School of Public Health

The Potential Association between Thiamin, Hyperglycemia and Chronic Diseases

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: ...F. Alaei Shoeili.....

Date: .....18/10/12.....

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Statement of candidate contribution

The thesis has been completed during the course of enrolment in this degree at Curtin University.

Subject recruitment and all processes mentioned in the methods sections of the cross - sectional and intervention studies (Sections 3.3 & 4.3) including measuring anthropometric variables; blood pressure, energy expenditure and vascular function assessments; taking blood samples and other participant interactions as well as the preparation of supplement and placebo capsules, and aliquot preparation were carried out by the candidate with no assistance. She was also involved in analysing all data. No professional proof-reader/editor was used in preparing the thesis.

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Abbreviations

Cardiovascular disease - CVD

Vascular cell adhesion molecule -1 - VCAM-1

Reactive oxygen species - ROS

Sphingomyelinase - SMase

Secretory phospholipase 2 - sPLA2

Monocyte chemoattractant protein-1 - MCP-1

Macrophage colony-stimulating factor - M-CSF

Smooth muscle cells - SMCs

Matrix metalloproteinase - MMP

C-reactive protein - CRP

Cellular adhesion molecules - CAMs

Soluble CD40 ligand - sCD40L

Interleukin - IL

Tumor necrosis factor-α -TNF-α

Interferon-γ -IFN-γ

Colony stimulating factors - CSF

High density lipoproteins - HDL

Low density lipoproteins - LDL

Intermediate density lipoproteins - IDL

Very low density lipoproteins - VLDL

Chylomicrons - CM

Apolipoprotein A- apoA

Apolipoprotein A- apoB

lipoprotein lipase - LPL

Lecithin cholesterol acyltransferase - LCA

Chylomicrons remnants - CMR

Reverse cholesterol transport - RCT

Nitric oxide - NO

Endothelial NO synthase - eNOS

Asymmetric dimethylarginine - ADMA

Mitogen-activated protein kinase - MARK

Pulse wave velocity - PWV

Cardiovascular magnetic resonance - CMR

Magnetic resonance imaging - MRI

Digital volume pulse - DVP

Reflection index - RI

Stiffness index - SI

World Health Organization -WHO

Adult Treatment Panel III - ATP III

International Diabetes Federation - IDF

Oral glucose tolerance test - OGTT

Australian Bureau of Statistics - ABS

Impaired glucose tolerance - IGT

Impaired fasting glycemia - IFG

Australian Diabetes Society - ADS

National Health and Medical Research Council - NHMRC

Insulin–dependent diabetes mellitus - IDDM

Non–insulin–dependent diabetes mellitus - NIDDM

Gestational diabetes mellitus - GDM

Malnutrition–related diabetes mellitus - MRDM

Glutamine:fructose-6-phosphate amidotransferase - GFAT

UDP-N- acetylglucosamine - UDP-GlcNAc

N-acetylglucosamine - GlcNAc

Acetyl-CoA carboxylase - ACC

Glycerophosphate dehydrogenase - GPDH

Fatty acid synthase - FAS

Plasminogen activator inhibitor-1 - PAI-1

Diacylglycerol - DAG

Transforming growth factor-β - TGF-β

Thiamin monophosphate - TMP

Thiamin diphosphate - TDP

Thiamin pyrophosphate - TPP

Thiamin triphosphate - TTP

Recommended dietary intakes - RDIs

Human immunodeficiency virus-acquired immunodeficiency syndrome - HIV-AIDS

Thiamin-responsive megaloblastic anemia - TRMA

α-ketoglutarate dehydrogenase -αKGDH

Transketolase -TK

Pyruvate dehydrogenase complex - PDHC

Glyceraldehyde-3-phosphate - G3P

Endothelium dependent vasodilatation - EDV

Erythrocyte transketolase activity - ETKA

Thiamin pyrophosphate effect - TPPE

Hemostatic model assessment - HOMA

High- performance liqid chromatography - HPLC

Trichloroacetic acid - TCA

Fasting whole blood glucose - FWBG

Fasting plasma glucose - FPG

Glomerular filtration rate - GFR

Body surface area - BSA

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Abstract

Chronic diseases, such as cardiovascular disease and diabetes, are the leading causes of morbidity and mortality worldwide. Recent studies have shown that in addition to diabetes mellitus, non-diabetic degrees of fasting and postprandial hyperglycemia are also directly linked to accelerated risks of cardiovascular diseases. Thiamin is a water soluble vitamin playing a key regulatory role as a co-enzyme in metabolic pathways implicated in the glucose metabolism. There is some evidence that diabetic patients are prone to thiamin deficiency possibly because of an increased excretion of thiamin in the urine. However, there has been no published study to investigate thiamin status in individuals with pre-diabetic range of hyperglycemia. Given this gap, we undertook a cross-sectional study, evaluating blood thiamin concentration of subjects with impaired glucose regulation compared with healthy people.

To examine the main objective of this study, data of 64 subjects (29 men and 35 women) were analysed. These subjects consisted of 39 normal healthy volunteers and 25 hyperglycemics with plasma glucose levels at pre-diabetic ranges (16 IGT, 9 IFG). The mean intake of thiamin in both groups as assessed by a validated semi-quantitative food frequency questionnaire was 1.37±0.72 mg/day for normal subjects and 1.46±0.51 mg/day for those with hyperglycemia. These values exceeded the Australian RDI for thiamin. There was no significant difference in the levels of RBC thiamin in hyperglycemic subjects relative to those in the normoglycemic group (0.95±0.17 vs. 0.88±0.24 nmol/g Hb, p=0.22).

The two groups were also evaluated for a range of risk factors for CVD, including arterial stiffness. Hyperglycemic subjects had higher levels of fasting DVP parameters (SI & RI), accompanied with tendencies toward blunted response to ingested glucose load relative to normoglycemic group. These results suggest that screening of individuals with hyperglycemia by using a Pulse Trace machine may be a means of recognising cardiovascular complications at early stages. Further research with a larger sample size is recommended to extend these interesting results.

Hyperglycemia is known to induce a variety of biochemical alterations at the cellular level, resulting in a range of vascular and tissue damages. The mechanism of action

of supplemental thiamin seems to involve the diversion of "excess" metabolic load (glycolytic intermediates) away from glycolysis and toward the reductive pentose pathway, a secondary pathway for glucose catabolism. Thiamin supplementation was also shown to improve cardiovascular risk factors in diabetic rats, suggesting the potential effects of thiamin in prevention of diabetic complications.

To date there has been no published study to investigate these effects in individuals with pre-diabetic range of hyperglycemia (IGT). Therefore, our objective in the second study was to assess the chronic effect of high dose thiamin supplement (300 mg/d) on glucose tolerance and some cardiovascular risk factors in individuals with hyperglycemia at an early stage. In this intervention study with a double blind cross - over design, the hyperglycemic subjects (n=12) were randomly allocated into two groups to receive either placebo for 6 weeks followed by a 14-week washout period and then thiamin for 6 weeks; or thiamin for 6 weeks, a 14-week washout period and placebo for 6 weeks.

The results of our intervention study showed that after 6 weeks of supplementation, RBC thiamin increased from 0.93 (±0.17) nmol/g Hb to 1.56 (±0.31) nmol/g Hb. In subjects receiving placebo, fasting plasma glucose increased significantly from baseline after six weeks (6.11±0.70 vs. 5.87±0.63 mmol/L, p=0.003). This significant change was accompanied with concomitant increases in fasting plasma insulin (7.67± 4.39 vs. 6.64± 3.45 μIU/mL, p=0.04), and HOMA score (2.10±1.32 vs. 1.75±1.01, p= 0.02). However in the supplement arm, there was no significant change in fasting plasma glucose (6.01±0.79 vs. 6.02±0.68 mmol/L, p=0.83), fasting insulin (7.46 ±4.67 vs. 7.36± 4.40 μIU/mL, p> 0.05) or HOMA score (2.05±1.51 vs. 2.00±1.32, p=0.75) determined at week 6 compared to baseline (week 0), indicating that supplementation with high dose thiamin may have prevented the natural progression of hyperglycemia toward diabetes mellitus in individuals with impaired glucose metabolism at early stages. We also found that high dose thiamin therapy can improve glucose tolerance (week 0: 9.89±2.50 vs. week 6: 8.78±2.20 mmol/L, p=0.004), and attenuate diastolic blood pressure (week 0: 71.42 ±7.41 vs. week 6: 79.2± 5.84 mm Hg, p=0.005) in patients with impaired glucose metabolism. The findings of the present study suggest that thiamin therapy may be effective in patients with hyperglycemia at early stages.

Previous studies examining the potential effects of thiamin under hyperglycemic condition have mainly been limited to animals. The current clinical study suggests that thiamin supplementation may be beneficial in humans with pre-diabetic ranges of hyperglycemia. Further studies are required to confirm these results and investigate the impact of thiamin supplementation on insulin secretion.

The findings of this research have the potential to inform food formulations and dietary recommendations for people who are at risk of developing diabetes mellitus, and may have a role in the prevention of hyperglycemic complications. The findings of this study serve as a base for further research investigating the effectiveness of different doses of thiamin on cardiovascular risk factors.

1. Introduction

Chronic diseases, such as heart disease, stroke and diabetes, are known as the major cause of morbidity and mortality in the world, with an increasing rate over the recent decades (World Health Organization 1998, 2002). There is now a general consensus that the dramatic increase in chronic diseases has been mainly due to lifestyle changes, which in turn results in increasing rates of obesity and insulin resistance, and subsequently hyperglycemia in diabetic or pre-diabetic ranges. The findings of recent studies have shown that in addition to diabetes mellitus, even non-diabetic degrees of fasting and postprandial hyperglycemia are directly linked to accelerated risks of cardiovascular diseases (Levitan et al. 2004).

Thiamin is a water soluble vitamin playing an essential regulatory role as a co-enzyme in metabolic pathways involved in glucose metabolism. Evidence shows that both type 1 and type 2 diabetic patients are prone to thiamin deficiency, resulting mainly from an increased urinary excretion (Thornalley et al. 2007), however there are no published studies evaluating thiamin status in individuals with pre-diabetic range of hyperglycemia.

In addition, studies have demonstrated that thiamin suppresses key pathogenic pathways involved in biochemical abnormalities associated with hyperglycemia in vitro (Thornalley, Jahan, and Ng 2001) and improves cardiovascular risk factors in diabetic rats (Babaei-Jadidi et al. 2003), suggesting the possible benefits of thiamin supplementation in the prevention of diabetic complications. Despite these preliminary findings, there is limited literature investigating the effects of thiamin supplements on metabolic outcomes in humans, particularly among individuals with hyperglycemia at early stages.

The present research work was designed in two studies, to assess the status and effects of thiamin on cardiovascular risk factors in subjects with abnormal glucose metabolism.

1.1 Hypotheses

1. The blood thiamin concentration is decreased in subjects with impaired glucose regulation, relative to healthy people.

2. The chronic consumption of high doses of thiamin decreases the risk of hyperglycemic complications by reducing fasting plasma glucose, blood pressure and serum lipids, and enhances glucose tolerance, insulin sensitivity, renal function and energy expenditure relative to control.

1.2 Objectives

The study hypotheses were addressed through the following studies:

1. The first hypothesis was addressed with a cross-sectional study, evaluating thiamin status in subjects with pre-diabetic ranges of hyperglycemia compared to normoglycemic group. Subsequently, potential associations between thiamin status and different cardiovascular risk factors were investigated.

The variables of interest for this study were anthropometric factors; biochemical measurements of fasting and 2-h blood glucose, fasting and 2-h insulin, serum lipids and CRP; systolic and diastolic blood pressure; vascular endothelial function indices (RI, SI); renal function measurements (urinary albumin and GFR); and thiamin status parameters.

2. The second hypothesis was tested with a randomised, double blind, cross-over design study, investigating the chronic effect of high dose thiamin supplement (300 mg/d) on cardiovascular risk factors in subjects with early-stage hyperglycemia, relative to control.

The endpoints of this intervention study were anthropometric parameters; biochemical measurements of fasting and 2-h plasma glucose, fasting and 2-h insulin, serum lipids and CRP; systolic and diastolic blood pressure; renal function measurements (urinary albumin and GFR); resting and postprandial energy expenditure.

2. Literature review

2.1 Chronic diseases

Chronic disease refers to a range of long-lasting disorders with usually slow progression. Chronic diseases such as cardiovascular disease and diabetes are known as the main causes of morbidity and mortality globally. It is predicted that by 2020, chronic diseases will be responsible for approximately 75% of all deaths in the world, compared to an approximate rate of 60% in 2001 (World Health Organization 2002, 1998).

2.2 Cardiovascular diseases

Cardiovascular disease (CVD) includes a group of diseases which involve the heart and blood vessels, consisting of coronary heart disease, heart failure, hypertension, cerebrovascular disease and peripheral artery disease. CVD is identified as the leading cause of deaths worldwide (World Health Organization 2009). In Australia during 2008, over 48000 deaths were attributable to CVD, representing 34% of all registered deaths (Australian Bureau of Statistics 2008). The main underlying cause of CVD is atherosclerosis, a progressive disease characterised by the remodelling of the arterial system. Moreover, it is now known that there is a link between cardiovascular diseases and diabetes mellitus (Ruderman and Haudenschild 1984), and hyperglycemia in either diabetic or pre-diabetic ranges (Balkau, Forhan, and Eschwege 2002; Balkau et al. 2004; Barzilay et al. 1999; Coutinho et al. 1999; DECODE Study Group 2001, 2003; Gabir et al. 2000; Levitan et al. 2004; Meigs et al. 2002; Saydah et al. 2001; Stern et al. 2002; Tominaga et al. 1999) is also associated with accelerated atherosclerosis.

2.2.1 Pathophysiology of atherosclerosis

Atherosclerosis is a chronic inflammatory process, in which deposits of lipid are accumulated in the inner lining of large and medium-sized arteries, resulting in thickening and hardening of the arterial wall. Pathophysiology of atherosclerosis is complex and results from reciprocal actions between plasma atherogenic lipoproteins, cellular elements (inflammatory cells and arterial endothelial and smooth muscles cells) and arterial wall extracellular matrix (Fan and Watanabe 2003).

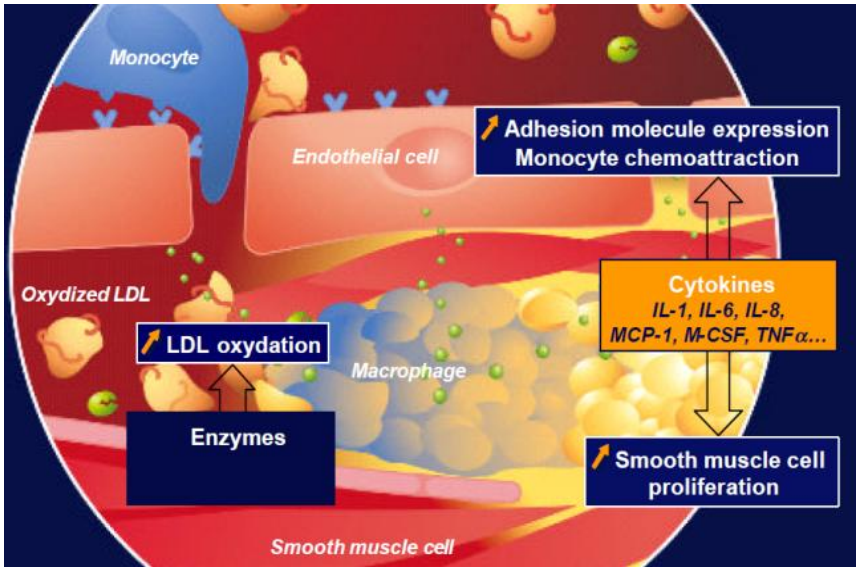


Figure 1. Pathophysiology of atherosclerosis: attachment and penetration of leucocytes into intima. *White blood cells poorly bind to the normal endothelium. However, in the presence of atherogenic stimuli, endothelial cells express selective adhesion molecules, mediating the attachment of various classes of leukocytes (Fruchart 2003 Feb 23)*

There are several hypotheses about the pathogenesis of atherosclerosis, which among them the theory of "response-to- injury" is now widely accepted (Ross 1993). According to this theory, the process of atherosclerosis begins with injury to the arterial wall which triggers a series of inflammatory reactions, mediating arterial plaque formation.

Various atherogenic stimuli have been shown to contribute to the arterial wall injury, including free radicals, high blood pressure, hyperglycemia, homocysteine, substances released from fat cells and especially, oxidised low density lipoprotein (LDL) (Sherwood 2009). In general, white blood cells poorly attach to the normal endothelium. However, pro-inflammatory factors can stimulate the endothelial cells to express adhesion molecules, such as vascular cell adhesion molecule -1 (VCAM-1), which mediate the binding of circulating monocytes and lymphocytes (Li et al. 1993) [Figure 1].

As a consequence of inflammation, endothelial cells begin to lose their integrity and allow the attached leucocytes to move into the intima (Woywodt et al. 2002). The

increased endothelial permeability resulting from inflammation also enables the plasma atherogenic lipoproteins, in particular particles of low density lipoprotein (LDL) to penetrate the endothelial layer. The retention in the intima isolates LDL particles from plasma anti-oxidants, facilitating their subsequent oxidative modification by reactive oxygen species (ROS) and enzymes (Lusis 2000; Packard and Libby 2008). Oxidatively modified LDL particles can in turn promote the arterial wall cells to secrete chemoattractant factors such as monocyte chemoattractant protein-1 (MCP-1), enhancing the migration of adherent leukocytes (Fan and Watanabe 2003; Ross 1999; Takeya et al. 1993). In parallel, the monocytes infiltrated in the endothelial layer differentiate into macrophages, in response to macrophage colony-stimulating factor (M-CSF). Additionally, M-CSF stimulates macrophages to express scavenger receptors, permitting them to endocytose the oxidised lipoproteins and take on a foamy appearance under a microscope, known as "foam cells" (Qiao et al. 1997; Smith et al. 1995). Accumulation of these lipid-laden macrophages (foam cells) in the subendothelial space forms a visible “fatty steak", a hallmark of atherosclerosis in its early stage.

The atherosclerosis process continues with the migration of smooth muscle cells (SMCs) from the tunica media to the endothelial layer, under the influence of platelet derived growth factor released by activated leukocytes and vascular endothelial cells. In response to inflammation, arterial wall smooth muscle cells express genes for matrix metalloproteinase enzymes, in particular matrix metalloproteinase - 9 (MMP-9), degrading extracellular matrix and permitting SMCs to pass through the elastic laminate and enter the subendothelial space (Mason et al. 1999).

Within the intima, various fibrogenic mediators, including a number of peptide growth factors, promote SMCs to proliferate, and subsequently produce extracellular matrix proteins, especially interstitial collagen, overlying the foam cells. This process may cause the early lipid-laden lesion to progress into a fibrotic plaque (atheroma), characteristic of the more advanced stage of atherosclerosis (Packard and Libby 2008).

As the atherosclerotic plaque grows, it progressively bulges into the arterial lumen and produces a critical vascular narrowing. Typically, two kind of atherosclerotic plaques can be identified: stable plaque containing a thick fibromuscular layer overlying a small lipid core, and unstable or vulnerable plaque which often comprises a large lipid core covered by a thin layer of smooth muscle cells, extracellular matrix and abundant inflammatory cells , rendering it prone to rupture and clot formation (Fan and Watanabe 2003) [Figure 2].

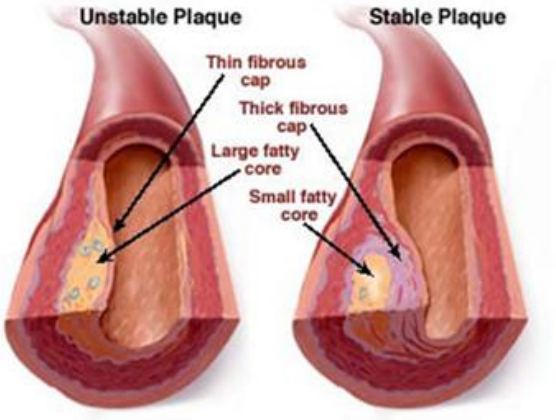


Figure 2. Stable and unstable plaques. *As plaque builds up, it can become either stable or unstable. Unstable plaque is more prone to sudden rupture, a potentially life-threatening event (Mangge et al.).*

Inflammatory processes have been shown to not only contribute to the initiation and progression of atheroma, but also participate in its destabilization, leading to the thrombotic complication of atherosclerosis (Libby 2002). Subsequent to the plaque evolution, activated T lymphocytes resident in the atheroma secrete pro-inflammatory cytokines, such as interferon (IFN)-γ, inhibiting smooth muscle cells in the synthesis of new collagen. In parallel, inflammatory mediators released by T lymphocytes can stimulate macrophages to produce matrix metalloproteinase enzymes, including MMP-1, MMP-8, MMP-13 and in particular MMP-9, breaking down interstitial collagen that exists in the fibrous layer of atheroma (Amento et al. 1991; Galis et al. 1994; Gough et al. 2006; Sukhova et al. 1999). This process causes the rupture of enlarged plaque through the weakened fibrous cap and its adjacent endothelial lining, permitting blood elements to make contact with the thrombogenic substance in the subintimal space at the site of arterial damage. This contact triggers the process of thrombus formation which can impede blood flow through the affected artery (Sherwood 2009).

2.2.2 Inflammatory biomarkers and atherosclerosis

Given the central function of inflammation in atherosclerosis, several studies over the last decade have focused on investigating the role of inflammatory biomarkers for risk stratification. These biomarkers mainly comprise adhesion molecules like VCAM-1; cytokines including tumor necrosis factor, interleukin-1, interleukin-12 and interleukin-18; matrix metalloproteinases such as MMP-9; and acute phase reactants such as PAI-1, fibrinogen and in particular C-reactive protein (CRP) (Packard and Libby 2008).

Cellular adhesion molecules

Cellular adhesion molecules (CAMs) including VCAM-1, ICAM-1 and E- selectin have been shown to play an essential role in atherosclerosis process (Cybulsky and Gimbrone 1991; Kawakami et al. 2006; Li et al. 1993). These adhesion molecules can be detected in serum in their soluble forms. Elevated levels of soluble adhesion molecules were found in coronary artery atherosclerosis (Blann, Amiral, and McCollum 1996; Damnjanovic et al. 2009), carotid sclerosis (Hwang et al. 1997), peripheral artery diseases (De Caterina et al. 1997) and in association with metabolic syndrome components, including diabetes mellitus (Doupis et al. 2010), hypertension (DeSouza et al. 1997), hyperlipidemia (Hackman et al. 1996) and impaired glucose tolerance (Ferri et al. 1998). This suggests a possible role for these inflammatory biomarkers in further cardiovascular risk assessment, although it is still a matter of controversy (de Lemos, Hennekens, and Ridker 2000).

Cytokines

Pro-inflammatory cytokines of the interleukin (IL) group and macrophage-associated cytokines, including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and colony stimulating factors (CSF), are known to be key players in the pathogenesis of atherosclerosis (Mallat et al. 2001; McKellar et al. 2009; Tedgui and Mallat 2006). However, the level of circulatory cytokines does not necessarily reflect their actual activity (Tedgui and Mallat 2006). Several studies suggested IL-18 as a useful maker for risk stratification in individuals with or without established cardiovascular diseases (Blankenberg et al. 2003; Blankenberg et al. 2002). Other interleukins, such as IL-1 and IL-6, cannot be easily measured in clinical laboratories, because of their

short half-lives in plasma (Packard and Libby 2008). TNF-α is another cytokine considered for predicting cardiovascular events (Skoog et al. 2002). Nevertheless, some researchers proposed soluble tumor necrosis factor receptor (sTNFR) as a more reliable marker than measuring TNF-α levels itself (Elkind et al. 2002).

MMP-9

Consistent with the role of matrix metalloproteinases in plaque formation (Gough et al. 2006; Mason et al. 1999), studies indicated that the circulatory concentrations of MMP-9 were higher among patients with stable coronary artery disease compared to control (Noji et al. 2001; Tayebjee et al. 2005). There was also a direct correlation between MMP-9 and the concentration of LDL- cholesterol (Noji et al. 2001). There is still limited evidence and further investigations are required to determine whether MMP-9 measurement provides more prognostic information compared to other markers.

Acute phase reactants

Acute phase reactants including PAI-1, fibrinogen and CRP have been shown to participate in the atherosclerosis inflammatory process (Bini and Kudryk 1994; Libby and Theroux 2005; Zwaka, Hombach, and Torzewski 2001).

In a prospective nested case-control study involving a population with high prevalence of coronary heart disease, elevated plasma PAI-1 concentrations were associated with the first myocardial infarction in both men and women (Thogersen et al. 1998). Evidence indicates that PAI-1 production can be directly influenced by key cardiovascular risk factors, including genetic predisposition (Dawson et al. 1991; Festa et al. 2003), obesity, insulin resistance (Juhan-Vague, Alessi, and Morange 2000) and neurohormonal causes (Brown et al. 1998). These findings have cast some doubts on PAI-1 as an independent predictor of cardiovascular risk (Packard and Libby 2008). Additionally, a short half- life in the circulation (6 minutes) and also meticulous precautions required for blood sampling process limit PAI-1 as a routine measurement in the clinical setting (Packard and Libby 2008).

Data from several prospective studies indicate that the concentration of fibrinogen at baseline was higher among individuals who experienced a subsequent myocardial infarction and stroke (Ma et al. 1999; Wilhelmsen et al. 1984). Furthermore, recent studies confirmed the association between fibrinogen and traditional and non-traditional cardiovascular risk factors (Maple-Brown et al. 2010). However, fibrinogen seems a less useful cardiovascular risk marker than CRP (Ridker, Stampfer, and Rifai 2001), partly due to its diurnal variations, and problems in its measurement reproducibility and standardisation (Packard and Libby 2008).

Among various biomarkers, CRP is accepted as the strongest non-lipid predictor of cardiovascular events (Ridker, Hennekens, et al. 2000), including myocardial infarction (Ridker et al. 2002), stroke (Ridker et al. 2002; Ridker 2003), cardiac sudden death (Albert et al. 2002) and peripheral artery diseases (Ridker et al. 2008), particularly in patients with diabetes mellitus (Linnemann et al. 2006; Pfutzner and Forst 2006). In addition, it was shown to predict the risk of recurrent adverse events in patients with established cardiovascular disease (Haverkate et al. 1997). Elevated levels of CRP also correlated with body weight and presence of metabolic syndrome (Linnemann et al. 2006; Zuliani et al. 2009). Interestingly, some non-diabetes drugs known to reduce the risk of atherosclerosis were shown to modify CRP concentrations (Fisher et al. 2008; Ridker et al. 2008; Ridker et al. 2001).

With regard to laboratory and clinical aspects, CRP circulates with a long half-life and minor seasonal and diurnal fluctuations (Packard and Libby 2008; Pearson et al. 2003). It can be measured simply by standardised high-sensitive immunoassays providing accurate and similar results in fresh, stored or frozen samples (Packard and Libby 2008; Pearson et al. 2003).

Based on available evidence, CRP measured by a high sensitive method (hsCRP) has been identified as an independent inflammatory marker for predicting the risk of coronary disease, and the analyte of choice by the Center for Disease Control and Prevention and the American Heart Association (Pearson et al. 2003).

According to the issued guideline, the cutpoints in clinical practice are: hsCRP levels < 1mg/L for low risk, 1.0 to 3.0 mg/L for average risk, and >3.0 mg/L for high risk [Table 1]. The risk of cardiovascular events was predicted to increase about 2 fold in individuals categorised as high risk compared to the low-risk group (Pearson et al. 2003).

Table 1. Cardiovascular risk assessment according to hs-CRP concentrations

hsCRP concentration	Cardiovascular risk assessment
0 to <1mg/L	Low risk
1-3 mg/L	Intermediate risk
>3 to 10 mg/L	High risk
>10 mg/L	Unspecific elevation

Given these findings, hs-CRP was the inflammatory marker chosen for evaluating the relationship between thiamin status and cardiovascular risk factors in this study.

2.2.3 Risk factors of cardiovascular disease

2.2.3.1 Lipids and lipoproteins

Atherogenic dyslipidemia commonly presents as the raised levels of triglyceride and LDL-cholesterol, and reduced HDL-cholesterol [although isolated low HDL-cholesterol occur rarely in some individuals] (Grundy 1998). The role of this lipid triad in cardiovascular events has been confirmed by a large number of experimental and epidemiological studies (Austin, Hokanson, and Edwards 1998; Gordon et al. 1989).

2.2.3.1 A Lipoproteins structure, metabolism and atherogenisity

Lipoproteins are macromolecular structures containing two major parts: lipids (triglyceride, cholesterol and phospholipids) and proteins. The function of lipoproteins essentially is to transport the hydrophobic triglyceride and cholesteryl esters in the circulation (Smith, Pownall, and Gotto 1978). With regard to the lipoproteins structure, the amphipathic phospholipids on the surface of lipoprotein

particles enable the hydrophobic core lipids to be soluble in the aqueous blood stream (Chahil and Ginsberg 2006).

Lipoproteins can be divided into five main classes, in terms of their densities, namely: high density lipoproteins (HDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL), very low density lipoproteins (VLDL) and chylomicrons (CM) (Garrett and Grisham 1995) [Table 2].

In addition to the core lipids, these lipoproteins classes are different in their surface proteins, named apolipoproteins. Two main subclasses of apolipoproteins are apolipoprotein A (apoA) and apolipoprotein B (apoB), which exists in two isoforms, apoB48 and apoB100 (Chahil and Ginsberg 2006).

The triglyceride-rich lipoproteins are assembled and secreted exclusively in the intestine or the liver through two distinct pathways, exogenous and endogenous.

In the exogenous pathway, the dietary lipids absorbed by intestinal epithelial cells are combined with apoB100 to form chylomicrons. These nascent lipoproteins are then released into the blood via lymphatic circulation. In the bloodstream, the nascent chylomicrons receive ApoC and ApoE from HDL particles, and develop into the mature form which carry triglyceride and, to a lesser extent, cholesterol to non-hepatic tissues.

Table 2. Classification of lipoproteins based on density (Garrett and Grisham 1995)

Density (g/mL)	Class	Diameter (nm)	%protein	%cholesterol	%phospholipids	% triacylglycerol
>1.063	HDL	5-15	33	30	29	4
1.019-1.63	LDL	18-28	25	50	21	8
1.006-1.019	IDL	25-50	18	29	22	31
0.95-1.006	VLDL	30-80	10	22	18	50
<0.95	CM	100-1000	<2	8	7	84

In the endogenous pathway, the triglyceride and cholesterol constituents of lipoproteins are primarily synthetised in the liver, assembled with ApoB100 into VLDL particles. Like chylomicrons, these nascent VLDLs require ApoC and ApoE to mature in the circulation. Subsequent to maturation, circulating chylomicrons and VLDL are catabolised under the influence of the enzymes, lipoprotein lipase (LPL) and lecithin cholesterol acyltransferase (LCA) (Smith, Pownall, and Gotto 1978).

