acid hydrolysis | - predominantly glucuronidated | (not measured) | - glucuronidated metabolites of oleuropein |
| Virgin oil (enriched) [441] | - mainly oleuropein e and elenolic acid linked to hydroxytyrosol<br>- some hydroxytyrosol<br>- minimal tyrosol | oleuropein extensively hydrolysed | - predominantly sulphated<br>- not glucuronidated | - predominantly sulphated hydroxytyrosol and tyrosol acetate<br>- some homovanillic acid<br>- minimal sulphated homovanillic acid | (not measured) |
| Virgin oil (enriched) [450] | - mainly oleuropein and elenolic acid linked to hydroxytyrosol<br>- some tyrosol<br>- minimal hydroxytyrosol | oleuropein extensively hydrolysed | - predominantly sulphated<br>- some methylation<br>- some glucuronidated | - mainly methylated and sulphated metabolites of tyrosol<br>- minimal homovanillic acid<br>- minimal oleuropein glucuronides and aglycone<br>- minimal sulphated and methylated metabolites of hydroxytyrosol | (not measured) |
| Virgin oil Khymenets, 2010 #4} | - mainly oleuropein (presumed due to increased hydroxytyrosol after acidic hydrolyisis)<br>- some hydroxytyrosol<br>- some tyrosol | oleuropein aglycone extensively hydrolysed | - predominantly glucuronidated<br>- some methylated | (not measured) | - predominantly glucuronidated metabolites of tyrosol, hydroxy homovanillic acid<br>- minimal free tyrosol, hydroxytyrsosol, homovanillic acid |
| Virgin oil [451] | - mainly oleuropein aglycone<br>- hydroxytyrosol<br>- tyrosol | oleuropein aglycone extensively hydrolysed | - predominantly glucuronidated | (not measured) | - predominanly glucuronidated metabolites of oleuropein, hydr<br>- some methlyated metabolites of oleuropein and hydroxytyros<br>- minimal homovanillic alcohol |
| Oil [452] | - mainly oleuropein aglycone<br>- some hydroxytyrosol | oleuropein aglycone extensively hydrolysed | - predominantly glucuronidated<br>- some other conjugation | - predominantly glucuronidated hydroxytyrosol<br>- some other conjugated hydroxytyrosol.<br>- methylated hydroxytyrosol<br>- some oleuropein aglycone | - predominantly glucuronidated hydroxytyrosol<br>- some other conjugated hydroxytyrosol<br>- methylated hydroxytyrosol<br>- some oleuropein aglycone |
| Labelled hydroxytyrosol[1] [449] | - labelled hydroxytyrosol | | - predominantly sulphated<br>- some glucuronidated | (not measured) | - predominantly sulphated hydroxytyrosol<br>- some glucuronidated hydroxytyrsosol and homovanillic acid |
| Labelled hydroxytyrosol[1] [448] | - labelled hydroxytyrosol | | - predominantly sulphated<br>- some glucuronidated<br>- some methylation | - predominantly sulphated hydroxytyrosol metabolites<br>- some glucuronidated and methylated metabolites | - predominantly sulphated hydroxytyrosol metabolites<br>- some glucuronidated and methylated metabolites |

derivatives in the consumed sample) [441, 450]. Different forms of oleuropein and derivatives consumed may influence the pathway of conjugation [445].

In our study mean time to peak for conjugated hydroxytyrosol metabolites following ingestion of liquid OLE was 64 minutes. Peak plasma concentrations following olive oil ingestion occurred much earlier, for example at 30 minutes [450] and 32 minutes [452]. We observed a rather large inter-individual variability in absorption and metabolism of polyphenols, which may result from differences in human enzymatic activity [453]. Importantly, despite our small sample size, there were marked gender differences, which is novel observations that needs to be confirmed by larger studies. Such differences are important, as the effects of olive polyphenols in the organism may consequently vary somewhat between males and females. Further, there could also be considerable day to day variation within individuals, which may also have important clinical implications.

We also observed that compared to capsules, OLE in a liquid formulation led to greater oleuropein peak and AUC levels in plasma. There was also an earlier metabolite peak associated with liquid formulation, which is also seen in other compounds that are primarily absorbed in the small intestine (e.g. paracetamol) [454]. However, the 6-fold higher peak oleuropein concentrations following ingestion of OLE in liquid form was notable, even if oleuropein concentrations were considerably lower than that of conjugated hydroxytyrosol metabolites. The observed differences may be associated with a number of factors, such as time taken for capsules to be dissolved, the polyphenol suspension (glycerine vs safflower oil), or the rapid delivery of the liquid formulation allowing escape from hydrolysis. Nonetheless, our results indicate that the OLE formulation is likely to be an important issue for nutraceutical companies, and possibly influence choice among better-informed consumers.

Conjugated metabolites of hydroxytyrosol are the main olive polyphenol metabolites found in biological fluids *in vivo*, and their bioactivity has been examined. Glucuronidated-hydroxytyrosol is five times more potent as a free-radical scavenger than the parent hydroxytyrosol [449]. Although glucuronidated-hydroxytyrosol is inactive against LDL-cholesterol oxidation [455], it protected renal tubular cells against oxidative damage *in vitro* [456]. However, the long held dogma that reactive oxygen species are associated with the progressive development of systemic disease has been questioned, as superoxides are essential

for normal metabolome and physiological function [457]. Further, *in vitro* experiments typically use concentrations several fold higher than those achieved *in vivo*.

An alternate explanation for olive polyphenols bioactivity was proposed by Konstantinidou et al, who demonstrated down regulation of proatherogenic genes (such as IFN-γ, ARHGAP15, and IL7R) following olive oil consumption [458]. There is also *in vitro* evidence of nutrigenomic interaction on several cell lines covering cancer [459] and insulin sensitivity [460] genes.

In summary, conjugated (sulphated and glucuronidated) metabolites of hydroxytyrosol were the primary oleuropein metabolite recovered in plasma and urine following OLE consumption. Absorption and metabolism of olive polyphenols to plasma is rapid, as is renal clearance. Bioavailability and metabolism of oleuropein is highly dependent on a number of factors, including preparation (capsule vs liquid) and individual factors such as gender. The form of oleuropein and the delivery method (OLE vs oil) also affect absorption and metabolism. The bioactivity of the conjugated metabolites needs to be further defined, and possible toxicity levels explored. Chronic ingestion may influence enzymatic activity and hence absorption and metabolism. Future research also needs to examine the tissue distribution of labelled olive polyphenols, and identification of individuals with greater absorption – as they might have the greatest biological affect.

# ONLINE SUPPLEMENT

## MATERIALS AND METHODS

### Ethics approval

Ethics approval for this study was provided by the Northern Y Regional Ethics Committee (Ministry of Health, New Zealand), approval number NTY/11/02/015. Written informed consent was obtained from participants.

### Subjects

Male and female subjects were recruited in equal numbers within the University of Auckland via email advertising. Inclusion criteria required that all subjects were healthy, free of chronic illnesses, and not regular drug users (including tobacco). Women were excluded if menstruation occurred during the study. A total of five men and five women entered the study, and were aged 42.8 ± 7.4 years (range 31.7–54.0 years) and with BMI 26.9 ± 1.9 kg/$m^2$ (range 24.6–29.8 kg/$m^2$).

### Study design

The study was designed to evaluate the concentrations of polyphenol metabolites in both plasma and urine, following acute ingestion of OLE according to dose (low vs high) and preparation (capsule vs liquid). Each participant was randomized (by computer random number allocation) to receive both doses of OLE as either capsules or liquid. Each participant ingested OLE at two different doses a week apart, whose order of intake was also randomized.

All clinical assessments were carried out at the Maurice & Agnes Paykel Clinical Research Unit (Liggins Institute, University of Auckland). Prior to assessments, participants were instructed to avoid all olive products and alcohol for at least 24 hours (as previous literature shows olive metabolites are eliminated well within this time), and fast for a minimum of 8 hours before each ingestion of OLE (to eliminate effects of dietary intake and standardize participants). On arrival, participants were weighed and measured, and asked to void urine. An intra-venous cannula was then placed, and the OLE preparation was ingested.

Blood samples were drawn at 0, 10, 30, 60, 90, 120, 150, 180, 240, 360, 480 minutes, and 24 hours post-ingestion. Samples were collected into heparin tubes on ice, centrifuged, and plasma was stored at -20°C until analysis. Urine samples were collected for 24 hours after OLE ingestion. Once participants ingested the OLE preparation, they remained in the Maurice & Agnes Paykel Clinical Research Unit fasted for the initial 8 hours, during which only water was to be consumed. Subsequently, over the next 16 hours, participants could eat and drink *ad libitum*, but were instructed to avoid alcohol and olive-containing products, and were instructed to collect all urine.

**Olive leaf extract**

The polyphenol contents of the OLE (Comvita, Auckland, New Zealand) used in capsules and liquid preparations were independently verified (Conmac Laboratory Services, Queensland, Australia) (Table 20). Each OLE capsule contained 400 mg of the extract, as well as 672.5 mg safflower oil, 150 mg lecithin, and 27.5 mg silica-colloidal anhydrous. The liquid OLE preparation was suspended in glycerol. Participants taking capsules received either four (51.1 mg oleuropein, 9.7 mg hydroxytyrosol) or six (76.6 mg oleuropein, 14.5 mg hydroxytyrosol) capsules, taken a week apart. Those randomized to receive the liquid preparation were matched to receive similar polyphenol doses (Table 20).

**Outcome measures**

Parameters of interest were peak oleuropein, oleuropein area under the curve (AUC), peak hydroxytyrosol, and conjugated metabolites of hydroxytyrosol AUC. For safety assessment, plasma was collected for liver function tests at each assessment, with measurements of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT), and international normalised ratio (INR) for liver synthetic function.

**Chemical Analysis**

Plasma and urine quantification of oleuropein and its metabolites were determined by LC-ESI-MS/MS, as described previously [444]. Methodological exceptions were:

a) ß-glucuronidase hydrolysis: 0.05M acetate buffer used.
b) Solid phase extraction: Phenomenex (Auckland, New Zealand) Strata C18-E 100mg/3mL cartridges were used, and supernatant transferred to tubes containing 25 ng/ml of internal

standard (2-Hydroxyphenylethanol (2-HPE), Sigma-Aldrich, St Louis, MO, USA); analytes were eluted with 2.5 ml of ethyl acetate and evaporated to dryness under vacuum for several minutes; residue was reconstituted with 9:1 $H_20$ (pH 3.7):acetonitrile (Merck, KGaA, Germany)

c) Instrumentation: all LC-HESI MS/MS analysis was performed on a Finnigan TSQ Quantum Ultra Accurate Mass Triple-quadrupole system (Thermo Fisher Scientific, San Jose, CA, USA); chromatographic separations were carried out using a Accela autosampler and 1250 pump (Thermo Fisher Scientific).

The separation was performed at 30$^{o}$C on a Luna 2.5µm C18(2) – HST 100x3mm column (Phenomenex) preceded by a KrudKatcher (Phenomenex) in-line filter. A gradient was used to separate the analytes, solvent A was water (adjusted to pH 3.7) and solvent B was acetonitrile (Merck, Darmstadt, Germany) (Table 18). A flow rate of 250 µl/min was maintained throughout the separation.

**Table 18: Solvent gradient for the separation of oleuropein and hydroxytyrosol.**

| Time (min) | % Solvent A | % Solvent B |
|---|---|---|
| 0.0 | 85 | 15 |
| 3.0 | 85 | 15 |
| 10.0 | 25 | 75 |
| 11.0 | 85 | 15 |
| 15.0 | 85 | 15 |

Mass spectral analyses were carried out in negative ion mode for all analytes using the following tune parameters: spray voltage 3500V, vaporiser temperature 320$^{o}$C, and capillary temperature 250$^{o}$C. Nitrogen gas was used as sheath, ion sweep and auxiliary gas at 50, 1, and 2 psi, respectively, with argon as the collision gas. Single reaction monitoring (SRM) parameters and the retention time were established for each compound, and their collision pressures and energies are listed in Table 19. Quantification of the analytes was established by assessing the peak area ratios of each analyte to that of the internal standard and comparing them with the corresponding ratios of spiked control plasma (range 0.25–50 ng/ml), the intra

and inter-assay %CV's for HT were 9 and 12%, respectively. Pre-filtered baseline and 8-hour urine samples were injected unextracted into the mass spectrometer to identify the presence of oleuropein and hydroxytyrosol metabolites following OLE consumption using single ion monitoring (SIM).

**Table 19: Liquid chromatography and mass spectrometer parameters.**

| | Oleuropein | Hydroxytyrosol | 2-hydroxyphenylethanol |
|---|---|---|---|
| **Molecular weight (g/mol)** | 540.51 | 154.16 | 138.16 |
| **Single reaction monitoring (m/z)** | 539.11-> 275.08 | 153.04 -> 123.10 | 137.06 -> 107.10 |
| **Retention time (min)** | 8.5 | 3.4 | 6.7 |
| **Collision E (V)** | 23 | 18 | 14 |
| **Collision P (mTorr)** | 1.2 | 1.2 | 1.2 |

AST, ALT, ALP, and GGT concentrations were measured on a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) by enzymatic colorimetric assay (Roche, Mannheim, Germany), with inter-assay CV's of less than 2.5%. INR was assessed using a point of care Coagucheck XS system (Roche Diagnostics, New Zealand).

**Statistical analysis**

Data were analysed in SAS v.9.2 (SAS Institute, Cary, NC, USA) using a linear mixed model design with repeated measures. Models accounted for preparation (capsule vs liquid), dose (high vs low), sex (male vs female), and between-subject differences. Models were also run accounting for a possible interaction between preparation and dose. If necessary, data were log-transformed to approximate normality. Data are expressed as the means and 95% confidence intervals adjusted for confounding factors in the multivariate models (back-transformed where appropriate).

**Supplementary results**

SIM results revealed the presence of hydroxytyrosol sulphate, hydroxytyrosol glucuronide, and two open-ring oleuropein phase II metabolites (at m/z 553.15 and 555.17, respectively) (Figure 16), which have been previously described [446]. SRM analysis further confirmed the identity

of the two hydroxytyrosol derivatives with parent – daughter ion transitions of 233-153 m/z and 329-153 m/z, respectively (Figure 19). It was not possible to determine the absolute amounts of any of these individual metabolites, because of the lack of commercially available standards.