In this process initially, LPL catalyses the hydrolysis of triglyceride in these triglyceride - rich lipoproteins, which results in a remnant from the chylomicrons and an intermediate density lipoprotein (IDL) from the VLDL particles. The products of triglyceride hydrolysis (fatty acid and acylglycerol) can be taken up by peripheral tissues for utilisation or storage.

The catabolic process continues with the conversion of IDL to LDL, and ultimately the uptake and degradation of chylomicrons remnants (CMR) by the liver and LDL particles by the liver and other tissues via a receptor- mediating mechanism.

All apoB-lipoproteins except nascent chylomicrons have the potential to penetrate into the endothelial layer (Chahil and Ginsberg 2006) and induce the inflammatory atherogenic process in an injured artery, as described before (see Section 2.2.1). However, HDL particles containing ApoA are known to be antiatherogenic, mainly because of their key role in reverse cholesterol transport (RCT). HDL particles are thought to be the only lipoproteins able to receive the cholesterol accumulated in peripheral cells, including macrophages in atherosclerotic lesions, and return them to the liver for subsequent excretion in the bile (Chahil and Ginsberg 2006).

2.2.3.1.B Prediction of CVD risk using serum lipids and lipoproteins

To evaluate the risk of further cardiovascular disease, only serum total cholesterol was measured initially. Subsequent studies considered serum LDL cholesterol as the most evident atherogenic lipoprotein and consequently, the main target for cholesterol lowering interventions (National Cholesterol Education Program (NCEP) Expert Panel on Detection 2002).

The relationship between LDL cholesterol and coronary heart disease has been shown to be log linear (Law, Wald, and Thompson 1994; Law 1999).

Table 3. Classification of triglyceride levels (National Cholesterol Education Program (NCEP) Expert Panel on Detection 2002)

Serum triglyceride (mg/dL) [mmol/L]	
Normal triglyceride	< 150 [1.7]
Borderline - high triglyceride	150-199 [1.7- 2.25]
High triglyceride	200-249 [2.26- 2.81]
Very high triglyceride	≥ 500 [5.65]

The risk of cardiovascular disease appears to be very low when serum LDL concentration is below the optimal level of 100 mg/dl (2.59 mmol/L). However, LDL cholesterol at higher levels is directly associated with the accelerated risk of atherosclerosis (National Cholesterol Education Program (NCEP) Expert Panel on Detection 2002).

In contrast with LDL, population based evidence indicates that higher levels of HDL cholesterol are associated with a reduced risk of coronary heart disease (Wilson et al. 1998). This inverse relationship was shown to be continuous, namely any reduction in HDL can result in a concomitant increase in coronary heart disease risk (Gordon et al. 1989).

Table 4. Classification of Total, LDL and HDL Cholesterol levels (National Cholesterol Education Program (NCEP) Expert Panel on Detection 2002)

Total Cholesterol (mg/dL) [mmol//L]		LDL Cholesterol (mg/dL) [mmol//L]		HDL Cholesterol (mg/dL) [mmol//L]	
		< 100 [2.59]	Optimal		
< 200 [5.17]	Desirable	100-129 [2.59-3.34]	Near optimal	< 40 [1.03]	Low HDL cholesterol
200-239 [5.17- 6.19]	Borderline	130-159 [3.46- 4.11]	Borderline		
≥ 240 [6.20]	High	160-189 [4.13- 4.89]	High	≥ 60 [1.55]	High HDL cholesterol
		≥ 190 [4.91]	Very High		

Accordingly, serum HDL levels are classified as two categories of "low HDL cholesterol" and "high HDL cholesterol" (National Cholesterol Education Program (NCEP) Expert Panel on Detection 2002) [Table 4].

Elevated serum triglyceride is another component of the lipid triad, indicated as an independent risk factor for coronary heart disease (Austin, Hokanson, and Edwards 1998). Raised levels of triglyceride were shown to be strongly associated with several lipid (high LDL and low HDL particles) and non-lipid risk factors, in particular hyperglycemia (Grundy 1998).

While the pathogenesis of hyperlipidemia associated with hyperglycemia is not fully understood, an increase in the hexosamine pathway in the liver due to hyperglycemia has been elucidated (Rumberger et al. 2003; Veerababu et al. 2000). Given the possible effect of thiamin on the hepatic hexosamine pathway (see Section 2.5.9), it is proposed that supplementation with thiamin may alter this effect.

2.2.3.2 Hypertension

Hypertension, chronic elevation of systemic arterial blood pressure, is established as another powerful and independent risk factor for atherosclerosis (MacMahon et al. 1990; Stamler, Stamler, and Neaton 1993). According to the categorical classification, hypertension is defined as a systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg or recent use of antihypertensive medication (Chobanian et al. 2003; National Cholesterol Education Program (NCEP) Expert Panel on Detection 2002; The sixth report of the Joint National Committee on prevention, detection, evaluation,

Table 5. Classification of blood pressure for adult (Chobanian et al. 2003)

Blood Pressure Classification	SBP mm Hg	DBP Mm Hg
Normal	<120	and < 80
Prehypertension	120-139	or 80 - 89
Stage 1 Hypertension	140- 159	or 90- 99
Stage 2 Hypertension	≥ 160	or ≥ 100

SBP, systolic blood pressure; DBP, diastolic blood pressure

and treatment of high blood pressure 1997). Even patients with blood pressure at prehypertension level [Table 5] are shown to have a higher risk CHD relative to those with normal blood pressure (Vasan et al. 2001).

The unequivocal association between hypertension and CVD events was indicated to be consistent and continuous (Chobanian et al. 2003). Therefore, any decrease in blood pressure is accompanied with a reduced risk for blood pressure-related endpoints (Cutler et al. 1995).

The link between hypertension and atherosclerosis can be essentially explained by the new understanding of central role of inflammation in CVD events. It is suggested that inflammation may participate in the initiation of hypertension through the secretion of several mediators, such as TNF-α, IL- 6 and CRP, resulting in an impaired capacity of the endothelium to produce vasodilating factors, in particular nitric oxide (NO), thus endothelial dysfunction and ultimately hypertension (Bautista 2003). The raised blood pressure can, in turn, damage the arterial wall (Sherwood 2009) and enhance the vascular inflammation (Boos and Lip 2005). Furthermore, the inflammatory process was indicated to be influenced by the renin - angiotensin system involved in hypertension pathology (Brasier, Recinos, and Eledrisi 2002). Angiotensin II, has a vasoconstricting effect, but can also induce the expression of arterial SMCs proinflammatory cytokines, including IL-6 and MCP-1, and the endothelium - derived VCAM-1 (Hernandez-Presa et al. 1997; Kranzhofer et al. 1999; Tummala et al. 1999). Angiotensin II can also promote the production of reactive oxygen species by arterial endothelial and smooth muscle cells (Griendling et al. 1997). A direct relationship between inflammatory markers, particularly CRP, and blood pressure was reported by several studies (Bautista et al. 2005; King et al. 2004), confirming the role of inflammation in the development of hypertension and subsequent atherosclerosis.

Thiamin supplementation was shown to have beneficial effect on systolic blood pressure of individuals with subclinical thiamin deficiency (Wilkinson et al. 1997)

2.2.3.3 Overweight/obesity

The prevalence of overweight and obesity has reached epidemic proportion worldwide, with approximately 1.6 billion adults overweight (World Health Organization 2006). The National Health Survey in Australia in 2007-2008 found 67.7% of men and 54.6% of women to be overweight or obese, peaking among those aged 65-74 years (78.9% of men and 71.4% of women) (Australian Bureau of Statistics 2009). Overweight is defined as a body mass index (BMI, weight in kg divided by the square of height in meters) of 25-29.9 kg/m^2 and obesity as BMI$\geq$ 30 (National Institutes of Health 1998). Overweight and obese people are not only at a raised risk of CVD, they have also higher all-cause mortality (Hubert et al. 1983; Wilcosky et al. 1990). Obesity is also strongly associated with other CVD risk factors, including hyperglycemia (Hartz et al. 1983; Straczkowski et al. 2003), hypertension (Berchtold et al. 1981; Blair et al. 1984) and in particular dyslipidemia (high LDL, VLDL and triglycerides and low HDL cholesterol) (Denke, Sempos, and Grundy 1994, 1993; Garrison et al. 1980; Olefsky, Reaven, and Farquhar 1974). This risk is known to be more significant in the presence of abdominal obesity, which is defined as a waist circumference of more than 88 cm in women and 102 cm for men (National Institutes of Health 1998). In people with central obesity, excess free fatty acids originating from intra-abdominal fat stimulate the production of VLDL particles in the liver, resulting in a high hepatic lipase activity and subsequent increase in the concentration of LDL (Sibley et al. 2003). The elevated VLDL can also lead to HDL particle reduction through enhancing exchange from HDL to VLDL, mediated by cholesteryl ester transfer protein (Libby 2002). Furthermore, adipose tissue was implicated as a major source of cytokines, such as IL-6 (Yudkin et al. 1999), suggesting a contribution to the inflammatory atherogenic process independent of its effects on lipoprotein metabolism or insulin sensitivity.

2.2.3.4 Arterial stiffness

Arterial stiffness is a general term used to define the stiffing of the arterial wall, resulting in a declined elasticity of the blood vessel. Arterial stiffness is an independent risk factor for cardiovascular disease and occurs mainly as a pathological consequence of ageing (Benetos et al. 2002; Wilkinson and McEniery 2004). However, its progress may be accelerated in the presence of other risk factors,

including hypertension, dyslipidemia, obesity and hyperglycemia in either diabetic or pre-diabetic ranges (Henry et al. 2003; Liu et al. 1989; Safar, Czernichow, and Blacher 2006; Salomaa et al. 1995; Schram et al. 2004; van Popele et al. 2006; Wilkinson et al. 2002; Xu et al. 2010). Furthermore, arterial stiffness is known as a strong predictor of cardiovascular events and all-cause mortality (Adji, O'Rourke, and Namasivayam 2011; Vlachopoulos, Aznaouridis, and Stefanadis 2010).

2.2.3.4.A Pathogenesis of arterial stiffness

Although the pathogenesis of arterial stiffness is not fully understood, multiple mechanisms have been implicated in its aetiology. These mechanisms include

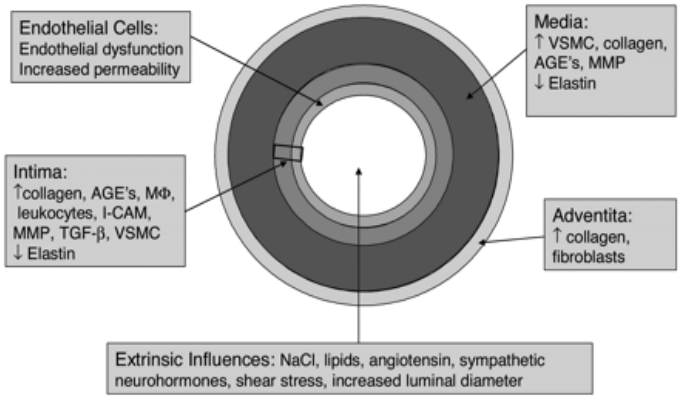


Figure 3. Summary of the multiple causes and locations of arterial stiffness (Zieman, Melenovsky, and Kass 2005)

changes in the structure of extracellular matrix, endothelial function and vascular smooth muscle tone (Zieman, Melenovsky, and Kass 2005) [Figure 3].

The integrity and elasticity of the vascular wall are respectively dependent on its two extracellular proteins, collagen and elastin (Zieman, Melenovsky, and Kass 2005). The proportion of these molecules in the arterial wall is balanced by a slow dynamic process of formation and degradation. Disturbance in this balance results in an increased production of collagen and concomitant reduction in elastin proportion, with in turn a decline the vessel elasticity (Johnson et al. 2001). Different factors contributing to arterial stiffness mostly affect the degradation of elastin and collagen,

which are in turn regulated by matrix metalloproteases (MMPs) (Zieman, Melenovsky, and Kass 2005).

The histological examination of stiffened vessel specimens reveal disarranged endothelial cells, increased collagen content, broken elastin molecules, infiltration of inflammatory cells, hypertrophied vascular smooth muscle layer, and increased matrix metalloproteases (Lakatta 2003; Virmani et al. 1991).

The tensile strength of the vessel wall is mainly due to the enzymatic cross - linking of collagen and elastin molecules, making them insoluble to hydrolytic enzymes (Reiser, McCormick, and Rucker 1992). However, due to the slow rate of hydrolytic turnover, these proteins are also prone to non-enzymatic glycation cross-linking, forming stable and irreversible cross - linked components of AGEs (Bailey 2001; Lee and Cerami 1992).

This process can lead to an increase in the content of collagen fibres which are now stiffer, and distributed irregularly (Verzijl et al. 2000). Non-enzymatic glycation reaction can also weaken the integrity of elastin bonds and also predispose the protein to mineralisation by calcium and phosphate, accelerating the arterial stiffness (Cattell, Anderson, and Hasleton 1996; Konova et al. 2004; Spina and Garbin 1976; Watanabe et al. 1996; Winlove et al. 1996). Furthermore, the binding of AGEs to their specific receptors (RANGE) on the surface of several cell types augments the production of oxidants, pro-inflammatory cytokines and other inflammatory mediators, increasing arterial stiffness via activation of MMPs (Kuzuya et al. 2001; Yan et al. 1994). Receptor-mediated interaction of AGEs with RAGE on smooth muscle cells can increase cellular proliferation mediated by cytokine or growth-factor (Vlassara, Bucala, and Striker 1994).

In addition to the structure of the extracellular matrix, arterial stiffness is strongly influenced by the endothelial cell function and vascular smooth muscle tone. Vascular tone is determined by a mechanostimulation mechanism activated via cell stretch and a calcium signalling system, by circulatory vasoactive mediators (i.e. bradykinin and thrombin) and by several vasoactive molecules, such as nitric oxide (NO), angiotensin II, endothelin, which are mainly produced by the endothelium, and

oxidant stress (Dzau 1986; Gurtner and Burke-Wolin 1991; Yanagisawa et al. 1988; Zieman, Melenovsky, and Kass 2005). Indeed, endothelial cells are able to release a wide range of factors, regulating vascular tone, smooth muscle cell proliferation and arterial wall inflammation (Deanfield, Halcox, and Rabelink 2007).

The endothelium refers to a single layer of cells that line all the vessels in the body, including conduit vessels, resistance vessels, pre-capillary arterioles and capillaries (Vane, Anggard, and Botting 1990). In recent years, the endothelium has been shown to be a key protective barrier against the progress of atherosclerosis. Endothelial dysfunction, characterised by "inadequate vasodilatation and/or paradoxical vasoconstriction in coronary and peripheral arteries", has been generally considered as one of the earliest markers of cardiovascular disease (Cersosimo and DeFronzo 2006). Emerging evidence shows that almost all conventional risk factors for atherosclerosis, such as smoking (Celermajer et al. 1996), dyslipidemia (Sorensen et al. 1994) and arterial hypertension (Panza 1994) as well as coronary artery disease (Tousoulis, Charakida, and Stefanadis 2008), congestive heart failure (Zelis, Mason, and Braunwald 1968) and hyperglycemia (Kawano et al. 1999; Tooke and Goh 1999) are associated with an abnormal endothelial vasoactivity. Endothelial dysfunction is essentially attributable to a decrease in NO bioavailability, which can be because of a reduced production, increased degradation or decreased sensitivity of the endothelium to NO (De Caterina 2000).

In normal vascular physiology, NO plays a key role in regulating arterial tone. NO is produced in endothelial cells from the oxidation of L-arginine by endothelial NO synthase (eNOS) (Forstermann and Munzel 2006). This gas can diffuse throughout the arterial smooth muscle cells and stimulate cGMP - mediated vasodilation via guanylate cyclase activation (Deanfield, Halcox, and Rabelink 2007). Decreased NO bioavailability reduces vascular smooth muscle cell relaxation, resulting in the functional stiffness of arteries.

Endothelial dysfunction can be also due to an increased production of ROS, which in turn leads to hydrogen peroxide generation. Hydrogen peroxide, similar to NO, diffuses to the vascular cells, targets main regulatory proteins and impairs their functions (Rhee 2006). Several cardiovascular risk factors such as dyslipidemia,

hypertension and hyperglycemia are thought to induce endothelial dysfunction and consequently arterial stiffness through the chronic dysregulation of NO and ROS production (Celermajer et al. 1994). There is also convincing evidence indicating that resistance to insulin plays a fundamental role in the pathogenesis of endothelial dysfunction induced by various risk factors (Caballero 2003; Cohn, Valdes, and Capuzzi 2001).

In general, insulin has a vasodilating effect, particularly on skeletal muscle vessels, to increase glucose delivery to this insulin-sensitive tissue (Petrie et al. 1996). Insulin is suggested to stimulate arterial dilation through the release of NO (Scherrer et al. 1994). In the insulin resistant state, insulin-mediated vasodilation may be impaired, due to a decrease in NO synthesis or an abnormal response of vascular smooth muscle cells to NO (Williams et al. 1996). Insulin resistance may lead to endothelial dysfunction through multiple mechanisms, including: the increased levels of asymmetric dimethylarginine (ADMA) which is an endogenous NO synthase inhibitor (Stuhlinger et al. 2002), defect in the phosphatidylinositol 3-kinase pathway which contribute to insulin-dependent endothelial NO production (Hsueh and Quinones 2003; Zeng et al. 2000), activation of mitogen-activated protein kinase (MARK) pathway mediating VSMC cell growth as well as migration of endothelial cells and monocytes (Pessin and Saltiel 2000), and inflammation (Sriraman and Tooke 2004). Resistance to insulin can be also associated with an increased production of ROS (Inoguchi et al. 2000) and the activation of the rennin-angiotensin system which in turn increases superoxide production (Rajagopalan et al. 1996).

2.2.3.4.B Measurement of arterial stiffness

Arterial stiffness can be noninvasively evaluated by a range of devices which are essentially designed based on three main methodologies ; 1) estimation of pulse wave velocity (PWV), 2) assessment of vessel distensibility by measuring the change in arterial diameter in relation to distending pressure, 3) pulse pressure waveform analysis.

Assessment of PWV is theoretically derived from Moens-Kortweg equation: $PWV^2=E*h/2r\rho$, where E is Young's Modulus of the arterial wall, *h* is wall thickness,

r is arterial radius at end - diastole, and ρ is the density of blood (Oliver and Webb 2003). PWV can be practically computed by measuring the time taken for the arterial pulse wave to pass the distance between two determined points. Various methods are applied to detect the pulse wave, such as Doppler ultrasound or applanation tonometry. PWA of large elastic arteries, such as the aorta, has emerged as the gold standard marker for regional arterial stiffness (Laurent et al. 2006). Central pulse wave velocity determined by measuring carotid-to-femoral transit time, is commonly used for evaluating aortic stiffness. However, given a relatively small inter-subject variation between subjects with arterial stiffness and healthy individuals, the results may not be obvious except in large scale studies (Woodman and Watts 2003). Recently, several methods of cardiovascular magnetic resonance (CMR) have been used for measuring aortic pulse wave velocity (Ibrahim el et al. 2010).

The change in arterial diameter in relation to distending pressure is a measure of local arterial stiffness, and is frequently measured on superficial arteries, such as carotid artery. The internal diameter of an artery can be evaluated by the eco-tracking technique and applanation tonometry is commonly used for measuring local pulse pressure (Benetos et al. 1993). Vessel distensibility can be also evaluated directly by magnetic resonance imaging (MRI), measuring the distension of deeper arteries, such as the ascending and descending thoracic, and abdominal aorta (Resnick et al. 1997). However, this technique is not in common use, because of its expense and also limitations regarding accurate and concurrent local arterial pressure measurement (Sakuragi and Abhayaratna 2010).

The arterial pressure waveform is composed of a forward pressure wave arising from the left cardiac ventricle contraction and a backward travelling wave reflected from the periphery, mainly at the branching points of arteries and the sites of increased stiffness. In elastic arteries, the backward wave tends to return to the aorta during diastole, enhancing diastolic pressure. However, in stiffened vessels, due to an increased pulse wave velocity, the reflected wave arrives back earlier in the systolic phase of the cardiac cycle, thus augmenting the systolic pressure instead of diastolic pressure (Mackenzie, Wilkinson, and Cockcroft 2002). This phenomenon is the basis of several methods of measuring arterial stiffness through the analysis of pressure pulse waveforms.

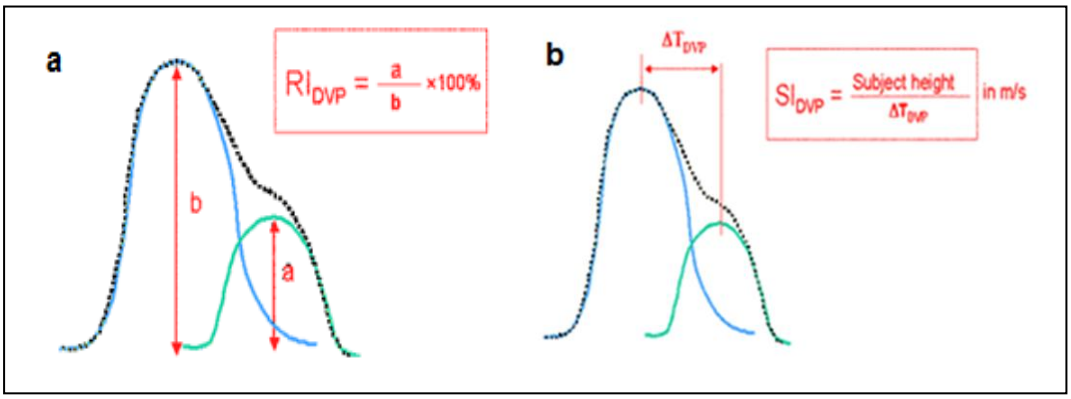
Pulse wave analysis (PWA) is a non - invasive method of measuring arterial stiffness mainly at the central level, such as the ascending aorta (O'Rourke, Pauca, and Jiang 2001). Aortic pressure waveform can be assessed either from the radial artery waveform via a transfer function or the common carotid waveform recorded by applanation tonometry. Pulse waves are analysed through three main measurements: central pulse pressure, central systolic pressure, and augmentation index, which is defined as " the difference between the second and first systolic peak (P2-P1) expressed as a percentage of the pulse pressure " (Laurent et al. 2006; London et al. 1992; Mackenzie, Wilkinson, and Cockcroft 2002; O'Rourke 1971).

Analysis of digital volume pulse (DVP) waveform produced by the Pulse Trace system is another technique of evaluating arterial stiffness. The system is based on the finger photoplethysmography, and validated in different setting and diseases (Chowienczyk et al. 1999; Millasseau et al. 2000; Takazawa et al. 1998).

The device is made of an infra-red sensor (photoplethysmograph), which detects the pulse waveforms at the finger. The sensor is connected to an analogue-to-digital converter, recording waveforms for 15-30 seconds. DVP consists of two components; a forward wave (systolic component) resulting from the transmission of pressure along a direct path from the aortic root to the finger, and a backward pressure wave (diastolic component), reflected back from the lower body along the aorta to the finger. Accordingly, both the directly transmitted pressure wave and the reflected wave can be detected in the upper limb, minimising the contour of DVP. Reflection index (RI) and stiffness index (SI) are the two main indices derived from the DVP [Figure 4].

RI is computed by the % ratio of the heights (amplitude) of reflected wave (diastolic component) to the systolic peak, and is a measure of pulse wave reflection and the small artery tone. SI can be calculated from the subject's height divided by the time between systolic and diastolic peaks, and is a marker of large arterial stiffness (Woodman and Watts 2003).

Figure 4. Indices derived from DVP; *a) RI, a measure of small arterial tone, b) SI, a surrogate marker of large arterial stiffness (Micro Medical Ltd 2007)*



SI was shown to be strongly correlated with the augmentation index as well as central pulse wave velocity (Woodman et al. 2003).

In comparison with other established methods for arterial stiffness assessment, pulse trace system is the simplest technique, and independent of operator skill. Also, its reproducibility is consistent with other accepted methods, with a CV of about 15% (Woodman and Watts 2003). Given these advantages, in the present study this technique was chosen to evaluate arterial stiffness in subjects with abnormal glucose regulation compared to normoglycemic volunteers.

2.2.3.5 Hyperglycemia

It is well known that diabetes mellitus is linked to an increased prevalence of cardiovascular diseases (Ruderman and Haudenschild 1984); however evidence from more recent studies has shown that pre-diabetic degrees of fasting and postprandial hyperglycemia are also associated with accelerated atherosclerosis (Balkau, Forhan, and Eschwege 2002; Balkau et al. 2004; Barzilay et al. 1999; Coutinho et al. 1999; DECODE Study Group 2001, 2003; Gabir et al. 2000; Levitan et al. 2004; Meigs et al. 2002; Saydah et al. 2001; Stern et al. 2002; Tominaga et al. 1999), resulting in the inclusion of hyperglycemia in diabetic or pre-diabetic ranges as a required component of metabolic syndrome (Alberti and Zimmet 1998).

2.3 Diabetes mellitus and other categories of hyperglycemia

2.3.1 Diabetes mellitus

Diabetes mellitus (DM) is a chronic disease with an epidemic prevalence worldwide (Colagiuri et al. 2005). According to the estimation of World Health Organization (WHO), there are now more than 180 million diabetic patients in the world and it is projected to increase more than double by 2030 (World Health Organization 2009). In Australia, the prevalence of diabetes mellitus has been based on two main national data sources. According to the AusDiab 1999-2000 study which collected blood samples, the prevalence of diabetes was estimated to be 7.4% of adults aged 25 or over (Dunstan et al. 2001). The second source of information was self-reported data collected from the Australian Bureau of Statistics (ABS) series of National Health Surveys (NHS). The latest NHS data show that in 2007-2008, 4.4% of Australians reported as being diabetic, which was substantially more than the self-reported rate of 2.4% in 1995 (Australian Institute of Health and Welfare 2011; Australian Bureau of Statistics 2006). Studies indicate that this increase in the prevalence of diabetes is associated with an accelerated risk of developing CVD and other major health problems (Australian Institute of Health and Welfare 2008).

2.3.1.1 Definition and diagnosis of diabetes mellitus

Diabetes mellitus refers to a metabolic disorder characterized by chronic hyperglycaemia, due to defects in insulin secretion, resistance to insulin or both (Alberti and Zimmet 1998). Hyperglycemia may be detected in an asymptomatic person with blood glucose just above the diagnostic cut–off values or be found in a subject with severe symptoms and blood glucose levels considerably more than normal ranges. Additionally, some conditions such as acute infections, trauma or surgery may be associated with a transitional hyperglycemia. So, given the lifelong consequences of diabetes mellitus, establishing an accurate diagnosis is very important and critical.

Table 6. Values for diagnosis of diabetes mellitus and other categories of hyperglycemia (Alberti and Zimmet 1998)

	Glucose concentration, mmol l^{-1} (mg dl^{-1})		
	Whole blood		Plasma*
	Venous	Capillary	Venous
Diabetes Mellitus:			
Fasting	≥ 6.1 (≥ 110)	≥ 6.1 (≥ 110)	≥ 7.0 (≥ 126)
or			
2-h post glucose load	≥ 10.0 (≥ 180)	≥ 11.1 (≥ 200)	≥ 11.1 (≥ 200)
or both			
Impaired Glucose Tolerance (IGT):			
Fasting (if measured)	< 6.1 (< 110)	< 6.1 (< 110)	< 7.0 (< 126)
and			
2-h post glucose load	≥ 6.7 (≥ 120) and < 10.0 (< 180)	≥ 7.8 (≥ 140) and < 11.1 (< 200)	≥ 7.8 (≥ 140) and < 11.1 (< 200)
Impaired Fasting Glycaemia (IFG):			
Fasting	≥ 5.6 (≥ 100) and < 6.1 (< 110)	≥ 5.6 (≥ 100) and < 6.1 (< 110)	≥ 6.1 (≥ 110) and < 7.0 (< 126)
and (if measured)			
2-h post glucose load	< 6.7 (< 120)	< 7.8 (< 140)	< 7.8 (< 140)

For diagnostic propose, blood glucose may be measured in venous plasma, venous whole blood or capillary whole blood [Table 6]. However, fasting plasma glucose (FPG) and 2-h OGTT are the tests recommended by WHO and IDF to identify all states of hyperglycemia (World Health Organization 2006). Also, for standardisation in Australia, venous plasma glucose was chosen as a recommended method of diagnosing diabetes and impaired glucose regulation state (IFG, IGT) by the Australian Diabetes Society (ADS) (Diabetes Australia Western Australia 2005; Welborn 1996). Additionally, according to the guidelines provided by National Health and Medical Research Council (NHMRC), venous plasma glucose measurements should be performed by an accredited laboratory, and blood glucose meters do not have sufficient accuracy required for definitive diagnosis (Diabetes Australia Western Australia 2005; National Health and Medical Research Council 2001; Poirier et al. 1998).

There is now a general consensus (Alberti and Zimmet 1998; Diabetes Australia Western Australia 2005; World Health Organization 2006) that for the symptomatic person, a single fasting plasma glucose concentration greater than or equal to 7 mmol/L or a value greater than or equal to 11.1 mmol/L, 2 hours after a 75 g oral glucose load is sufficient to establish the diagnosis [Table 6]. However, in asymptomatic subjects, at least two diagnostic glucose results in excess of the

diagnostic values on different days are essential, either fasting, from a random sample, or following oral glucose tolerance tests (OGTT).

2.3.1.2 Classification of diabetes mellitus

The first classification of diabetes mellitus was offered by the WHO Expert Committee in 1980 (World Health Organization 1980). In the 1980 Expert Committee report, diabetes mellitus was classified into two major types, "insulin–dependent diabetes mellitus" (IDDM) or Type 1 and "non–insulin–dependent diabetes mellitus" (NIDDM) or Type 2, as well as other classes of diabetes including: other specific types, impaired glucose tolerance (IGT) and gestational diabetes mellitus (GDM). In 1985, the WHO Study Group recommended a modified form of classification, in which the terms type 1 and type 2 diabetes were eliminated and the terms of insulin-dependent and non-insulin dependent diabetes (IDDM and NIDDM) retained (World Health Organization 1985). Additionally, a new class of malnutrition–related diabetes mellitus (MRDM) was introduced.

The latest classification of diabetes mellitus was established in 1997 to encompass both clinical stages and etiological types of hyperglycemia (Kuzuya and Matsuda 1997). In the new classification, which is universally accepted (Alberti and Zimmet 1998; Welborn 1996; World Health Organization 2006), the terms "insulin–dependent diabetes mellitus" (IDDM) and "non–insulin–dependent diabetes mellitus" (NIDDM) have been considered as confusing, because insulin treatment may be required at some stage of any type of diabetes. Accordingly, in the revised classification, these terms are omitted and the terms type 1 and type 2 diabetes reintroduced. Other aetiological types, such as diabetes caused by genetic defects or infections are classified as "other specific types", and gestational diabetes is the fourth class.

2.3.1.3 Risk factors of type 2 diabetes mellitus

It is believed that a range of genetic, behavioral and biomedical conditions can contribute to the onset and development of diabetes. Among them, the following factors have been considered as playing a more significant role.

- Age. Evidence indicates that the risk of developing diabetes increases with age (Australian Bureau of Statistics 2006; Dunstan et al. 2001). Based on measured data from the AusDiab 1999–2000 study, among adults aged 35–44 years the prevalence of diabetes was fewer than 3%. However, this rate increased to 23% for people aged 75 years and over (Dunstan et al. 2001).
- Obesity and low of physical activity. It is believed that individuals with extra body fat or/and insufficient physical activity have a greater likelihood of developing diabetes mellitus, resulting from the increased risk of resistance to insulin and defects in insulin secretion (Chan et al. 1994; Sharma 2006; Telford 2007). Based on the National Physical Activity Guidelines for Australians, at least 30 minutes of moderate physical activity on at least five days of the week, or 150 minutes spread out over five sessions in a week is recommended as the sufficient level for prevention of chronic diseases (Australian Institute of Health and Welfare 2003; Department of Health and Ageing; Australia 1999).
- Smoking. Longitudinal studies indicate that the incidence of diabetes mellitus among smokers was significantly higher than non-smokers. Additionally, in was shown that quitting smoking could reduce the increased risk of developing diabetes to that of non-smokers after some years (Eyre et al. 2004).
- Being from a high-risk racial/ethnic population. Epidemiological studies indicate that diabetes mellitus is more common among some certain ethnic groups (Harris et al. 1995; Valle, Tuomilehto, and Eriksson 1997; Zimmet 1992). According to the Australian Bureau of Statistics (ABS) series of National Health Surveys (NHS) data, in 2004-2005 the rate of diabetes among Aboriginal and Torres Strait Islander peoples was three times more than non-indigenous Australians (Australian Bureau of Statistics 2006). In addition, it has been shown that Australians who were born in the South Pacific Islands, Southern Europe, Middle East, North Africa and Southern Asia have higher rates of diabetes compared to Australian-born people (Australian Institute of Health and Welfare 2003).
- Past history of gestational diabetes mellitus (Alberti and Zimmet 1998).

- Having a relative with diabetes mellitus (de Courten et al. 1997; Knowler et al. 1993; Valle, Tuomilehto, and Eriksson 1997).
- Being recognized as having impaired glucose regulation [impaired glucose tolerance (IGT) or impaired fasting glucose (IFG)] on previous testing. According to existing evidence, individuals with impaired glucose regulation are 10-15 times as prone to developing diabetes relative to those with normal blood glucose levels (Dunstan et al. 2001) and most of them eventually (perhaps after many years) develop diabetes (de Vegt et al. 2001; Knowler et al. 2002; Larsson et al. 2000; Santaguida et al. 2005; Shaw et al. 1999; Tuomilehto et al. 2001; Twigg et al. 2007; Vendrame and Gottlieb 2004). Studies investigating the role of impaired glucose regulation as a predictor of developing diabetes indicate that the majority of people with diabetes (60%) had a history of either IGT or IFG during five years before being diagnosed as diabetics (Unwin et al. 2002).

2.3.2 Impaired glucose regulation

Impaired glucose regulation describes an intermediate stage of abnormal glucose regulation between normal glucose state and diabetes (Alberti and Zimmet 1998). This disordered carbohydrate metabolism may be represented in the fasting condition (IFG) or post– prandial (IGT), with different physiological bases (see Section 2.3.2.3). In Australia, 16.4% of adults aged 25 and over, participating in the 1999–2000 AusDiab study, were found to have impaired glucose regulation, with IGT being more common than IFG (10.6% and 5.8%, respectively) (Dunstan et al. 2001) [Table 7].

Table 7. Prevalence of impaired glucose regulation among Australians aged 25 years and over, 1999–2000 (per cent) (Dunstan et al. 2001)

Measure	Males	Females	Persons
Impaired glucose tolerance	9.2	11.9	10.6
Impaired fasting glucose	8.1	3.4	5.8
Total impaired glucose regulation	**17.4**	**15.4**	**16.4**

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2.3.2.1 Impaired fasting glycaemia (IFG)

Impaired fasting glycaemia (IFG) is defined as fasting blood glucose values above the normal range, but below those diagnostic of diabetes (Alberti and Zimmet 1998). It was estimated that in 1999-2000, 5.8% of Australians aged 25 years and above had IFG, being more prevalent among men (Dunstan et al. 2001) [Table 7].

The diagnostic values for IFG are defined as the fasting plasma glucose concentration of 6.1 mmol/L (110 mg/ dl) or greater, but less than 7.0 mmol/L (126 mg/dl) (Alberti and Zimmet 1998) [Table 6]. Individuals with IFG may be reclassified as IGT or diabetic if an OGTT is carried out. Therefore, performing an OGTT is recommended for all individuals with IFG to rule out the diagnosis of diabetes (Alberti and Zimmet 1998; Diabetes Australia Western Australia 2005).

2.3.2.2 Impaired Glucose Tolerance (IGT)

Impaired glucose tolerance represents abnormalities of glucose metabolism in the post-prandial condition (Alberti and Zimmet 1998).

The worldwide prevalence of IGT was estimated to be 8.2% of the adult population (314 million people) in 2003. It is predicted that this rate will increase to 9% (472 million people) by 2025, with the highest prevalence rate of 13.5% in South-East Asia (International Diabetes Federation 2009).

In Australia, according to measured data from 1999-2000 AusDiab study, 9.2% of men and 11.2% of women aged 25 and over had IGT [Table 7], peaking among Australians aged 75 years and over. This rate was substantially higher than the reported prevalence of 3% for both men and women from the 1981 Busselton Study (Dunstan et al. 2001).

IGT is diagnosed by means of performing a standard OGTT. In individuals with IGT, blood glucose levels 2 hours after consuming 75 g glucose are greater than normal (7.8 mmol/L), but not as high as diagnostic levels for diabetes mellitus (11.1 mmol/L). Fasting blood glucose levels in people with IGT are normal or moderately increased (lower than diabetes levels) [Table 6] (Alberti and Zimmet 1998). Given

the considerable day-to-day variability in 2-h plasma glucose level following a glucose load, a mean value of two OGTTs is required for diagnosis of IGT by IDF (Unwin et al. 2002). Studies indicate that IGT is a major independent risk factor for diabetes mellitus (Dunstan et al. 2001). Therefore, it is recommended that in individual with IGT, blood glucose measurements should be retested annually (Diabetes Australia Western Australia 2005).

It is now established that both IGT and diabetes mellitus are associated with higher risks for cardiovascular complications, with diabetes being a stronger risk predictor. However, given that the prevalence of IGT is considerably higher than diabetes, it has been shown that with some cardiovascular events such as premature deaths, the role of IGT was even more significant than diabetes (DECODE Study Group 1999)

2.3.2.3 Pathogenesis of impaired glucose regulation (IFG & IGT)

Although IFG and IGT are classified under the same category of impaired glucose regulation, the pathophysiologic mechanisms behind these disorders are different (Abdul-Ghani et al. 2006; Abdul-Ghani, Tripathy, and DeFronzo 2006; Festa et al. 2004; Hanefeld et al. 2003). Evidence indicates that IFG and IGT differ mainly based on the pattern of insulin secretion and the site of insulin resistance (Abdul-Ghani et al. 2006; Abdul-Ghani, Tripathy, and DeFronzo 2006; Hanefeld et al. 2003; Qiao et al. 2003).

It is shown that in IFG, hyperglycemia is essentially because of defects in early-phase insulin secretion as well as hepatic insulin resistance, resulting in the excessive fasting hepatic glucose production. Subsequently, during a standard OGTT an individual with isolated IFG, the high fasting plasma glucose continues to increase throughout the first 30-60 min. Thereafter, due to the normal late-phase insulin secretion combined with the appropriate muscle insulin sensitivity, glucose levels returns to near fasting values at 120 min [Figure 5].

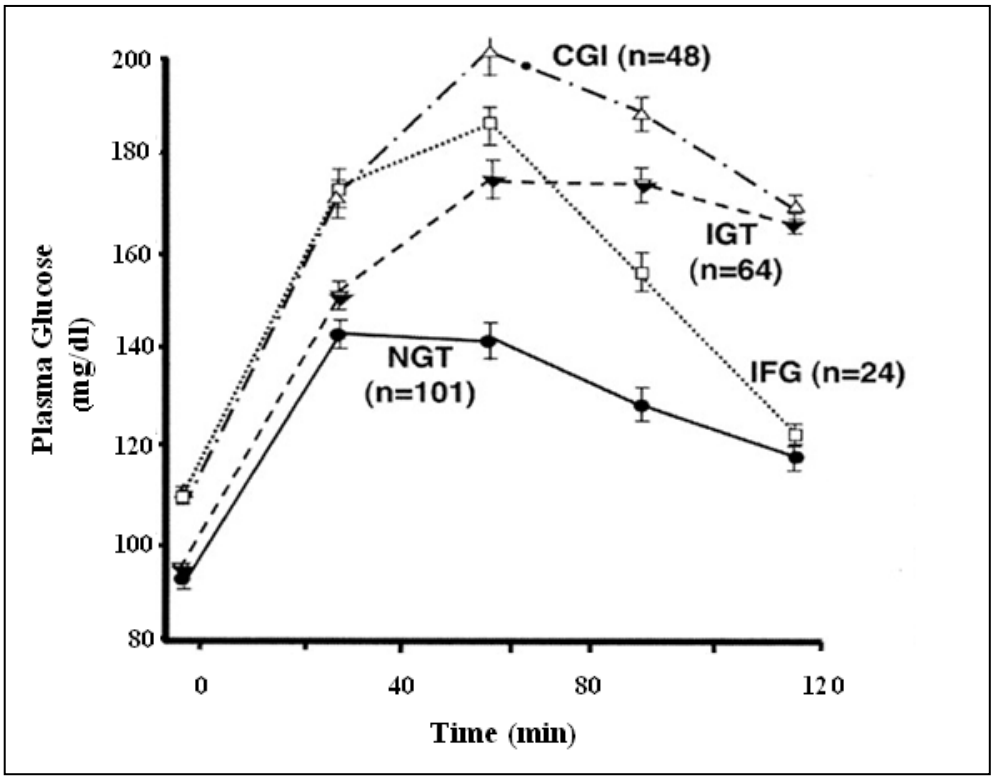

Figure 5. Plasma glucose concentration during an OGTT performed in subjects with NGT, IGT, IFG or combined IFG/IGT (CGI) (Abdul-Ghani, Tripathy, and DeFronzo 2006)

In contrast, people with isolated IGT have a moderate to severe insulin resistance at the levels of skeletal muscle rather than hepatic cells. This resistance to insulin combined with deficits in insulin secretion in both early and late phases leads to a prolonged hyperglycemia in IGT subjects (Abdul-Ghani et al. 2006; Abdul-Ghani, Tripathy, and DeFronzo 2006; Hanefeld et al. 2003; Nathan et al. 2007; Qiao et al. 2003).

2.3.2.4 Risk factors for impaired glucose regulation (IFG & IGT)

As with diabetes, the risk of impaired glucose regulation increases with age, overweight/obesity, physically inactivity, positive family history of diabetes, dyslipidemia or high blood pressure (Amini and Janghorbani 2007; Dunstan et al. 2001; Harris et al. 1998; International Diabetes Federation 2009; Lim et al. 2000; Mohan, Shanthirani, and Deepa 2003). In addition, impaired glucose regulation is more prevalent among certain ethnic groups such as Mexican Americans, African

Americans and South East Asians (Harris et al. 1998; International Diabetes Federation 2009).

2.3.3 Normoglycaemia

A fasting venous plasma glucose concentration of <6.1 mmol/ l (110 mg/ dl) and a 2–h plasma glucose of <7.8 mmol/l (140 mg/dl) has been determined as "normal" (Alberti and Zimmet 1998; World Health Organization 2006). Values above this are related with a progressively higher risk of developing micro and macrovascular complications (Alberti 1996; Engelgau et al. 1997).

2.4 Nutrition and hyperglycemia

The findings of studies have shown that in addition to diabetes mellitus, even non-diabetic degrees of fasting and postprandial hyperglycemia are directly linked to the accelerated risks of cardiovascular diseases (Coutinho et al. 1999; DECODE Study Group 2001; Levitan et al. 2004; Meigs et al. 2002). Accordingly, as a part of prevention strategies for chronic diseases, studies over the last decade have investigated the impact of macro and micronutrients on hyperglycemia in either diabetic or pre-diabetic ranges (Anderson 1998; Baliga et al. 2000; Boden et al. 1996; de Lordes Lima et al. 1998; Greenbaum, Kahn, and Palmer 1996; Paolisso et al. 1993; Pittas et al. 2007). For example, Pittas and his colleagues have suggested that 3-year supplementation with calcium and vitamin D may modify rises in fasting blood glucose and insulin resistance in elderly subjects with IFG, though there was no effect on the markers of inflammation (Pittas et al. 2007).

The attention of researchers has recently been attracted by thiamin. The administration of thiamin has been implicated to have an array of benefits in the prevention of hyperglycemia complications (see Sections 2.5.9&2.5.10).

2.5 Thiamin

2.5.1 Chemistry

Thiamin (also known as vitamin B1 or aneurin) is a water soluble vitamin with a chemical formula $C_{12}H_{17}N_4OS$, containing a pyrimidine and a thiazole ring connected by a methylene bridge. Thiamin monophosphate (TMP), thiamin diphosphate (TDP) [called also thiamin pyrophosphate (TPP)] and thiamin triphosphate (TTP) are natural derivatives of thiamin in the blood and tissues. Synthetic forms of thiamin include the hydrochloride and mononitrate salts, and non-water-soluble derivatives (e.g. benfothiamine).

Thiamin is stable at acidic pH and during frozen storage, but becomes increasingly unstable at high pH and temperature, and when exposed to ultraviolet light (Brody 1999; Butterworth 2006; Institute of Medicine 1998; Tanphaichitr 1999).

2.5.2 Dietary sources

Thiamin is found in a wide range of foods such as yeast, grain products, meat products, vegetables, dairy products, legumes, fruits and egg. Of these, bread and cereal products are considered the major dietary sources of thiamin, due to their staple role in most people's diets. Thiamin is highly concentrated in the pericarp and germ of cereals (Butterworth 2006; Institute of Medicine 1998; Tanphaichitr 1999). Thus, the thiamin content of whole grains is significantly more than refined grains. In some countries, such as Canada, UK and Australia, wheat flour used for making bread is fortified with thiamin mononitrate to restore losses during processing. In Australia, mandatory fortification of bread-making flour with thiamin was started in January 1991 (Harper and Butterworth 1997; The National Health and Medical Research Council 1990). According to the Australia New Zealand Food Standards Code, bread-making flour must contain at least 6.4 mg/kg of thiamin (Food Standards Australia New Zealand 2009). The National Nutrition Survey 1993 showed that, as a result of thiamin enrichment program in Australia, only 3% of women and 7% of men had a low thiamin intake (Baghurst et al. 1996), while studies in the 1980s indicated that thiamin intakes of 10%-20% of Australians were below the recommended values (Wood and Breen 1980).

2.5.3 Recommended amounts & upper intake level

The storage of thiamin in the body is limited and it also has a high turnover. Therefore, a regular supply is necessary. Because thiamin is involved in energy utilisation pathways, requirements are related to energy intake. In most countries, a daily requirement of at least 0.4 mg/1000 kcal is recommended for healthy adults (Department of Health 1991). In Australia, the recommended dietary intakes (RDI) for healthy men and women with moderate activity are set on 1.2 and 1.1 mg/day, respectively (National Health and Medical Research Council 2006).

The oral toxicity of the water-soluble forms of thiamin found in foods or dietary supplements in humans is very rare and its symptoms are reported mainly at very high doses (≥ 7000 mg) (Meador et al. 1993). However, some significant adverse effects have been reported following its inappropriate parenteral or intramuscular administration (Morinville, Jeannet-Peter, and Hauser 1998; Stephen, Grant, and Yeh 1992; Wrenn, Murphy, and Slovis 1989). Existing data from clinical studies are insufficient to estimate a tolerable upper intake level (UL) for thiamin (National Health and Medical Research Council 2006).

2.5.4 Physiology

Following ingestion of food or supplements containing thiamin, uptake occurs mainly in the proximal part of small intestine (Rindi and Laforenza 2000). Intestinal uptake of thiamin is dose-dependent, through an active, carrier-mediated transport system at physiological concentrations (less than 1 μmol/L) and passive diffusion at high concentrations (Rindi and Ferrari 1977). In the intestinal lumen, free thiamin is normally taken up across enterocyte membranes using high affinity transporters, THR1and THR2 (Thornalley 2005). These thiamin transporters are encoded by two members of the SLC (solute carrier) 19A gene family, *SLC19A2* and *SLC19A3* (Dutta et al. 1999; Rajgopal et al. 2001). THR1 is the transporter on the basolateral surface of gastrointestinal epithelial cells, and in skeletal muscle, placenta, heart, liver, kidney and red blood cell plasma membrane (Dutta et al. 1999; Reidling et al. 2002; Thornalley 2005; Thornalley et al. 2007). The second thiamin transporter, THR2, is present on the luminal surface of enterocytes, and in placenta, kidney and

liver (Eudy et al. 2000; Said et al. 2004; Thornalley 2005). Another member of the SLC19 family (*SLC19A1*) is known to encode the reduced folate transporter RFC-1, transporting folic acid as well as TMP and TDP into cells (Matherly 2001; Zhao, Gao, and Goldman 2002). RFC-1 is expressed in various human tissues including: erythrocytes and renal brush border membranes, mediating re-absorption of filtered TMP and TDP (Thornalley 2005; Thornalley et al. 2007). As a result, these metabolites of thiamin are not present in the urine under normal circumstances.

Thiamin absorption is generally enhanced following thiamin deficiency (Laforenza et al. 1997), which is suggested to be because of an increased expression of tissue THR1 (Laforenza et al. 1997). Thyroid hormones and insulin can influence the intestinal tissue and regulate the uptake of thiamin (Patrini et al. 1996). Following uptake, thiamin is phosphorylated in the intestinal epithelial cells. However, it is released into the blood mainly as its free form. This process is dependent on Na^+ and a transcellular H^+ gradient (Combs 2008; Rindi and Laforenza 2000). Thiamin is distributed in the blood within both plasma (10%) and cells (90%). In plasma, less than 10% of thiamin is phosphorylated, and most of plasma thiamin is bound to proteins, mainly albumin (Royer-Morrot et al. 1992). About 80% of total whole blood thiamin is present in the erythrocytes, mainly in the form of TDP (Kimura and Itokawa 1985; Schrijver et al. 1982). In tissues, thiamin is mainly converted to its phosphorylated esters and the highest concentrations are present in the liver, heart, kidney, muscles and brain. The thiamin (in its free form) is excreted in the urine and thiamin intake in excess of requirements results in an increased urinary excretion (Butterworth 2006; Tanphaichitr 1999).

There are a few clinical studies which have investigated the kinetics of thiamin. For example, Davis and colleagues reported that after an oral test dose of 10 mg, the mean serum thiamin increased moderately (42%) and then returned to baseline within 6 hours (Davis et al. 1984). With higher pharmacological dosages, Royer-Morrot and colleagues showed that following 11-day administration of thiamin orally (250 mg every 12 hours) or intramuscularly (500 mg/once a day) in healthy young men, plasma concentrations of thiamin [3.2 ±1.7 μg/L (11±5.7 nmol/L) at baseline] reached a steady state after 5.6 [23 μg/L (76 nmol/L)] and 7 days [29 μg/L (96 nmol/L)], respectively. The mean elimination half-life value of 1.8 days was

calculated (Royer-Morrot et al. 1992). In another clinical study (Rabbani et al. 2009) conducted in type 2 diabetic patients, a median (range) plasma thiamin concentration of 98.2 (2.6-294.5) nmol/L was found after supplementation with 300 mg/day thiamin for 3 months [10.6 (0.8-84.5) nmol/L at baseline]. In these studies, the plasma thiamin concentrations were considerably low relative to the administrated doses, indicating the very low bioavailability of thiamin and its poor oral absorption, its wide distribution in the body and/or its high turnover (Rabbani et al. 2009; Royer-Morrot et al. 1992; Smithline, Donnino, and Greenblatt 2012; Tallaksen et al. 1993).

2.5.5 Interactions and anti-thiamin factors

It is known that the uptake and metabolism of thiamin can be impaired following alcohol consumption (Hoyumpa 1980). Ethanol was shown to reduce the active transport of thiamin into enterocytes via inhibition of Na/K-ATPase (Rindi and Ferrari 1977), and alters the phosphorylation of thiamin to TDP (Rindi, Imarisio, and Patrini 1986). These effects are the likely explanation for the higher prevalence of thiamin deficiency among alcoholics (Butterworth 2006; Wood and Breen 1980).

The availability of thiamin for uptake can be also reduced by anti-thiamin factors present naturally in the diet [Table 8] (Prinzo 1999). These include the thiamin-degrading enzyme (thiaminase) present in raw or fermented fish, shellfish, ferns and certain bacteria, and thiamin antagonists found in some foods (e.g. tea, coffee and betel nuts) (Butterworth 2006; Prinzo 1999). In vitro, ascorbic acid (vitamin C) has been shown to protect the modification of thiamin by tea and other tannin-containing products (Kositawattanakul et al. 1977).

The metabolism of thiamin can be also affected by magnesium status. Magnesium acts as a cofactor in the transketolase reaction (see Section 2.5.7) (Fattal-Valevski 2011; Lonsdale, Shamberger, and Obrenovich 2011) and the phosphorylation of thiamin to TDP (Voskoboyev and Ostrovsky 1982). It was shown that magnesium deficiency may occur in patients with known diabetes (type1&2) (Tosiello 1996). However, there has been no indication of hypomagnesemia among those with pre-diabetes (IGT&IFG) (Simmons, Joshi, and Shaw 2010).

Table 8. Types of anti-thiamin factors and their actions (Prinzo 1999)

Anti-thiamine factor	Mechanism	Source
Thiaminase [1]		
Type I	alters the structure of thiamine	raw or fermented fish, shellfish, ferns, some bacteria
Type II	reduces biological activity of thiamine	certain bacteria
Thiamine antagonists [2]		
polyphenols (e.g. caffeic acid, chlorogenic acid, tannic acid)	interferes with absorption or digestion of thiamine	tea, coffee, betel nuts, red cabbage, blueberries, red currants, red beets , also in cereals, pulses, oilseeds
flavonoids (e.g. quercetin, rutin)	"	widely distributed in edible fruits and vegetables, buckwheat plants
haemin	"	animal tissues

[1] heat labile enzyme
[2] heat stable non-enzymatic factors

2.5.6 Thiamin deficiency

As mentioned above, thiamin is taken up from gastrointestinal tract, transported through the portal blood to the liver, taken up into cells and excess is excreted mainly in the urine. So, thiamin deficiency may be caused by an inadequate dietary intake of the vitamin, reduced absorption, defective uptake into cells, increased requirements and enhanced losses (Butterworth 2006; Institute of Medicine 1998; Tanphaichitr 1999).

The biological half-life of thiamin is approximately between 9 to 18 days (Ariaey-Nejad et al. 1970) and given the limited storage of thiamin, a sub-clinical deficiency can develop rapidly (Brin 1963). The symptoms in the early stage of deficiency are non-specific, including anorexia, weakness, aching, burning sensation in hands and feet, indigestion, irritability and depression (Prinzo 1999). Clinical deficiency of thiamin can affect the cardiovascular and nervous systems, leading to the major diseases of Beriberi and Wernicke-Korsakoff syndrome (Prinzo 1999).
A genetic defect in the intracellular transport of thiamin can cause the autosomal recessive disorder of TRMA syndrome (thiamin-responsive megaloblastic anemia),

which is characterised by the triad of megaloblastic anemia with ringed sideroblasts, diabetes mellitus, and progressive sensorineural deafness (Neufeld et al. 2001).

Thiamin deficiency has been primarily reported in some parts of South-East Asia, where polished rice is the main dietary ingredient, and the diet generally contains anti-thiamin factors, such as tea leaves, herring, shellfish, raw carp, fermented fish and betel nuts (Kositawattanakul et al. 1977; Lee 1994; Vimokesant et al. 1982; Vimokesant et al. 1975). In Western countries, thiamin deficiency has been mostly demonstrated in association with the regular high alcohol intake (Institute of Medicine 1998). High alcohol consumption is recognized to alter the uptake and metabolism of thiamin (Hoyumpa 1980). Also, studies indicate that patients with human immunodeficiency virus-acquired immunodeficiency syndrome (HIV-AIDS) (Butterworth et al. 1991), gastrointestinal and liver diseases, persistent vomiting (Michel et al. 2010), and appetite disorders as well as those receiving parenteral nutrition (Rastenyte et al. 2003) or using certain drugs, such as diuretics, increasing excretion of thiamin in the urine (Kwee and Nakada 1983; Suter and Vetter 2000), have been recognised as being at high risk for the development of thiamin deficiency.

Some recent studies demonstrated that both type 1 and type 2 diabetic patients are also at risk of thiamin deficiency (Jermendy 2006; Saito et al. 1987; Thornalley et al. 2007; Valerio et al. 1999; Vindedzis et al. 2008). The findings of Saito et al (1987) indicate that only 23.9 % of 46 diabetic outpatients studied had a total blood thiamin concentration more than the normal lower limit. Low levels of plasma thiamin were found by Valerio et al. (1999) who compared thiamin status of 10 children with type 1 diabetes (35.3 ± 3.6 pmol/mL) with that of six age-matched normal subjects (53.2 ± 2.3 pmol/mL, $p < 0.05$). This was confirmed by Thornalley et al. (2007) who showed that thiamin concentration in blood plasma was decreased by 76 per cent in type 1 (26 subjects) and by 75 per cent in type 2 diabetic patients (48 subjects). However, studies investigating red blood cell thiamin levels in diabetic patients reveal opposing results. While, some studies reported low RBC thiamin levels in diabetic patients (Vindedzis et al. 2008), it was normal in another study (Thornalley et al. 2007). These controversial results could be due to differences in sampling and assay techniques, as well as problems in the studies' methodology. For instance, only

a few authors (Jermendy 2006; Thornalley et al. 2007) considered excess alcohol consumption as an exclusion criteria in their studies, or no data were provided on thiamin intake in the study conducted by Thornalley et al. (2007).

The mechanisms responsible for thiamin deficiency in diabetics are not well known. In the study conducted by Thornalley et al. (2007) low plasma thiamin was associated with an increased excretion of thiamin in the urine. In that study, subjects had diabetes of long duration (22.1 ± 13.3 and 13.0 ± 8.9 y for type 1 and type 2, respectively). The renal damage resulting from diabetes was clearly evident in approximately 1/3 of subjects who had urinary albumin excretion characteristic of microalbuminuria and incipient nephropathy. The same group showed that the increased fractional excretion of thiamin in diabetics was more significant in patients with microalbuminuria and early GFR decline than in individuals with stable renal function (Adaikalakoteswari et al. 2008). In addition to an increased renal excretion, a decreased intestinal thiamin absorption may also contribute to the low thiamin levels observed in diabetic patients (Patrini et al. 1996). With regard to the role of thiamin in glucose metabolism, diabetes-associated thiamin deficiency is expected to amplify the cellular damage induced by hyperglycemia. It is also suggested that the consequences of thiamin deficiency described in diabetics could be exaggerated in vascular endothelial cells, which are at risk of high intracellular glucose concentrations (Kaiser et al. 1993) and subsequent reactive oxygen species (ROS) overproduction. Such accumulated ROS could oxidise the limited thiamin available in the cells and produce components such as thiochrome and oxydihydrothiochrome, which in turn, have inhibitory effects on thiamin-dependent enzymes (Stepuro et al. 1997; Zimatkina et al. 1991).

2.5.7 Role of thiamin in cell function

Thiamin plays an essential regulatory role as a co-enzyme in metabolic pathways involving sugars, amino acids and fatty acids (Bender 1999; Butterworth 1993; Foulon et al. 1999; Gibson and Zhang 2002). It also modulates neural and neuro-muscular transmission, likely via activation of the chloride ion channel (Bender 1999). Following uptake from gastrointestinal tract into the cell, thiamin is rapidly phosphorylated to its active form, TDP. TDP is a cofactor for enzymes in glucose

and energy metabolism (Bender 1999; Butterworth 1993; Gibson and Zhang 2002), including transketolase, pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase (αKGDH) [Figure 6].

Transketolase (TK) is a key enzyme in the pentose phosphate pathway, generating pentose sugars which in turn are required for synthesis of nucleic acids and their precursors. In fact, this TDP-dependent enzyme catalyses the transfer of two-carbon (glycolaldehyde group) fragments from one sugar phosphate to another, leading to the lengthening of the carbon chain of the acceptor sugar phosphate at the expense of the donor (Bates 2009) [56]:

xylulose-5-phosphate(C5) + ribose -5-phosphate(C5)
⟷ glyceraldehyde-3-phosphate(C3) + sedoheptulose-7-phosphate (C7)

and

xylulose-5-phosphate (C5) + erythrose-4-phosphate (C4)
⟷ glyceraldehyde- 3-phosphate (C3) + fructose-6-phosphate (C6)

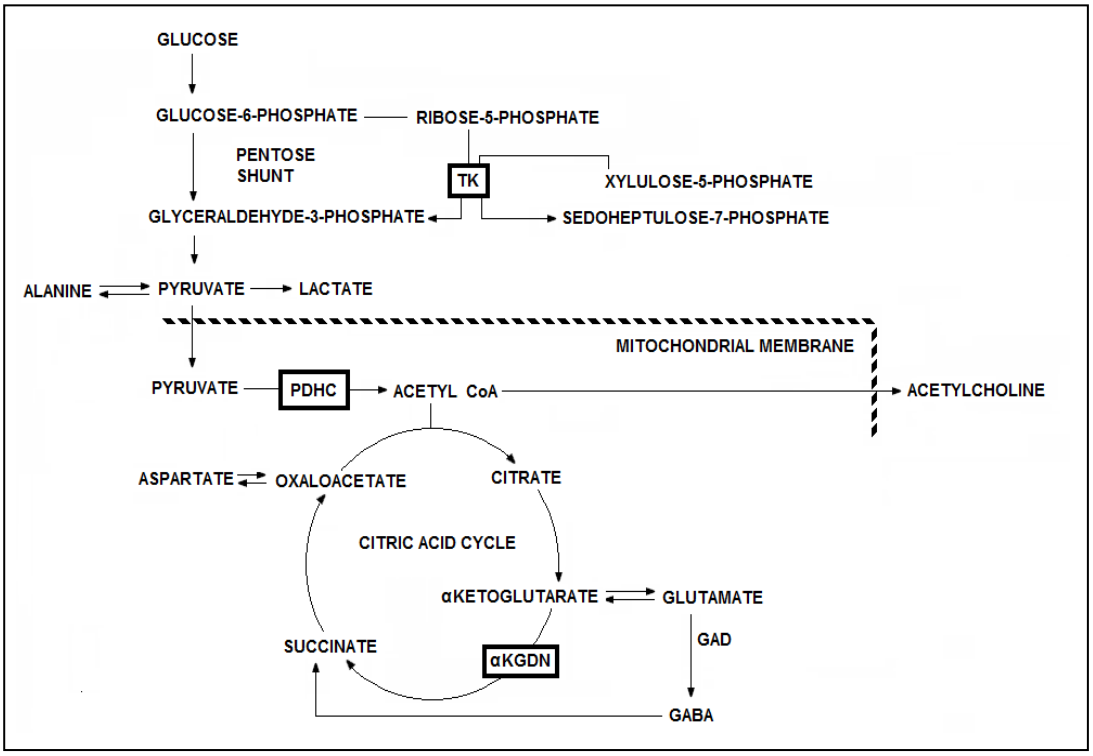

Figure 6. Thiamin diphosphate-dependent enzymes. *αKGDH, α-ketoglutarate dehydrogenase; PDHC, pyruvate dehydrogenase complex; TK, transketolase (Butterworth 2006)* .