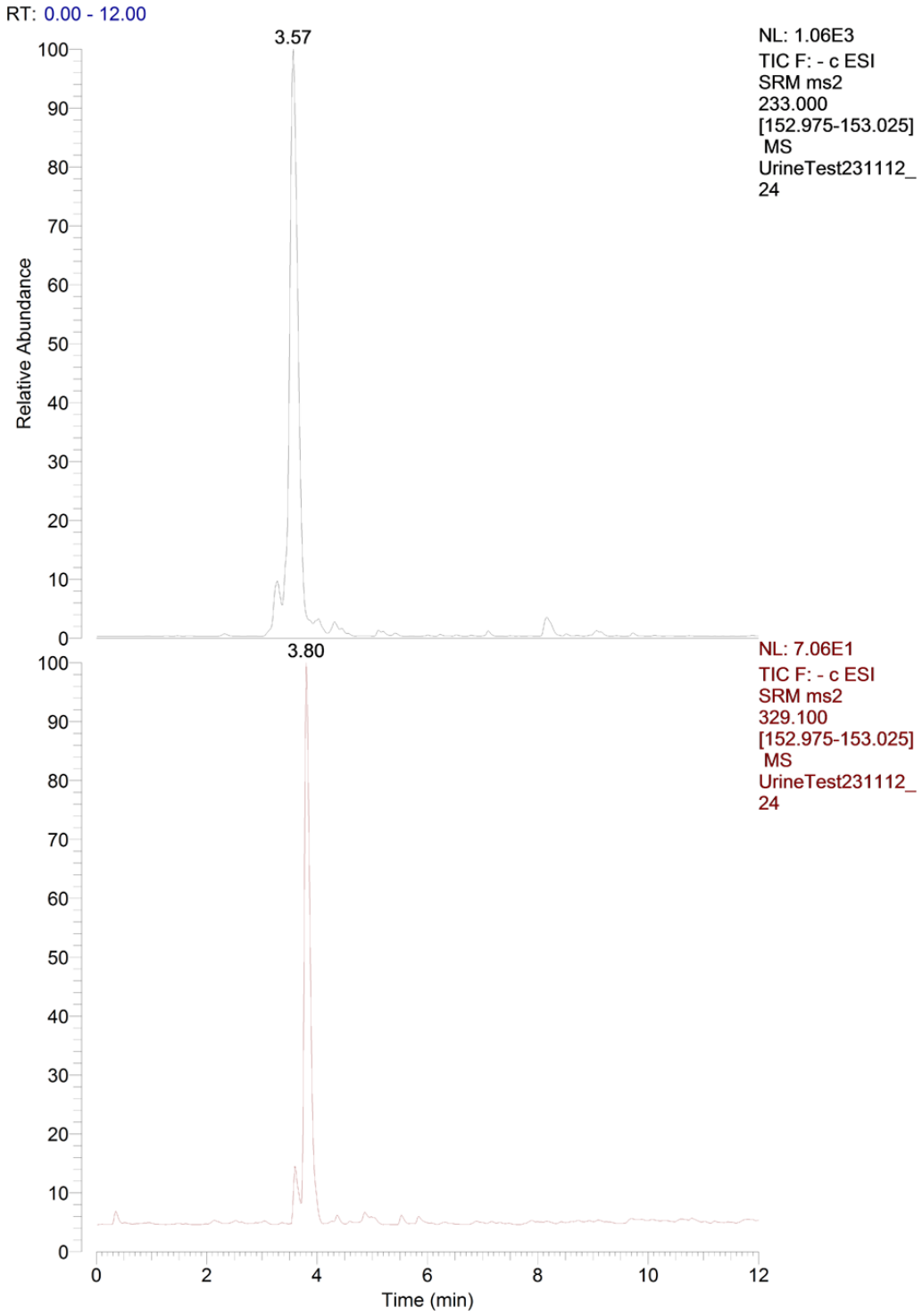


Figure 19: Sample single reaction monitoring (SRM) from urine analyses.

*Sulphated (A) and glucuronidated (B) hydroxytyrosol metabolites*

End of Manuscript

# Chapter 5. Does olive leaf extract improve glucose homeostasis in humans?

**Preface to publication:**

Despite the common use of the olive plant in traditional folk medicine in diabetes, there is a lack of scientific literature investigating if such use is justified. In chapter 4 we established that meaningful plasma oleuropein and hydroxytyrosol metabolites could be reached after ingesting olive leaf extract.

The following manuscript describes our investigation examining glucose homeostasis and other cardiovascular disease parameters before and after olive leaf extract supplementation in a group of middle aged overweight males. We chose a dose of 4 capsules of olive leaf extract, containing 51.1mg of oleuropein and 9.7mg of hydroxytyrosol, as this is the manufacturers current recommended daily dose, and therefore what consumers are already taking.

The following manuscript is an unaltered version of that which has been accepted for publication in *PLOS one, and is currently in press.*

# Olive (*Olea europaea* L.) leaf polyphenols improve insulin sensitivity in middle-aged overweight men: a randomized, placebo-controlled, crossover trial

Martin de Bock[1], José G B Derraik[1], Christine M Brennan[1], Janene B Biggs[1], Philip E Morgan[2], Steven C Hodgkinson[1], Paul L Hofman[1,3], Wayne S Cutfield[1,3*]

[1] Liggins Institute, University of Auckland, Auckland, New Zealand

[2] Heart Research Institute, University of Sydney, Sydney, Australia

[3] National Centre for Growth and Development (NRCGD), University of Auckland, Auckland, New Zealand

*Author for correspondence: Liggins Institute, University of Auckland, Private Bag 92019, Auckland, New Zealand; Ph: +64.9.923.5118; Fax: +64.9.373.8763; Email: w.cutfield@auckland.ac.nz

**Short title:** Olive leaf polyphenols & insulin sensitivity

**Keywords:** *Olea europaea;* diabetes; insulin; β-cell; olive; glucose homeostasis; polyphenols

**Trial registration:** #336317 (Australian New Zealand Clinical Trials Registry). http://www.anzctr.org.au/trial_view.aspx?id=336317

## ABSTRACT

**Background:** Olive plant leaves (*Olea europaea* L.) have been used for centuries in folk medicine to treat diabetes, with very limited data examining the effects of olive polyphenols on glucose homeostasis in humans.

**Objective:** To assess the effects of supplementation with olive leaf polyphenols (51.1 mg oleuropein, 9.7 mg hydroxytyrosol per day) on insulin action and cardiovascular risk factors in middle-aged overweight men.

**Design:** Randomized, double-blinded, placebo-controlled, crossover trial in New Zealand. 46 participants (aged 46.4 ± 5.5 years and BMI 28.0 ± 2.0 kg/m$^2$) were randomized to receive capsules with olive leaf extract (OLE) or placebo for 12 weeks, crossing over to other treatment after a 6-week washout. Primary outcome was insulin sensitivity (Matsuda method). Secondary outcomes included glucose and insulin profiles, cytokines, lipid profile, body composition, 24-hour ambulatory blood pressure, and carotid intima-media thickness.

**Results:** Treatment evaluations were performed on intention-to-treat. All participants took >96% of prescribed capsules. OLE supplementation was associated with a 15% improvement in insulin sensitivity (p=0.024) compared to placebo. There was also a 28% improvement in pancreatic β-cell responsiveness (p=0.013). OLE supplementation also led to increased fasting interleukin-6 (p=0.014), IGFBP-1 (p=0.024), and IGFBP-2 (p=0.015) concentrations. There were however, no effects on interleukin-8, TNF-α, ultra-sensitive CRP, lipid profile, ambulatory blood pressure, body composition, carotid intima-media thickness, or liver function.

**Conclusions:** Supplementation with olive leaf polyphenols for 12 weeks significantly improved insulin sensitivity and pancreatic β-cell secretory capacity in overweight middle-aged men at risk of developing the metabolic syndrome.

## INTRODUCTION

It is estimated that 20–50% of the European population use complementary or alternative therapy to treat disease or to help prevent its onset [461]. In Britain, approximately 40% of general practitioners provide complimentary therapies for their patients [462]. With respect to type 2 diabetes, one third of patients actively use alternative medicine to manage their disease, despite the paucity of scientific evidence to support its use [253]. The leaves of the olive plant (*Olea europaea* L.) have been used for centuries in folk medicine to treat diabetes

[463]. Recently, the medicinal properties of olive products have focussed on its polyphenols (particularly oleuropein and hydroxytyrosol), which according to animal and *in vitro* studies have anti-oxidant, hypoglycaemic, antihypertensive, antimicrobial, and anti-atherosclerotic properties [38]. Polyphenols are found in most edible plants, and are reportedly responsible for the health benefits associated with the consumption of chocolate, coffee, green tea, and red wine [464].

The nutraceutical market exploring the potential health benefits of olive products is expanding. As the concentration of polyphenols is far greater in the olive leaves than the olive fruit or fruit oil, this once discarded by-product of tree pruning is now considered a valuable commodity. However, while the cardiovascular health benefits of a Mediterranean diet rich in olive oil is well established [465], clinical studies examining the effects of olive polyphenols supplementation on cardiovascular disease risk are scarce, flawed, or contradictory. Thus, although the European Food Safety Authority has endorsed the health claim that "the consumption of olive oil polyphenols contributes to the protection of blood lipids to oxidative damage", it has rejected several other health claims [466].

There are very limited data examining the effects of olive polyphenols on glucose homeostasis in humans. In this study, a randomized, double-blinded, placebo-controlled, crossover trial was conducted to assess whether supplementation with olive leaf polyphenols would affect modifiable cardiovascular risk factors in overweight males, who by virtue of their body mass are likely to be insulin resistant. In addition, plasma markers involved in the development of cardiovascular disease were investigated. Potential mechanisms underpinning the clinical outcomes were also examined.

## METHODS

### Ethics statement

Ethics approval for this study was provided by the Northern Y Regional Ethics Committee (New Zealand Ministry of Health), and written informed consent was obtained from all participants. This study was registered with the Australian New Zealand Clinical Trials Registry (#336317).

## Subjects

Overweight males (BMI 25–30 kg/m$^2$) aged 35–55 years were eligible to participate. Volunteers were recruited in February 2011 via advertisements in local newspapers that circulate freely in the central Auckland metropolitan area. Exclusion criteria included drug use (including tobacco), diabetes, or being on medications likely to affect insulin sensitivity. Subjects taking anti-hypertensive or lipid-lowering medications were included, but were required to have been on a stable dose for at least 6 months prior to start of the study. These subjects were also encouraged not to change dose throughout the trial, and doses were checked at each assessment. Further, participants were asked not to make any substantial alterations to their lifestyle for the duration of the trial. Specifically, participants were instructed not to make changes to their diet and physical activity levels.

## Randomization and masking

Randomized allocation was done using computer random number generation. The code was kept by an independent third party, and was not released until after statistical analysis. Both researchers and subjects were 'blinded' to the contents of capsules being taken. To maintain integrity of the trial evaluation, statistical analyses were carried out on encoded data, such that the analyst (JGBD) was also 'blinded' to treatment.

## Study design

This was a 30-week randomized, double-blinded, placebo-controlled, crossover trial. Participants were randomized to receive capsules with olive leaf extract (OLE) or placebo (Comvita, Auckland, New Zealand) for 12 weeks, which is the minimum study period that can reliably detect a sustained effect of dietary intervention [467]. Participants then switched over to the other treatment after a 6-week washout period. The polyphenol content of the OLE was independently verified Table 20. Participants were instructed to take four capsules as a single dose, once a day, with a glass of water, equating to a dose of 51.1 mg oleuropein and 9.7 mg hydroxytyrosol when participants were on active treatment. OLE was suspended in safflower oil, while placebo capsules contained safflower oil only. Importantly, placebo and active capsules were both odourless and identical in appearance (opaque green soft capsules), size, and grade.

**Table 20: Polyphenol content of in each dose of olive leaf extract.**

| Compound | Content in 4 capsules (mg) |
|---|---|
| Oleuropein | 51.124 |
| Hydroxytyrosol | 9.666 |
| Kaempferol | 0.021 |
| Apigenenin | 0.046 |
| Flavonoid | 0.028 |
| Verbascoside | 0.344 |
| Phenolic acids (calculated as caffeic acid) | 0.233 |
| Oleic acid | 0.013 |
| Quercetin | 0.038 |
| Luteolin | 0.249 |
| Rutin | 0.150 |

We have shown that following ingestion of an identical dose of OLE, olive polyphenol metabolites in plasma peak after 80 minutes and are cleared by 240 minutes (de Bock et al, unpublished data). Nonetheless, we chose a generous 6-week washout period, after which participants crossed to the opposite intervention (Figure 20). All clinical assessments were carried out between 06:30 and 08:30 at the Maurice & Agnes Paykel Clinical Research Unit (Liggins Institute, University of Auckland), after an overnight fast and no strenuous activity over the previous 24 hours. Participants were instructed not to take their assigned capsules on the morning of investigation. Subjects were assessed at the start of the study, and at the end of each intervention phase. Blood samples collected and placed on ice; following separation, plasma and serum were stored at -20 and -80°C, respectively, for later analysis.

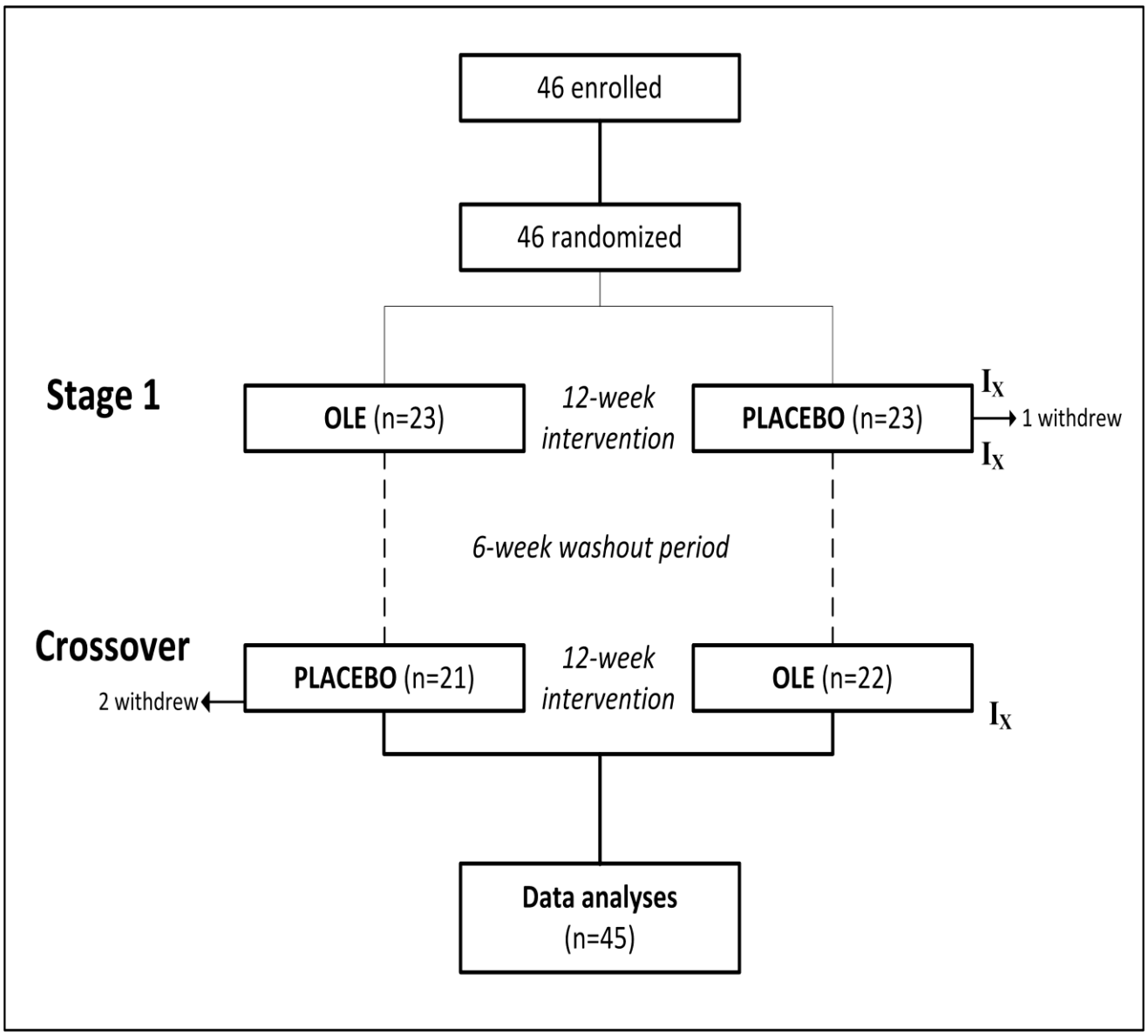


Figure 20: Summary of study's recruitment process and trial execution.

*$I_X$ indicates timing of assessments. One participant withdrew from the study during stage 1 due to injury, while the two subjects that withdrew after crossover were either lost to follow up or the developing of acne.*

**Primary outcome**

The primary outcome was insulin sensitivity, assessed via a 75 g oral glucose tolerance test. Insulin sensitivity (ISI) was assessed using the Matsuda method, with glucose and insulin samples collected at 0, 30, 60, 90, and 120 minutes [388]. The Matsuda method has a strong correlation with the hyperinsulinemic euglycaemic clamp (r=0.77) [129], and excellent reproducibility during multiple measures [132].

**Secondary outcomes**

Other parameters of glucose homeostasis assessed included pancreatic β-cell function, also calculated from the oral glucose tolerance test: the product of insulin sensitivity (derived by the Matsuda method) and the change in glucose and insulin over the first 30 minutes (oral disposition index) [139]. Glucose and insulin profiles after the glucose challenge were calculated and expressed as the area under the curve (AUC).

To identify potential underpinning mechanisms, fasting blood samples were used to assess cytokines known to influence glucose metabolism: insulin-like growth factor I (IGF-I), IGF-II, IGF binding protein 1 (IGFBP-1), IGFBP-2, IGFBP-3, ultra-sensitive C-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α), interleukin-6, and interleukin-8.

Fasting blood samples were also used to assess lipid profile, namely triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C). Liver function tests were also performed at each assessment, with measurements of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT).

Auxological assessments included height measurement using a Harpenden stadiometer. Weight and body composition were assessed using whole-body dual-energy x-ray absorptiometry (DEXA, Lunar Prodigy 2000, General Electric, Madison, WI, USA). Body composition data of interest were total percentage body fat and the ratio of android fat to gynoid fat. Note that android and gynoid fat values were determined by the manufacturer's software, based on an automated sectioning of specific areas of the body [416].

24-hour ambulatory blood pressure was assessed prior to each clinical assessment. Participants were fitted with a Spacelabs 90207 or 90217 (Spacelabs Medical Inc., Redmond, USA), with each subject being assigned the same device model for all assessments. Measurements were performed every 20 minutes from 0700–2200, and every 30 minutes from 2200–0700. Only profiles with a total of at least 40 readings over a 24-hour period were included for analysis [418].

Carotid intima-media thickness (cIMT) was also measured to assess possible treatment effects, as it is a validated and reproducible measure that is predictive of cardiovascular and cerebrovascular risks [401]. cIMT was measured using an M-Turbo ultrasound system (Sonosite, Bothel, USA) by a trained investigator [MdB], with images attained using a standard protocol [402]. The far wall of the right common carotid artery was used for all three assessment points. Digitally stored images were analysed by a single reader [MdB] using computer software automated callipers (SonoCalctm v.4.1, Sonosite). A maximal cIMT measurement approximately 10 mm proximal to the carotid bulb was used for comparative analysis. To assess reproducibility, triplicate measures were taken of seven healthy volunteers over a 7-day interval, and resulted in an intra-observer CV of 3.7% (unpublished data).

Lifestyle factors were recorded with an itemised food diary and a physical activity recall. Three-day dietary records were collected at baseline and at clinical assessment following each 12-week intervention. Each dietary report encompassed an itemized nutritional intake recorded during two week days (Monday to Friday) and one weekend day. Nutritional intake was recorded using standard household measures, as well as the information from food labels where appropriate. Participants were instructed by a trained investigator [MdB], who also reviewed all food records with each participant to address unclear descriptions, errors, omissions, or doubtful entries. Records were subsequently entered into Foodworks software (v6.0, Xyris Software, Brisbane, QLD, Australia) by the trained investigator [MdB]. Physical activity levels were assessed using the International Physical Activity Questionnaire (IPAQ) [400], covering four domains of physical activity: work-related, transportation, housework/gardening, and leisure time.

In addition, subjective measures of wellbeing were assessed by the Medical Outcomes Study Short Form (SF-36: New Zealand / Australia adaptation). The SF-36 is a validated tool that measures perception of health on eight multi-item dimensions covering functional status, wellbeing, and overall evaluation of health [468].

**Assays**

Insulin concentrations were measured using an Abbott AxSYM system (Abbott Laboratories, Abbott Park, IL, USA) by microparticle enzyme immunoassay with an inter-assay coefficient of variation (CV) of 5.4%. Glucose concentrations were measured on a Hitachi 902

autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) by enzymatic colorimetric assay (Roche, Mannheim, Germany) with a CV of 2.1%. Commercially available ELISAs were used to measure plasma IGF-I, IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3 (Meddiagnost, Reutlingen, Germany) with CV of 3.5, 0.9, 3.6, 8.8, and 8.5%, respectively). Commercially available ELISA kits were used to evaluate TNF-α, interleukin-6, and interleukin-8 (Invitrogen, Carlsbad, CA, USA) with CV of 9.3%, 7.4, and 3.4%, respectively, and oxidised LDL-C (Mercodia, Uppsala, Sweden) with a CV 5.7%. Commercially available ELISA kits were used to evaluate ultra-sensitive CRP (USCN Life Science, Wuhan, China) with a CV of 10%. Triglycerides, total cholesterol, HDL-C, LDL-C, AST, ALT, ALP, and GGT concentrations were measured on a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation) by enzymatic colorimetric assay (Roche) with an interassay CV of less than 2.5%.

**Sample Size**

The power calculation was based upon a known mean adult Matsuda ISI of 15.6 and standard deviation of 8.7 [420]. A total sample size of n=46 would have at least 80% power at 5% level of significance (two-sided) to detect a 25% difference in ISI with and without OLE. This has assumed a correlation of 0.5 between measurements on the same subject, and a 10% drop out during the study.

**Statistical analysis**

Treatment evaluations (i.e. OLE vs placebo) were performed on the principle of intention to treat (ITT). All statistical tests were two-sided and a 5% significance level maintained throughout the analyses. Statistical analyses were performed in SAS v.9.2 (SAS Institute, Cary, NC, USA). Linear mixed models were used to assess the main treatment effect accounting for randomization sequences and time periods. Importantly, regression models also adjusted for the baseline value of the outcome response to gain statistical efficiency and power (i.e. baseline data were included in the model as covariates). Other confounders that were considered in the analysis included: on-going use of medication (for cholesterol or hypertension), IPAQ scores, age, and total body fat percentage (from DEXA scans). When necessary, response variables were log-transformed to approximate normality. Baseline

descriptive data are presented as mean ± standard deviation (SD). The results from multiple regression analyses are expressed as model-adjusted means and 95% confidence intervals.

## RESULTS

Forty-six eligible participants were randomized into the trial (Figure 20). Four participants were on cholesterol lowering medication, three were on anti-hypertensives, and two were on both. Compliance with the study protocol was very high (>96% as measured by counting capsules in regularly returned containers), and no participants missed more than 3 days of capsules.

One participant dropped out of the study during stage 1 (due to injury), and two withdrew after crossover (one was lost to follow up, another due to developing acne) (Figure 20). All three subjects that withdrew were taking placebo at the time. Thus, data from 45 participants were included into 'intention to treat' analyses.

All participants were overweight, most were New Zealand Europeans (89%), and aged 46.5 years (range 34.5–55.6) (Table 21). Their metabolic profiles at baseline are itemized on Table 21. Daily energy intake among participants prior to study is show in Table 21, and was mostly unchanged throughout the trial. There was however, an increased energy intake from sugars during OLE supplementation (17.3 vs 14.7%; p=0.036). There were no changes in physical activity over the study period as assessed by the IPAQ (Placebo = 4651 vs OLE = 4649 METs; p=0.85)*.

* This value is MET-minutes per week

**Table 21: Baseline data on the study population. n=45, data are mean ± SD, or adjusted means and 95% confidence intervals.**

| | |
|---|---|
| **Demographics** | |
| Age (years) | 46.5 ± 5.5 |
| BMI (kg/m$^2$) | 28.0 ± 2.0 |
| **Diet & Lifestyle** | |
| Daily energy intake (kcal) | 2331 ± 525 |
| Daily energy intake from saturated fat (%) | 13.3 ± 3.2 |
| **Glucose homeostasis** | |
| Insulin sensitivity (Matsuda index) | 5.12 (4.31–6.09) |
| Disposition index | 5.17 (2.73–7.74) |
| **Plasma Lipids** | |
| Total cholesterol (mmol/l) | 5.09 (4.78–5.40) |
| LDL-C (mmol/l) | 3.18 (2.91–3.46) |
| Oxidised LDL-C (mU/ml) | 62552 (57691–67413) |
| HDL-C (mmol/l) | 1.05 (0.97–1.14) |
| Triglycerides (mmol/l) | 1.46 (1.32–1.61) |
| **Adiposity** | |
| Total body fat (%) | 29.4 (27.7–31.0) |
| Android fat to gynoid fat ratio | 1.31 (1.25–1.37) |
| **Ambulatory (24-hour) blood pressure** | |
| Mean diastolic (mmHg) | 80.9 (78.7–83.1) |
| Mean systolic (mmHg) | 127.6 (124.4–130.8) |
| Nocturnal diastolic dipping (%) | 18.9 (16.6–21.5) |
| Nocturnal systolic dipping (%) | 13.7 (11.7–15.7) |

**Insulin sensitivity and other parameters on glucose homeostasis**

The assessment of treatment effect (i.e. OLE vs placebo) showed that OLE supplementation was associated with a 15% improvement in insulin sensitivity (5.46 vs 4.73; p=0.024). Supportive findings included a 28% improvement in pancreatic β-cell function (5.45 vs 4.26; p=0.013) (Table 3). Further, OLE supplementation also led to a reduction in the area under the curve for both glucose (6%; p=0.008) and insulin (14%; p=0.041) (Figure 21). These findings were consistent with observed reductions following OLE treatment in glucose concentrations at 30 (6%; p=0.008) and 60 (10%; p=0.005) minutes, as well as a 23% reduction in insulin concentrations at 60 minutes (p=0.004) (Figure 21).

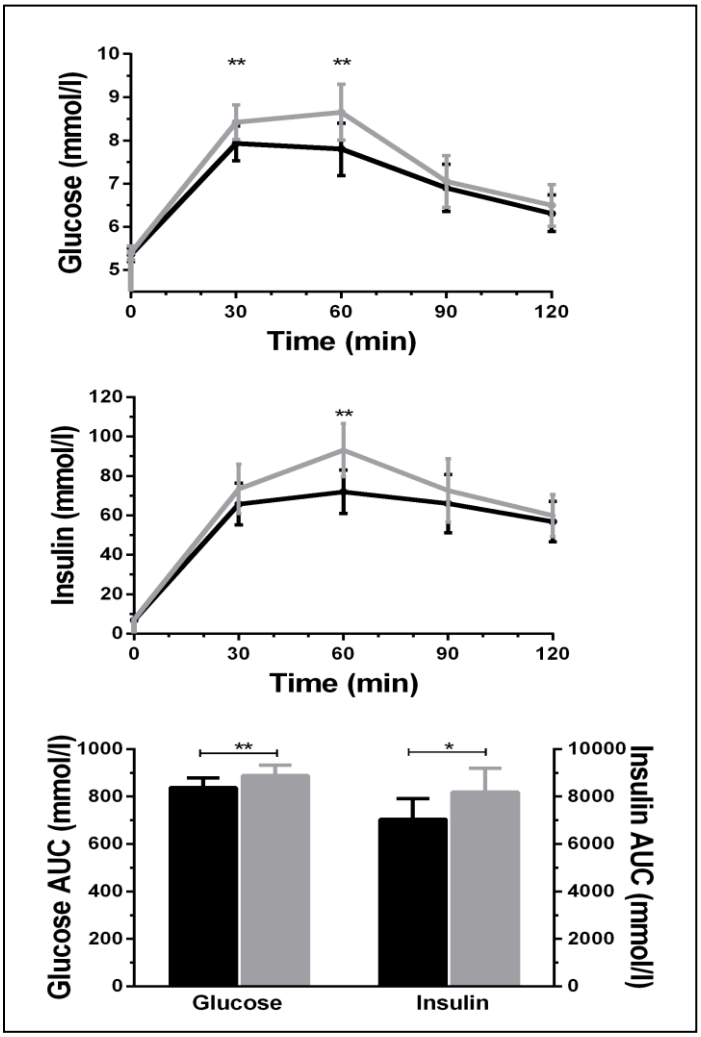


Figure 21: Insulin and glucose responses to oral glucose tolerance tests and respective areas under the curve (AUC), following supplementation with placebo (gray) and olive leaf extract (black).

*Data are adjusted means from multivariate models, and 95% confidence intervals.*

Subjects on OLE also experienced a 32% increase in interleukin-6 (p=0.014), but there were no observed changes in interleukin-8, TNF-α, or ultra-sensitive CRP (Table 22). While there were no differences in IGF-I, IGF-II, or IGFBP-3 plasma concentrations, OLE supplementation was associated with an increase of 20% in IGFBP-1 (p=0.024) and 13% in IGFBP-2 (p=0.015) concentrations (Table 22). There were no significant changes in lipid profile (including oxidised LDL-C), ambulatory blood pressure, body composition (Table 22), or carotid intimal thickness (OLE 0.820 (0.782–0.859) vs Placebo 0.832 (0.795–0.871) mm; p=0.40). There were also no significant changes in subjective assessment of wellbeing (data not shown).