Under hyperglycemic condition, activation of TK can decrease the accumulation of triosephosphates, such as glyceraldehyde-3-phosphate, and of fructose-6-phosphate, and subsequently increase intracellular concentration of ribose-5-phosphate. A deficit in thiamin is known to reduce transketolase activity. The reduced activity of transketolase in erythrocytes is commonly used as a marker for thiamin deficiency (Nixon et al. 1990; Warnock 1970).

Similarly, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (αKGDH) complex are respectively responsible for the oxidative decarboxylation of pyruvate, the end product of glycolysis, and oxidative decarboxylation of a-ketoglutarate into the citric acid cycle (Bates 2009). Given the fundamental role of these TDP-dependent enzymes in cellular glucose metabolism, thiamin deficiency can lead to the accumulation of alanine (Butterworth and Heroux 1989), lactate and pyruvate (Hakim 1984; Horwitt and Kreisler 1949) as well as reducing synthesis of high-energy phosphates and citric acid cycle intermediates (Aikawa et al. 1984).

Thiamin is also a co-enzyme for branched chain keto-acid dehydrogenase, an enzyme contributing to the oxidation of the branched-chain amino acids, valine, leucine, and isoleucine (Bender 1999), and 2, hydroxyphytanoyl-CoA lyase in fatty acid alpha-oxidation reactions (Foulon et al. 1999).

2.5.8 Assessment of thiamin status

Thiamin status is generally assessed indirectly by measuring the erythrocyte transketolase activity (ETKA) and the thiamin pyrophosphate effect (TPPE), or directly by determining thiamin and its phosphorylated esters concentrations in blood components or thiamin in the urine. Other less specific and practical methods include thiamin concentrations in the cerebrospinal fluid, and the carbohydrate metabolism index, which is based on measuring blood pyruvate and lactate levels following a glucose load (Bates 2009). A summary of methods which are generally used for assessing thiamin status are as follows:

- Erythrocyte transketolase activity: The measurement of erythrocyte transketolase activity (ETKA) is considered the best functional test for evaluating the thiamin

status in humans (McCormick and Greene 1994). However, it has some limitations because factors other than diet affect it, for example alteration in binding of apo-enzyme and co-enzyme resulting from the presence of transketolase iso-enzymes (Kaczmarek and Nixon 1983), and reduction in the synthesis of apoenzyme in patients with liver diseases (Fennelly et al. 1967) and diabetes mellitus (Friedrich 1988). ETKA may not also be an accurate assay in diabetic patients, because of being affected by changes in the levels of thiamin transporter protein (THTR-1, RFC-1) (Thornalley et al. 2007). It is proposed that low levels of plasma thiamin found in diabetes may cause increases in expression of genes encoding thiamin transporters and their protein levels in erythrocytes, which in turn can maintain ETKA in normal ranges (Thornalley et al. 2007). Thiamin pyrophosphate effect (TPPE) is an assay for transketolase, revealing the saturation status of transketolase with its coenzyme in erythrocytes (Takeuchi et al. 1990). This assay is carried out before and after addition of TPP to the erythrocytes and reflects the percentage increase in the basal ETKA after stimulation with added TPP and is stated as the activity coefficient (Prinzo 1999). Low TPPE and high ETKA are diagnostic criteria for thiamin deficiency [Table 9].

- Urinary thiamin excretion: Determination of urinary thiamin excretion rate has been widely used as an index of thiamin status in humans for many years (Mason and Williams 1942; Oldham, Sheft, and Porter 1950). The correlations between thiamin intake and urinary excretion are mainly significant when the intake is in adequate range, however this indicator is less sensitive under thiamin deficiency conditions (Bates 2009). Concentration of thiamin in urine increase sharply above the thiamin intake threshold of about 0.3-0.4 mg/1000 kcal in adults (Interdepartmental Committee on Nutrition for National Defense 1963). The measurement of total thiamin in urine or other body fluids and tissues has been traditionally quantitated by microbiological methods (Baker et al. 1964) or by chemical assay procedure, involving extraction, oxidation of thiamin to thiochrome and measurement of its fluorescence (Leveille 1972; Pelletier and Madere 1972). More recently, the development of high performance liquid chromatography (HPLC) with pre-column or post-column conversion to thiochrome has provided more sensitive, specific and accurate assays of thiamin

status, particularly in deficient individuals (Baines 1985; Talwar et al. 2000; Warnock 1982).

- Blood thiamin: Thiamin status can be also determined by the direct assay of thiamin in plasma or serum (Tallaksen et al. 1991; Weber and Kewitz 1985), washed red blood cells or whole blood (Baines 1985; Talwar et al. 2000). The measurement of thiamin in plasma/serum is less frequently used to assess tissue storage, because, in contrast to erythrocyte thiamin (Brin 1964), the extracellular concentration of thiamin is mainly a reflection of the recent intake. Also, approximately 10% of total blood thiamin is found in the plasma and 80% is in erythrocytes (Talwar et al. 2000). As a result, plasma thiamin concentration is difficult to assay precisely without being contaminated by the leakage of thiamin from the blood cells during the preparation of samples (Bates 2009). Accordingly, some investigators have identified the concentration of TDP in erythrocytes determined by the HPLC assay as the most sensitive indicator of thiamin status in individuals who are at risk of thiamin deficiency (Baines and Davies 1988; Brin 1964; Howard 2000; Talwar et al. 2000). However, Thornalley et al. (2007) suggested using plasma thiamin for evaluating thiamin status in diabetic patients, because the measurement of erythrocyte thiamin as well as the erythrocyte transketolase activity may be affected by the increased thiamin transporter protein levels.

Table 9. Reference values for the primary measures of thiamin status in adults (Institute of Medicine 1998)

Indicator	Marginal deficiency	Severe deficiency
ETKA (Schrijver 1991)	1.20-1.25	>1.25
TPPE % (Brin 1970)	15-24	≥25
Urinary thiamin (Schrijver 1991)		
(nmol[μg]/g creatinine)	90-220 [27-66]	<27
(nmol[μg]/d)	133-333[40-100]	<40
Erythrocyte thiamin (Schrijver 1991)		
(nmol/L)	70-90	<70

2.5.8.1 Analytical methods for thiamin measurement

Several methods have been described for the determination of thiamin in biological fluids and tissues. These techniques can be classified into two categories:

1. Microbiological determinations: Some species of Lactobacilli, such as L. viridescents (Hankin and Squires 1960) and L. fermenti (Icke and Nicol 1994), or Ochromonas danica (Baker et al. 1964) have been used to measure total thiamin in biological materials. These methods are feasible and sensitive to detect 5-50 ng amounts of thiamin (Hurst 2008). Icke & Nicol described a microbiological assay of thiamin with an inter-batch precision (CV%) ranging from 7.4% to 10% and recovery of thiamin pyrophosphate between 101 - 110 % (Icke and Nicol 1994).

Microbiological methods are not now generally used due to being time consuming compared to high performance liquid chromatography (HPLC) methods, and methodological complexities.

2. Physicochemical methods: These methods are used to measure thiamin and its phosphate esters in the biological samples using fluorimetry or HPLC.

In fluorimetric assays, following enzymatic hydrolysis of the thiamin phosphate esters, a clean-up procedure using ion-exchange chromatography is applied. Subsequently, thiamin is oxidised by potassium ferricyanide or other oxidant reagents (such as $KMnO_4$ or MnO_2) to form thiochrome required for fluorimetric detection. Thiochrome derivatives are then extracted into an organic solvent such as isobutanol prior to final analysis by a fluorophotometer (Myint and Houser 1965). Nowadays, the traditional fluorimetric assays are generally replaced by new HPLC techniques.

HPLC methods are now the most common techniques used for the measurement of thiamin or its phosphate esters in blood and urine samples. In some methods, thiamin is determined directly by HPLC with UV detector (Hilker and Clifford 1982). However, methods using HPLC with fluorescence detection (after conversion of thiamin to fluorescence thiochrome) are much more sensitive than UV detection

(Botticher and Botticher 1986). The thiochrome procedure can be carried out either before the chromatographic separation or after the column. Both pre- and post-column derivatisation techniques are robust, sensitive and precise (Baines 1985; Botticher and Botticher 1986; Schrijver et al. 1982; Talwar et al. 2000; Warnock 1982), however post column methods are less time consuming (analysis time <15 min) compared to pre-column techniques (Talwar et al. 2000). In contrast, HPLC with pre-column derivatisation provide results with sharper peaks and better resolution (Zempleni et al. 2007).

The best results can be achieved by reversed phase HPLC (Ake, Soko, and Malan 2006; Korner et al. 2009; Talwar et al. 2000). A reversed - phase HPLC method with post column derivatisation and fluoresence detection was used for determination of TDP in whole blood and washed red blood cells by Talwar et al. (2000). The inter-run coefficent variation (CV) was reported as < 8%, with recovery of TDP from blood samples > 90% and detection limit of 130 ng on column.

2.5.9 The potential effects of thiamin in preventing hyperglycemic complications: mechanisms of action

It is well known that hyperglycemia induces a variety of biochemical abnormalities at the cellular level (particularly in endothelial cells), resulting in a range of vascular and tissue damage. Existing evidence reveals that thiamin may modify specific mechanisms involved in hyperglycemic complications (Berrone et al. 2006). These mechanisms include: increased flux through the polyol pathway, the hexosamine pathway activity, protein kinase C (PKC) activation, advanced glycation end products (AGEs) formation, increased oxidative stress formation, and inflammation, which occur mainly because of the diversion of excess glycolytic intermediates to alternative pathways (Aronson 2008; Aronson and Rayfield 2002; Xu, He, and King 2005).

The polyol pathway

The shunting of excess intracellular glucose into the polyol pathway is one of the mechanisms suggested to contribute to the pathogenesis of hyperglycemia complications (Dagher et al. 2004; Kinoshita 1986) .

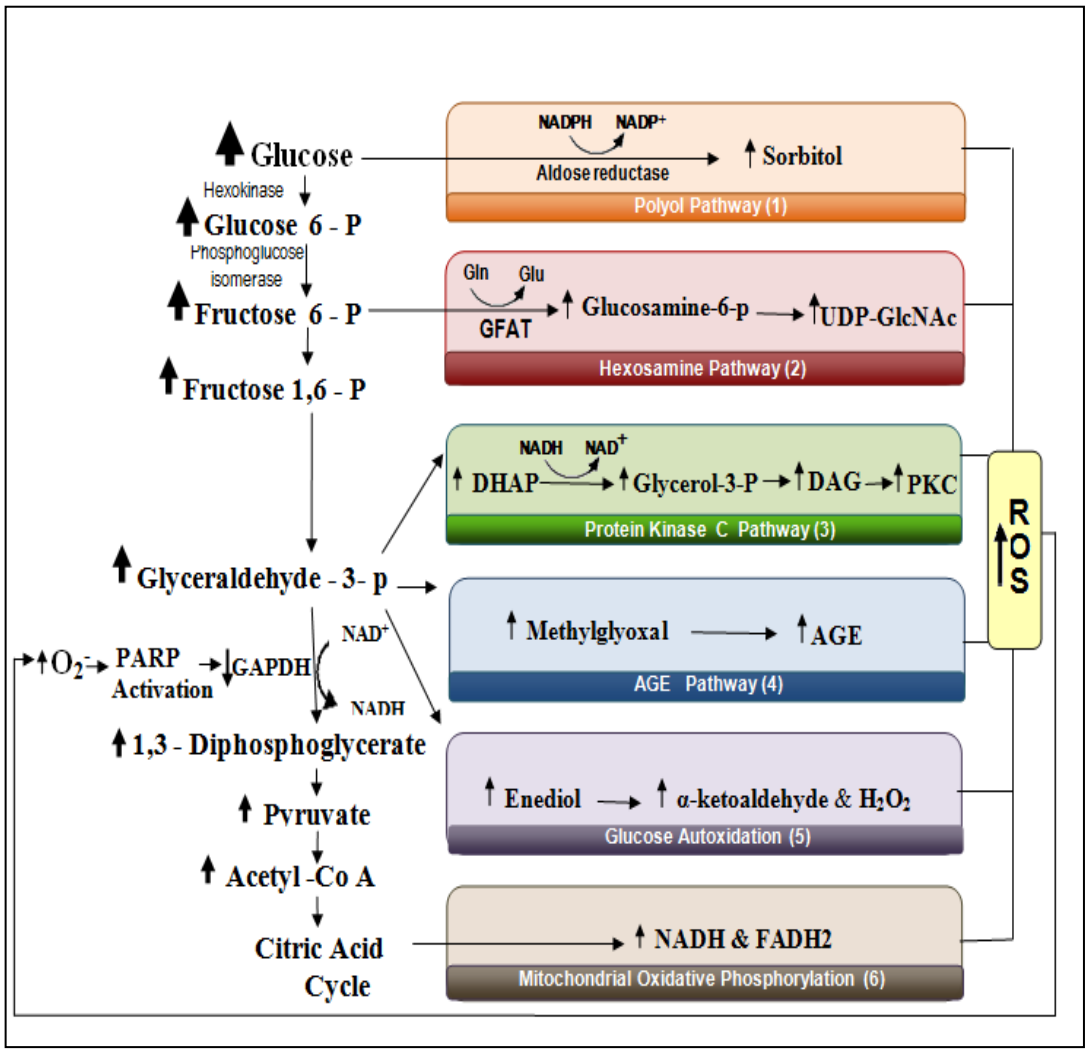

Figure 7. Pathological link between oxidative stress formation and other mechanisms involved in hyperglycemia-dependent cell damages. *GFAT; Glutamine:fructose-6-phosphate amidotransferase, DHAP; Dihydroxyacetone phosphate, DAG; Diacylglycerol , PKC;.Protein kinase C, PARP; Poly (ADP-ribose) polymerase GAPDH; Glyceraldehyde 3-phosphate dehydrogenase, AGE; Glycation end products. Reproduced from Brownlee (2001) with modification.*

In fact, glucose flux through the polyol pathway increases considerably when intracellular glucose concentrations are high and hexokinase, the enzyme responsible for converting glucose into glucose 6-phosphate, becomes saturated [Figure 7, pathway 1]. The excess glucose load is then available to aldose reductase, the rate-limiting enzyme in the polyol pathway, to be catalysed to sorbitol (Beyer and Hutson 1986; Cárdenas, Cornish-Bowden, and Ureta 1998).

With respect to the slow metabolism of sorbitol and also difficulty in its diffusion through cell membranes, accumulation of sorbitol and subsequent imbalance in intracellular homeostasis, have been proposed as the likely explanation for hyperglycemia-dependent damages in many cell types (e.g. vascular pericytes and endothelial cells, corneal epithelial cells, ganglion and Muller glial cells and the epithelial cell lining renal tubules) (Chung and Chung 2003; Crabbe and Goode 1998; Dvornik 1987, 1987; Kinoshita and Nishimura 1988; Kinoshita 1986; Williamson et al. 1993).

Thiamin was demonstrated to be effective in preventing damages induced by hyperglycemia via normalisation of the polyol pathway. As mentioned before, the increased glucose flux through the polyol pathway is known to be due to the high intracellular concentrations of glucose and the subsequent hexokinase saturation (Beyer and Hutson 1986; Dagher et al. 2004). High dose thiamin is thought to be able to redirect the excess levels of metabolic intermediates, glyceraldehyde-3-phosphate (G3P) and fructose-6-phosphate, from the glycolytic pathway toward the reductive pentose shunt, as an alternative pathway. Consequently, alleviate pressure on the polyol pathway [Figure 8] (Hammes et al. 2003). Addition of thiamin (50-500 μM) to human red blood cells incubated in high glucose condition (50 mM glucose) was shown to increase the transketolase activity, increase the concentration of total sedoheptulose-7-phosphate and ribose-5-phosphate, and decrease the accumulation of triosephosphates pool glycolytic intermediates (Thornalley, Jahan, and Ng 2001). The positive effect of thiamin on transketolase activity and a consequent increase in the conversion of triosephosphate to ribose-5-phosphate were confirmed by an animal study conducted on STZ diabetic rats treated with high dose thiamin (7-70 mg/kg) for 24 weeks (Babaei-Jadidi et al. 2003).

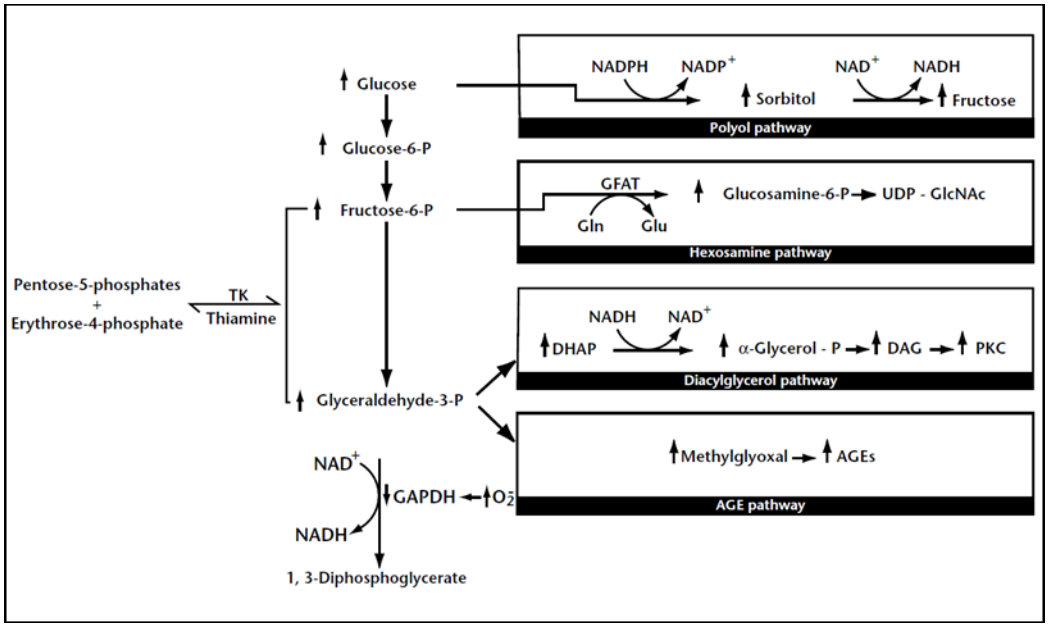

Figure 8. Potential mechanism by which benfotiamine blocks 4 pathways of hyperglycemic damage. *Hyperglycemia-induced mitochondrial superoxide overproduction partially inhibits the glycolytic enzyme GAPDH, thereby diverting upstream metabolites from glycolysis into glucose-driven signalling pathways of glucose overuse. Thiamin activates the thiamine-dependent pentose phosphate pathway enzyme transketolase (TK), which converts excess fructose-6-phosphate and glyceraldehyde-3-phosphate to pentose-5-phosphates and erythrose-4-phosphate. P, phosphate; GFAT, glutamine; fructose-6-phosphate amidotransferase; DHAP, dihydroxyacetone phosphate (Hammes et al. 2003).*

Thiamin was also shown to up regulate mRNA level for the thiamin-dependent enzyme pyruvate dehydrogenase in vitro, which results in accelerated glycolysis (La Selva et al. 1996; Pekovich, Martin, and Singleton 1998). Recently, it was found that addition of thiamin to vascular cells cultured in high glucose reduces aldose reductase mRNA and activity as well as the intracellular concentrations of glucose and sorbitol (Berrone et al. 2006).

The hexosamine pathway

Glucose metabolism via the hexosamine pathway is another mechanism implicated in hyperglycemia complications. In the hexosamine pathway, the glycolytic product fructose 6-phosphate, instead of conversion to fructose 1,6 biphosphate, the next intermediate of glycolysis pathway, is converted into glucosamine-6-phosphate by

the enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) [Figure 7, pathway 2]. Glucosamine-6-phosphate is then converted to UDP-N-acetylglucosamine (UDP-GlcNAc) which is a substrate for O-linked glycosylation (Brownlee 2001; Kolm-Litty et al. 1998) [Figure 9].

In O-linked glycosylation a hexosamine, usually N-acetylglucosamine (GlcNAc), is added to serine or threonine residues, which otherwise, under normal circumstance, are phosphorylated by serine/threonine kinases. Serine/threonine prosphorylation is known to play a critical role in the regulation of a number of cellular processes, including various enzymes. As a result, involving serine or threonine in O-linked glycosylation and producing O-linked GlcNAc may modify normal activity of many nuclear and cytoplasmic proteins including some transcription factors (Brownlee 2001; Wells, Vosseller, and Hart 2001).

According to cellular studies investigating the pathogenesis of hyperglycemia complications, the decrease of endothelial nitric-oxide synthase (eNOS) activity (Du et al. 2001), and an increase in the gene expression levels of TGF-β (Kolm-Litty et al. 1998) and PAI-1 (Du et al. 2000), are examples of perturbations mediated by the hexosamine pathway products. The hexosamine pathway was also demonstrated to be involved in glucose-mediated de novo lipogenesis in the liver and adipocytes by increasing mRNA levels of lipogenic enzymes acetyl-CoA carboxylase (ACC), glycerophosphate dehydrogenase (GPDH) and fatty acid synthase (FAS) in vitro (adipose cell culture) (Rumberger et al. 2003). The effect of increased hexosamine flux in the liver on the hepatic glucose and lipid metabolism was confirmed in animals. In the study conducted by Veerababu et al. (2000), overexpression of glutamine:fructose-6-phosphate amidotransferase in transgenic mice resulted in significant increase in hexosamine end product UDP-N-acetyl glucosamine concentration, elevated levels of free fatty acids and triglyceride, and higher content of hepatic glycogen relative to their non-transgenic littermates. In the long term (after 9 months) transgenic mice became also significantly heavier than the age-matched controls. This was accompanied with a significant increase in the area under the curve with performed intraperitoneal glucose tolerance test.

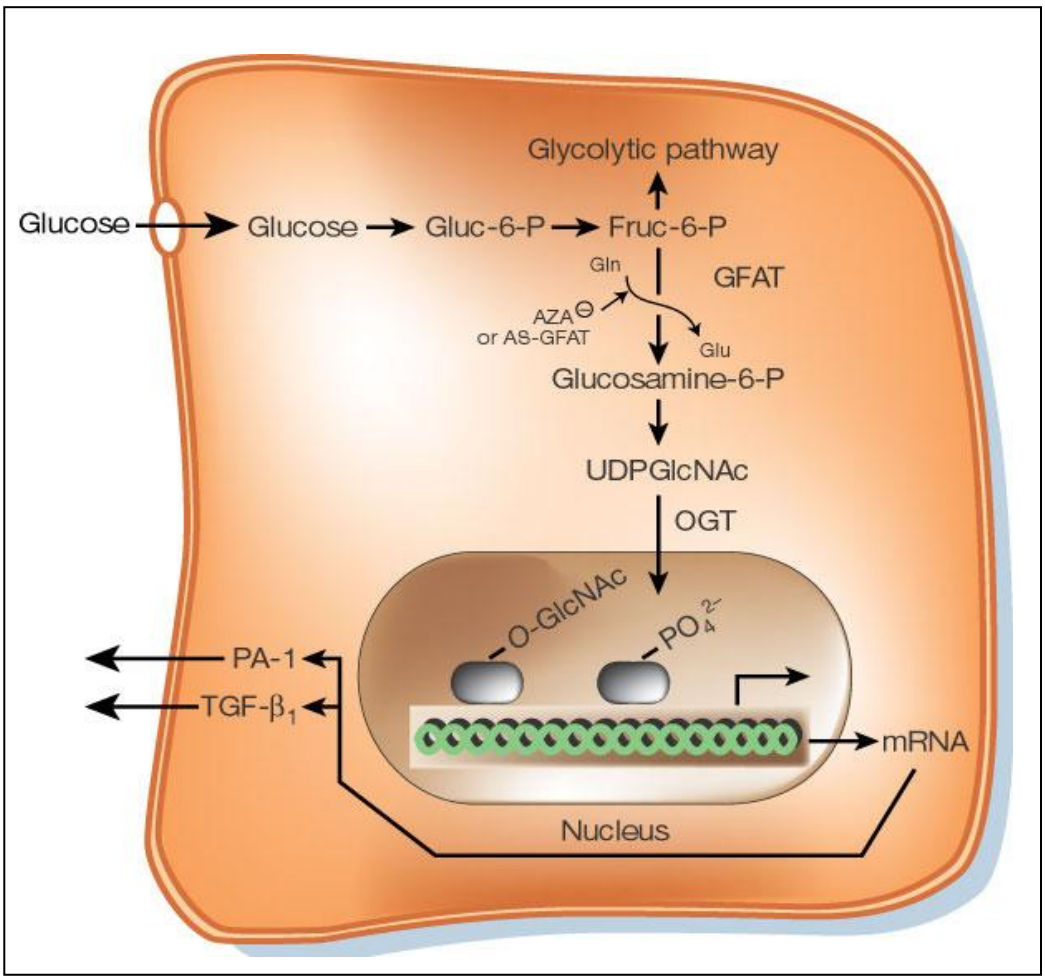

Figure 9. The hexosamine pathway. *The glycolytic intermediate fructose-6-phosphate (Fruc-6-P) is converted to glucosamine-6-phosphate by the enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT). Intracellular glycosylation by the addition of N-acetylglucosamine (GlcNAc) to serine and threonine is catalysed by the enzyme O-GlcNAc transferase (OGT). Increased donation of GlcNAc moieties to serine and threonine residues of transcription factors such as Sp1, often at phosphorylation sites, increases the production of factors such as PAI-1 and TGF-b1. AZA, azaserine; AS-GFAT, antisense to GFAT (Brownlee 2001).*

High dose thiamin has been suggested to divert the excess glycolytic intermediate of fructose 6-phosphate away from the hexosamine pathway to the pentose phosphate pathway (Thornalley 2005) [Figure 8]. This group (Babaei-Jadidi et al. 2004) proved that in STZ diabetic rats, increased TK activity resulting from high dose thiamin therapy (70 mg/kg, for 24 weeks) was associated with a decrease in hepatic UDP-N-acetylglucosamine, an intermediate of the hexosamine pathway, and fatty acid

synthase activity. In that study, thiamin therapy with a lower dose of 7mg/kg was ineffective.

Protein kinase C (PKC) activation

One of the mechanisms suggested for the damage induced by hyperglycemia is activation of protein kinase C (PKC) through diacylglycerol signalling pathway (Feener and King 1997; Ishii et al. 1996; Koya and King 1998). PKC is a family of serine/threonine kinases, consisting of at least ten isoforms.

This group of protein kinases can be activated by several signals such as increases in the formation of diacylglycerol from glycerol-3-phosphate, which is derived initially from glycolytic intermediates dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (Koya and King 1998) [Figure 7, pathway 3]. Activation of PKC enzymes can result in a wide range of effects on gene expression, and several signal transduction cascades through the phosphorylation of other proteins (Koya et al. 2000).

It is known that the effects of PCK activation are cell-type specific. For example, in vascular smooth muscle cells, activation of PKC modulates growth rate, DNA synthesis and growth factor receptor turnover (Koya and King 1998). In adipocytes and hepatic cells, PKC activation has been suggested to be involved in glycogenolysis and gluconeogenesis pathways (Fitzpatrick, Purves, and Augustine 2004). PKC activation has been also shown to increase the expression of transforming growth factor-β (TGF-β) gene, resulting in thickening of the capillary basement membrane (Koya et al. 2000) [Figure 10].

In the same way, PKC activation contributes to glucose-induced endothelial dysfunction by increasing oxidative stress and decreasing NO bioavailability (Beckman, Creager, and Libby 2002; Brownlee 2001). Activation of the protein kinase C was indicated to have an important role in vascular complications of hyperglycemia in retinal (Shiba et al. 1993), renal (Craven, Davidson, and DeRubertis 1990) and cardiovascular tissues (Inoguchi et al. 1992).

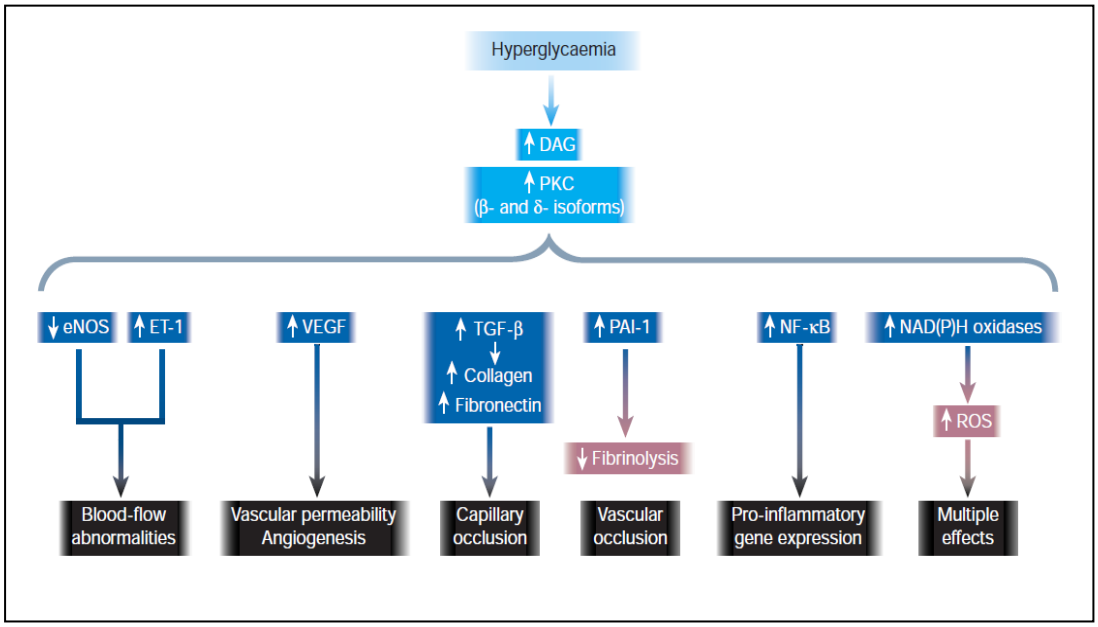

Figure 10. Consequences of hyperglycaemia-induced activation of protein kinase C (PKC). *Hyperglycaemia increases diacylglycerol (DAG) content, which activates PKC, primarily the b- and d-isoforms. Activation of PKC has a number of pathogenic consequences by affecting expression of endothelial nitric oxide synthetase (eNOS), endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), transforming growth factor-b (TGF-b) and plasminogen activator inhibitor-1 (PAI-1), and by activating NF-kB and NAD(P)H oxidases.(Brownlee 2001).*

This is consistent by experimental studies in rats showing that administration of a PKC beta inhibitor is able to ameliorate and reverse diabetic vascular dysfunction in the renal glomeruli and retina (Ishii et al. 1996; Koya et al. 2000).