**Table 22: Outcomes following a 12-week supplementation with olive leaf extract or placebo. n=45. Data are adjusted means from multivariate models, and 95% confidence intervals (CI).**

| | Placebo | Olive Leaf Extract | p-value |
|---|---|---|---|
| **Glucose homeostasis** | | | |
| Insulin sensitivity (Matsuda index) | 4.73 (4.13–5.41) | 5.46 (4.83–6.16) | **0.024** |
| Disposition index | 4.26 (3.28–5.54) | 5.45 (4.14–7.17) | **0.013** |
| **Hormones** | | | |
| IGF-I (ng/ml) | 176 (166–186) | 181 (172–191) | 0.13 |
| IGF-II (ng/ml) | 726 (698–754) | 7.09 (683–735) | 0.14 |
| IGFBP-1 (ng/ml) | 1.33 (1.02–1.73) | 1.59 (1.28–1.99) | **0.024** |
| IGFBP-2 (ng/ml) | 144 (126–164) | 162 (143–183) | **0.015** |
| IGFBP-3 (ng/ml) | 2345 (2203–2507) | 2324 (2187–2469) | 0.65 |
| **Plasma lipids** | | | |
| Total cholesterol (mmol/l) | 4.60 (4.39–4.82) | 4.72 (4.52–4.94) | 0.24 |
| LDL-C (mmol/l) | 3.06 (2.87–3.27) | 3.10 (2.93–3.28) | 0.63 |
| Oxidised LDL-C (mU/ml) | 62574 (57378–67770) | 62344 (57032–67655) | 0.90 |
| HDL-C (mmol/l) | 1.07 (1.01–1.13) | 1.04 (0.99–1.10) | 0.32 |
| Triglycerides (mmol/l) | 1.12 (1.01–1.24) | 1.16 (1.05–1.29) | 0.48 |

| | | | |
|---|---|---|---|
| **Proteins** | | | |
| Interleukin-6 (pg/ml) | 0.57 (0.44–0.75) | 0.75 (0.59–0.96) | **0.014** |
| Interleukin-8 (pg/ml) | 1.81 (1.63–1.99) | 1.93 (1.72–2.15) | 0.11 |
| Ultra-sensitive CRP (ng/ml) | 727 (540–978) | 702 (543–907) | 0.76 |
| TNF-α (pg/ml) | 7.57 (7.07–8.10) | 7.81 (7.28–8.39) | 0.46 |
| **Adiposity** | | | |
| Total body fat (%) | 30.3 (29.3–30.7) | 30.1 (29.3–30.8) | 0.89 |
| Android fat to gynoid fat ratio | 1.36 (1.33–1.38) | 1.36 (1.33–1.38) | 1.00 |
| **Ambulatory (24-hour) blood pressure** | | | |
| Mean diastolic (mmHg) | 78.2 (76.7–79.7) | 79.6 (77.8–81.5) | 0.088 |
| Mean systolic (mmHg) | 126.2 (124.0–128.4) | 127.3 (124.8–129.7) | 0.33 |
| Nocturnal diastolic dipping (%) | 17.6 (15.1–20.0) | 17.7 (15.7–19.7) | 0.89 |
| Nocturnal systolic dipping (%) | 13.6 (11.4–15.7) | 13.2 (11.1–15.3) | 0.70 |

**Adverse outcomes**

The only adverse event reported was a flare up of acne. The participant withdrew from the study and un-blinding showed that he was receiving placebo. Liver function tests showed no differences in AST, ALP, ALT, or GGT among participants in OLE vs placebo (data not shown).

**Subgroup analyses**

Data were also analysed on a subgroup of 36 participants, excluding 9 subjects who were on lipid-lowering and/or anti-hypertensive medications (

Table 23). The results changed very little, but importantly, there was evidence of an even greater effect of OLE on insulin sensitivity (20%) compared to placebo (5.94 vs 4.96; p=0.009) (Table 23).

**Table 23: Outcomes following a 12-week supplementation with olive leaf extract or placebo. Analyses excluded 9 participants on lipid-lowering and/or anti-hypertensive medications, so that n=36. Data are adjusted means from multivariate models, and 95% confidence intervals (CI).**

| | Placebo | Olive Leaf Extract | p-value |
|---|---|---|---|
| **Glucose homeostasis** | | | |
| Insulin sensitivity (Matsuda index) | 4.96 (4.35–5.65) | 5.94 (5.32–6.62) | **0.009** |
| Disposition index | 4.87 (3.80–6.25) | 6.03 (4.59–7.93) | **0.050** |
| **Hormones** | | | |
| IGF-I (ng/ml) | 175 (165–185) | 178 (169–188) | 0.43 |
| IGF-II (ng/ml) | 701 (670–732) | 689 (670–708) | 0.37 |
| IGFBP-1 (ng/ml) | 1.75 (1.38–2.11) | 2.28 (1.78–2.77) | **0.004** |
| IGFBP-2 (ng/ml) | 166 (149–185) | 194 (175–216) | **0.003** |
| IGFBP-3 (ng/ml) | 2317 (2200–2439) | 2324 (2197–2458) | 0.90 |
| **Plasma lipids** | | | |
| Total cholesterol (mmol/l) | 4.78 (4.60–4.98) | 4.84 (4.64–5.06) | 0.63 |
| LDL-C (mmol/l) | 3.28 (3.13–3.44) | 3.30 (3.13–3.47) | 0.89 |
| Oxidised LDL-C (mU/ml) | 66927 (62918–70936) | 67433 (61965–72902) | 0.82 |
| HDL-C (mmol/l) | 1.08 (1.03–1.14) | 1.04 (0.99–1.10) | 0.15 |
| **Proteins** | | | |
| Interleukin-6 (pg/ml) | 0.49 (0.37–0.63) | 0.64 (0.53–0.78) | **0.028** |
| Interleukin-8 (pg/ml) | 1.71 (1.58–1.84) | 1.92 (1.74–2.12) | **0.008** |
| Ultra-sensitive CRP (ng/ml) | 793 (640–983) | 586 (711–1037) | 0.45 |
| TNF-α (pg/ml) | 7.42 (7.01–7.84) | 7.86 (7.32–8.43) | 0.19 |
| **Adiposity** | | | |
| Total body fat (%) | 29.9 (29.3–30.6) | 30.1 (29.4–30.9) | 0.60 |

| | | | |
|---|---|---|---|
| Android fat to gynoid fat ratio | 1.33 (1.30–1.36) | 1.33 (1.30–1.35) | 0.72 |
| **Ambulatory (24-hour) blood pressure** | | | |
| Mean diastolic (mmHg) | 75.9 (74.6–7.3) | 77.2 (75.3–79.2) | 0.17 |
| Mean systolic (mmHg) | 122.7 (120.7–124.7) | 123.9 (121.4–126.5) | 0.34 |
| Nocturnal diastolic dipping (%) | 16.8 (14.5–19.1) | 18.0 (15.8–20.1) | 0.42 |
| Nocturnal systolic dipping (%) | 12.8 (10.8–14.7) | 12.6 (10.3–14.9) | 0.88 |

## DISCUSSION

We have shown that supplementation with olive leaf polyphenols for 12 weeks improves two aspects of glucose regulation (both insulin action and secretion) in a cohort of overweight middle-aged men. This novel finding was independent of lifestyle factors, such as dietary intakes, physical activity levels, BMI, or fat distribution. Importantly, the 15% improvement in insulin sensitivity observed with OLE supplementation is comparable to those seen with medications commonly used to treat diabetes. For example, metformin (250 mg TDS) improved insulin sensitivity by 17% in a group of sedentary overweight non-diabetics [469]. However, as Ou et al.'s cohort reported lower levels of physical activity than our participants [469], the use of metformin in our study group would likely have led to a comparatively smaller improvement in insulin sensitivity. Thus, we speculate that the observed improvement in insulin sensitivity with OLE is greater than would have otherwise been observed if our subjects have been treated with metformin instead. Another study demonstrated a 28% improvement in insulin sensitivity after treatment with 30 mg pioglitazone for 26 weeks [470]; but as their participants had type 2 diabetes, they are also likely to have shown an exaggerated response compared to our study group.

In addition, OLE also improved insulin secretion to further aid glucose regulation, which does not occur with the use of metformin. Type 2 diabetes generally involves defects in both insulin sensitivity and pancreatic β-cell secretory capacity [135, 136]. OLE supplementation was associated with a reduction in the glucose and insulin excursion after oral glucose challenge, suggesting an improvement in both pancreatic β-cell function and insulin sensitivity. The observed 28% improvement in disposition index is consistent with this

observation. Comparatively, studies in diabetic adults (who are likely to have an exaggerated response to therapy) have shown that mainstream medications affecting only β-cell secretion capacity have achieved improvements of 55% (dipeptidyl peptidase-4 antagonists) [471] and 100% (glucagon-like peptide-1 agonists) [472]. Hence, compared to these drugs that only improve insulin secretion, OLE improves both insulin sensitivity and pancreatic β-cell secretory capacity. Remarkably, the observed effects of OLE supplementation in our study population is comparable to common diabetic therapeutics (particularly metformin), and our results could have clinical significance for patients with type 2 diabetes.

Only one randomized placebo-controlled trial has previously investigated the effects of OLE on glucose metabolism in type 2 diabetes, finding an improvement in glycated haemoglobin (HbA1c) after 14 weeks of supplementation [473]. However, that study did not measure or discuss possible variations in diet or levels of physical activity among participants [473], so that the independent effect of OLE cannot be determined. Hence, our study is the first to show the independent effects of OLE on glucose homeostasis in humans, corroborating previous findings *in vitro* and in animal models[474].

We also found elevated interleukin-6 levels (a pro-inflammatory cytokine) with OLE supplementation. Interleukin-6 functions differently depending on its concentration and the tissue it acts upon. Acute increases improve the insulin-regulated glucose metabolism in the muscle [475], while chronically mildly elevated levels are associated with a pro-inflammatory insulin resistant state in the liver. Thus, OLE supplementation may improve insulin sensitivity and glucose uptake via interleukin-6, and possible mechanisms for this effect have been proposed [476, 477]. Further, we also observed that OLE supplementation led to increased IGFBP-1 and IGFBP-2 plasma concentrations. Increased IGFBP-2 concentrations are protective against the development of obesity and improve insulin sensitivity [478], while higher IGFBP-1 concentrations are associated with lower insulin levels [479].

In regards to other measured cardiovascular outcomes, OLE supplementation did not improve 24-hour ambulatory blood pressure, lipid profile, or cIMT. Previous studies have shown improvements in blood pressure with OLE supplementation [480, 481], but they did not involve 24-hour monitoring.. Similarly, our findings on lipid profile also contrast with those of previous studies [480-482]. However, Perrinjaquet-Moccetti et al. did not examine dietary

factors [480], Susalit et al. had a low cholesterol dietary component to the trial [481], and Fonolla et al. studied hypercholesterolemic subjects [482]. In addition, although we did not observe improvements in cIMT, this null result may be a result of our relatively short intervention. Nonetheless, consistent with our findings, the European Food Safety Authority recently concluded that there was insufficient evidence to substantiate health claims of improvements on blood pressure, lipid profile, or anti-inflammatory effects [466].

The strengths of this study lie with it being a randomized, double-blinded, placebo-controlled, crossover trial, using well-validated scientific methods (i.e. ambulatory blood pressure, Matsuda method, and cIMT). Although insulin sensitivity was not measured using the gold-standard euglycemic hyperinsulinemic clamp, it was assessed using the Matsuda method that is one of the best performing proxy methods [129]. In addition, we adopted a comprehensive approach to modifiable cardiovascular risk factors, including attention to dietary intakes and physical activity levels. The OLE supplement was well-tolerated, and compliance with the study protocol was excellent. Potential weaknesses include the relatively short intervention, which may have obscured pathophysiological changes that require longer periods of time to develop.

Overall, this is the largest and most comprehensive study to date examining the effect of supplemented olive leaf polyphenols alone on modifiable cardiovascular risk factors. We showed improvements in insulin sensitivity and pancreatic β-cell secretion capacity, in a cohort of overweight middle-aged men. Future research should evaluate the potential effects of olive leaf polyphenols on insulin sensitivity and glycaemic control (HbA1c) in patients with type 2 diabetes, and compare any such effects to conventional therapy (e.g. metformin).

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# Chapter 6. Discussion

The original research presented in this thesis has yielded novel results that add to the existing literature on macro- and micronutrient effects on insulin sensitivity. Importantly, while we have confirmed that psyllium appears to have no insulin sensitising properties, we have reinforced that it can improve cardiovascular disease risk by reducing LDL cholesterol. In contrast, the positive effect of olive leaf extract on insulin sensitivity has potentially validated its use as a folk medicine for treating diabetes, but requires confirmation in future studies, which should also attempt to define underlying mechanisms of action. Overall, while there are many limitations to the published studies contained within this thesis, these can inform future studies on dietary fibre and olive leaf extract to better advance our knowledge on their effects on insulin sensitivity.

## 6.1 Psyllium and olive leaf extract as potential nutritional supplements to improve insulin sensitivity

The null result in Chapter 3 has shown that supplemented psyllium alone does not improve insulin sensitivity in adolescents. Our hypothesis that psyllium would improve insulin sensitivity was based on: i) broad epidemiological data showing greater fibre consumption is associated with improved insulin sensitivity (section 1.4.1.2); ii) improved post-prandial glucose and insulin response to meals with supplemented psyllium[420, 421] for which repeated doses should up-regulate insulin receptors and secondary insulin signalling molecules[179], and iii) that psyllium is also 30% insoluble fibre (which confer protection from type 2 diabetes[181]). Despite these factors suggesting that psyllium supplementation would improve insulin sensitivity, our findings are consistent with results from a shorter term study (3 days) using a higher amount (10 g/day) of psyllium supplementation in adults, which adopted gold-standard methods to measure insulin sensitivity[421].

We can speculate that psyllium was ineffective at improving insulin sensitivity due to limitations in our study design (see later). Alternatively, the proportion of insoluble fibre in psyllium is poorly fermented in the gut, and therefore does not produce enough of the short-chain fatty acids in the colon, which have been identified as a likely mechanism for fibre to improve insulin sensitivity. Another potential factor contributing to the negative result was

the population tested, which was predominantly of Pacific Island ethnicity. The genetic propensity (the so-called “thrifty gene”) that Pacific peoples have for the development of type 2 diabetes when placed in a Western environment (abundance of food) is well established[483]. The population we studied are in such a 'diabetogenic' environment due to their genetics and food availability, that no possible improvements in insulin sensitivity could be observed with a relatively small intervention of 6 g/day of psyllium.

On the other hand, olive leaf extract (as investigated in Chapters 4 and 5) is a promising nutraceutical. It contains micro-nutrients (most likely the dominant constituent polyphenols oleuropein and hydroxytyrosol) that are sufficiently absorbed, and can significantly improve insulin sensitivity. The observed 15% improvement in insulin sensitivity following olive leaf extract supplementation is exciting, especially considering that this improvement is similar to what has been shown with metformin in a similar study population. Further, the 28% improvement in pancreatic β-cell function shows that olive leaf extract potentially shares mechanisms of action with other commonly prescribed diabetic medications, such as the dipeptidyl peptidase-4 antagonists and glucagon-like peptide-1 agonists. When put into context of the existing literature on micronutrients (reviewed in section 1.4.2), this magnitude of improvement appears impressive.

Naturally, interpretation of the olive leaf extract results remain guarded, and would likely not reach a “best evidence for” judgement from Yeh et al [253], until further studies have corroborated our findings. It is perhaps unfortunate that most data on macro- and micronutrient effects on insulin sensitivity are epidemiologically based, and hence it is difficult to truly gauge the significance of our results on a comparative basis.

## 6.2 Importance of our findings

Although we confirmed that psyllium supplementation does not improve insulin sensitivity, it should be disregarded in the clinical setting of impaired glucose homeostasis. While insulin sensitivity may not be affected, psyllium remains useful when impaired glucose homeostasis is established, as it is predominantly soluble and reduces post prandial glucose excursion[420, 421]. It is possible to speculate that repeated reductions in glucose excursion would improve overall glycaemia, which could be measured by glycosylated haemoglobin, but such a study is yet to be performed. Importantly, any effects of psyllium supplementation on glucose

homeostatis, even if relatively minor in isolation, cannot be under-estimated at a population level due to its ease of use. Commercial food producers already utilise psyllium to boost fibre content of food as it does not affect taste or palatability (being therefore probably more acceptable than anti-diabetic or lipid-lowering medications). Further, psyllium is cheap and can be incorporated into a vast amount of commonly consumed foods, so that intervention at a population level is achievable.

While our study did not show improvement in insulin sensitivity, it did confirm that psyllium reduces LDL cholesterol. Therefore, supplemented psyllium is useful at lowering the risks of cardiovascular disease. Further, had our study extended longer and shown a sustained improvement in body composition, it could be speculated that improvements in insulin sensitivity may have been observed and our hypothesis proven correct.

The ability of olive leaf extract to improve insulin sensitivity is important as one can speculate on several exciting prospects: i) by virtue of improving insulin sensitivity, it may slow the progression of impaired glucose tolerance to type 2 diabetes; ii) it may be a useful therapeutic adjunct to Western medicine when type 2 diabetes is already established; and iii) as the mechanism of action remain elusive, once established, it may open a new therapeutic target in the management of type 2 diabetes. However, as this is novel research that has yet to be reproduced, at best we can conclude that olive leaf extract has considerable potential as a nutraceutical and indeed as a lucrative commercial market for olive farmers.

The strength of our results is reinforced by the rigorous attention to detail, where all attempts were made to isolate the effect of the dietary intervention alone. In general, few papers have attempted to control for other dietary components or activity levels, which are potentially important confounders when measuring insulin sensitivity. Recording nutritional intake in particular, is considerably labour intensive, which is probably why it is not reported consistently. In both of our clinical trials, food inventories and physical activity levels were carefully recorded to ensure that the nutrition intervention of interest was primarily responsible for the observed clinical outcomes.

## 6.3 Limitations

Numerous aspects of the studies presented in this thesis may have influenced the results, and must be taken into consideration before overall conclusions can be drawn. These include study design (e.g. study size and length of intervention), the use of the Matsuda method to measure insulin sensitivity, the populations studied, and for the olive leaf trial our inability to identify a likely mechanism of action.

Both the psyllium and olive leaf extract trials involved a randomised controlled crossover design. While this study design is widely considered to be of high scientific validity, it has certain limitations (section 2.1). While there were no problems with patient drop out and the statistical analysis took into account sequence affects, residual or carry on effects could have been better accounted for in the experiments. Strictly speaking, each participant should have had a $4^{th}$ assessment (at the end of washout period) to ensure that metabolic profiles had returned to baseline. However, the associated additional burden on study participants could have led to patient drop-out. Therefore, a compromise was reached where washout periods used by other investigators were incorporated, i.e. the length of the washout was based on ancillary data, and in the olive leaf bioavailability study for example, metabolites were eliminated from the plasma within hours. Reviewers of our manuscripts have accepted this compromise.

The length of each individual study should also be considered a limitation. In the olive leaf extract trial, each participant was involved for 10 months, a considerable investment of time. The psyllium trial was much shorter, with each study phase only lasting six weeks. It remains a possibility that a longer study might have improved insulin sensitivity – as our results suggest that psyllium improves fat distribution..

All three clinical studies had quite small study populations, which reduced their power, and therefore the significance of the results. Nevertheless, both the psyllium and olive leaf extract trials were based on statistical power calculations to determine the sample size to examine their effects on insulin sensitivity. In regards to the bioavailability study, its rather small sample size is reflected in the heterogeneous results observed. While no other investigators in this field have performed larger studies, perhaps the repeated theme of large inter- and intra-patient variability reported by others authors is therefore not surprising. Had our experiment been conducted with more participants, it might have been possible to confidently establish differences in bioavailability of olive leaf extract on the basis of gender, age, or body habitus.

However, bioavailability studies such as this are expensive, and like any research, there are unavoidable budgetary limitations.

In section 2.2, the reasons for choosing the Matsuda method were established, but it does have important limitations (as previously described). Only through the hyperinsulinemic-euglycaemic clamp can insulin sensitivity be isolated at its point of action (primarily skeletal muscle). This is particularly important when considering the olive leaf extract clinical trial, as a mechanism of action could not be identified. Further, it is unknown at what level does olive leaf extract improve insulin sensitivity, which might have been partly answered if the hyperinsulinemic-euglycaemic clamp was utilised. Future research is discussed later, but with specific reference to this issue, a pilot study on a subset of participants using the clamp technique could have made our findings more robust.

Our study populations were specifically targeted to enrol participants carrying the highest rates of disordered glucose homeostasis. In the psyllium trial, recruitment was from schools in a poor socio-economic area, with a high proportion of Pacific Islanders – the most at-risk group in New Zealand for developing type 2 diabetes. As previously mentioned, it is possible that such a select group may be resistant to any improvement sustained from fibre supplementation, due to other factors associated with lifestyle and/or genetics that might have overwhelm any possible therapeutic advantage. Only large scale studies, covering a broad range of ethnicities and socio-economic groups would be able to identify if this was the case.

Another criticism is that in the psyllium trial we presented obesity rates in accordance to adult WHO criteria (30 $kg/m^2$). We could have instead adopted a reference database (such as UK Cole), which uses cut-offs that project a BMI greater than 30 $kg/m^2$ at 18 years of age. However, this might have been an inappropriate reference population, given our predominantly Pacific Island population. Regardless, using the adult WHO criteria rather than a population reference would only under-estimate the true rates of obesity in an adolescent population.

The olive leaf extract trial recruited through newspaper advertising, and resulted in the recruitment of predominantly affluent participants of New Zealand European ethnicity (and hence at lower risk of developing type 2 diabetes compared to ethnic minorities). However, we studied the very group who are the highest consumers of nutraceuticals. So, while the results of the olive leaf trial are applicable to the most likely buyers of nutraceuticals, they may not apply to the low socio-economic ethnic minority groups that suffer most at risk of

developing type 2 diabetes. It is also important to note that we used the IPAQ questionnaire in the olive leaf trial for assessment of physical activity, while we could have used the NZPAQ, which is a modified version of the IPAQ taking into account usual physical activities undertaken in New Zealand.

Lastly, the inability to find a mechanism of action to account for the improvement in insulin sensitivity due to olive leaf extract supplementation is a major limitation to this study. *In vitro* work has established many potential mechanisms, particularly around down-regulation of inflammatory cytokines. However, the raised IL-6 seen after olive leaf extract supplementation in our study potentially rules this out. Metabolomics and nutrigenomic effects are other possible mechanisms of action, and require attention in further research. As previously mentioned, had we chosen the clamp method to measure insulin sensitivity, the search for candidate mechanisms of action might have been narrowed.

## 6.3 Applicability of our findings

When considering applicability at a population level, there is a large contrast between the psyllium trial and olive leaf extract trial. Firstly, psyllium is comparatively cheap, and online shoppers can buy 10 kg of organic psyllium for US$ 125, (equating to 12.5c per day if 10 g was added to food); in contrast 500 mL of olive leaf extract cost approximately US$ 35 (, equating to US$ 1 per day when consumed at a commonly recommended daily dose of 15 mL). Therefore, the use of psyllium at an individual and population levels is feasible. Psyllium can be added to (and already is) to a wide variety of foods, unnoticed by the consumer, making it a tolerable and acceptable supplement. Of course, as the study showed no improvement in insulin sensitivity, any benefits to cardiovascular disease would likely be through sustained improved LDL cholesterol, or perhaps improved body composition.

Olive leaf extract is expensive and bitter to taste. Nonetheless, regardless of price or tolerability, given the willingness of diabetic patients to take complementary medicine, our findings may result in olive leaf extract becoming a common choice for these patients. Therefore, olive leaf extract may have potential for specific groups, but is unlikely to be useful at a population level.

There are many general factors with respect to nutritional interventions that need to be considered with regard to clinical applicability of our results. These are expanded upon in the final commentary.

## 6.4 Future research

Taking into account the limitations of our studies, a paradigm for future investigations can be envisaged. Futures studies on psyllium should considerably extend the duration of intervention, so that effects such as the improved fat distribution demonstrated in our own work have a chance to improve insulin sensitivity. Importantly, Anderson et al [421] who also showed a negative result, applied the hyperinsulinemic euglycemic clamp after just three days of psyllium supplementation. Further, a study in patients with established type 2 diabetes would be useful, given that previous literature showed that psyllium improved post-prandial glucose and insulin excursions, so that long-term supplementation could improve insulin signalling and measures of glycaemic control. Finally, a constant theme in nutrition literature in this field, is the need to define mechanisms through which foods improve health outcomes. For fibre, the search for these could be in secondary messengers such as G proteins nutrigenomics and metabolomics. Leading journals still ask that researchers investigate new mechanisms of action for dietary fibre, which could lead to new therapeutic targets[167].

As previously mentioned, supplementation with primarily insoluble fibre might have been a better choice to assess possible effects on insulin sensitivity. Weickert et al. have conducted such a study giving purified oat fibre supplementation to overweight and obese women, and observed an improvement in whole body insulin sensitivity (using gold standard measures) by 13%[484] However, this study was small (n = 13), the intervention was very short (72 hours), and the authors also failed to find a mechanism to explain the results. A quandary also exists when considering Weickert at al.'s study, as there was no improvement in lipid profile, as compared our study and others[485] using primarily soluble fibre. Therefore, from a practical perspective, different purified fibre sources with unique physiological activities may specifically improve certain cardiovascular disease risk factors – either lipids or insulin sensitivity. In addition, while studies using purified fibre sources like ours and that of Weickert et al. allow for a 'clean' scientific analysis, it should be remembered that in every day dietary intake, high fibre whole foods are rich in both insoluble and soluble fibre, and can therefore offer the diverse range of health benefits.

The step-wise research program establishing olive polyphenol bioavailability after olive leaf extract ingestion and the subsequent clinical trial demonstrating improved insulin sensitivity have answered many questions, but raised several more. With specific reference to the

bioavailability trial, future research should be directed at: i) finding an optimum dose to maximize absorption; ii) establishing tissue distribution of metabolites using cold labelled olive leaf polyphenols (which are currently unavailable); and iii) conducting a larger trial to more clearly define the effects of gender, age, or body habitus on absorption and metabolism of olive leaf polyphenols. Such research may also be able to identify individual groups most likely to benefit from olive leaf supplementation.

Due to the improvement in insulin sensitivity seen with olive leaf extract supplementation, planning is already underway for future work. This research will aim to establish mechanisms of action, target a different population (diet-controlled type 2 diabetics), and will directly compare any effects to western medicine (Metformin). Initial power calculations indicate that this study will require upwards of 200 participants, making it one of the largest and most robust studies to examine the effects of any micronutrient on glucose homeostasis. By directly comparing to a commonly prescribed medication such as metformin, it is hoped that health professionals and consumers may understand the relative benefits of both (either taken separately or combined).

New mechanisms through which macro- and micronutrients like fibre and olive leaf extract can improve insulin sensitivity should be an emphasis in future research. Perhaps the most exciting new and expanding field is that of nutrigenomics. Nutrigenomics can be summarised as the study of how nutrients modulate gene and protein expression, which then influences the metabolism[486]. A significant focus of the nutrigenomics field has been the interaction between nutrition and genes known to be involved in the metabolic syndrome – for example genes involved with inflammation, metabolic stress, insulin resistance and diabetes[486]. While there is still a lot of research to be done, commercial operators are already able to provide specific nutrition advice based on an individual's genotype and/or phenotype. In practice for example, commercial operators are able to genotype an individual from a buccal swab, and then deliver specific information – like variations in the apolipoprotein E (APOE) gene, where carriers of the APOE4 allele benefit most from a diet low in saturated fat and alcohol[487]. The hope is that this will eventually allow for strategic targeted nutritional advice. By doing so, the nutrigenomic field recognises that “one size fits all” is over-simplistic, and that there are unique individual responses to nutrients – a fact that has been mentioned several times during this thesis. Certainly, by incorporating likely individually tailored bioavailability profiles (which can only be achieved after doing lager trials), such personalized dietary interventions can be designed.

Future studies on fibre or olive leaf extract should employ gold standard methods whenever feasible and financially viable, as this was a significant limitation in our work. Food regulators are alert to this, and for example the European Food Safety Authority clearly indicates that gold standard measures or the Matsuda method are necessary for products to make a health claim on insulin sensitivity[141].

These recommendations for future research describe a scientific utopia, where research funding, time, and resources are unlimited. An idealistic view of what the literature on nutrition in the future should strive for has been painted. However, many aspects, especially with respect to careful planning and robustness of study design are easily attained. Other aspects such as finding new mechanisms of action, conducting long clinic trials, and using labour intensive and costly gold standard measures, are more challenging to overcome. Nevertheless, if we consider the nutraceutical industry, which is becoming increasingly more competitive, researchers need to engage with commercial companies, who may be in a position to provide financial backing for trials, allowing the use of some of the more expensive research tools discussed. Such research collaborations should aim to conduct research that will not only give credibility to the intervention (with the ultimate effect of benefiting the patient), but would also benefit commercial operators by providing robust scientific evidence on the effectiveness of their products (and hence improve market share and profits), whilst increasing the skill set and expertise of nutraceutical researchers.

## 6.5 Macro and micronutrient diet manipulation in context; insights and commentary.

Lifestyle modification (diet and exercise) is the principle method to prevent type 2 diabetes, and macro and micronutrient manipulation is just one way that the diet can be changed in order to improve insulin sensitivity. While most literature in the field has targeted weight loss through caloric restriction, there is growing evidence that diets which use isocaloric macronutrient manipulation – for example low CHO and high fibre diets - can improve insulin sensitivity. The evidence concerning the relationship between micronutrient intake and insulin sensitivity is still in its infancy. However, because the human diet is infinitely diverse, there are an equally diverse range of possible dietetic interventions that could be used to improve or alter insulin sensitivity. The human diet is influenced by external (for example geography, season, culture, social) and internal (for example appetite, wellbeing, physical activity, genetic, age) factors. Many of the patterns seen in population health have been analysed to try and determine which external and internal factors may influence overall population health e.g. rate of type 2 diabetes, rates of CVD. As a result, many of the factors outlined above have become candidates for research on healthy nutrition. Taking "culture" as an example of an external influence on diet, numerous nutritional programs have been designed to mimic diets particular to certain cultures that appear to protect from diabetes – for example a high fibre diet typical in sub-Saharan African tribes, the Mediterranean diet, or even the high oily fish diet seen in some populations. Similarly, internal factors have been targeted, for example specific nutrition plans to improve satiety and therefore caloric intake with an eventual aim to improve glucose homeostasis. In the following section, the potential role of macronutrient and micronutrient dietary manipulation will be contextualised within the current lifestyle intervention framework and amongst the tools currently used to treat and prevent type 2 diabetes.

## 6.5.1 The effect of diet alone as part of lifestyle modification on insulin sensitivity

### 6.5.1.1 Problems with nutrition interventions targeting weight loss through caloric restriction

Obesity and impaired glucose tolerance are inextricably linked, and thousands of studies using lifestyle intervention targeting weight loss have been published. The majority of caloric restriction diets have been studied as a component of a lifestyle intervention program that includes exercise. However, given that not all obese individuals are insulin resistant and that some non-obese individuals are insulin resistant, interventions targeting weight loss as the mode to improve insulin sensitivity will miss some individuals at risk of developing type 2 diabetes[488]. For those that are obese and insulin resistant, many studies have documented improved insulin sensitivity when weight loss has been the primary objective[488]. Unfortunately, the main diet approach to achieve weight loss is calorie restriction, and the common theme to the vast majority of these studies is an early response, and then eventual relapse. For example, after a 6 month hypocaloric diet in obese women, 96% lost greater than 5kg by the end of the intervention, but only 11% had maintained weight loss after 5 years[489]. While I am not attempting to write an opinion on eating behaviour and psychology (this is available elsewhere[490]), anyone who has attempted to remove a lolly from a child, has been employed as a health professional fighting obesity, or has personally tried to ignore the dessert table at a buffet, will know that applying dietary constraint on oneself or another person is a daunting task indeed. Experience will tell us that for the majority, attempts at dietary restraint are likely to fail – and even if it does succeed the first time, to consistently restrain approaches impossibility. The failure of our ability to self regulate total energy intake has fuelled the popularity of many fad diets and our expenditure on so-called super foods marketed as the saviour from metabolic disease, to rescue us after we have subjected ourselves to decades of overindulgence.