Potentially, thiamin can inhibit the PKC activation by shifting the excess glyceraldehyde-3-phosphate toward the alternative pentose shunt, and facilitating the utilisation of glycolysis products in the Krebs cycle. In support of this effect, Babaei-Jadidi et al. (2003) verified that high dose thiamin therapy can reverse increased glomerular PKC activity in STZ diabetic rats.

Advanced glycation end products

The non-enzymatic modification of proteins by glucose-derived advanced glycation end products (AGEs) has been shown to play a major role in the pathogenesis of vascular damages induced by hyperglycemia (Horie et al. 1997; Stitt et al. 1997).

The process of AGEs formation is referred as glycation to be distinguished from enzymatic glycosylation pathways (Aronson 2008) although not all authors follow this practice. AGEs arise from several parallel glycation pathways initiated by the metabolites of glucose in early glycation processes. In these pathways, AGEs are mainly produced through the biochemical reactions involving: the autoxidation of glucose to glyoxal, the formation of Amadori products, and methylglyoxal arising from non-enzymatic fragmentation of glyceraldehyde phosphate and dihydroxyacetone phosphate, two intermediates of glycolysis (Degenhardt, Thorpe, and Baynes 1998; Thornalley 1990; Wells-Knecht et al. 1995) [Figure 11].

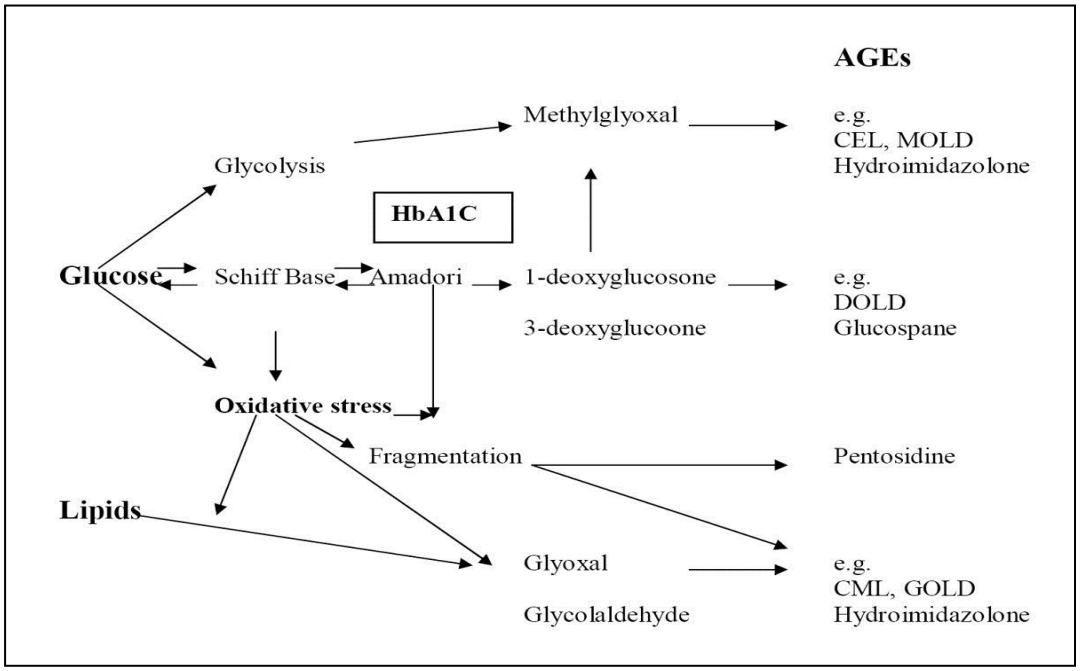

Figure 11. Simplified scheme of the complex Maillard reaction and formation of some advanced glycation end products (AGEs) in vivo. *CEL = carboxyethyllysine; MOLD = methylglyoxal lysine dimer; DOLD, 3-deoxyglucosone lysine dimer; CML, carboxymethyllysine; GOLD, glyoxal lysine dimer (Meerwaldt et al. 2008).*

Reducing sugars such as glucose and glyceraldehyde can react non-enzymically with the amino groups of proteins, which is known as the Maillard or non-enzymatic browning reaction (Maillard 1912). The early product of this process (Schiff bases) is highly unstable, and a lowered concentration of glucose can reverse this reaction within minutes [Figure 12]. Over the course of days, this process continues with the rearrangement of unstable Schiff bases to form more stable glycation intermediates, termed Amadori products. The same as the Schiff base process, Amadori products formation is reversible and dependent on the glucose concentration, but at a slower rate, reaching its equilibrium levels in weeks. As a result, measurement of Amadori products (e.g. hemoglobin A_1C) can reflect the degree of glucose control over several weeks. Subsequently, over a period of weeks to months, some of Amadori-type early glycation products undergo further complex rearrangement reactions and form stable and irreversible cross-linked compounds, called advanced glycation end products (AGEs) (Brownlee, Cerami, and Vlassara 1988). In the setting of hyperglycemia, accumulation of AGEs in vessel walls is known to be implicated in the atherosclerotic process through various mechanisms, which can be mediated by a receptor or progress without receptor mediation.

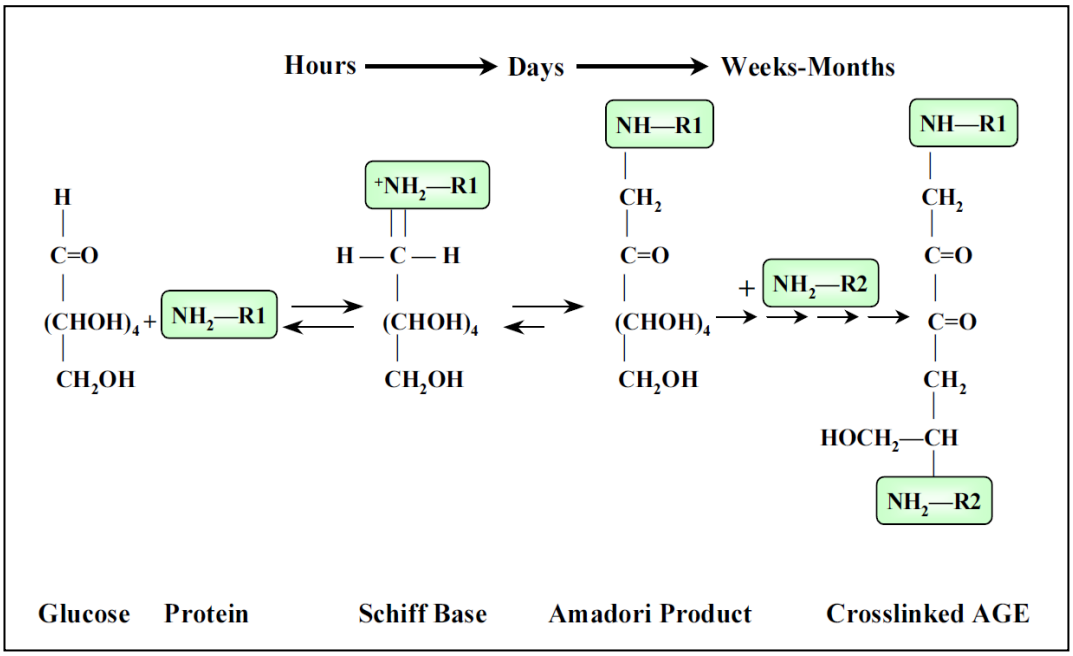

Figure 12. The formation of advanced glycosylation end products (Aronson and Rayfield 2002)

Activation of the pentose phosphate pathway by high dose thiamin and subsequent elimination of excess G3P from the cytoplasm was also shown to decrease AGE formation in bovine retinal and umbilical vein endothelial cells cultured in high glucose concentrations (La Selva et al. 1996). This effect was confirmed by Thornalley et al. (2001) who showed that the addition of thiamin to human red blood cells incubated in high glucose is able to decrease methylglyoxal, a key intermediate in the glycation process arising from non-enzymatic fragmentation of glyceraldehyde phosphate (Degenhardt, Thorpe, and Baynes 1998) (see Figure 8). Moreover, high dose thiamin therapy was indicated to decrease methylglyoxal, glyoxal and 3-deoxyglucosone concentrations in STZ diabetic rats (Babaei-Jadidi et al. 2003). The inhibitory effects of thiamin on AGE formation were described in both initial (Beltramo et al. 2002) and late (post-Amadori) stages (Booth, Khalifah, and Hudson 1996) of glycation.

Oxidative stress

There is abundant evidence supporting the pivotal role of oxidative stress in the pathogenesis of hyperglycemic complications (Baynes and Thorpe 1999; Baynes 1991; Nishikawa et al. 2000). Hyperglycemia has been shown to be involved in ROS formation through several mechanisms (Figure 7, pathways 1-6). The formed ROS include superoxide, hydrogen peroxide, nitric oxide, and hydroxyl radicals (Aronson and Rayfield 2002; Robertson et al. 2003; Robertson 2004).

It is known that oxidation of excess glucose can increase production of electron donors, such as NADH and $FADH_2$, resulting in the overproduction of the superoxide anion by the mitochondrial electron transport chain, particularly in endothelial cells. Increased mitochondrial superoxide production has been postulated to be the main source of oxidative stress induced by hyperglycemia (Nishikawa et al. 2000).

The transition metal- catalysed autoxidation of free glucose is shown to be an additional mechanism involved in oxidative stress. Through this pathway and in the presence of heavy metals, α-hydroxyaldehyde products of glucose (such as glyceraldehyde) can enolize and, via enediol radical anion production, generate

hydrogen peroxide (H202) and reactive intermediates, such as the highly toxic hydroxyl radical as well as α-ketoaldehydes (Wolff and Dean 1987; Wolff 1993).

Another mechanism involves the transition metal- catalysed autoxidation of protein-bound Amadori products, generating superoxide and hydroxyl radicals as well as highly reactive dicarbonyl components, such as 3-deoxyglucosone and methylglyoxal (Baynes and Thorpe 1999).

Finally, hyperglycemia is associated with increased oxidative products mediated by AGEs. As mentioned above, binding of AGEs to their specific receptors (RAGE) can result in the up regulation of oxidative stress response genes and increase oxidative stress (Yan et al. 1994).

There is convincing evidence that the tissue damage mediated by oxidative products may be exaggerated in diabetic patients because of compromised natural antioxidant defences associated with hyperglycemia (Aronson and Rayfield 2002). Free radicals are normally neutralized by antioxidants such as reduced glutathione, vitamin C and vitamin E. However, the levels of these antioxidants in diabetics have been reported to be significantly lower than normal individuals (Chen et al. 1983; Dominguez et al. 1998; Karpen et al. 1985; Sano et al. 1998; Yoshida et al. 1995; Yue et al. 1989).
It is shown that there is a close pathological relationship between oxidative stress generation and other pathways implicated in hyperglycemia complications, including the hexosamine and the polyol pathways, PKC activation and AGEs formation (Brownlee 2001; Garcia Soriano et al. 2001) [Figure 13]. In fact, the aberrations induced by hyperglycemia have been suggested to result from a single "unifying mechanism", which involves accelerated generation of reactive oxygen species (ROS) (Brownlee 2001). It is suggested that increased ROS formation induces DNA single-strand breakage resulting in the activation of nuclear enzyme poly (ADP-ribose) polymerase (PARP). Activation of PARP in turn, contributes to ribosylation and inactivation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and subsequent accumulation of glycolytic metabolites, particularly glyceraldehyde 3-phosphate (G3P) upstream.

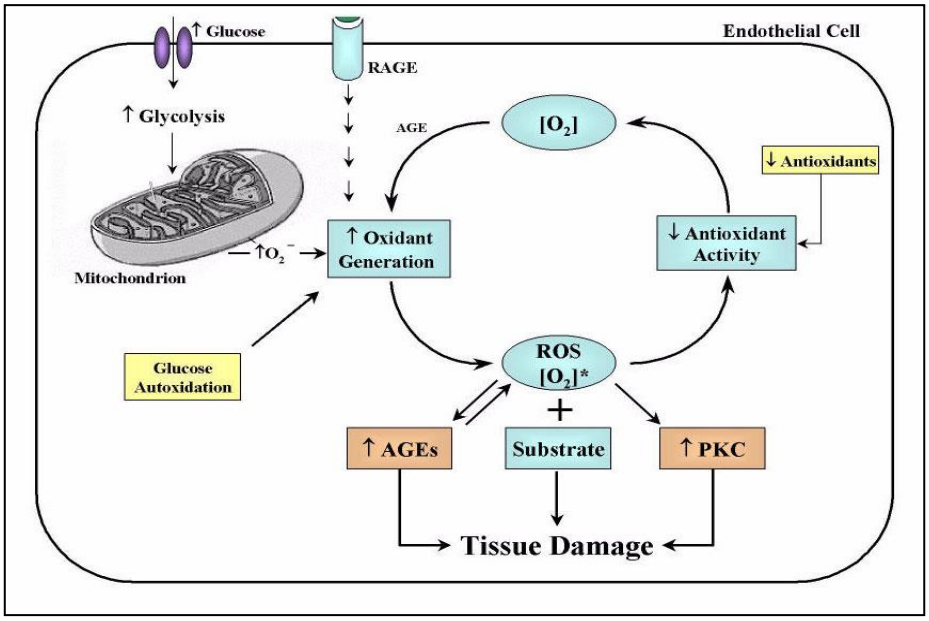

Figure 13. Relationship between rates of oxidant generation, antioxidant activity, oxidative stress, and oxidative damage in diabetes. *[O2]* represents various forms of reactive oxygen species [ROS]. The overall rate of formation of oxidative products leading to oxidative tissue damage is dependent on ambient levels of both [O2]* and substrate. Increased generation of [O2]* depends on several sources including glucose autoxidation, increased mitochondrial superoxide production, and as a result of the receptor for advanced glycosylation end products activation. [O2]* deactivation is reduced because antioxidant defences are compromised in diabetes. Note that oxidative stress also promotes other hyperglycemia-induced mechanisms of tissue damage. Oxidative stress activates protein kinase C (PKC) and accelerates the formation of advanced glycosylation endproducts (AGEs) (Aronson and Rayfield 2002).*

Finally, this process can increase mitochondrial superoxide production and also diversion of excess glycolytic intermediates to alternative pathways (Du et al. 2003; Garcia Soriano et al. 2001; Reusch 2003).

Reciprocally, as discussed before, activation of PCK (Brownlee 2001), AGEs formation (Yan et al. 1994), increased levels of sorbitol through the polyol pathway (Robertson 2004) and the hexosamine pathway activity (Kaneto et al. 2001; Robertson 2004) can promote oxidative stress [Figure 7]. Accordingly, if thiamin could have an inhibitory effect on each of hyperglycemia-dependent alternations, supplementation with thiamin would reduce oxidative stress markers.

Apart from re-directing the accumulated glycolytic intermediates toward the pentose phosphate pathway, the antioxidant properties of thiamin can be also considered as another likely explanation for the positive effects of thiamin on oxidative stress generation (Lukienko et al. 2000). Additionally, thiamin was suggested to act as an antioxidant indirectly by involving in the production of NADPH deriving from the pentose phosphate shunt. NADPH is a necessary cofactor for converting oxidised glutathione to its reduced form, required for neutralising oxidative stress. Over production of free radicals increases the demands for reduced glutathione and subsequently NADPH. This in turn, results in enhancing flux through the pentose phosphate shunt, requiring more thiamin (Bakker, Heine, and Gans 1997). The decreased levels of reduced glutathione were reported in the erythrocytes and tissues (heart and brain) of thiamin- deficient animals (Hsu and Chow 1960; McCandless, Schenker, and Cook 1968).

Importantly, given the reduced thiamin levels reported in diabetic patients (Thornalley et al. 2007) and also its critical role as an antioxidant, thiamin supplementation may improve the compromised natural antioxidant defences in individuals with hyperglycemia.

Inflammation

It is now established that low grade chronic inflammation plays a central role in mediating all stages of atherosclerosis (Libby 2002) (see Section 2.2.1). Prospective epidemiological studies have shown that the increased levels of cytokines such as IL-6 and TNF-α (Harris et al. 1999; Ridker, Hennekens, et al. 2000; Ridker, Rifai, Stampfer, et al. 2000; Ridker, Rifai, Pfeffer, et al. 2000), cell adhesion molecules such as soluble ICAM-1, P selectin, and E selectin (Hwang et al. 1997; Ridker, Hennekens, et al. 1998; Ridker, Buring, and Rifai 2001), and circulatory acute-phase reactants such as CRP, fibrinogen, and serum amyloid A (Danesh et al. 2000; Haverkate et al. 1997; Koenig et al. 1999; Kuller et al. 1996; Ridker, Hennekens, et al. 2000; Ridker et al. 1997; Ridker, Buring, et al. 1998; Tracy et al. 1997) may mark future risk for cardiovascular events. Moreover, elevation in inflammatory markers such as C-reactive protein has been proven to be associated with the adverse short-

term prognosis of acute coronary syndromes (Berk, Weintraub, and Alexander 1990; Biasucci et al. 1999; Liuzzo et al. 1994; Rebuzzi et al. 1998; Toss et al. 1997).

As previously mentioned, hyperglycemia has been shown to promote inflammation via the AGEs formation. Through this mechanism, binding of AGE-modified proteins with their specific receptors on the surface of several cell types can augment the production of pro-inflammatory cytokines and other inflammatory mediators involved in vascular damage (Kirstein et al. 1990; Kirstein et al. 1992; Ross 1999; Schmidt et al. 1995; Schmidt et al. 1993; Vlassara et al. 1988; Vlassara et al. 1992; Wautier et al. 1994; Yan et al. 1994). In addition to the AGE formation, other mechanisms implicated in hyperglycemia can contribute to the inflammatory response mediated by reactive oxygen species (Brownlee 2001; Garcia Soriano et al. 2001; Yan et al. 1994). Moreover, hyperglycemia has been implicated in promoting intracellular oxidative stress in adipocytes, resulting in the induction and secretion of acute phase reactants (Lin et al. 2005). The elevated levels of inflammatory markers such as CRP and interleukin-6 have been identified in both type 1 and type 2 diabetic patients (Aronson et al. 2004; de Rekeneire et al. 2006; Devaraj et al. 2006) as well as those with pre-diabetic ranges of hyperglycemia (Lin et al. 2009; Tan et al. 2003).

With regard to the potential role of thiamin in suppressing the key pathologic pathways contributing to inflammatory process discussed above (Beltramo et al. 2002; Booth, Khalifah, and Hudson 1996; Degenhardt, Thorpe, and Baynes 1998; La Selva et al. 1996; Thornalley, Jahan, and Ng 2001), supplementation with thiamin may have beneficial effects on the level of inflammatory markers, and improve outcomes in individual who are at the risk of hyperglycemia vascular complications.

2.5.10 The potential effects of thiamin in preventing hyperglycemia complications: from clinical aspects

2.5.10.1 Glycemic control

Effective control of blood glucose is a main goal in the management of hyperglycemia. Given the evidence suggesting a potential role for thiamin in preventing hyperglycemic complications discussed before (Babaei-Jadidi et al. 2003; Beltramo et al. 2002; Berrone et al. 2006; Booth, Khalifah, and Hudson 1996; La Selva et al. 1996; Thornalley, Jahan, and Ng 2001) (see Section 2.5.9), some experiments were undertaken to examine the effects of thiamin on blood glucose levels in vivo. Several animal and human studies examining the effects of thiamin on hyperglycemia are summarised in Table 10.

Babaei-Jadidi et al. (2004) showed that administration of high doses of thiamin (7 and 70 mg/kg/day) for 24 weeks had no effect on fasting blood glucose levels of the streptozotocin (STZ) - induced diabetic rats. This was consistent with the results of a clinical trial conducted by Rabbani et al. (2009) who showed no significant change in the fasting glucose of subjects with type 2 diabetes receiving either placebo or thiamin (300 mg/d) treatment for 3 months. These subjects had been diagnosed with diabetes for at least 5 years. Also, except one subject who achieved glycemic control with diet only, all other patients were on treatment with antihyperglycemic medications. However, since commencing this thesis, Gonzalez-Ortiz et al. (2011) have published a study on 24 Mexican overweight/obese subjects with type 2 diabetes. In this study, supplementation with 150 mg thiamin (once daily) for 1 month was shown to decrease the fasting plasma glucose (6.7 ± 1.0 mmol/L vs. 6.0 ± 1.0 mmol/L, $p = 0.024$) before and after intervention respectively. These subjects had fasting plasma glucose concentrations < 11.0 mmol/L and HbA_{1C} level < 8%, and were not treated with medications. These results suggest that thiamin therapy may be more effective on the fasting plasma glucose of patients with hyperglycemia at early stages.

As discussed earlier, diabetic patients are at risk of thiamin deficiency because of decreased absorption (Patrini et al. 1996) and increased excretion of thiamin in the

urine (Thornalley et al. 2007). Animal studies indicate that thiamin deficiency can in turn, reduce overall glucose utilisation in the tissues (Hakim, Carpenter, and Pappius 1983; Hakim and Pappius 1981) and thus increase fasting blood glucose (Rathanaswami and Sundaresan 1988). This is also associated with a decreased fasting insulin concentration (Rathanaswami and Sundaresan 1988). Basal secretion of insulin and insulin secretion in response to glucose and tolbutamide were also decreased in isolated pancreatic islets of thiamine deficient rats (Rathanaswami, Pourany, and Sundaresan 1991). Moreover, thiamin was shown to reduce hyperglycemia in a mouse model of TRMA (thiamin-responsive megaloblastic anemia syndrome) in which developed diabetes mellitus was associated with decreased insulin secretion (Oishi et al. 2002), These findings led to the conclusion that apart from the activation of thiamin-dependent enzymes and increased glucose utilisation, improvement of β-cell function and insulin secretion may be implicated in the beneficial effect of thiamin on glycemic control (Thornalley 2005). This was also considered as a possible explanation for the lack of significant effect of thiamin therapy on glycemic control in diabetic rats (Babaei-Jadidi et al. 2004), where permanent insulin deficiency was induced by streptozotocin (STZ) destroying most pancreatic β-cells (Thornalley 2005), and patients with long-term diabetes mellitus (Rabbani et al. 2009).

Thiamin deficiency was also shown to induce glucose intolerance in animals (Rathanaswami and Sundaresan 1989). A potential association between thiamin status and glucose tolerance in human was identified by Bakker et al. (1998) who showed that thiamin intakes of 2196 men and women, 50-75 years old without diabetes, were inversely correlated to 2-h postprandial glucose concentrations, suggesting that thiamin supplementation may have an important role in improving glucose tolerance. However, to date there has been no published study to confirm this effect in individuals with impaired glucose tolerance. Only in one publication, thiamin supplementation (50 mg/d for 30 days) was shown to improve glucose tolerance in 25 patients with hepatic cirrhosis (Hassan, Qureshi, and Zuberi 1991).

Table 10. Studies examining the effects of thiamin on hyperglycemia

Study	Mode	Method	Dose of thiamin	Results
Thornalley et al. (2001)	In vitro [human red blood cells (HRBCs)]	H RBCs (50% v/v) incubated in 5 mmol glucose (normoglycemic condition) and 50 mmol glucose (hyperglycemic condition)	50-500 µmol	Under hyperglycemic state, addition of 500 µmol thiamin significantly decreased the concentration of triosephosphates, and increased concentrations of sedoheptulose-7-phosphate and ribose-5-phosphate (R-5-P).
La Selva et al. (1996)	In vitro [human umbilical vein (HUVEC)&bovine retinal endothelial cells (BREC)]	HUVEC &BREC incubated in 5.6 mmol/L glucose (normoglycemic con.) and 28mmol/L glucose (hyperglycemic con.)	150 µmol/L	Thiamin decreased production of lactate and AGEs in endothelial cells cultured in high glucose
Berrone et al.(2006)	In vitro [HUVEC & BREC]	HUVEC &BREC incubated in 5.6 mmol/L glucose (normoglycemic con.) and 28mmol/L glucose (hyperglycemic con.)	50 & 100 µmol/L	Thiamin (50 & 100 µmol/L) reduced significantly aldose mRNA expression and activity, normalised sorbitol concentrations (at dose 100 µmol/L) and intracellular glucose, and increased the expression and activity of TK.
Oishi et al. (2002)	In vivo (mouse)	Thiamin repletion for 6 weeks in a mouse model of TRMA, diabetes was developed by disruption of the gene encoding Thtr-1	22mg/kg	Glucose intolerance resolved after 6 weeks of thiamin repletion
Babaei-Jadidi et al. (2004)	In vivo (rat)	Thiamin therapy for 24 weeks in the streptozotocin (STZ) - induced diabetic and normal control rats	7 &70 mg/kg/d	High dose thiamin therapy (7&70mg/kg/d) increased glomerular TK expression, increased the conversion of triosephosphate to R-5-P in RBCs, and decreased plasma glycating agents. No effect on plasma glucose and Hb A_1.
Hassan et al. (1991)	In vivo (human)	Non - controlled clinical study. Thiamin therapy for 30 days in patients with hepatic cirrhosis.	50 mg/d	Thiamin therapy decreased fasting blood glucose and improved glucose tolerance significantly.
Rabbani et al. (2009)	In vivo (human)	Placebo - controlled clinical trial (parallel design). Thiamin suppl. for 3 Mon. in diabetics with microalbuminuria	300 mg/d	No significant change in the fasting blood glucose of diabetic subjects receiving either placebo or thiamin treatment.
Gonzalez-Ortiz et al. (2011)	In vivo (human)	Placebo - controlled clinical trial (parallel design) -Thiamin suppl. for 1 month in drug-naive diabetic patients	150 mg/d	Administration of thiamin decreased fasting blood glucose concentrations significantly.

2.5.10.2 Lipids and lipoproteins

It is now well established that both fasting and postprandial dyslipidemia are independent predictors of future cardiovascular events (Eberly, Stamler, and Neaton 2003; Gaziano et al. 1997) . The association of hyperglycemia at the levels below the current diagnostic thresholds for diabetes with dyslipidemia has been increasingly recognized (Blake et al. 2004; Lim et al. 2000; Meigs et al. 1998; Novoa et al. 2005; Rodriguez et al. 1996). Evidence indicates that the adverse effects of postprandial hyperglycemia may be potentiated when it is associated with hyperlipidemia (Anderson et al. 2001).

While the pathogenesis of hyperlipidemia associated with impaired glucose metabolism is unknown, a switch from lipid oxidation to lipogenesis in the liver has been considered as a possible mechanism (Taskinen 2003). This is linked to an increase in the production of apolipoprotein B-100 (apo B100) (Packard and Shepherd 1997) and alteration in the assembly of very low density lipoprotein (VLDL) in the liver (Adiels et al. 2006). As indicated in Section 2.5.9, an increase in the hepatic hexosamine pathway in the liver due to hyperglycemia has been linked with lipogenesis (Rumberger et al. 2003; Veerababu et al. 2000) and it is proposed that supplementation with thiamin may alter this effect.

High dose thiamin therapy (70 mg/kg/d) was shown to normalise food intake and prevent diabetes-induced increases in plasma triglycerides and cholesterol in diabetic rats, but there was no effect on the decrease of HDL (Babaei-Jadidi et al. 2004). These positive effects on diabetic dyslipidemia were due to thiamin repletion and increase in transketolase activity in the liver of rats. This was associated with a significant reduction in hepatic UDP-N-acetylglucosamine and fatty acid synthase activity. These effects were not confirmed by the studies carried out in humans. In the study conducted by Rabbani and her colleagues (2009), supplementation with high dose thiamin (300mg/day) for 3 months had no significant effect on dyslipidemia in type 2 diabetic patients (n= 20) with microalbuminuria, compared with controls (n=20). Similarly, Gonzalez-Ortiz et al. (2011) reported no changes on serum total cholesterol, HDL, LDL, VLDL and triglyceride of patients with type 2 diabetes receiving thiamin orally (150 mg/day) for 1 month (n=12) with respect to

placebo (n=12). There has been no published literature so far to investigate the effect of serum lipid profile of individuals with pre-diabetic range of hyperglycemia.

2.5.10.3 Endothelial function

Evidence from more recent studies has shown endothelial dysfunction in patients with diabetes or IGT, and in healthy subjects with transient increases in plasma glucose, indicating that hyperglycemia even in the absence of overt diabetes is a cardiovascular risk factor (Akbari et al. 1998; Kawano et al. 1999; Salomaa et al. 1995; Tooke and Goh 1999).

In contrast to most cell types, vascular endothelial cells are unable to down regulate glucose transport in the presence of hyperglycemia, leading to an increased intracellular glucose concentration (Kaiser et al. 1993). As a result, endothelial cells are among the first targets of glucose-mediated damage.

The exact mechanisms responsible for endothelial dysfunction under hyperglycemic status are not fully understood. However, reduced release and/or synthesis of endothelium-derived nitric oxide (NO) resulting from the overproduction of ROS, and an imbalance between NO release and superoxide anion formation (Bucala, Tracey, and Cerami 1991; Cersosimo and DeFronzo 2006; Giugliano et al. 1997; Srinivasan et al. 2004) have been suggested as likely mechanisms.

Thiamin has been shown to improve endothelial and smooth muscle cell function in vitro (Chen, Okada, and Okeda 1997; La Selva et al. 1996). Additionally, it was shown to protect against glucose and insulin-mediated proliferation of human vascular smooth muscle cells (Avena et al. 2000). Thiamin was also demonstrated to mitigate or delay hyperglycemia-induced endothelial dysfunction through the improvement of endothelial cell migration, and reversing the increased secretion of the von Willebrand factor in cultured endothelial cells (Ascher et al. 2001).

The potential effect of thiamin on vascular function in humans was initially investigated in patients with beriberi heart disease, in which hemodynamic function and systemic vascular resistance were significantly improved following intravenous

administration of thiamin (Attas et al. 1978; Ayzenberg, Silber, and Bortz 1985). Recently, the same effect was reported by Arora et al. (Arora et al. 2006) who showed that administration of 100 mg intravenous thiamin improved endothelium dependent vasodilatation (EDV) in the presence of hyperglycemia. Little is known, however, about the inhibitory role of thiamin on hyperglycemia-induced ROS production, and if its antioxidant properties contribute to these vasomotive effects (Bakker, Heine, and Gans 1997; Lukienko et al. 2000).