### 6.5.1.2 The role of a low carbohydrate diet

One of the most successful fad diets (in terms of popularity) in recent times has been the low CHO “Atkins” diet. This approach was initially unconventional and controversial as it

directly opposed the long held belief (and public policy) doctrine that a low fat diet was the best way to prevent CVD. However, as already mentioned in section 1.4.1, this macronutrient approach has produced consistent and prolonged results with respect to weight loss. The irony of course, it that this is just restraint in disguise, as many studies show that total energy intake on a low CHO diet is dramatically reduced despite having unrestricted fat and protein intake (see [491] for example). However, distinct from energy intake (and the effects on weight loss) there is a powerful argument that limiting CHO intake is very important when diabetes is already established. This is because CHO directly affects plasma glucose and insulin requirements, and the published evidence on insulin sensitivity and glycaemic control while on a low CHO diet is compelling[492, 493]. From a preventive perspective, proponents of the low CHO diet have connected public policy on low fat diets to greater intakes of highly refined CHO intake (and therefore increased rates of type 2 diabetes). However, such claims must be balanced against safety concerns when a low CHO diet is implemented long term. These concerns include nutrient inadequacy (fibre, thiamin, folate, iron, calcium, magnesium, and vitamins A, E and $B_6$), the lack of sustained lipid profile improvement, lack of cancer preventing food groups like fruit and vegetables, poor bone health due to low calcium, and poor kidney health due to high protein load[494]. Overall, the low CHO diet has taught us three things; i) improved insulin sensitivity and glucose homeostasis on a low CHO diet is improved more than one can predict from weight loss alone in the setting of diabetes, ii) popular diets that are fashionable can be useful and have powerful motivational factors, and iii) traditionally held views can be overly simplistic and restrictive, but new methods must be monitored long term for safety.

#### 6.5.1.3 The role of a high fibre diet

Promoting a high fibre diet, which arguably has the best evidence for improving insulin resistance and preventing type 2 diabetes, seems to be lost in the world of healthy nutrition messages. Even in a highly educated Scandinavian population, surveyed individuals were poor estimators of fibre intake, and fibre did not feature spontaneously as part of a healthy diet[495]. This reflects that the current nutrition education model which is based upon whole foods (eat more fruit and vegetables), does not effectively communicate what it is about those foods (i.e. fibre) that confers health benefit.

There are several misconceptions about dietary fibre which likely contribute to the low intakes common in Western diets. For example adolescent males are typically considered to have an inherently high fibre diet due to the huge volumes of bread they consume, but the results in our study mirror those around the world [496]. Another typical myth is that infants cannot tolerate whole grains or high fibre, so that rice cereal is commonly introduced as a first food for babies – a practice that many believe trains infants to favour highly refined CHOs which ultimately leads to obesity and type 2 diabetes[497].While our own study using 6g of supplemented psyllium did not improve insulin sensitivity, the intervention was well tolerated and one could speculate that a longer study would show improved insulin sensitivity given the improvement in fat distribution we observed. Psyllium as a form of fibre is extremely versatile because it is well tolerated, and easily added to foods without significantly altering texture or taste. Specifically targeting foods that adolescents living in poor socio-economic areas commonly consume (pastries, pizza, refined bread), and enriching them with fibre such as psyllium could conceivably have wide ranging long term health benefits. At least in an adolescent population, such an approach would likely be more acceptable and tolerated than calorie restriction, or very low CHO diets.

## 6.5.2 Impact of dietetic manipulation on insulin sensitivity in comparison to other nutritional and non nutritional interventions

### 6.5.2.1 Macro and micronutrient diet manipulation compared to pharmaceuticals

Head to head comparisons of dietary fibre versus metformin are rare, but at least one author has found that the use of a supplemented high fibre diet was more effective than education alone, but not as effective as metformin at preventing impaired glucose tolerance progressing to type 2 diabetes[498].

The improvements in insulin sensitivity due to some micronutrients and traditional herbal remedies are comparable to pharmaceuticals. Our results with olive leaf extract showed a 15% improvement in insulin sensitivity, which is very comparable to an observed 20%

improvement seen after a typical metformin dose, in a very similar overweight male population. The weakness here, as is applicable to the vast majority of literature in this field, is that direct comparisons to anti-diabetic medicine (for example metformin, or sulfonylureas), or even physical activity have not been made. Efforts to gain such data may be hampered by the ethics of non-treatment. It is therefore impossible to rank treatment responses.

Individuals respond to dietary interventions in a heterogeneous way, and therefore a one size fits all approach is inappropriate. For example, in the published commentary on polyphenols (section 1.4.2.6), interindividual differences in absorbance and physiological response were highlighted. Our own results investigating the absorbance (Chapter 4) and bioactivity (Chapter 5) of olive leaf extract are consistent with this observation. Modern technology (for example nutrigenomics) will help predict individual responses to nutritional interventions in the future. In practice, the consumer or the health practitioner has to consider what benefit a traditional remedy, micronutrient, or a concentrated nutraceutical may have for themselves or their patient, and this must be weighed up against tolerability, side effects, and individual preferences. This introduces the concept that dietary approaches often need to have an individualised strategic targeted focus, or at least form a significant part of a multi-facet intervention, which is expanded upon later.

#### 6.5.2.2 Nutrition intervention compared to exercise

Increasing physical activity is another established mechanism by which insulin sensitivity can be improved. Compared to exercise, macronutrient diet manipulation is equally effective at preventing the progression of impaired glucose tolerance to type 2 diabetes. Several large studies, with very long follow up, and conducted in very different cultural and environmental settings (China and Finland, United States) have shown that diet and exercise intervention has sustaining effects on preventing type 2 diabetes, long after the intervention has occurred[147, 499, 500]. The nutrition advice in all these studies was targeted to reduce total calories, reduce saturated fat, and to increase dietary fibre intake. Importantly, in the Chinese study, the initial randomization separated the cohort into 4 groups; control, exercise, diet, or combination. The diet alone intervention had a hazard rate ratio for diabetes of 0·58, compared to 0.51 for exercise, and 0.66 for the combination after 20 years of follow up. A highlight from the Chinese study is that their effects appeared to be independent of weight

change during the intervention period, which provides evidence that diet manipulation can improve insulin sensitivity beyond any effect on weight, and does so over a sustained period of time. Unfortunately, the detail required to see if there was a specific aspect of the diet which conferred the protection from type 2 diabetes was not collected or reported. Our own results in Chapter 5 which demonstrated improved insulin sensitivity independent of weight loss are consistent with this study, and add weight to our speculation that olive leaf extract could ameliorate the progression of impaired glucose tolerance to type 2 diabetes.

#### 6.5.2.3 Overall lifestyle modification compared to pharmaceuticals

There is comparative literature on pharmaceutical interventions versus the lifestyle approach. For example, the United States diabetes prevention program found that lifestyle (a "healthy" diet and increased physical activity) intervention was far superior at improving insulin sensitivity, preserving pancreatic β-cell function, and preventing progression to type 2 diabetes compared to metformin[501]. Furthermore, in a meta-analysis of 21 trials, lifestyle programs performed better than anti-diabetic drugs, and orlistat (a weight reduction drug commonly known by its trade name Xenical) at preventing type 2 diabetes[502]. Again, the limitation here is that the independent effect of diet over exercise is not possible to determine, let alone specific aspects of the diet which contributed most to the observed benefit.

#### 6.5.2.4 The role of bariatric surgery

Bariatric surgery is reserved for the obese, and the associated improvement in glucose homeostasis is dramatic and sustained[503]. Not only is it health effective, it is cost effective[503]. However, from a population standpoint, it is seen as rescue therapy after lifestyle interventions have failed. Indeed, there is usually public outrage when it is disclosed that tax payer money is funding bariatric surgery. As a counter argument, proponents of bariatric surgery will argue whilst we live in an diabetogenic environment it is necessary for governments to take responsibility for its citizens not only at a population level, but also for the individual (for example bariatric surgery) when the benefits outweigh the risks and costs. However, bariatric surgery currently only has a very limited role in terms of preventing diabetes, with most publicly funded surgical entry criteria having type 2 diabetes as a required co-morbidity.

#### 6.5.2.5 The role of complementary and alternative therapy

The fact that over 30% of type 2 diabetics actively use complementary and alternative therapies (many of which are nutrition based) to treat their condition[253] is an important point. Indeed, considering optimal compliance with conventional oral anti-diabetic drugs is less than 50%[504] even small benefits (assuming they are validated) from complementary therapies could be considered useful in the overall scheme. The popularity of alternative nutritional remedies is evidence for their tolerability, and that they are perceived to be beneficial (as if this were not the case, people would likely to stop using such therapies given they are generally not prescribed and are purchased at the cost of the consumer). While some would argue that given the lack of hard scientific evidence proving their efficacy, endorsing the use of alternative treatments (like nutraceuticals) is modern day quackery, the reality is that many individuals strongly believe that they work. Therefore, health care workers discussing nutrition and the effects on glucose homeostasis need to ask about alternative treatments their patients may be taking, and to keep a relatively open mind.

In summary, a healthy diet as part of a healthy lifestyle is the best proven way to prevent type 2 diabetes. Unfortunately, applying dietary change beyond the research setting appears to defy the desires of human nature with relapse rates comparable to behaviour modification programs targeting drug, smoking and alcohol addiction[505]. The fact that the incidence of type 2 diabetes continues to increase despite the knowledge we have testifies that application of theory has so far been ineffective at a population level.

## 6.5.3 What prevents nutritional interventions improving diabetes incidence at a population level?

Globally, type 2 diabetes is increasing; a clear indication that current nutrition based strategies are failing. Why is it that despite the well-funded and well-informed knowledge health strategists have, efforts to curb obesity and rates of type 2 diabetes have been largely ineffective? To attempt to answer this question, several aspects that resist social change can

be considered. These include; cost and availability, education, marketing and regulation, and civil liberty.

### 6.5.3.1 The cost and availability of healthy nutrition

The enormous cost impact of type 2 diabetes on health budgets provides a fiscal motivation for health promotion and administrative policy targeted at preventing its onset. Hundreds of millions of dollars are used in large scale clinical trials, with modest benefits. However, the fact that obesity and type 2 diabetes rates continue to climb suggest that costly administration driven health initiatives that attempt to apply theory from clinical trials at a population level are failing.

After taste, cost is the most influential factor on food choice[506]. It is an unfortunate fact that healthy foods like fresh fruit and vegetables are more expensive than energy dense high sugar and high fat foods[507]. It is well established that those from the most deprived socio-economic status have the highest rates of poor nutrition and type 2 diabetes, yet these are the same individuals expected to consume a more expensive healthy diet. Our results in Chapter 3 are consistent with the established literature showing that low socio-economic status is associated with unhealthy food choices. For the individual living on a restricted budget, it is extremely challenging to understand and justify an extra expense today to prevent disease in the distant future. The effect of food price on purchasing behaviour is complex, but it appears that increasing the price of unhealthy foods (for example through 'fat tax'), rather than lowering the cost of healthy foods (for example by removing the Goods and Services Tax [GST] from fruit and vegetables) is most effective at promoting healthy food choices[508].

"Fat Tax" – applying taxation to food high in fat, has been identified as a possible way to increase the cost of unhealthy food, and has been subject to vigorous debate over the last decade. Taxation on unhealthy food is unpopular, but this attitude is mitigated when applied to foods marketed to young children[509]. Evidence demonstrating the effect of taxing food on purchasing behaviour at a population level is lacking, and currently relies on statistical models. These models highlight the regressive nature of taxing unhealthy food – that is, it impacts the poor the most (as a proportion of income)[510]. Attempts to apply tax to unhealthy food have been made, but are often repealed after opposing arguments from food manufacturers. It is possible that tax on unhealthy foods could be part of a public health initiative, but this strategy is probably best applied in closed systems (e.g. in schools and workplaces), and mixed with subsidies on healthy lifestyle alternatives (food and

exercise)[509]. It is likely that there will be ongoing debate in this contentious area and that many associated issues will remain unresolved until the results of any specific policies are properly evaluated in a real world setting.

There is a socio-political juxtaposition when considering policies regarding food costs and health. For example in the United States, farm policies have resulted in the over production of corn crops, leading to artificially low prices for high fructose corn syrup, which is now commonly added by food manufacturers to improve taste, and therefore consumption[507]. This of course is in direct contrast to health policies targeted at increasing fruit and vegetable intake. There is a strong argument that sweetened beverages should be targeted by specific taxes in order to improve health, in much the same way tobacco has[511]. However such moves are often resisted by the politically influential sweetened beverage corporations. Perhaps if governments provided more incentives for farmers to grow fresh fruit and vegetables, this would make healthy eating more affordable to all (and simultaneously increase the price of sweetened beverages). Low socio-economic status communities also have reduced access to healthy foods due to an over representation of limited choice convenience stores in comparison to chain supermarkets that offer greater variety and lower prices[507]. There are positive associations between proximity to large supermarkets and improved BMI, and there is evidence that improving access to supermarkets in poor communities leads to healthier food purchasing behaviour[507]. Urban planning needs to take this into consideration, especially in high density cheap housing subdivisions.

The disadvantage that individuals from poor socio-economic areas have is compounded when you consider that emerging nutraceuticals are generally expensive and pitched at a more affluent demographic. Many traditional remedies for diabetes are cheap, which partly explains their common use. However, when nutraceutical companies identify bioactive products and concentrate them, the cost exponentially increases. For example, it costs nothing to find an olive tree, pick some leaves and brew a cup of olive leaf tea, but to take olive leaf extract on a daily basis at the recommended dose costs several dollars a day. It is extremely unlikely that the low socio-economic groups at highest risk of developing type 2 diabetes would purchase nutraceuticals to prevent disease. Indeed the core market of the multi-billion dollar nutraceutical market is highly educated and middle aged[512]. In fact, in this demographic, purchasing of nutraceuticals is influenced more by health claims and brand trust than cost[513]. In part, the recruitment in our trials reflects this, where for the psyllium trial we targeted a poor socio-economic group, and for the olive leaf extract trial the middle aged men that volunteered were from middle and high socio-economic backgrounds. While it

is inevitable that nutraceutical companies are profit driven and need to pass on the cost of food technology, it would appear that on a population basis those who are set to gain the most from nutraceutical products are unable to afford them. To keep perspective though, if the macronutrient nutrition message of less saturated fat and sugar, and more fibre was to actually occur at a population level, it would be likely to confer a greater diabetes preventative benefit than nutraceutical supplementation.

Improved nutrition is a cost effective way of preventing type 2 diabetes compared to other methods. For example, as a cost to society, comparing metformin, and lifestyle intervention to placebo, lifestyle intervention was three times cheaper than metformin[514]. One must remember however that these analyses have been done on a population level. In New Zealand, where many medications such as metformin are subsidised, it may appear to the individual that cost of buying healthy food regularly could in fact be more to the individual than taking metformin. However, if one considers that healthy eating not only encompasses buying healthy food but also *not* buying unhealthy food, simply not buying sweetened beverages or reducing the frequency of high fat take-out meals would result in household saving. The difficulty of course, is convincing the population to do so, which requires education.

Finally, while it is not possible to dissect out the specific cost of nutrition interventions compared to physical activity, one can speculate. On an individual level, increasing physical activity by walking is free, and may even save costs if it means less vehicle use. However, physical activity programs, are not. Thus it would seem that using self motivated exercise would be an extremely cost effective way to improve insulin sensitivity, however the efficacy of such an approach relies on high levels of participant compliance and motivation. As such although self motivated exercise may be a cost effective intervention, when compliance is factored in it may be relatively ineffective overall meaning that healthy eating, although possibly more costly, is comparatively a more effective solution overall. Of course in reality whether exercise or healthy eating impacts on insulin sensitivity more, the advice to patients will always be to try to eat a more healthy diet and exercise more frequently.

### 6.5.4 Education

Eating patterns are set early in life, and therefore education on making healthy food choices needs to start early. In the context of young children, it is the parent who needs to be targeted. For older children, the school environment is an ideal place to educate healthy food messages. Most Western countries now have healthy nutrition as part of the school curriculum. School based initiatives are effective as a teaching tool, but it is unclear if this translates into any improvement in obesity rates (for which they are targeted)[515]. Health administrations spend a lot of money and time on promoting healthy nutrition through a variety of media (food labelling, posters, internet, television, radio, food etc.) in an attempt to educate the general public. When populations are surveyed about healthy nutrition, there is little doubt that nutrition education achieves its objective; that is people know what a healthy diet should be. However, at-risk low socio-economic groups have poor retention of healthy eating messages[516]. Further, despite knowing what food choices should be made, translation into purchasing behaviour is compromised by opposing forces – particularly product marketing, and of course, our taste preferences and cost. The current trend of increasing waist lines despite increased education indicates that the current education strategies do not translate into healthy eating behaviour or improved health outcomes.

School based nutrition education programs provide an interesting snapshot of the problems faced when trying to change the eating patterns of a community. In schools that provide meals (as is common in the United States and Europe), the two main barriers to providing a healthy meal were a lack of funding (reiterating that healthy food is expensive), and student preference for unhealthy food[517]. A common occurrence was that when only healthy food was served, the children did not eat it (leading to a lot of food wastage) and parents complained that when there wasn't food their children enjoyed to eat, the children would not eat at all. This is an excellent example of how taste is a primary driver of food choices. While in New Zealand schools generally don't provide food, it is likely that parents in New Zealand homes face the same challenges as the food providers in United States schools, striving to find the balance between packing a nutritious lunch and one that will actually be eaten. Nevertheless, as nutrition education has only recently been emphasised in the curriculum, it may take a generation for benefits at a population level to show. Some emerging literature in New Zealand has shown that a lifestyle intervention program (food and exercise) provided in

schools over a large demographic range and geographical area can improve body fat accumulation and systolic blood pressure[518].

A practical alternative to educating people about what foods are healthy is to label the food directly (and then educate people to eat food that is labelled as healthy). The Australian New Zealand Food Standards Code enforces all packaged food to carry a nutrition information panel. However, current nutrition information panels are confusing, difficult to interpret, are seldom used for making food choices, and are often hidden at the back or side of the food packaging[519, 520]. Front of package food labelling, which is more easily and quickly interpreted (for example, the traffic light system where each macronutrient component of the food is interpreted as being good (green) bad (red) or somewhere in between (orange)) is far superior at positively influencing purchasing behaviour towards healthier food choices. If such labelling were to become mandatory, it would be in the best interest of food manufacturers to try and improve the nutrition content of their foods. Increasing fibre content without negatively affecting taste and texture is cheap and easily done, but as yet there is no clear motivation for food manufacturers to do so. Instead, the consumer is at the mercy of ingenious marketing campaigns designed to encourage our self indulgent tendencies.

### 6.5.5 Marketing

Food manufacturers use contrasting marketing techniques in their attempt to lure the consumer to make a purchase, depending on whether it is a healthy or unhealthy product. An excellent example of an incredibly successful marketing strategy of an unhealthy food is the recent “share a coke” campaign by Coca-Cola. Citing that 50% of teens and young adults in Australia had not even tasted a full sugar ‘Coke’, bottles and cans emblazoned with a personalised name were produced to “reconnect Coke with Australians”. After 3 months 7% more full sugar “coke” was drunk by young adults, 5% more people were drinking full sugar ‘coke”, sales transactions were up 3%, volume of full sugar ‘coke’ consumed was up 4%, there was an 870% increase in Facebook traffic, and 378,000 personalised cans of full sugar coke were purchased at kiosks  In general terms, in the United States $100 million is spent every four days marketing unhealthy foods, compared to a government fund targeting obesity having a paltry $100 million to be spent over 5 years[521]. With this discrepancy in monetary

backing, is it any wonder that large multinational companies marketing their unhealthy foods win over state efforts to promote healthy eating?

Some food manufacturers cleverly target knowledge gaps in order to make their product appear healthy, while it is not. The 99% fat free catch phrase has been used extensively, and plays on to the fact that for decades we were informed about the dangers of fat in the diet. However, the total energy content of such foods is often just as high or higher than other 'fattier' foods due to substitution with sugars, and in particular high fructose corn syrup. This is also the problem with the glycaemic index, as although a food might be "low GI" it can still be energy dense and full of sugar and fat. Such advertising is often printed boldly on the front of a packet, while the confusing nutrition information panel is hidden away from the consumer. This gives more weight to the argument that front of package interpreted labelling should be encouraged, if not mandatory.

There is some regulation of unhealthy food marketing, particularly through television. The New Zealand Children's Code for Advertising Food has a number of principles which regulate what messages can be delivered with food advertising, particularly in regard to using low fat, or low sugar messages if the energy content is still high due to unhealthy ingredient substitution[522] Unfortunately, it is apparent that the code has been ineffective at improving marketing of unhealthy food to children through television. Many examples of code breaches are itemized and the lack of repercussions for the offenders have been highlighted elsewhere[523]. Further regulation is required.

A specific barrier to safely delivering the possible benefits of functional foods and nutraceuticals at a population level is the current poor regulation of the industry. For example, in the United States of America, nutraceuticals are classed as dietary supplements, and are therefore not subject to the same degree of regulation that medicines are[294]. The consequence of this is that there is less scrutiny on safety and quality control of nutraceuticals that are available to the consumer. The irony here is that nutraceuticals are perceived by the public as being natural, safe, and free of side effects, yet without industry regulation and rigorous scientific investigation, these perceptions could be far from the reality. In countries where nutraceuticals are common, there are regulatory bodies who endeavour to enforce frameworks around "health claims", for example the Food Standards Australia New Zealand (FSANZ) in New Zealand and Australia, the Food and Drug Administration (FDA) in the United States, the Food for Specified Health Uses (FOSHU) in Japan, and the European Food

Safety Authority (EFSA) in Europe. In an effort to curtail marketing based on the addition of a 'healthy' ingredient to unhealthy foods (for example adding pomegranate to a high sugar or high fat food), which can fool consumers into believing a food is healthy, these regulations also apply to functional foods (rather than the more specific nutraceuticals). However, it is clear that food manufacturers are still taking advantage of the current regulatory framework. For example Kellogg's Nutri-Grain cereal is still able to claim it is "high in protein", a "source of calcium", a "good source of B1, B2 & niacin" and a "good source of iron", despite being low in fibre, high in sugar and high in salt. Tightened regulation, as is occurring in most developed countries, is critical in order for functional foods including nutraceuticals to gain credibility (with respect to action, and safety) so they can then be endorsed by health professionals with confidence.

### 6.5.6 Civil Liberty

The developed world has built its empire on capitalism, where the success is due to hard work and motivation, and lack of success is due to personal failing. Applying this to obesity and nutrition, it could be viewed that it is the fault of the individual if they become obese and diabetic through unhealthy eating. While it follows that education should then rectify this, as already mentioned, this strategy has already been conducted for long enough and is obviously failing. An alternative argument would be that if the current food environment is diabetogenic, it is the responsibility of government to protect its citizens by creating laws and policy around nutrition.

History has taught us that enforcing dietary change at a population level through strict governmental policy is met with staunch resistance. A recent example is the public back lash and subsequent governmental U-turn which occurred in New Zealand with regard to fortification of bread with folic acid. When health agendas reach "crisis" levels, governmental agencies are more justified in intervening through authoritative legislation, and the public more accepting of them. This in essence is the problem, because there is such a range between those whom are obese and developing type 2 diabetes, seismic shifts in food regulation will affect not only those who are likely to benefit, but those who are not at risk at all. As food has such cultural significance, and is a necessity for living (rather than tobacco

for example), there are strong civil libertarian arguments that claim such authoritative legislation is “unconstitutional” (for United States citizens anyway).

The recent decision by the New York Board of Health, supported by mayor Michael Bloomberg, to ban the sale of sweetened beverages in containers larger than 16 ounces (470mL) in restaurants, fast-food outlets, theatres, and office cafeterias (but not grocery and convenience stores) is courageous. Six out of 10 New York residents oppose the idea, and say that it is an infringement of civil liberty[497]. It is not the first state level intervention to have occurred in the United States, and each time there has been considerable resistance from the sweetened beverage industry which has at times been successful at overturning such decisions at a federal level[521]. The New Zealand government has also succumbed to the civil liberty argument, and school tuck shop profit margins, by recently removing National Administration Guideline five which told schools that "where food and beverages are sold on school premises, to make only healthy options available".

Of course, not all government regulation which attempts to force healthy eating is guaranteed to be successful. For example, when calorie information was enforced to be placed on menus in New York, the results were less than impressive, and in some situations, more calories were actually consumed[524]. Recently, there has been debate in New Zealand regarding removing GST on fruit and vegetables. While on the face of it, this does appear to be good initiative, if one recalls the previous discussion regarding food purchasing, higher taxes on sweetened beverages and fatty foods would probably be more effective, but requires real world evaluation. This highlights the fact that if legislation is to be made, it must be based on excellent evidence. Robust research is required to provide such evidence, to give health administrators the confidence to make effective legislative changes, and for the general public to accept them.

### 6.5.6 The future of science, industry, and clinical practice

The nutraceutical industry is estimated to be worth $US176.7 billion in 2013, with an annual compound growth of 7.4%[294]. This market growth is so impressive that many large pharmaceutical companies are investing in creating new nutraceuticals[294, 525]. However, if we consider industry, researchers and consumers as the primary stakeholders, the

relationship is currently unbalanced, favouring direct marketing from industry to the consumer, with only a fraction of profit margins being spent on research. Given that consumer choice is influenced by trust in the brand and product, investment in research will become increasingly important for companies attempting to market nutraceuticals. There is a potential win-win situation, where the scientific nutraceutical field gains credibility and forwards knowledge, but the research industry partner can also gain a competitive edge. Researchers must be careful to guard the integrity of the science by maintaining ownership of the data, and therefore have the freedom to publish negative results as well as positive.

In practice, the clinician is currently left in the middle, having to balance the interests from the consumer, the available scientific literature, and industry claims (Figure 22). The physician (or any health advisor) is required to interpret scientific data, critically appraise commercial claims, and help guide the consumer to efficacious and safe products. From the discussion above, it is apparent that health advisors face several challenges in providing good advice, these include:

i) The lack of randomized, placebo controlled human trials that definitively show an extension of in-vitro findings to clinical practice.
ii) The lack of regulation, which currently allows for direct industry to consumer delivery.
iii) Current lack of quality control for marketed products due to lack of regulation.
iv) The enormous range of products, with no easily accessed clinical study outcome database for a clinician to access and be guided by.
v) Consumer demand and lay interpretation of industry claims.

Until such a time that there is robust data, and regulation governing safety and quality control of such compounds, an individualised nutrition prescription remains distant.

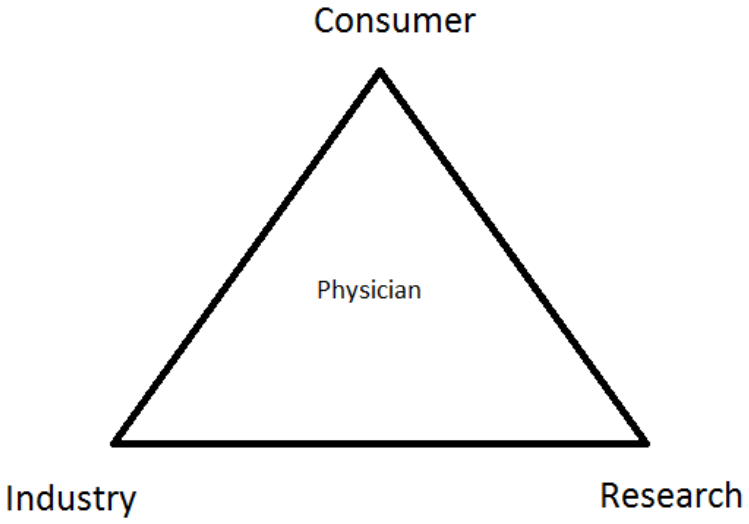



Figure 22: The relationship between the nutraceutical industry, research, consumer, and the physician.

### 6.5.6 Concepts on future nutrition plans to improve glucose homeostasis

We can conceptualize the future role of nutrition (in terms of macro- and micronutrients) in the prevention and management of disordered glucose homeostasis. In doing so, we need to consider the knowledge acquired to date, the barriers that currently impede nutrition intervention, and also look forward to future innovation. We can do this at two levels – population, and individual.

For a great part of the 20$^{th}$ century nutrition population based nutrition legislation has targeted malnutrition (for example iodine, vitamin D, and iron deficiency), but it is plain that the impact of metabolic disease is now having more and more impact on health systems, and action is required. The current education model is failing when we consider that rates of obesity and type 2 diabetes continue to rise, and therefore education models need to change or additional legislative measures need to be instated. Such interventions are most likely to influence macronutrient intakes. For example, food choices at schools may be restricted again, regulations on the size of sweetened beverages as seen in New York may be enforced, or common foods may be fortified with soluble and insoluble fibre. Urban planning should consider location of supermarkets in poor socio-economic areas as opposed to convenience stores. Placing higher taxes on unhealthy foods, and front of pack interpreted food labelling

could also help to impact purchasing behaviour. However, in order for the public to accept such authoritative interventions and encroachment on civil liberty, science has a critical role in establishing the evidence for which interventions are likely to improve nutrition and subsequent disease. Scientific evidence needs to underpin all such options, and the scientific fraternity also needs to improve communication with the population, administrators, and industry. While some of the proposed interventions may be initially considered extreme, we can learn from other models that have gained acceptance. The best example would be tobacco, where taxes have increased prices exponentially, labelling on packets highlights the health dangers of smoking, and the outlawing of smoking in restaurants bars and workplaces has made access difficult, resulting in a significant drop in smoking rates[526]. Until we consider and manage unhealthy eating as an addiction leading to poor health outcomes in the same way as tobacco, we are unlikely to see changes in obesity and disordered glucose metabolism at a population level.