2.5.10.4 Renal function

Nephropathy is a known complication of diabetes and cardiovascular diseases (Sharma 2003; Stratton et al. 2000; The Diabetes Control and Complications Trial Research Group 1993). However, there is emerging evidence that hyperglycemia in non-diabetic ranges (IGT) is also associated with renal injury (Gabir et al. 2000; Metcalf et al. 1993; Pan et al. 1993). In a cross- sectional study on more than 5,000 Maori and European subjects, microalbuminuria was recognised in 21% of those with diabetes and 16% of those with IGT, but only 4% of normoglycemic individuals (Metcalf et al. 1993). In another large population-based study in China, the rate of albumin excretion was significantly higher among IGT subjects compared with the non-diabetic control group (7.2 ± 5.7 vs. 4.5 ± 2.8) (Pan et al. 1993).

The increased level of plasma glucose can cause a high cytosolic glucose concentration in renal endothelial cells and pericytes. This in turn can result in the biochemical dysfunctions of: protein kinase C, hexosamine and polyol pathways activations; metabolic pseudohypoxia; oxidative stress production; and advanced glycation end products (AGEs) formation (Brownlee 2001).

Given the potential role of thiamin under hyperglycemic status (see Section 2.5.9), accumulating evidence has demonstrated the suppressive effect of thiamin on the multiple key pathogenic pathways involved in diabetic nephropathy in vitro (La Selva et al. 1996). Additionally, it was shown that the high dose of thiamin inhibits the development of incipient nephropathy (the initial stage of development of nephropathy) in the streptozotocin (STZ)-induced diabetic rats, and increases the thiamin-dependent enzyme transketolase activity in renal glomeruli (Babaei-Jadidi et

al. 2003). In the same work, these effects were associated with deceases in cytosolic and membrane fraction PKC activities, markers of glycation (AGEs in glomerular protein) and oxidative stress (plasma protein thiols), and concentrations of dicarbonyl compounds of methylglyoxal, glyoxal and 3-deoxyglucosone. More recently, the beneficial effect of high dose thiamin supplementation on microalbuminuria was confirmed in human subjects with type 2 diabetes mellitus (Rabbani et al. 2009; Riaz, Skinner, and Srai 2011). However, there has been no literature so far investigating the chronic effect of thiamin on the renal function of individuals with pre-diabetic range of hyperglycemia.

2.5.10.5 Body weight & energy expenditure

Only a few studies have investigated the effect of thiamin on body weight. Tanaka et al. (2010) showed that thiamin intervention prevents obesity in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, displaying obesity and metabolic abnormalities similar to those found in humans. In Tanaka et al' study, OLETF rats were given free access to water (control group) or water containing 0.2% thiamin (thiamin group) for 21 and 51 weeks. In a clinical trial conducted by Wilkinson et al. (1997), the weight of subjects with persistent subclinical thiamin deficiency receiving thiamin supplement (10 mg daily) decreased significantly after 3 months. No exact explanation has been offered by Wilkinson et al. for this finding. They explained a likely improvement in the heart failure of participants taking Furosemide (n=3, 17%) and a subsequent weight loss indicating diuresis as a notable point. However, there is no information in the article indicating whether this significant reduction in body weight has been limited to these subjects. Also, no data about the energy intake of subjects in this study has been provided. By contrast, Smidt et al.(1991) reported that supplementation with 10 mg/d thiamin for 6 weeks resulted in a considerable increase in the body weight of elderly subjects with marginal thiamin deficiency. This change was associated with significant improvements in their appetite, and energy intake assessed by weekly 24-hour recalls. There has been no significant change in physical activities of subjects participating in either Smidt et al's or Wilkinson et al's study.

In general, changes in body weight result from an imbalance between the energy intake and energy expenditure. Total energy expenditure is comprised of three main components: basal metabolic rate (BMR), diet-induced thermogenesis (DIT) and energy expenditure physical activity. BMR represents the minimal rate of energy required for normal body function in a resting and awake state, accounting for approximately 50% to 70% of total energy expenditure (Ravussin and Bogardus 1992).

BMR is mainly dependent on the body size and composition (Tzankoff and Norris 1977). Existing evidence indicates that body composition and subsequently energy expenditure can be influenced by the change in macro and micronutrient intake. Some studies have shown that administration of thiamin increases muscle mass and energy expenditure in experimental animals (Hamano, Okada, and Tanaka 1999; Huang et al. 2010). There has been no published study so far to evaluate the effect of thiamin on the energy expenditure in humans.

2.6 Summary

Impaired glucose regulation plays a critical role in the pathogenesis of type 2 diabetes mellitus and its cardiovascular complications. Thiamin is a water soluble vitamin playing a key regulatory role as a co-enzyme in metabolic pathways involving glucose. According to the findings of recent studies, people with hyperglycemia in the diabetic range are prone to thiamin deficiency. For example, the findings of Thornalley and his colleagues indicate that due to an increased excretion of thiamin in the urine, thiamin concentration in blood plasma was decreased by 76 per cent in type 1 and by 75 per cent in type 2 diabetic patients (Thornalley et al. 2007). In addition to the increased thiamin renal clearance, a reduced intestinal absorption was also suggested to contribute to the thiamin-deficient state reported in diabetics (Patrini et al. 1996).

In spite of these findings and also the essential role of impaired glucose metabolism in the pathogenesis of biochemical abnormalities associated with diabetes, there has been no published study to investigate thiamin status in individuals with pre-diabetic range of hyperglycemia.

Studies also showed the presence of impaired glucose tolerance in thiamin-deficient laboratory animal (Oishi et al. 2002), suggesting that thiamin deficiency could be considered as both cause and effect of hyperglycemia. Therefore, supplementation with thiamin may break this vicious circle and improve glucose tolerance.

In vitro studies reveal that thiamin can inhibit certain biochemical pathways that lead to abnormalities associated with hyperglycemia (Berrone et al. 2006; La Selva et al. 1996; Thornalley, Jahan, and Ng 2001). The mechanism of action seems to involve the diversion of "excess" metabolic load (glycolytic intermediates) away from glycolysis and toward the so-called reductive pentose pathway, a secondary pathway for glucose catabolism.

Given these findings some studies have been undertaken to examine the effects of thiamin on hyperglycemia complications in vivo. Thiamin supplementation was shown to improve cardiovascular risk factors in diabetic rats (Babaei-Jadidi et al.

2003) and increase muscle mass and energy expenditure in experimental animals (Hamano, Okada, and Tanaka 1999; Huang et al. 2010). Recently, a clinical study investigating the effects of thiamin on hyperglycemia showed that supplementation with thiamin decreased the fasting plasma glucose of diabetic patients with fasting plasma glucose concentration <11 mmol/L and HbA_{1C} level < 8% (Gonzalez-Ortiz et al. 2011). Thiamin supplementation was also shown to improve glucose tolerance in patients with hepatic cirrhosis (Hassan, Qureshi, and Zuberi 1991). Additionally, thiamin is found to have a beneficial effect on microalbuminuria in subjects with type 2 diabetes mellitus (Rabbani et al. 2009). However, to date there has been no published study to confirm these effects in individuals with pre-diabetic range of hyperglycemia (IGT).

Therefore, the present research work was designed in two studies, to assess the status and effects of thiamin on some cardiovascular risk factors in subjects with abnormal glucose metabolism.

3. The cross - sectional study

As mentioned before (see Section 2.5.6), recent studies indicate that individuals with hyperglycemia in the diabetic range are at higher risk of thiamin deficiency (Jermendy 2006; Thornalley et al. 2007; Valerio et al. 1999), because of an increased excretion of thiamin in the urine (Thornalley et al. 2007) and possible decreased absorption (Patrini et al. 1996). For example, Thornalley and his colleagues (2007) showed that thiamin concentration in blood plasma was decreased by 76 per cent in type 1 and by 75 per cent in type 2 diabetic patients relative to control subjects. However, there has been no published study to investigate thiamin status in individuals with other degree of abnormal glucose levels.

Given this gap and also the essential regulatory role of thiamin in metabolic pathways involving glucose metabolism, it was hypothesised that the level of thiamin is also decreased in people with pre-diabetic range of hyperglycemia.

3.1 Study design

Our first hypothesis was addressed through a cross - sectional study, evaluating thiamin status in individuals with abnormal glucose regulation (IFG and IGT) relative to healthy subjects. Subsequently, potential associations between thiamin status and different cardiovascular risk factors were investigated. As indicated below, we were only able to evaluate thiamin status by RBC thiamin.

3.2 Subjects

Subjects were recruited by advertising in community and The West Australian newspapers, WA Practice Nurse Association newsletter, Curtin University web site and radio as well as outpatient clinics and divisions of general practice. Additionally, printed information sheets and flyers [Appendix 1] were sent to Community Diabetes Education Services for distributing among the people attending the educational programs for prevention of diabetes.

Following recruitment, potential volunteers were asked to complete an online screening questionnaire available on the web site of Curtin University

The prospective subjects who didn't have access to the internet were screened via telephone. Both on-line and telephone screening questionnaires began with some questions related to the subjects' general information, such as sex, age, address, weight and height. The second part of the forms consisted of the questions relating to the study's inclusion criteria [Appendix 2].

Volunteers were excluded based on the following criteria:

- Smoking
- Known impaired renal or liver function
- Major gastrointestinal disorders
- Hypo and hyperthyroidism
- Known diabetes mellitus
- Cardiovascular event over the last 6 months
- Known allergy or intolerance to thiamin
- Females who are pregnant or lactating
- Use of steroids, thyroxin or other supplements and medications that affect glucose metabolism
- Taking diuretics with possible effects on urinary excretion of thiamin.

Subjects who were taking supplements containing thiamin (e.g. multivitamins preparations) or consuming more than 2 standard alcoholic drinks per day, but met other inclusion criteria, were asked to undergo a washout period of at least 4 weeks before attending the clinical day.

The sample was based on an expected 15% reduction in plasma thiamin concentration in the hyperglycemic group relative to the healthy group. Based on the results of previous studies (Thornalley et al. 2007), a sample size of 50 subjects (25 subjects in each group) could provide 80% power at the 5% significance level using a two-sided independent groups t-test.

An initial seventy five subjects (31 males and 44 females) were recruited to allow for drop out/non-compliance. Of these, six female subjects dropped out, because of difficulties in undergoing a 4-week wash - out period for multivitamin supplements before attending the clinical visit and also an inability to commit the time. In addition, given the main objectives of this study, data of 1 known case of pre-diabetic who became normal based on her results in our study, and four other subjects (2 males, 2 females), being identified as new cases of diabetes mellitus based on their plasma glucose concentrations, were excluded for final analysis.

For evaluating the main objective of this study (see Section 3.1), data of sixty four subjects (29 men and 35 women) were analysed, except for vascular function measurements which included data of forty four subjects (21 males and 23 females) . Also, data of 61 subjects (26 men, 35 women) were used for assessment of glucose tolerance by comparing two sampling systems of venous whole blood and venous plasma (see Section 3.5).

The sixty four subjects consisted of thirty nine normal healthy volunteers (14 males, 25 females) and twenty five hyperglycemics (15 males, 10 females) with blood glucose levels at pre-diabetic ranges (16 IGT, 9 IFG), who were classified as normal and hyperglycemic based on venous plasma glucose measurements (FPG & 2 h-GTT), the recommended tests for detecting all stages of hyperglycemia (Diabetes Australia Western Australia 2005; World Health Organization 2006). Two of IGT subjects were on long term cholesterol- lowering medicine (Zocor& atorvastatin) and one IGT subject was taking medication for hypertension.

Participation in this study was completely voluntary and subjects were informed that they were able to withdraw from the study at any point in time, without prejudice. This study was approved by the Curtin University Human Research Ethics Committee (Approval number HR 118/2008) and a written consent form [Appendix 3] was obtained from the subjects during the introductory short visit.

3.3 Methods

3.3.1 Introductory short visit

After an initial screening, the subjects who met inclusion criteria attended an introductory short visit to receive the study's procedure and protocol in details and to provide written consent [Appendices 3&4].

In addition, during the short visit, participants were given a standard light-resistant container (greiner bio-one GmbH, Kremsmunster, Austria) to collect their urine for 24 hours, starting the morning before the clinical day. A light-resistant urine container was provided because of the light sensitivity of thiamin (Interdepartmental Committee on Nutrition for National Defense 1963). Boric acid (3 g) was added to the collection containers as a preservative to ensure thiamin stability (Tasevska et al. 2008). It is known that thiamin is easily destroyed at pH 8 or above, particularly in high-temperature environments (Brody 1999).

Subjects were instructed to discard their first urine of the day and record the time on the container label. From then, they should collect all urine for the next 24 hours, including the first urine of the following day (clinical day) at the same time as the recorded commencement time [Appendix 5]. The urine samples collected in a collection time outside the range of 23-25 h, or with a ratio of urinary creatinine (mg/d) to body weight (kg) outside the range of 10.8-25.2, calculated based on the strategy developed by WHO (World Health Organization 1984), were considered as incomplete collections and excluded.

3.3.2 Meal standardisation

Participants were asked to be on an unrestricted carbohydrate diet of at least 150-200 g of carbohydrate daily, during three days prior to the test as recommended by WHO (Alberti and Zimmet 1998) [Appendices 5&6]. Moreover, on the evening before the clinical day, they were required to consume a standard meal provided by investigators. These requirements were to ensure subjects were consuming a reasonable amount of carbohydrate before the glucose tolerance test. Studies have shown that fasting and 2-hour blood glucose levels can be influenced by the source

and amount of carbohydrate consumed the evening prior to the test (Alberti and Zimmet 1998; Wolever et al. 1988).

The evening meal was Vegetable Korma with rice (Crafty Chef Pty. Ltd., NSW, Australia), containing about 1720 kJ with a macronutrient composition of 68% of energy from carbohydrate, 10% from protein and 21% from fat [Table 11]. Following consumption of the standard evening meal, subjects were required to fast overnight.

Table 11. Information from Nutrition Information Panel for Vegetable Korma

	Per 100 g	**Per meal (375g)**
Energy (kJ)	459	1721
Protein (g)	2.5	9.4
Fat (g)		
Total	2.5	9.4
Saturated	1.5	5.6
Carbohydrate (g)		
Total	18.2	68.3
Sugars	0.8	3.0
Sodium (mg)	217	814

3.3.3 Experimental protocol

On the morning of clinical day, subjects attended the out-patient clinic, School of Public Health, Curtin University, following an overnight fast of 10-14 hours and brought their completed 24 - hour urine collection.

Upon arrival, participants were asked to empty their bladder and collect a midstream urine sample. The 24-h urine volume was measured and recorded. Twenty mL of collected midstream urine sample and 30 mL of 24-h urine sample were stored in separate 5 mL plain plastic vials at -70°C until being analysed for urinary thiamin, albumin and creatinine.

Anthropometric measurements were taken with subjects dressed in a gown with no shoes and empty bladder. Body weight was measured using a calibrated body composition monitor (Model BC 541, Tanita Corporation, China) to the nearest 0.1 kg, and height was measured with a portable stadiometer (Mentone Education Centre, design number 1013522, Victoria, Australia) to the nearest 0.5 cm.

Waist circumference was measured at mid-exhalation, midpoint between the lateral lower rib margin and the ileac crest in the standing position (Gibson 2005; Jones et al. 1986) and hip circumference measurement was taken at the widest point over the buttocks (Lohman, Roche, and Martorell 1988). The average of two readings was recorded. Participants were then asked to rest 30 minutes prior to the measurements of blood pressure and vascular endothelial function, and blood sampling (see Sections 3.3.4& 3.3.5).

After taking fasting measurements, subjects were required to consume the full bottle of a standard glucose tolerance test beverage (Glucaid: Fronine Pty. Ltd., NSW, Australia), containing 75 g of glucose (75 g/300 mL). The glucose beverage was lemon - flavoured and kept refrigerated prior to ingestion, to increase palatability. Subjects were instructed to drink the test load as rapidly as they comfortably could, but no more than 5 minutes. Following consumption of the beverage, subjects remained at rest during the test. They were allowed to drink water either before or during the test. Two hours after ingesting the beverage, another blood sample was collected for measuring glucose level (glucose tolerance test).

3.3.4 Blood pressure & vascular function measurements

Following a mandatory rest period of 30 minutes, systolic and diastolic blood pressures were measured on subjects' right arms in supine position, using a vital signs patient monitor (Cardiocap II, Datex, Helsinki, Finland) with a standard cuff for adults. Three blood pressure measurements, with 2 minutes intervals, were recorded in the fasting state and the average value was reported.

Vascular endothelial function was assessed by a Pulse Trace machine (PCA2, Micro Medical Ltd., UK) [Figure 14]. Pulse Trace PCA2 is a non-invasive device used to assess endothelium function and vascular stiffness by recording digital volume pulse (DVP). The technique of DVP is based on measuring infra-red light transmission through the finger (photoplethysmography) (Chowienczyk et al. 1999).

Figure 14. Pulse Trace PCA2 machine used to assess endothelium function

After an initial 30-minute rest, a photoplethysmography sensor transmitting infra-red light was placed on the index finger of subjects. The digital volume pulse measurements were recorded with subjects supine in a temperature-controlled room (22±2°C). Two readings, with at least 2 minute apart, were taken in fasting state. Each of these measurements was the average of three recordings with intervals of 30 seconds. The average of six measurements was reported as baseline value. In addition to the fasting measurement, postprandial endothelial function changes were assessed intermittently at 30, 60, 90 and 120 minutes after ingestion of the test load.

3.3.5 Biomedical measurements

Fasting blood samples were collected via venipuncture into serum, heparin and EDTA tubes (BD Vacutainer®, NJ, USA). After taking blood samples, the tubes were inverted gently between 8-10 times, with no delay. The serum tube containing clot activator was then left at room temperature for 20-30 minutes to clot before centrifugation. The blood collected into EDTA was centrifuged at 1100 g for 10 minutes at 4°C immediately (within minutes). The isolated plasma was stored in separate 1.5 mL microcentrifuge tubes at -80 °C until analysed for glucose and insulin concentrations. The same protocol was also administrated for the blood sample collected 2 hours after ingestion of glucose beverage repeating the plasma glucose measurement.

Venous blood collected into the tube containing lithium heparin was used to assess thiamin status. For the measurement of thiamin in plasma, the collected blood was centrifuged immediately (1100 × g, 10 min, 4°C) and the separated plasma was frozen at temperature of -70 °C until analysis. The erythrocytes were prepared for thiamin analysis. After separating the plasma, buffy coat layer was removed from the erythrocytes and discarded. The packed red blood cells (RBC) were then washed three times with normal saline (9 g/L NaCl). For hemolysing the cells, 1 mL of de-ionised water was added to 1 mL of washed RBC and mixed by vortex mixer (Australian Scientific, Australia) for 1 min (Talwar et al. 2000). The mixture was then aliquoted and frozen at -80 °C until assayed. After a period of about half an hour from collecting fasting blood samples, the blood clotted into serum tube was centrifuged. The separated serum was then stored at -80 °C until analysis for total cholesterol, HDL-cholesterol, LDL cholesterol, triglyceride, hs-CRP and creatinine.

In addition to measuring glucose in plasma, fasting and postprandial glucose levels were assessed in whole blood by using a blood glucose meter (Accu-Chek Active, Roche Diagnostics , NSW, Australia). To measure glucose level in whole blood, immediately after taking fasting blood samples, one drop of fresh whole blood left-over from the used blood collection set was placed in the test zone of a glucose meter strip. This test was repeated with no delay. The average of two readings was recorded. The concentration of glucose in whole blood reported for 2-h post glucose load was determined according to the same procedure.

3.3.6 Blood and urine analysis

Aliquot of plasma (separated from the blood collected into EDTA tube), serum and spot urine samples were sent to Royal Perth Hospital for analysing glucose, insulin, total cholesterol, HDL-cholesterol, LDL cholesterol, triglyceride, highly sensitive C-reactive protein, serum and urine creatinine, and urinary albumin.

Plasma glucose levels were determined with the hexokinase method. Insulin levels were measured by the Architect insulin assay. Enzymatic colorimetic assays were used to determine the levels of serum triglyceride, total cholestrol and creatinine. Concentrations of HDL cholestrol were directly measured in the serum samples by

the Ultra HDL assay. These analyses were performed using the Abbott diagnostic kits (Abbott Laboratories, IL, USA) with a within- and between-run coefficient of variation of < 4.3%. LDL cholesterol was calculated using a modified version of Friedewald equation (Friedewald, Levy, and Fredrickson 1972) with quanitites in mmol/L:

$$\text{LDL- cholesrerol} = \text{total cholesterol} - (0.46 \times \text{triglyceride}) - \text{HDL- cholesrerol}$$

Serum hs-CRP was measured by nephelometry using a BNII system (Siemens Healthcare Diagnostic inc. Newwark, DE, USA) with between- and within-run coefficient of variation of 8.35% and 5.7% respectively. The levels of albumin in urine were assessed by the Abbott Microalbumin assay, a turbidimetric immunoassay using polyclonal antibodies againt human albumin, with between- and within-run coefficient of variation of 12.19% and 0.74% (Wako Pure Chemical Industries, Ltd. Osaka, Japan).

Blood glucose was also determined in whole blood using the blood glucose meter (Accu-Chek Active, Roche Diagnostics , NSW, Australia), immediately after collecting blood samples (fasting & 2-h post glucose load) as described before.

The results of fasting plasma glucose and insulin were also used to estimate the hemostatic model assessment (HOMA) score, as an index of insulin resistance by the following equation (Bonora et al. 2000):

$$\frac{\text{Fasting insulin (mIU/L)} \times \text{fasting glucose (mmol/L)}}{22.5}$$

A HOMA score > 2.5 indicates low insulin sensitivity (IR) in adults (Keskin et al. 2005).

In the present study, subjects were screened for albuminuria by determing albumin/creatinine ratio in the collected fasting spot urine sample. Microalbuminuria was detected in the presence of an albumin excretion ≥ 30 and < 300 mg/g creatinine.

An albumin/creatinine ratio ≥ 300 mg/g represents clinical albuminuria (Molitch et al. 2004).

Renal function was also assessed by estimating GFR (glomerular filtration rate), using Cockroft- Gault formula adjusted for BSA (body surface area) (Cockcroft and Gault 1976):

$$\text{eGFR (ml/min/1.73 m}^2\text{)} = \frac{[140 - \text{age (years)}] \times \text{weight (kg)} \times K \times C}{\text{Serum creatinine (μmol/L)}}$$

Where K is 1.23 for men and 1.04 for women, and C corrects for BSA, equal to 1.73/BSA. BSA was calculated by using DuBois formula (Du Bois and Du Bois 1989):

$$\text{BSA(m}^2\text{)} = [\text{weight (kg)}]^{0.425} \times [\text{height(cm)}]^{0.725} \times 0.007184$$

Concentration of creatinine is commonly used for measuring GFR. However, it is known to be influenced by muscle mass and diet. Accordingly, Cockroft- Gault formula is recommended as a useful surrogate tool, which employs patient's age, weight and serum creatinine to estimate glomerular function (Chudleigh et al. 2008; Guimaraes et al. 2007; Perlemoine et al. 2003).

In this study, assessment of thiamin status was attempted in urine and plasma. However, there were some technical problems in establishing the methods for this analysis at Curtin University. The washed red blood cells separated from the blood collected into the heparin tube were sent to Royal Perth Hospital (RPH) to be analysed for thiamin.

Thiamin (TPP) status was determined by high-performance liquid chromatography (HPLC) with fluoresecent detection (pre-column derivatisation) using the Chromsystems reagent kit (Chromsystems Instruments and Chemicals GmbH, Munich, Germany). The retention time, limit of quantification and recovery values for TPP were: 5.3 min, 0.7 nmol/L and 88-97%, respectively.

3.3.7 Assessment of dietary intake

Dietary intake of thiamin and alcohol were assessed by the validated semi-quantitative food frequency questionnaire of the Anti-Cancer Council Victoria (Giles and Ireland 1996; Hodge et al. 2000), containing 74 food items with 10 frequency response options ranging from 'Never' to '3 or more times per day' [Appendix 8]. The used food frequency questionnaire also included 3 photographs of scaled portions for four foods (to calculate a portion size calibrator), questions on the overall frequency of consumption of fruits and vegetables (to calibrate the overestimation of these foods in the food list), and questions on consumption of foods such as bread that could not be simply grouped by the used frequency format. The intake of alcoholic beverages was assessed by a separate set of questions. The output of the questionnaire compromised 35 nutrients (Cancer Council Victoria 2009).

On the assessment day, after taking fasting measurements, subjects were instructed how to complete the questionnaire (self-administrated method). Subsequently, the completed questionnaires were checked by the study investigator for completeness, and any probable errors were corrected on the spot. The collected forms were then optically scanned, and nutrients were computed by the Cancer Council Victoria, based on Australian food composition data from NUTTAB 95 (Cancer Council Victoria 2009; Lewis, Milligan, and Hunt 1995).

3.4 Statistical analysis

Statistical analysis was done using SPSS for Windows (version 16, SPSS Inc., USA). Data were assessed for normality to ensure that the assumptions for the analysis are met and transformations were used if required. Two different sampling systems for assessment of glucose tolerance were compared using a paired samples t-test and Bland-Altman analysis. An independent sample t-test was used to compare anthropometric and metabolic measurements of the hyperglycemic subjects with those of normal group. Data are presented as mean ± standard deviation (SD) (in the text and tables) and mean ± standard error of mean (SEM) (in charts). Statistical tests were two-tailed and a $p<0.05$ was considered as statistically significant.

3.5 Results

Assessment of glucose tolerance - a comparison of methods

In this study, participants were assessed for glucose tolerance through two different sampling systems, namely venous plasma analysed by the WHO reference glucose oxidase method (Royal Perth Hospital), and venous whole blood measured using a blood glucose meter (Accu-Chek Active, Roche Diagnostics , NSW, Australia) (methods - see Section 3.3.5).

Data of 61 subjects [26 (43%) men, 35 (57%) women] with a mean (± SD) age of 47.7 (±14.6) and a BMI of 27.54 (± 4.3) kg/m2 were analysed for comparing two sampling systems of venous whole blood and venous plasma used for assessment of glucose tolerance.

As shown in Table 12, the minimum fasting and 2-h post glucose load (2-h PG) blood glucose measured in venous whole blood were 4.50 and 3.20 mmol/L, while those of venous plasma were 4.70 and 3.40 mmol/L. The maximum fasting and 2-h PG blood glucose values were 6.35 and 12.50 mmol/L in venous whole blood, and 6.70 and 13.80 mmol/L in plasma samples, respectively.

The mean (± SD) of fasting whole blood glucose (FWBG) (5.33± 0.40 mmol/L) was found to be significantly lower than the mean of fasting plasma glucose (FPG) (5.51 ± 0.54 mmol/L) using a paired samples t-test (p-value < 0.001). This finding was consistent with the results of comparing the mean values of 2-h PG blood glucose measured in venous whole blood and plasma samples (6.69± 1.63 vs. 6.87 ± 1.83 mmol/L, p = 0.007).

There was a strong positive linear relationship between venous whole blood and plasma glucose concentrations measured in both fasting state (r = 0.85, p < 0,001) and 2-h post glucose load (r = 0.97, p < 0,001) [Figure 15].

Table 12. Descriptive statistics of venous whole blood glucose and venous plasma glucose concentrations

Parameters	Venous whole blood glucose concentrations (n=61)		Venous plasma glucose concentrations (n=61)	
	Fasting	2-h Post glucose load	Fasting	2-h Post glucose load
Minimum (mmol/L)	4.50	3.20	4.70	3.40
Mean	5.33	6.69	5.51	6.87
Maximum (mmol/L)	6.35	12.50	6.7 0	13.80
Standard deviation (SD)	0.40	1.63	0.54	1.83

Agreements between the two sampling methods for both fasting and 2-h post glucose load measurements were assessed by Bland-Altman analysis. For the fasting values, the bias (mean difference score between whole blood and plasma glucose values, FPG minus FWBG) was calculated 0.19 and 95% limit of agreement (mean difference scores ± 2SD) was obtained as - 0.39 to 0.77mmol/L. [Figure 16].

The bias decreased slightly to 0.17, and 95% limit of agreement was - 0.79 to 1.13 mmol/L, when the results of 2h-PG blood glucose values measured in two different sampling systems were compared.

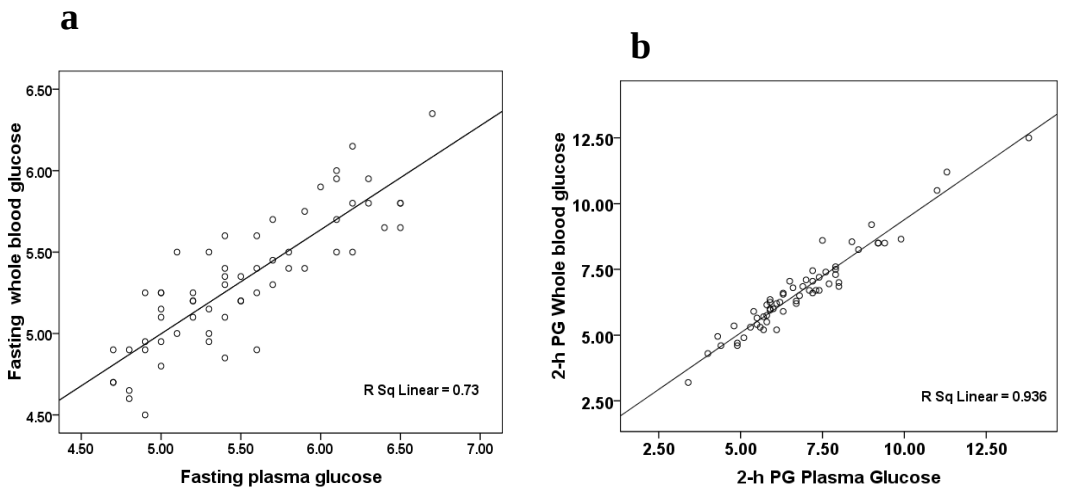

Figure 15. Scatter plots of venous whole blood and plasma glucose concentrations (mmol/L) measured (a) in fasting state and (b) at 2-h post glucose load.

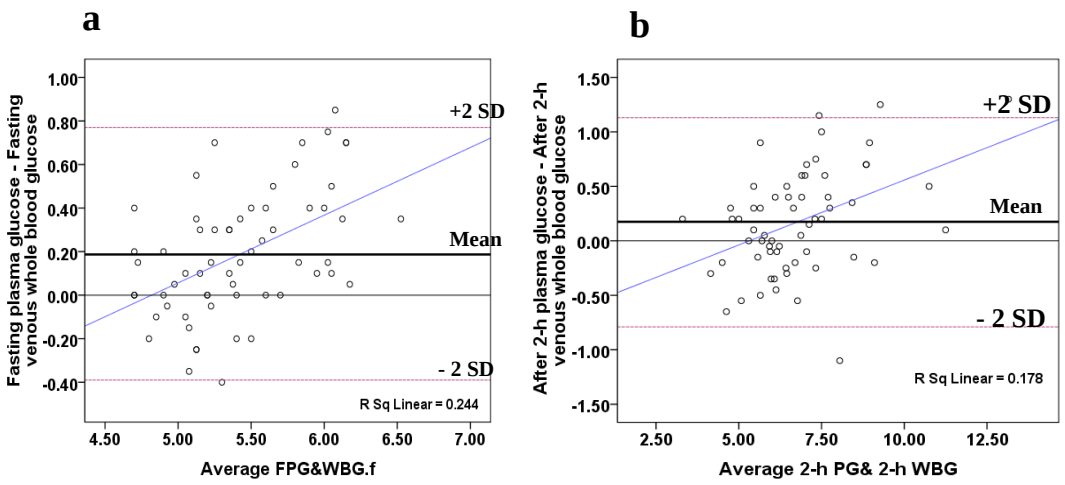

Figure 16. Bland-Altman graphs of difference scores for venous whole blood and plasma glucose concentrations (mmol/L) measured (a) in fasting state and (b) at 2-h post glucose load.