When we consider the macro- and micronutrient constituents of an individual diet, future health professionals will need to integrate advice and knowledge not only of the food an individual ears, but of their genetics, and the social/cultural environment in which they live. An individualised nutrition plan including macro- and micronutrient supplements (such as nutraceuticals) that are likely to be effective in that individual could be devised. It is likely that such a plan will sit alongside other non nutritional approaches – for example physical activity or pharmaceuticals (especially if glucose homeostasis is already significantly impaired). Co-therapy will be even more common place than it already is, but with proven efficacy and safety. It is conceivable that nutraceuticals will potentiate the effects of medications, or exercise, and even each other. A final targeted and focused nutrition plan will take into account all such factors. As our knowledge expands, and the complexities of the field are revealed, future health care workers giving nutrition advice will need to be highly trained.

The emerging data from whole foods on insulin sensitivity – particularly traditional based herbs, has resulted in a dilemma where there are conflicting strategies between using a whole food based approach or isolating and concentrating a single component micronutrient (as was alluded when considering studies using purified sources of fibre). It is easier to research a single micronutrient, as results are not contaminated by possible other bioactive micronutrients that are present in whole foods. Also, food manufacturers and scientists are able to isolate single nutrients and concentrate them well beyond that achieved in whole

foods, in order to try and exert a biological affect. Whole foods by nature are complex, and vary greatly in nature, which makes reproducibility of research using whole foods near impossible. Whether there are additive beneficial effects of multiple micronutrients in whole foods is very plausible, and has two important implications: i) will the best approach in the future involve several concentrated micronutrients, as a "multi-nutraceutical" (indeed many available products already market themselves in this fashion, and ii) the lay person perception that a concentrated nutraceutical may offer a "golden bullet" beyond that from eating whole foods is probably ill-founded. Currently there is no clear distinction between the relative benefits of single ingredient nutraceuticals compared to that provided by good candidate whole foods, and this will remain so until more literature is available.

## 6.6 Concluding remarks

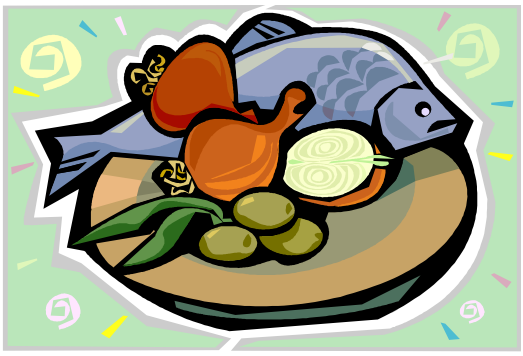
The objective of this thesis was to understand the mechanisms through which insulin resistance causes disease, review the current knowledge on which macro and micronutrient aspects of the diet can be manipulated to improve insulin resistance, and then through novel investigations, add to this knowledge base. The pathogenic processes through which insulin resistance, and compensatory hyperinsulinism contribute to a wide variety of diseases were established. Manipulating macro- and micronutrients are acceptable and potentially potent ways improve glucose homeostasis, and in particular, insulin sensitivity. To add to this field the ability of psyllium fibre to improve insulin sensitivity in adolescents at risk of developing type 2 diabetes was investigated. This study showed no improvement in insulin sensitivity, however, an improved lipid profile and fat distribution was demonstrated, which may have long term benefits in terms of cardiovascular disease risk. A step-wise investigation into a new emerging product – olive leaf extract – a rich source of micronutrient polyphenols – namely oleuropein and hydroxytyrosol was subsequently conducted. Firstly,following ingestion of the olive leaf extract, it was shown that oleuropein and hydroxytyrosol metabolites could be detected in human plasma in potentially meaningful concentrations. Following this, a randomized controlled trial found that the same olive leaf extract improved insulin sensitivity in overweight adults by 15%. Both these studies applied robust methodology in order to provide credible results, which are often lacking this field. The future role of emerging micronutrients is particularly exciting, especially when new

technology may allow strategic individualised nutrition interventions to improve glucose homeostasis. From a population point of view, macro- and micronutrient diet changes could certainly improve the burden of disease from the type 2 diabetes epidemic, but will require collaboration between researchers, administrators, health care workers, industry, and the community as a whole. When Hippocrates, the father of modern medicine proclaimed "Let food be thy medicine and medicine be thy food" over two and half thousand years ago, he couldn't possibly have conceived the lengths that researchers, medical practitioners, lay individuals, industry and health administrators would go to in order to understand the truth behind this statement over the following millennia.

# Chapter 7. Appendices

## Appendix I: Food diary

# FOOD INTAKE FOR FIBER STUDY



# 3-day Diet Record

## Intructions

- Use the pad provided to keep a record of **All** the food and drink you have during three days. Include everything you ate and drank at home and away.
- Choose **2 week days** and **1 weekend day**

---

**Date started** _____

**Date finished** _____

**Age** _____

**Sex** _____

**Study number** _____ **(Dr Martin will put this number in)**

## Keeping a Food Diary

**Some basic rules to remember:**

- Write down everything:
- Keep your form with you all day, and write down everything you eat or drink. A piece of chocolate or a couple of biscuits may not seem much at the time but they could be significant.
- Do it now: Don't depend on your memory at the end of the day. Record your eating as you go.
- Don't change your eating habits while you're keeping your food diary.

**Type of food**

Write down the types of food you ate. Use separate lines to include, sauces, gravies, and other “extras,” like salad dressing, mayonnaise, butter, sour cream, sugar, or tomato sauce.

- Remember to write down things you add, like milk and sugar in tea.
- Remember to include information on the name and brand of any packaged or take-away food items.

**Description of preparation**

If you can, say how the food was cooked: fried, baked, grilled etc.

Don’t forget to include the amount of oil or butter used for cooking if you can. You will have to ask the cook about this!

**Amount**

In this space, indicate the amount of the particular food item you ate. Measure or estimate the size (e.g., 6cm x 3cm x 3cm), the volume (e.g., 1/2 cup, I teaspoon), the weight (e.g., 120g), and/or the number of items (e.g., 12) of that type of food. For more information on estimating portion size when exact weight or measures are not available, these rules of thumb may help:

• 100g of meat, chicken, or fish = a deck of playing cards

• One-half cup of fruit, vegetables, pasta, or rice = half a tennis ball, a small fist, or a light bulb

• 30g of cheese = your thumb or two dominos

• One cup of milk, yogurt, or chopped fresh greens = a small hand holding a tennis ball

• A teaspoon of butter or margarine = the tip of your thumb to the first joint

• Two tablespoons of peanut butter = a ping pong ball

• One-half teaspoon of oil = 1 thimble

Based on amount eaten, fill in the number of approximate weight, cup-equivalents, or spoons.

**Name** _____

# DIET RECORDS

**Example**

Day 1 Date Page 1

| Eating Time | Meal type | Food item | Food and Beverage Name, Brand, Description, Preparation i.e. boiling, frying, microwave etc, and Recipe if necessary. | Amount |
|---|---|---|---|---|
| 7.15 | breakfast | Milo | Milk: trim milk | 1 cup |
| | | | Milo | 2 teaspoons |
| | | Toast with margarine | Bread: wholemeal toast, Tiptop | 2 slices<br>ate it all except crusts |
| | | | Margarine: olivio | 2 teaspoons |
| | | Cornflakes | Cornflakes | ½ bowl |
| | | | Trim milk | 1 cup |
| | | | Sugar | 1 tsp |

# Diet records

Day _____ Date _____ Page ____

| Eating Time | Meal type | Food item | Food and Beverage Name, Brand, Description, Preparation i.e. boiling, frying, microwave etc, and Recipe if necessary. | Amount |
|---|---|---|---|---|
| | | | | |
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# Appendix II: Physical activity questionnaire for adolescents.

## PAQ-A

Name

Participant number

Age

Date

PRE X-OVER END

We are trying to find out about the level of activity in the last 7 days (in the last week). This includes sport, dancing or games that make your legs feel tired, sweat, or breathe hard.

There are no right or wrong answers, this is not a test

Please answer all questions as honestly and accurately as you can – this is very important.

QUESTION 1: Physical Activity in your spare time (not at school). Have you done any of the following in the past week, and if you have, how many times?

| | none | 1 - 2 | 3 - 4 | 5 - 6 | 7 or more |
|---|---|---|---|---|---|
| Rowing, Waka Ama | | | | | |
| In-line skating | | | | | |
| Tag | | | | | |
| Fast walking for exercise | | | | | |
| Bicycling | | | | | |
| Running | | | | | |
| Aerobics | | | | | |
| Swimming | | | | | |
| Softball / cricket | | | | | |
| Dance | | | | | |
| Rugby (union or league or touch) | | | | | |

Badminton

Skateboarding

Soccer

Hockey

Skiing

Other (specify)

Other (specify)

QUESTION 2:

In the last 7 days during PE at school, how often were you very active (playing hard, running, jumping, throwing)

| none | Hardly ever | Sometimes | Quite Often | Always |
|---|---|---|---|---|
| ○ | ○ | ○ | ○ | ○ |

QUESTION 3:

In the last 7 days during lunch at school, apart from eating, what did you do (only one answer)

| Sat down (talking, reading, schoolwork) | Stood around or walked around | Ran or played a little bit | Ran around and played quite a bit | Ran and played hard most of the time |
|---|---|---|---|---|
| ○ | ○ | ○ | ○ | ○ |

QUESTION 4:

In the last seven days, on how many days ***right after school***, did you do sports, dance or play games in which you were really active?

| none | One time | 2 or 3 times | 4 times | 5 times |
|---|---|---|---|---|
| ○ | ○ | ○ | ○ | ○ |

QUESTION 5:

In the last 7 days, on how many evenings did you do sports, dance or play games in which you were really active?

| none | one | 2 or 3 times | 4 or 5 times | 6 or 7 times |
|---|---|---|---|---|
| ○ | ○ | ○ | ○ | ○ |

QUESTION 6:

On the last weekend on how many times did you do sports, dance or play games in which you were really active?

| none | one | 2 or 3 times | 4 or 5 times | 6 or 7 times |
|---|---|---|---|---|
| ◯ | ◯ | ◯ | ◯ | ◯ |

QUESTION 7:

Which one of the following describes you best for the last seven days? Read all five statements before deciding on the one answer that describes you:

1. All, or most of my time was spent doing things that involve little physical effort ◯

2. I sometimes (1 – 2 times per week) did physical things in my free time (eg played sports went running, went biking, swimming, did aerobics) ◯

3. I often (3 – 4 times per week) did physical things in my free time ◯

4. I quite often (5-6 times per week) did physical things in my free time ◯

5. I very often (7 times or more per week) did physical things in my free time ◯

QUESTION 8:

Mark how often you did physical activity (like playing sports, games, doing dance, or any other physical activity) for each day of the last week

| | None | Little bit | medium | often | Very often |
|---|---|---|---|---|---|
| Monday | ◯ | ◯ | ◯ | ◯ | ◯ |
| Tuesday | ◯ | ◯ | ◯ | ◯ | ◯ |
| Wednesday | ◯ | ◯ | ◯ | ◯ | ◯ |
| Thursday | ◯ | ◯ | ◯ | ◯ | ◯ |
| Friday | ◯ | ◯ | ◯ | ◯ | ◯ |
| Saturday | ◯ | ◯ | ◯ | ◯ | ◯ |

| | | | | | |
|---|---|---|---|---|---|
| Sunday | ◯ | ◯ | ◯ | ◯ | ◯ |

QUESTION 9:

Were you sick last week? Or did anything prevent you from doing your normal physical activities?

Yes ◯

No ◯

If yes, what prevented you? _____

# Appendix III: Physical activity questionnaire for adults

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

(October 2002)

LONG LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is encouraged to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

## Further Developments of IPAQ

International collaboration on IPAQ is on-going and an International Physical Activity Prevalence Study is in progress. For further information see the IPAQ website.

## More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). Assessment of Physical Activity: An International Perspective. Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the last 7 days. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the vigorous and moderate activities that you did in the last 7 days. Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Moderate activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

Yes ☐

No ☐ → Skip to PART 2: TRANSPORTATION

The next questions are about all the physical activity you did in the last 7 days as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, heavy construction, or climbing up stairs as part of your work? Think about only those physical activities that you did for at least 10 minutes at a time.

_____ days per week

☐ No vigorous job-related physical activity → Skip to question 4

3. How much time did you usually spend on one of those days doing vigorous physical activities as part of your work?

_____ hours per day

_____ minutes per day

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate physical activities like carrying light loads as part of your work? Please do not include walking.

_____ days per week

☐ No moderate job-related physical activity → Skip to question 6

5. How much time did you usually spend on one of those days doing moderate physical activities as part of your work?

_____ hours per day

_____ minutes per day

6. During the last 7 days, on how many days did you walk for at least 10 minutes at a time as part of your work? Please do not count any walking you did to travel to or from work.

_____ days per week

☐

No job-related walking → Skip to PART 2: TRANSPORTATION

7. How much time did you usually spend on one of those days walking as part of your work?

_____ hours per day

_____ minutes per day

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the last 7 days, on how many days did you travel in a motor vehicle like a train, bus, car, or tram?

_____ days per week

☐ No traveling in a motor vehicle → Skip to question 10

9. How much time did you usually spend on one of those days traveling in a train, bus, car, tram, or other kind of motor vehicle?

_____ hours per day

_____ minutes per day

Now think only about the bicycling and walking you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the last 7 days, on how many days did you bicycle for at least 10 minutes at a time to go from place to place?

_____ days per week

☐ No bicycling from place to place → Skip to question 12

11. How much time did you usually spend on one of those days to bicycle from place to place?

_____ hours per day

_____ minutes per day

12. During the last 7 days, on how many days did you walk for at least 10 minutes at a time to go from place to place?

_____ days per week

☐ No walking from place to place → Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

13. How much time did you usually spend on one of those days walking from place to place?

_____ hours per day

_____ minutes per day

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the last 7 days in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, chopping wood, shoveling snow, or digging in the garden or yard?

_____ days per week

☐ No vigorous activity in garden or yard → Skip to question 16

15. How much time did you usually spend on one of those days doing vigorous physical activities in the garden or yard?

_____ hours per day

_____ minutes per day

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, sweeping, washing windows, and raking in the garden or yard?

_____ days per week

☐ No moderate activity in garden or yard → Skip to question 18

17. How much time did you usually spend on one of those days doing moderate physical activities in the garden or yard?

_____ hours per day

_____ minutes per day

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, washing windows, scrubbing floors and sweeping inside your home?

_____ days per week

☐ No moderate activity inside home → Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY

19. How much time did you usually spend on one of those days doing moderate physical activities inside your home?

_____ hours per day

_____ minutes per day

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the last 7 days solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the last 7 days, on how many days did you walk for at least 10 minutes at a time in your leisure time?

_____ days per week

☐ No walking in leisure time → Skip to question 22

21. How much time did you usually spend on one of those days walking in your leisure time?

_____ hours per day

_____ minutes per day

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like aerobics, running, fast bicycling, or fast swimming in your leisure time?

_____ days per week

☐ No vigorous activity in leisure time → Skip to question 24

23. How much time did you usually spend on one of those days doing vigorous physical activities in your leisure time?

_____ hours per day

_____ minutes per day

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis in your leisure time?

_____ days per week

☐ No moderate activity in leisure time → Skip to PART 5: TIME SPENT SITTING

25. How much time did you usually spend on one of those days doing moderate physical activities in your leisure time?

_____ hours per day

_____ minutes per day

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the last 7 days, how much time did you usually spend sitting on a weekday?

_____ hours per day

_____ minutes per day

27. During the last 7 days, how much time did you usually spend sitting on a weekend day?

_____ hours per day

_____ minutes per day

This is the end of the questionnaire, thank you for participating.

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