In this study, the mean of plasma /whole blood glucose ratio was 1.03 with a range of 0.93 - 1.15 in fasting samples, which was in line with 2-h post glucose load measurements (mean ratio: 1.02, range: 0.87-1.17, p = 0.15).

After converting glucose concentrations in venous whole blood to the equivalent concentrations in participants' plasma, using the conversion factor of 1.11 recommended by the International Federation of Clinical Chemistry (IFCC), the mean (±SD) values of converted fasting plasma glucose values were still significantly different from fasting plasma glucose concentrations measured in the laboratory (5.91 ± 0.45 vs. 5.51 ± 0.54 mmol/L, $p < 0.001$), with a bias of - 0.40 and 95% limit of agreements of - 0.96 to 0.16. This significant difference was also found for 2-h PG glucose values (7.43 ± 1.81 vs. 6.87 ± 1.83 mmol/L, $p <0.001$). The bias from this was - 0.56 and 95% limit of agreements were -1.48 to 0.36.

To evaluate the two different sampling systems for identifying various stages of hyperglycemia, blood glucose measurements were stratified on the basis of WHO cut-off values for venous plasma and venous whole blood samples (Alberti and Zimmet 1998): namely, fasting venous plasma glucose concentrations into <6.1, 6.1 - 6.9 and > 6.9 mmol/L, fasting venous whole blood glucose concentrations into <5.6, 5.6 - 6.0 and > 6.0 mmol/L, 2-h PG venous plasma glucose concentrations into < 7.8, 7.8 - 11.0 and >11.1 mmol/L, and 2-h PG venous whole blood glucose concentrations into < 6.7, 6.7- 9.9 and > 9.9 mmol/L.

Accordingly, 72.1%, 24.6% and 3.3% of participants were categorised as normoglycemic, IFG and diabetic, respectively, based on the fasting whole blood glucose measurements. However, these percentages changed to 77%, 23% and 0% respectively, when individuals were classified with regard to the fasting plasma glucose concentrations. Furthermore, there was an increased discrepancy when only 2-h post glucose load measurements were used as the basis of classifications, namely 54.1%, 41.0 and 4.9% of individuals categorised as normoglycemic, IGT and diabetic according to 2-h PG whole blood measurement, compared to 75.4%, 21.3% and 3.3% based on 2-h PG plasma glucose concentrations. When both fasting and 2-h post glucose load measurements were considered for classification, 42% (13 out of 31) of participants diagnosed as IFG or IGT according to WHO venous whole blood cut-off values, had normal plasma glucose concentrations and only one normoglycemic participant was misclassified as IFG. Furthermore, among the three

participants classified as diabetic, based on the venous whole blood values, one subject had plasma glucose concentration at the pre-diabetic level (IGT).

Assessment of anthropometric and metabolic factors

To achieve the main objective of this study, data of 64 subjects (29 men and 35 women) with an overall mean (± SD) age of 48.2 (±14.3) years and BMI of 27.4 (±4.2) kg/m^2 were analysed, except for vascular function measurements which included data for forty four subjects (21 males and 23 females).

According to the WHO classification [Appendix 7], 10 (6 men, 4 women) of 64 subjects were identified as having metabolic syndrome, and 11 subjects (6 men, 5 women) were insulin resistant based on the calculated HOMA score (HOMA score > 2.5) (Keskin et al. 2005).

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The sixty four subjects consisted of 39 normal healthy participants (14 men, 25 women) and twenty 25 hyperglycemics (15 men, 10 women) with blood glucose levels at pre-diabetic ranges (16 IGT, 9 IFG). These classification were based on venous plasma glucose measurements (FPG& 2-h GTT), as recommended by WHO, IDF and ADS for detecting glucose intolerance.

Of eleven participants classified as insulin resistant, 10 subjects were hyperglycemic (6 IGT, 4 IFG) and 1 subject had normal blood glucose levels.

As presented in Table 13, hyperglycemic subjects had an average (±SD) age of 57.96±11.08 years which was significantly higher than the average age of normoglycemic group (41.92 ± 12.64, $P<0.001$).

Table 13. Anthropometric and metabolic characteristics of subjects in each group

Subjects' characteristics	Hyperglycemic Group		Normoglycemic Group		P-Value
	Mean(SD)	Range	Mean(SD)	Range	
Age (y)	57.96(11.08)	27-73	41.92(12.64)	22 - 71	<0.001
Weight (kg)	83.0(12.55)	61.6-105.9	74.8(16.02)	51.6-105.4	0.03
Body mass index (kg/m^2)	28.30(3.69)	21.1-36.4	26.88(4.46)	19.9-35.8	0.19
Waist circumference (cm)	99.94(10.18)	86.0- 122.0	90.4(12.74)	68.0- 124.0	0.003
Waist/hip ratio	0.94(0.07)	0.81- 1.06	0.85(0.11)	0.45-1.05	0.001
Systolic blood pressure (mmHg)	118.7(11.3)	97 - 142	109.9(11.93)	91 -140	0.005
Diastolic blood pressure (mmHg)	71.0(8.05)	60 - 91	63.5(7.62)	48 - 90	<0.001
Fasting plasma glucose (mmol/L)	6.04(0.37)	5.30 - 6.50	5.21(0.34)	4.7- 6.0	<0.001
2-h plasma glucose (mmol/L)	8.08(1.26)	6.1 - 11.0	5.93(1.01)	3.40 - 7.70	<0.001
Fasting insulin (μIU/mL)	8.74(4.78)	3.0 - 21.0	6.03(2.35)	0.9 - 11.40	0.01
2-h insulin (μIU/mL)	81.03(55.78)	24.7- 233.0	52.84(36.12)	15.0 -205.0	0.02
HOMA score	2.37(1.33)	0.83-6.07	1.40(0.56)	0.20-2.53	0.002
Triglyceride (mmol/L)	1.49(0.53)	0.84 - 3.02	1.38(0.79)	0.48 - 4.58	0.56
Total cholesterol (mmol/L)	4.90(1.15)	2.95 - 6.63	5.12(1.13)	1.44 - 8.88	0.40
Fasting LDL (mmol/L)	2.96(0.75)	1.40 - 4.62	3.07(0.92)	1.82 - 5.82	0.61
Fasting HDL (mmol/L)	1.22(0.24)	0.82 - 1.94	1.30(0.30)	0.81 - 6.03	0.30
Fasting hs-CRP (mg/L)	1.60(1.25)	0.16 - 3.93	1.60(1.43)	0.16 - 6.98	0.10
eGFR * ($ml/min/1.73\ m^2$)	93.47(20.7))	52.4-146.8	112.6(20.8)	67.4-163.5	0.001

* Estimated by using Cockroft - Gault formula adjusted for body surface area

When all subjects were stratified into three age groups, namely: < 45, ≥ 45 and < 65, and ≥ 65 years, the mean (± SD) values of fasting and postprandial glucose increased with age, peaking among those aged 65 years and older [Table 14]. Moreover, subjects aged 45-64 years and ≥ 65 years had higher insulin levels (fasting & 2-h PG) and HOMA score, compared to subjects who were younger than 45 years.

Table 14. Plasma glucose and insulin levels and HOMA score in different age groups

Measurements - Mean(SD)	< 45 years (n=24)	≥45 and <64 years (n=27)	≥ 65 years (n=8)
Fasting plasma glucose (mmol/L)	5.18(0.39)	5.66(0.48)	6.08(0.47)
2-h PG plasma glucose (mmol/L)	6.22(1.42)	7.05(1.58)	7.33(1.29)
Fasting insulin (μIU/mL)	6.15(2.40)	7.82(3.86)	7.06(5.83)
2-h PG insulin (μIU/mL)	53.57(37.14)	69.55(46.51)	71.91(69.17)
HOMA score	1.42(0.57)	2.01(1.07)	1.96(1.72)

Normal and hyperglycemic groups had mean BMI (± SD) of 26.88 (± 4.46) and 28.30 (± 3.69) kg/m^2, respectively. The tendency for an increased levels of fatness (indicated by BMI) in hyperglycemic subjects became significant when two groups were compared for waist circumference (P = 0.003) and waist/hip ratio (P = 0.001), as two indices of abdominal obesity. There were also significant correlation between waist circumference and plasma glucose levels (fasting: r = 0.41, p = 0.001; 2-h PG: r = 0.33, P = 0.007) as well as other cardiovascular risk factors: SBP (r = 0.32, P = 0.009), HDL- cholesterol (r = - 0.29, P = 0.02), triglyceride (r = 0.27, P = 0.03) and CRP (r = 0.31, P = 0.01).

Hyperglycemic subjects had a significantly higher HOMA score (2.37±1.33 vs. 1.40±0.56, p=0.002), compared to the normoglycemic group. There were also significant associations between HOMA score and blood glucose measured in both fasting (r = 0.49, p<0.001) and postprandial (r=0.29, p<0.05) states, and between HOMA score and indices of central obesity [WC: r = 0.53, p < 0.001; W/H ratio: r = 0.43, P <0.001].

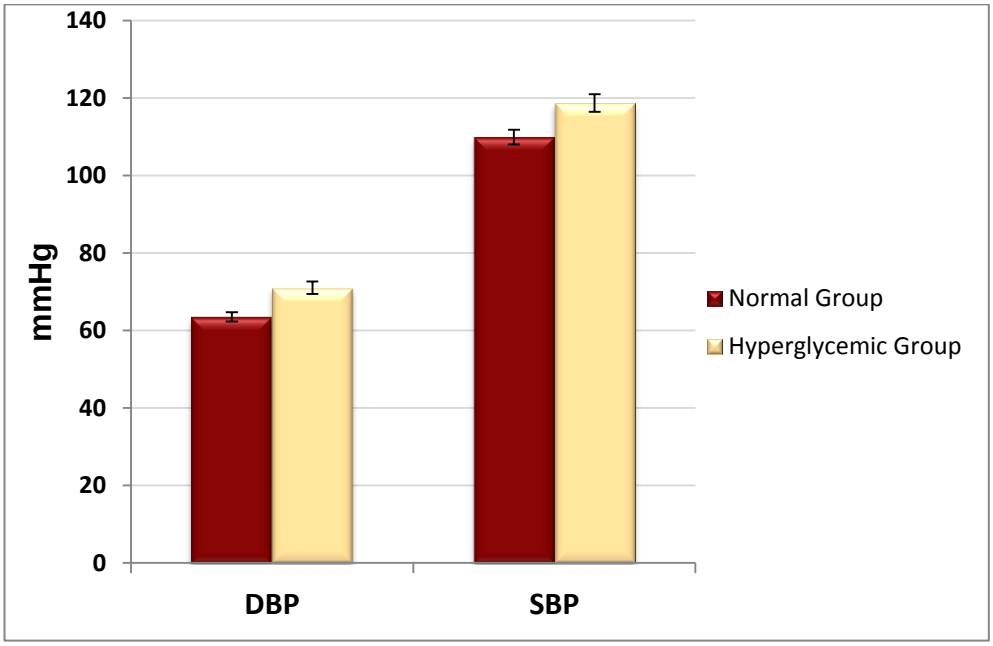


Figure 17: Systolic and diastolic blood pressure in hyperglycemic and normal groups. *Compared to normal group, hyperglycemic subjects had a significantly higher systolic (p=0.005) and diastolic (p<0.001) blood pressure. Data are presented as mean ± SEM*

Subjects with impaired glucose regulation had also a significantly higher resting systolic (118.7±11.3 vs. 109.9±11.93 mmHg, P = 0.005) and diastolic (71.00±8.05 vs. 63.5±7.62 mmHg, P< 0.001) blood pressure, relative to normal volunteers [Figure 17].

In comparison with normoglycemic group, hyperglycemic subjects had a tendency toward higher mean (± SD) value of triglyceride (1.51±0.57 vs. 1.38±0.78 mmol//L), associated with lower mean values of total cholesterol (5.08±1.07 vs. 4.90±1.37 mmol//L), LDL-cholesterol (3.23±0.53 vs. 3.07±0.91 mmol//L) and HDL-cholesterol (1.40±0.70 vs. 1.52±1.06 mmol//L) [Table 13].

Microalbuminuria (detected by albumin to creatinine ratio ≥ 30 µg/mg) was present in 12 % of those with hyperglycemia in pre-diabetic ranges (3 of 25 subjects), but 7.7% of normal individuals (3 of 39 subjects). Moreover, there was a significant difference between glomerular filtration rate (GFR) estimated in hyperglycemic and normal participants (93.47±20.7 vs. 112.63±20.8 ml/min/1.73 m^2, P = 0.001).

Subjects with metabolic syndrome had a tendency toward higher BMI (29.64±3.74 vs. 27.03±4.19 kg/m^2, P = 0.07), as well as significantly higher WC (103.30±12.37 vs. 92.46±12.02 cm, P = 0.01), and W/H ratio (0.96±0.06 vs. 0.87±0.10, P = 0.01). Also, there was a significant association between metabolic syndrome and insulin resistance (χ^2 = 23.23, p < 0.001).

Arterial stiffness was assessed in a subgroup of participants including 27 normal and 17 hyperglycemic (12 IGT, 5 IFG) volunteers, by using a Pulse Trace machine recording RI and SI as two main indicates derived from digital volume pulse (DVP). In the present study, in addition to the fasting measurement, post prandial vascular function was evaluated at 30, 60, 90 and 120 minutes after ingestion of the glucose load. Areas under the curves were calculated using the trapezoidal rule. Changes in SI and RI responses were expressed as SI Postprandial minus SI Fasting, and RI Postprandial minus RI Fasting, respectively.

The mean value (±SD) of (Ln) fasting SI measured in hyperglycemic group was significantly higher than those measured in the normoglycemic group (2.19±0.32 vs. 1.96 ±0.22 m/s, p = 0.005) [Figure 18]. The observed difference was borderline after adjustment for age as the main potential confonder (p=0.05). SI measured in fasting state was significantly correlated with age (r = 0.78, p<0.001), W/H ratio (r = 0.47, p = 0.001), SBP (r = 0.60, p < 0.001), DBP (r = 0.55, p<0.001), total cholesterol (r = 0.32, p=0.04) and fasting plasma glucose (r=0.57, p<0.001).

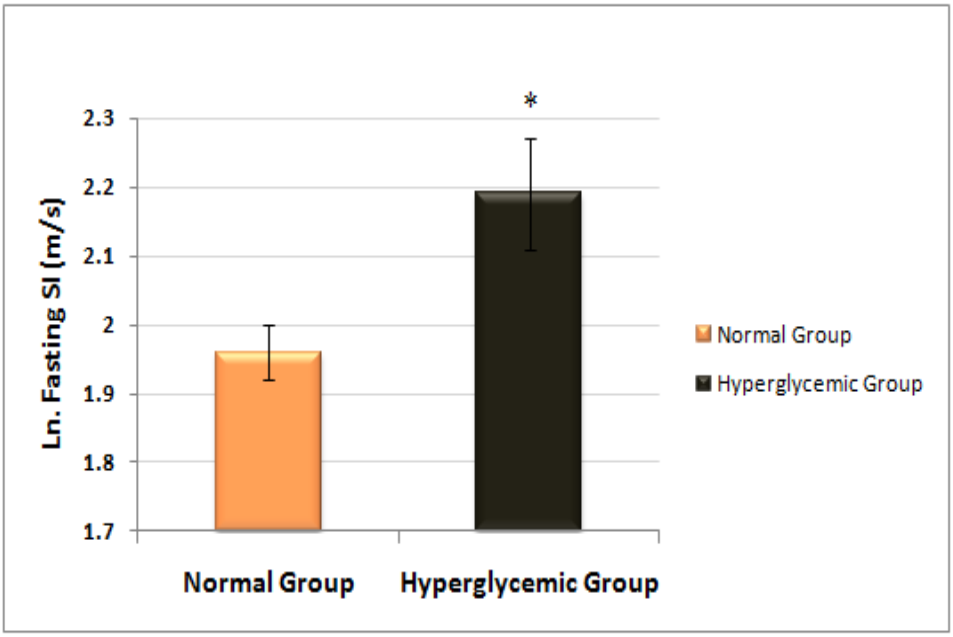


Figure 18. Comparison between (Ln) Fasting SI measured in hyperglycemic and normal groups. (*Ln) Fasting SI measured in hyperglycemic group was significantly higher than those measured in the normoglycemic group (p=0.005). Data are presented as mean ± SEM.*

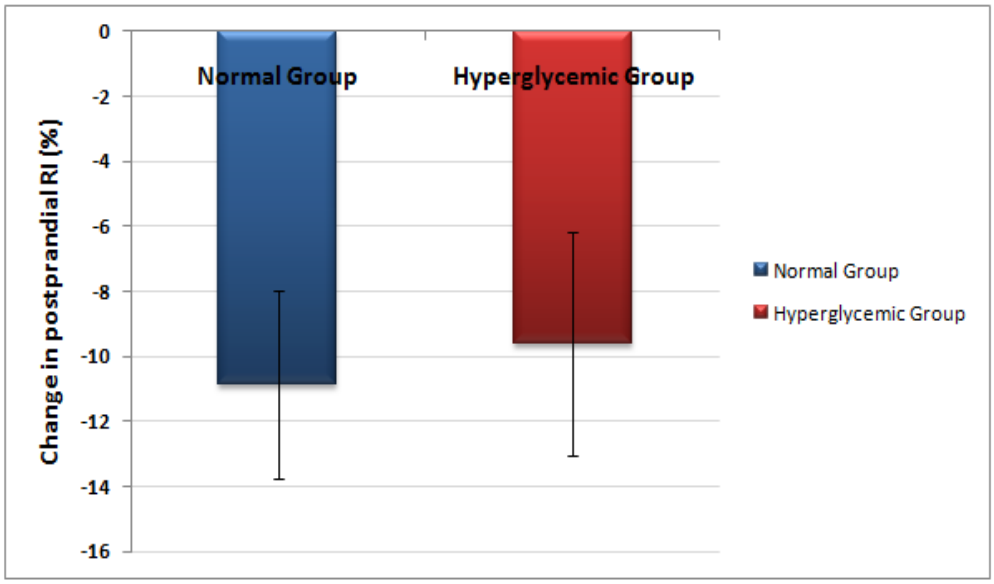


Figure 19. Change in postprandial RI observed in hyperglycemic and normal groups. *Hyperglycemic subjects had tendency to a smaller change in the post-glucose challenge RI values, when compared with normoglycemic group. Data are presented as mean ± SEM.*

Subjects with hyperglycemia also had a higher fasting RI (71.62% ±10.62 vs. 68.96% ±10.50, p=0.42) relative to normoglycemic individuals, although it was not statistically significant. There was also a weak but significant correlation between fasting RI and age ($r = 0.34$, $p<0.05$).

Furthermore, hyperglycemic subjects had tendencies to a smaller change in SI (-0.37 ± 2.93 vs. -0.80± 1.90 m/s, p=0.56) and RI (-9.61% ± 14.15 vs. -10.87% ±14.89, p=0.78) values following the glucose callenge, when compared with normoglycemic group [Figure 19].

Subjects participating in this study were also classified on the basis of HOMA score. According to our data, there were trends toward higher (Ln) fasting SI (2.22 ± 0.24 vs. 2.01 ± 028 m/s) and fasting RI (73.14 % ± 8.08 vs. 69.39% ± 10.89), associated with bunted changes in postprandial SI (-0.21 ± 2.7 vs. -0.72 ± 2.3 m/s) and RI (-10.26% ± 15.50 vs. -10.41% ± 14.48) in the insulin resistant group (n=7) relative to subjects without insulin resistance (n=37).

Assessment of thiamin status

In the present cross-sectional study, dietary intake of thiamin was estimated by a validated semi-quantitative food frequency questionnaire (Anti-Cancer Council, Victoria). There was no significant difference between the mean (± SD) of thiamin intake in normal subjects and those who were diagnosed with hyperglycemia (1.37±0.72 vs. 1.46±0.51 mg/day, p=0.56) [Figure 20]. Also, there were no significant difference between females and males in thiamin intake (1.29±0.62 vs. 1.54±0.66 mg/day, p=0.13). Thiamin intake was not significantly associated with BMI, WC, W/H ratio, SBP, DBP, FPG, 2-h plasma glucose, HOMA score, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride, hs-CRP, fasting SI and RI, and changes in postprandial SI and RI. Similarly, no significant correlations between thiamin intake and these variables found for each gender group. There was a strong and significant correlation between dietary intakes of thiamin and fibre ($r = 0.63$, $p<0.001$).

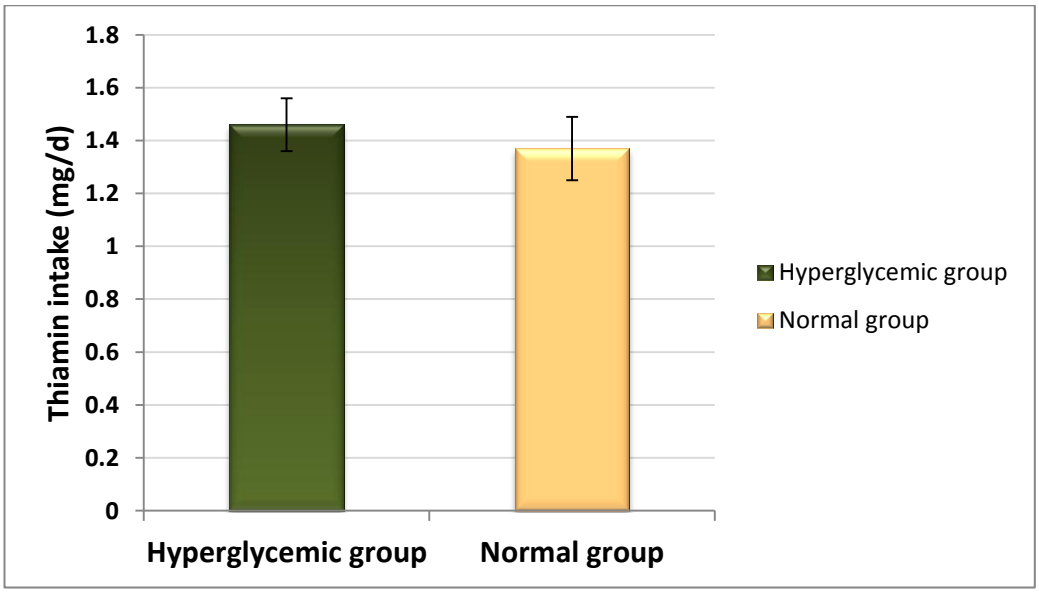


Figure 20: Dietary intake of thiamin in hyperglycemic and normal groups. *There was no significant difference between thiamin intake in normal subjects and hyperglycemic group (p=0.56). Data are presented as mean ± SEM.*

In this study, it was planned to assess thiamin status by measuring thiamin in urine, plasma and RBC samples. However, we encountered technical problems in establishing the HPLC methods for this analysis at Curtin University. Therefore, only the results of RBC thiamin (which were assayed at RPH) are presented.

No significant difference was observed between the mean (± SD) of RBC thiamin levels measured in hyperglycemic and normal group (159.76±29.08 vs. 142.59±39.58 nmol/L, p= 0.07). Also, when RBC thiamin levels were converted to nmol/g hemoglobin, no significant difference was found between the hyperglycemic and normal groups (0.95±0.17 vs. 0.88±0.24 nmol/g Hb, p=0.22) [Figure 21]. The levels of RBC thiamin reported as nmol/g Hb were strongly associated with those reported as nmol/L (r=0.95, p<0.001). RBC thiamin level (nmol/g Hb) was not significantly correlated with BMI, WC, W/H ratio, SBP, DBP, HOMA score, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride, fasting insulin, hs-CRP, 2-h plasma glucose, 2-h insulin, urinary albumin/creatinine ratio, , fasting SI and RI, changes in postprandial SI and RI, and thiamin intake [Figure 22]. There was a weak but significant correlation between RBC thiamin level (nmol/g Hb) and FPG (r=0.25, p=0.049).

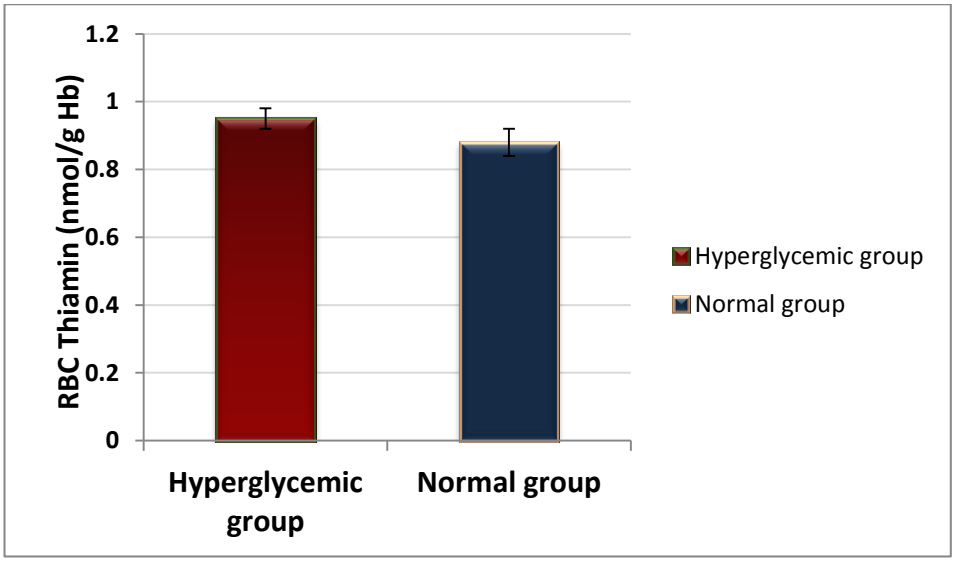


Figure 21: RBC thiamin levels in hyperglycemic and normal groups. *No significant difference was observed between RBC thiamin levels measured in hyperglycemic subjects and normal group (p= 0.22) Data are presented as mean ± SEM.*

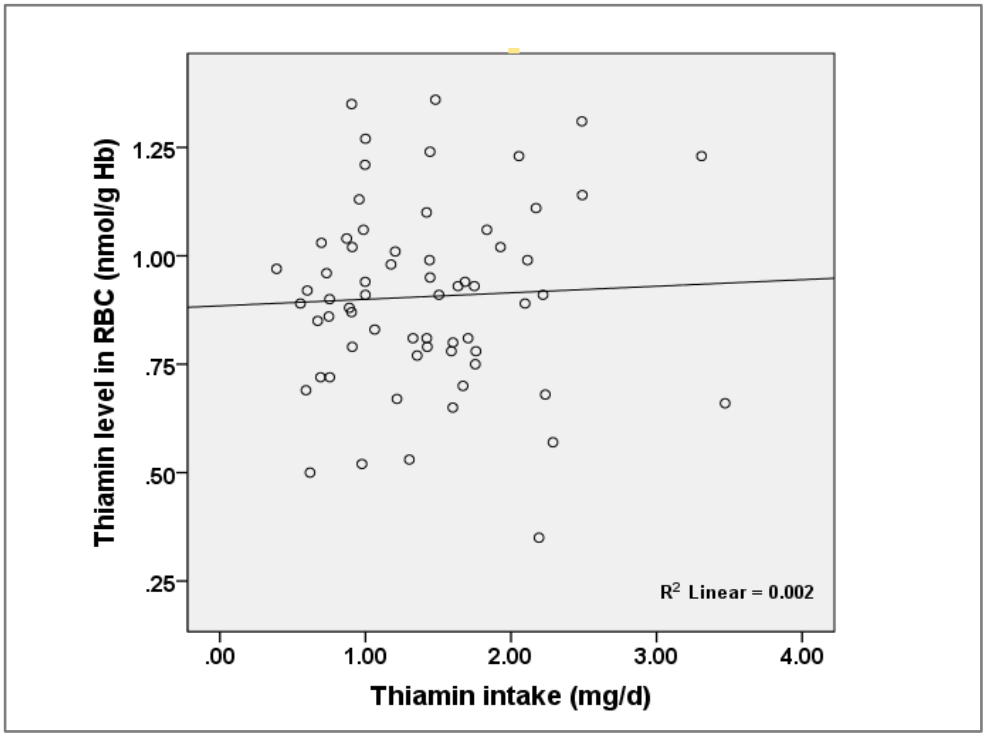


Figure 22: Scatter plot of RBC thiamin levels and dietary intake of thiamin. *No significant correlation was observed between RBC thiamin levels (nmol/g Hb) and thiamin intake (mg/d) (r=0.046, p=0.72).*

3.6 Discussion

Comparison of methods used for assessment of glucose tolerance

Glucose concentration can be measured in plasma or whole blood. Concentration of glucose measured in whole blood is generally lower than its pertinent plasma (Holtkamp, Verhoef, and Leijnse 1975; Kempe et al. 1997), as confirmed by this study. This is mainly due to a higher content of proteins (i.e. hemoglobin) and cellular elements in whole blood, and a concomitant decrease in the water portion (Dungan et al. 2007; Marks 1996). Several factors have been shown to influence blood glucose concentrations in different sample types, including patients variables (i.e. feeding, hematocrit, temperature and drugs) and the method of measurement (Dungan et al. 2007; Haeckel et al. 2002; Oberg and Ostenson 2005; Smith and Kilpatrick 1994). The significant difference detected in this study as well as a clinically poor agreement between plasma and venous whole blood values are in line with the finding of other authors who recommended avoiding the use of glucose concentrations measured in the two systems interchangeably (D'Orazio et al. 2005; Dungan et al. 2007).

The Bland-Altman graphs [Figure 16] indicate that the difference scores for blood glucose measured in two sampling systems proportionally increased with the glucose concentrations. In the present study, the measured fasting and 2-h PG glucose concentrations were limited to the described ranges. Hence, it is not possible to discuss the impact of sampling type on detecting hyperglycemia beyound the mentioned values.

Given the discussed discrepancy, the use of fixed factors for converting glucose concentrations from one sampling system to another has been suggested by some investigators (Cederholm and Wibell 1990; D'Orazio et al. 2005; Dungan et al. 2007; Stahl and Brandslund 2003). These conversion factors are determined based on the water content of different compartments, or derived from the statistical analysis (ratio of mean values or regression lines equation) of glucose concentrations measured in different systems (Haeckel et al. 2002).

Accordingly, considerable variation in suggested conversion factors may be attributable to a large discrepancy between the mean values of plasma/whole blood glucose ratio reported in previous studies (Holtkamp, Verhoef, and Leijnse 1975; Ingram et al. 1971; Lind et al. 1972; McDonald, Fisher, and Burnham 1964; Morrison and Fleck 1973; Tustison, Bowen, and Crampton 1966). To provide harmonised results, IFCC recommended a constant factor of 1.11 for converting whole blood glucose concentrations to equivalent plasma values, as was used here.

The significant difference between glucose concentrations measured in venous plasma samples and the converted values observed in this study, together with a relatively wide limit of agreement, indicate that the conversion of glucose concentrations using a fixed factor may result in unpredictable rates of clinical misinterpretation. The findings of the present study are in agreement with previous studies that reject the practice of converting glucose concentration, and recommend to use a single system of venous plasma for all glucose measurements (Holtkamp, Verhoef, and Leijnse 1975; Morrison and Fleck 1973; Wiener 1995). Holtkamp et al. (1975) showed that the use of conversion factors can be helpful only when the samples are deproteinized.

In this study, glucose concentrations measured in two different sampling systems were also compared for glucose tolerance assessment, using the cut-off values recommended by WHO. According to our findings, the percentage of individuals classified as having impaired glucose tolerance or diabetes using venous whole blood was higher compared to venous plasma (χ 2 = 22.002, $p < 0.001$), and evaluating glucose tolerance using venous whole blood values caused misclassification in up to 25% of the GTTs. In agreement with this, Neely et al. (1991) found that, based on the WHO criteria, the use of venous whole blood values may give a higher incidence of gestational impaired glucose tolerance relative to venous plasma glucose.

By contrast, in an epidemiological survey conducted by Farrer et al. (1995), no significant difference was reported in the proportion of individuals classified as having impaired glucose tolerance or diabetes using venous whole blood glucose, when compared with venous plasma glucose. The inconsistency between the findings of Farrer et al. and the present study may be explained in part by the difference in the

method of glucose measurements. In the present study venous whole blood samples were analysed by means of a blood glucose meter (Accu-Chek Active, Roche Diagnostics), wheras in the study carried out by Farrer et al, venous whole blood samples were assayed using a bench top analyser. The Accu-Chek device used for this study was a glucose dehydrogenase-based meter, measuring glucose concentration in whole blood sample by the photometric metod. According to the information provided by the manufacturer, the Accu-Chek glucose meter works acceptably with both venous and capillary blood samples (Roche Diagnostics Corporation 2001). However, in general the applicability of glucose meters for detecting glucose intolerance is still a matter of debate (Tonyushkina and Nichols 2009).

At present, fasting plasma glucose and 2-h OGTT are recommended by WHO and IDF to identify all states of hyperglycemia (World Health Organization and International Diabetes Federation 2006). Also, for standardisation in Australia, venous plasma glucose was chosen as a recommended method of diagnosing diabetes and impaired glucose regulation state (IFG, IGT) by the Australian Diabetes Society (ADS)(Diabetes Australia Western Australia 2005; Welborn 1996). Moreover, according to the guidelines provided by National Health and Medical Research Council (NHMRC), venous plasma glucose measurements should be performed by an accredited laboratory, and blood glucose meters do not have sufficient accuracy required for definitive diagnosis (National Health and Medical Research Council 2001; Poirier et al. 1998).

Anthropometric and metabolic factors

Based on the results of the present study, subjects with impaired glucose regulation were significantly older than healthy group. This was in line with previous studies, indicating that the prevalence of abnormal glucose tolerance and diabetes increases with age (Cowie et al. 2006; DECODE Study Group 2003). Additionally, in this study the mean of HOMA score (a measure of insulin resistance) increased with age. In keeping with this, the increased risk of hyperglycemia with age is thought to be mainly attributable to a decline in insulin-mediated glucose uptake by peripheral tissue, and a delay in insulin-induced suppression of hepatic glucose production

(Couet et al. 1992). Bryhni et al. (2010) also suggested a reduced secretion of insulin as the possible mechanism, which was not confirmed by our study (see table 13). The increased insulin levels showed in the current study could be as a result of compensatory hyperinsulinemia induced by insulin resistance. Consequently, changes with age could be due to a reduced capacity of pancreatic beta cells to secrete adequate insulin to compensate for insulin resistance, rather than the absolute decrease in insulin secretion.

Hyperglycemic subjects participting in the current study also had a higher BMI relative to the normoglycemic group, although it was not signifiant. This tendency became significant when two groups were compared for the indices of abdominal obesity (waist circomfrence & W/H ratio). These findings confirm several studies showing a relationship between obesity and various cardiovasular risk factors including: hypertension (Berchtold et al. 1981; Blair et al. 1984), dyslipidemia (Garrison et al. 1980; Olefsky, Reaven, and Farquhar 1974) and in particular hyperglycemia (Hartz et al. 1983; Stern and Haffner 1986). This risk is known to be more significant in the presence of central obesity (National Institutes of Health 1998). Moreover, in agreement with our study, excess adipose tissue has been shown to induce the production of cytokines such as CRP, suggesting that it contributes to the inflammatory atherogenic process independent of its effects on lipoprotein metabolism or insulin sensitivity (Yudkin et al. 1999). CRP is accepted as the strongest non-lipid predictor of cardiovascular events (Ridker, Hennekens, et al. 2000), including myocardial infarction (Ridker et al. 2002), stroke (Ridker et al. 2002; Ridker 2003), cardiac sudden death (Albert et al. 2002) and peripheral artery diseases (Ridker et al. 2008), particularly in patients with diabetes mellitus (Linnemann et al. 2006; Pfutzner and Forst 2006).

Hyperglycemic subjects participating in this study also had a significantly higher HOMA score compared to the normoglycemic group. There were also significant correlations between HOMA score and indices of central obesity. These results are in agreement with other studies (Despres 1993; Jellinger 2007; Westphal 2008) which indicate that abdominal obesity may play a key role in the pathogenesis of hyperglycemia via enhancing insulin resistance.

In this study, we also observed that not all of hyperglycemic subjects were IR as estimated by HOMA. This could be partly because of the method used for the assessment of insulin sensitivity. There are several measures of insulin resistance (DeFronzo, Tobin, and Andres 1979; Galvin et al. 1992; Harano et al. 1977; Matthews et al. 1985). Of these, hyperinsulinemic euglycemic clamp is considered as the gold standard method to quantify insulin resistance (American Diabetes Association 1998). Given that glucose clamp technique is difficult, time consuming and expensive, homeostasis model assessment (HOMA) of insulin sensitivity was proposed as an acceptable alternative measure of insulin resistance (Bonora et al. 2000). However, HOMA measures liver resistance rather than peripheral resistance (Bonora et al. 2000).

In the present study, more hyperglycemic subjects had microalbuminuria than normal group. This was accompanied by raised levels of serum lipids and blood pressure. Consistent with these results, impaired glucose metabolism was shown to be strongly asscociated with other lipid (Zhang et al. 2009) and non-lipid (hypertension, obesity and microabuminuria) risk factors (Deedwania 2011; Zelmanovitz et al. 2009). Consequently, to improve cardiovascular risk prediction, these risk factors are generally considered as a cluster, what is now known as the "metabolic syndrome". Statistical data indicate that metabolic syndrome occurs in approximately one out of four of the world’s adult population and those who have it are three times as prone to having a heart attack or stroke, compared with people without the syndrome (Alberti, Zimmet, and Shaw 2006; International Diabetes Federation 2006).

According to our results, there was a significant correlation between metabolic syndrome and insulin resistance. Also, subjects with metabolic syndrome had significantly higher BMI, WC and W/H ratio relative to those who were without metabolic syndrome. These findings are in line with previous studies suggessting insulin resistance and obesity (specifically abdominal obesity) as the main underlying contributors to the development of metabolic syndrome (Despres et al. 2008; Despres 1993).

Arterial stiffness was another risk factor evaluated in the present study. According to our results, fasting SI was correlated more strongly with age, while there was a weak

relationship between fasting RI and age. In agreement with this, Millasseau et al. suggested SI as a more reliable index for evaluating vascular aging than RI (Millasseau et al. 2003). Furthermore, Woodman and Watts indicate that the stiffness index derived from DVP analysis provides an estimation of large artery stiffness which is strictly linked to age-related changes (Woodman and Watts 2003).

Although arterial stiffness occurs mainly as a pathological consequence of aging (Benetos et al. 2002), other risk factors including central obesity, hypertension, dyslipidemia and hyperglycemia can accelerate its progress (Henry et al. 2003; Liu et al. 1989; Safar, Czernichow, and Blacher 2006; Salomaa et al. 1995; Schram et al. 2004; van Popele et al. 2006; Wilkinson et al. 2002; Xu et al. 2010). Hyperglycemia was shown to enhance arterial stiffness through several mechanisms (Henry et al. 2003; Salomaa et al. 1995; Schram et al. 2004; van Popele et al. 2006; Xu et al. 2010), causing endothelial dysfunction as well as alternations in the structure of vascular extracellular matrix.

In the present study, fasting stiffness index measured in subjects with hyperglycemia was significantly higher than those measured in normoglycemic subjects. This result confirms a previous study indicating that hyperglycemia even in pre-diabetic ranges can augment arterial stiffness (Henry et al. 2003). It was also consistent with the findings of Gunarathne et al. (2008) who designated SI derived from digital volume as a potent discriminator of increasing cardiovascular complications in high- risk subjects including diabetics. The prevalence of hyperglycemia increases with age (Australian Bureau of Statistics 2006; Dunstan et al. 2001), which is also the main determinant of arterial stiffness, as discussed above. Accordingly, in the present study, higher fasting SI value observed in hyperglycemic subjects could be partially explained by the age difference between the two groups. It was shown by the increase in the p-value after adjustment for age (see Section 3.5).

We reported that hyperglycemic subjects had also a higher fasting RI value relative to normoglycemic subjects. The difference in fasting RI value between the two groups was not statistically significant, but it may be considered significant from a clinical view.

RI is mainly related to the tone of small arteries which can be altered in the presence of hyperglycemia (Gunarathne et al. 2009; Rizzoni, Porteri, Guelfi, Muiesan, Valentini, et al. 2001; Rizzoni, Porteri, Guelfi, Muiesan, Piccoli, et al. 2001; Rosei and Rizzoni 2010). Nitric oxide released from endothelium can influence smaller arterioles and branch vessels more than larger arteries, due to a thinner media of smaller vessels (Cohn 1999; de Koning and Rabelink 2002). RI was also shown to be a reliable parameter for evaluating the effect of different vasoactive substances, such as glyceryl trinitrate, angiotensin II and salbutamol (Chowienczyk et al. 1999; Gopaul et al. 2001; Millasseau et al. 2003). However, there is no published literature so far to show the changes in RI after a glucose challenge in subjects with hyperglycemia.

The effect of oral glucose (usually 75 g administrated in a standard OGTT) on endothelial function has been mainly assessed by the change in the flow-mediated vasodilation (FMD). FMD was shown to decrease within 1 hour after ingestion of the glucose load by healthy subjects as well as patients with IGT and diabetes (Akbari et al. 1998; Kawano et al. 1999). However, the change in the post-prandial FMD was more significant in diabetic or impaired glucose tolerant patients, compared to subjects with normal glucose metabolism.

Several mechanisms are thought to account for the temporary impairment of endothelium-dependent vasorelaxation observed following an oral glucose challenge, including generation of oxidative stress (Kawano et al. 1999) and free radicals (Title et al. 2000), a transient loss of NO bioavailability (Title et al. 2000), and a reduction in antioxidant defence systems (Ceriello et al. 1998).

According to our data, the insulin resistant group had tendencies toward higher SI and RI measured in fasting state, accompanied by reduced changes in postprandial responses, compared to individuals without insulin resistance. Consistent with these results, Gopaul et al. (2001) showed that in diabetic patients, the blunted response of RI following inhalation of salbutamol was significantly correlated with insulin sensitivity and plasma 8-epi-prostaglandin F2 alpha (8-epi-PGF2α), a product of oxidative stress. Insulin has been suggested to cause arterial dilation through the release of NO (Scherrer et al. 1994). In the insulin resistant state, insulin-mediated

vasodilation may be impaired due to a decrease in NO bioavailability (Williams et al. 1996), occurring temporarily after ingestion of an oral glucose load.

In the present study, a tendency toward a blunted response of DVP-derived parameters (RI & SI) to the oral glucose challenge in subjects with impaired glucose metabolism (IFG & IGT) confirms an impaired endothelium-dependent vasodilation in these patients, as shown by others in the FMD assessments. Moreover, in agreement with our results, Gopaul et al. (2001) reported a trend toward a reduced change in the RI derived from DVP analysis following inhalation of salbutamol in subjects with IGT, which became significant in newly diagnosed and established diabetes. Similarly, Chowienczyk et al. (1999) showed an attenuated response of RI to inhaled albuterol (salbutamol) in patients with type 2 diabetes mellitus, compared to control subjects. Given the evidence of changes in FMD following an oral glucose challenge in subjects with hyperglycemia as well as the reduction in photoplethysmographic digital volume pulse parameters under salbutamol inhalation, it can be expected that evaluating DVP analysis following an oral glucose challenge could provide additional information in the assessment of vascular function, particularly in diabetic patients. Further studies are required to confirm these findings in individuals with an increased risk of cardiovascular diseases.

Thiamin status

Hyperglycemic subjects participating in the present study had thiamin intakes similar to normoglycemic group as assessed by FFQ. The thiamin intake in both groups were greater than Australian Recommended Dietary Intakes (RDIs) (1.2 mg/d males, 1.1 mg/d females) (National Health and Medical Research Council 2006), as expected. Subsequent to mandatory fortification of bread-making flour with thiamin in Australia which started in January 1991, almost all Australians were shown to have an intake of at least the recommended values (Baghurst et al. 1996; The National Health and Medical Research Council 1990). However, RDIs are set for healthy men and women, and the adequacy of thiamin intake at these levels is still a matter of debate about patients with diabetes (Vindedzis et al. 2008). Recent studies indicated that diabetic patients are at risk of thiamin deficiency, which is mainly due to an increased excretion of thiamin (Jermendy 2006; Thornalley et al. 2007).

In our study, the level of RBC thiamin in hyperglycemic subjects was not significantly different to the normoglycemic subjects. Also, the means of RBC thiamin in both groups were in the normal range (Schrijver 1991), although they were less than the values [mean value of 433 ng/g Hb (1.44 nmol/g Hb), with 95 % reference interval of 290-590 ng/g Hb (0.93-1.96 nmol/g Hb)] reported in another study using the same method (Talwar et al. 2000).

The results of the current study are in line with the findings of Thornalley et al. (2007) who showed a normal concentration of RBC thiamin in diabetic patients (normal volunteers 0.318±0.028 pmol/mg Hb; type 1 diabetes 0.345±0.025 pmol/mg Hb; type 2 diabetes 0.328±0.137 pmol/mg Hb), despite a significant increase in their urinary thiamin excretion (fourfold and threefold in type 1 and type 2 diabetic subjects relative to normal participants). These authors found that the normal level of RBC thiamin in diabetic patients was accompanied by significant increases in erythrocyte membrane thiamin transporters THTR-1 and RFC-1, normal activity of erythrocyte transketolase, and severe decreases in plasma thiamin (76% and 75% in type 1 and type 2 diabetic subjects, respectively). This brings into question the recommendation of the concentration of TDP in erythrocytes as the most sensitive indicator of thiamin status (Baines and Davies 1988; Brin 1964; Howard 2000; Talwar et al. 2000). Thornalley et al. (2007) suggested plasma thiamin for evaluating thiamin status in diabetic patients, because erythrocyte thiamin levels and also the erythrocyte transketolase activity may be influenced by the increased levels of thiamin transporter protein.

Measurement of plasma thiamin is less frequently used to evaluate tissue storage, because, contrary to erythrocyte thiamin (Brin 1964), the extracellular concentration of thiamin can be influenced largely by the recent intake. Also, approximately 10% of total blood thiamin is found in the plasma and 80% is in erythrocytes (Talwar et al. 2000). Therefore, plasma thiamin concentration is difficult to assay precisely without being contaminated by the leakage of thiamin from the blood cells during the preparation of samples (Bates 2009). With respect to these facts and also the findings of Thornalley and his colleagues (2007), whole blood may be the preferred specimen for thiamin assessment in individuals who are at risk of thiamin deficiency, including diabetics.

It was not possible to evaluate thiamin urinary excretion and plasma status in our participants. However, evidence indicates that hyperglycemia even in non-diabetic ranges is associated with some degrees of renal injury (Gabir et al. 2000; Metcalf et al. 1993; Pan et al. 1993), as confirmed in our study. Therefore, it can be predicted that patients with impaired glucose regulation (IFG, IGT) would be also prone to thiamin deficiency as judged by plasma levels, due to increased urinary excretion. Further analysis of plasma and urinary thiamin is required to confirm this prediction in patients with pre-diabetic hyperglycemia.

In the current cross-sectional study, there was no significant correlation between RBC thiamin and thiamin intake. This could be partly due to the use of FFQ for estimating dietary intake of thiamin. FFQs are useful tools for ranking individuals' usual dietary intakes. However, they may not be adequately accurate to determine the absolute intakes of energy and nutrients. The lack of correlation may also be as a result of the likely renal damage in some hyperglycemic subjects and subsequent variation in the loss of thiamin.

In this study, thiamin intake was not significantly associated with other measurements of this study including the plasma glucose determined in postprandial state. In contrast with our results, Bakker et al. (1998) reported a strong correlation between thiamin intake and 2-h plasma glucose in the Hoorn Study. They hypothesised that the effect of fibre consumption on glucose tolerance is in part attributable to concomitant intake of thiamin. The Hoorn Study consisted of Caucasian men and women aged 50-74 years, with a mean (±SD) dietary thiamin intakes of 1.2±0.3 and 1.1±0.3 mg/d, respectively, assessed using a semi-quantitative food frequency questionnaire (Grootenhuis et al. 1995). In that study, the intake of dietary fibre was reported as 28.7±8.7 g/d in men and 26.2±6.9 g/d in women, which were greater than those observed in the present study (23.2±10.3 g/d in men, 20.8±8.1 g/d in women). The inconsistency between the findings of Bakker et al. and our study may be explained partly by the considerable difference in the number of subjects participating in the Hoorn Study (n=2196) and the present study (n=64).

3.7 Conclusion

In addition to diabetics, patients with the pre-diabetic range of hyperglycemia have been proposed to be prone to thiamin deficiency, due to increased urinary excretion. This hypothesis was not accepted in this study, when thiamin status of hyperglycemics was compared with normal subjects, based on the erythrocyte thiamin levels. On the other hand, we cannot reject it completely. Measurement of erythrocyte thiamin has already been recommended as the most sensitive indicator of thiamin status in individuals who are at risk of thiamin deficiency; however it may be less useful in patients with hyperglycemia, due to the possibility of changes in the levels of thiamin transporter proteins. Because of some technical limitations, it was not possible to analyse thiamin urinary excretion and plasma levels of our participants in this study. Further evaluation of plasma and urinary thiamin is needed to make better judgment about thiamin status in patients with impaired glucose regulation. This gives us an opportunity to extend our investigation in the future.

In the current study, a subgroup of subjects was also evaluated for arterial stiffness via a Pulse Trace device. According to our findings, hyperglycemic subjects had higher levels of fasting DVP parameters (SI & RI), accompanied with tendencies toward blunted response to ingested glucose load relative to normoglycemic group. These results suggest that screening of individuals with hyperglycemia by using a Pulse Trace machine may be a means of recognising cardiovascular complications at early stages. Further research with a larger sample size is recommended to extend these interesting results.

The results of this study also confirm that the use of different sampling systems as well as converting glucose concentrations from one system to another using fixed factors may lead to misclassification of hyperglycemia, particularly in cases where the blood samples are analysed by glucose meters. Glucose meters may be helpful to obtain an idea about patients' blood gluose or for an inital screening of hyperglycemia. However, glucose concentrations determined in venous plasma by a qualified laboratory should be consided in situations requiring an accurate glucose measurement for definite diagnosis of glucose intolerance or diabetes mellitus, or for making treatment decisions.

4. Intervention study

As discussed previously (sections 2.5.9 & 2.5.10), in vivo studies suggest that thiamin supplementation can inhibit certain biochemical pathways leading to abnormalities associated with hyperglycemia (Berrone et al. 2006; La Selva et al. 1996; Thornalley, Jahan, and Ng 2001). These findings were supported by some experiments undertaken in animals (Babaei-Jadidi et al. 2003; Babaei-Jadidi et al. 2004; Oishi et al. 2002). For example, Babaei-Jadidi et al.(2003) showed that thiamin supplementation improves cardiovascular risk factors in diabetic rats. Despite these findings, there is limited literature investigating the effects of thiamin on metabolic outcomes in humans, particularly among individuals with hyperglycemia at early stages. In a clinical study investigating the effects of thiamin on hyperglycemia in humans, supplementation with thiamin decreased the fasting plasma glucose of diabetic patients with HbA_{1C} level < 8%, who were not treated with medications (Gonzalez-Ortiz et al. 2011). Thiamin supplementation was also shown to improve glucose tolerance in patients with hepatic cirrhosis (Hassan, Qureshi, and Zuberi 1991). To date there has been no published study to examine the effects of thiamin supplementation on glucose tolerance in individuals with impaired glucose metabolism.

4.1 Study design

A randomised, double blind, cross-over design study investigated whether in hyperglycemic adults, chronic consumption of thiamin improves glucose tolerance and some cardiovascular disease risk factors. The study design is illustrated in Figure 23. Hyperglycemic subjects were randomly allocated into two groups (Groups A and B) by the investigator (Dr. Fariba Alaei), to consume either 100 mg (2 × 50 mg) thiamin hydrochloride or placebo three times a day (300 mg/d) before meals for six weeks (Part 1). Cardiovascular disease risk factors were assessed at weeks 0 (baseline), 3 and 6. Following completion of the first part and a 14-week wash out period, subjects came back to receive alternative capsules for another six weeks (Part 2). Subsequently, clinical measurements were repeated on another three separate visits at weeks 0, 3 and 6, according to the same protocol as the first part.

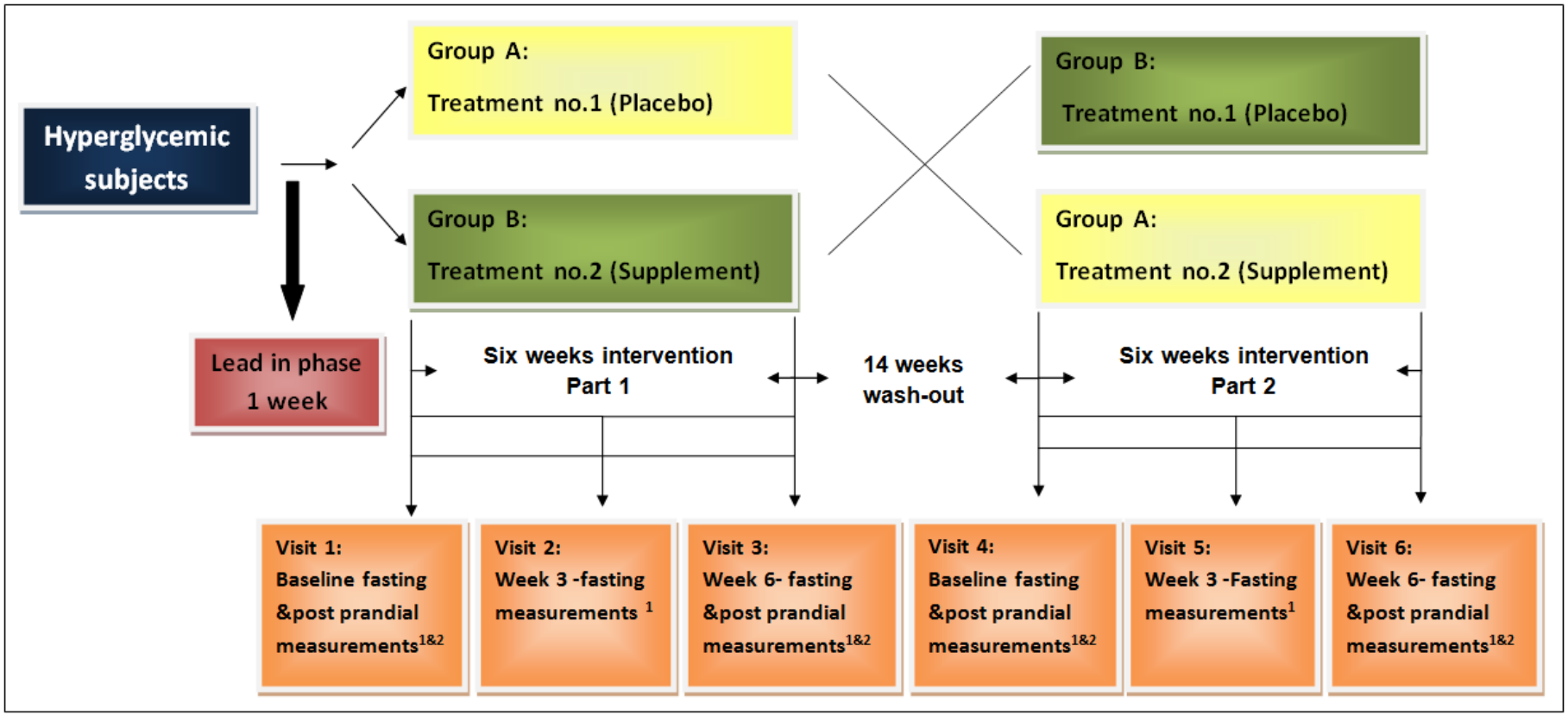


Figure 23. Intervention study- study design

1. Fasting measurements: anthropometric measurements, BP, fasting indirect calorimetry (used for predicting REE), urinary albumin, urinary urea and creatinine (used for estimating urinary nitrogen), plasma and venous whole blood glucose, plasma insulin, serum total cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride, hs-CRP and creatinine (used for estimating eGFR), and RBC thiamin (except in visits 2&5)

2. Post prandial measurement: post prandial indirect calorimetry (used for predicting post glucose load EE), urinary urea and creatinine (used for estimating urinary nitrogen), 2-h plasma and venous whole blood glucose, 2-h plasma insulin

The allocation of treatments to Groups A and B was done by a person who did not know anything about the subjects or their data. Thus, the randomisation was double blind.

4.2 Subjects

Subjects were recruited as described for the cross - sectional study (see Section 3.2). The eligible subjects diagnosed as glucose intolerant during the cross-sectional study, were invited to participate in the intervention study as well. Seventeen of them agreed to participate.

Volunteers were asked to complete the online screening questionnaire available on the web site of Curtin University. The prospective subjects who had no access to the internet were screened by using a telephone screening tool [Appendix 2]. Volunteers were included according to the criteria considered for the cross - sectional study, and if they could be available during the period of the study.

Subjects who were taking supplements containing thiamin (i.e. multivitamins supplements) or consuming more than 2 standard alcoholic drinks per day were asked to cease the supplement and reduce the alcohol intake during their participation, starting 4 weeks before attending the first clinical day. One subject on long-term medication (Allopurinol) with no impact on the endpoints of the study was allowed to continue his medication.

The power calculation was based on the predicted change of 20 % in blood glucose level measured 2 hours after ingestion of glucose syrup in an OGTT (Hassan, Qureshi, and Zuberi 1991). A sample of 12 subjects in a cross-over design provides sufficient power (95%) to detect the predicted change in 2-h plasma glucose at a 5% significance level.

For the intervention study, a total of seventeen subjects (7 males and 10 females) were recruited to allow for drop out/non-compliance.

Participation in this study was completely voluntary and subjects were informed that they were able to withdraw from the study at any point in time without prejudice. This study was approved by the Curtin University Human Research Ethics Committee (Approval number HR 161/2008) and a written consent form [Appendix 9] was obtained. This trial was registered on the Australian New Zealand Clinical Trials Registry (ACTRN12611000051943).

4.3 Methods

4.3.1 Preparation and characterization of capsules

The thiamin supplement and placebo were provided in the form of capsules with similar physical characteristics [Table 15], and distributed in bottles of same shape and colour.

Table 15. Specifications of prepared thiamin and placebo capsules

Capsule Specifications	Thiamin Capsule	Placebo Capsule
Colour (cap/body)	White/White	White/White
Closed length (mm)	15.40 - 16.00	15.40 - 16.00
External diameter (mm)		
Cap	5.84 - 5.87	5.84 - 5.87
Body	5.59 - 5.62	5.59 - 5.62
Capsule weight (mg)		
Mean(SD)	246.96(9.81)	229.60 (15.66)

To prepare supplement capsules, tablets of vitamin B1 (Betamin, Sanofi-Aventis Australia Pty Ltd., Australia) were ground into a powder using a coffee grinder. The vitamin tablets contained thiamin hydrochloride as active component as well as inactive ingredients of starch (wheat) and lactose. The vitamin B1 powder was then put into the empty white/white gelatine capsules (Capsuline®Inc, FL, USA) [Figure 24] using a capsule filler machine (Capsuline®Inc, FL, USA), according to the manufacturer's instructions. The prepared thiamin capsule had a mean (± SD) weight of 246.96 ± 9.81 mg, containing 50.74 ± 2.46 mg thiamin hydrochloride with a CV of 4.84% in random samplings (n=25).

Figure 24. The used white/white gelatine capsules

The capsules provided as control were matched with the supplement, containing the inactive ingredients of thiamin capsules. Accordingly, for preparation of

placebo, the same empty gelatine capsules were filled with a mixture of wheat starch (Nursesfoods Pty Ltd., WA, Australia) and lactose (Ramprie Laboratories, WA, Australia) powders, with a ratio of 1:1.

4.3.2 Introductory visit

After an initial screening, eligible subjects were invited to attend a short introductory visit. This visit occurred before commencement of the study to explain the study's procedures and protocol in detail and how to complete a weighed food record for three consecutive days before baseline and week 6 clinical visits (see Section 4.3.8), and to obtain written consent [Appendices 9&10].

During the introductory visit, participants were also given a standard light-resistant container to collect their urine for 24 hours, starting the day before the first clinical day. A light-resistant urine container was provided because of the light sensitivity of thiamin (Interdepartmental Committee on Nutrition for National Defense 1963). Acid was not added to the container as a preservative because this urine was used to determine albumin excretion. Thus, subjects were instructed to keep the container refrigerated (or in an esky with ice) during the collection. Subjects were instructed to discard their first urine of the day and record the time on the container label. From then, they should collect all urine for the next 24 hours, including the first urine of the following day (clinical day) at the same time as the recorded commencement time [Appendix 5].

At the completion of the introductory visit, subjects were supplied with a standard frozen meal to consume the evening before attending in the first clinical visit (see below).

4.3.3 Meal standardisation

Participants were asked to follow an unrestricted carbohydrate diet of at least 150-200 g per day for the three days before each the clinical visits [Appendix 5]. Additionally, on the evening before each of these six clinical visits, they were required to consume the standard meal provided. As described in Section 3.3.2, these were to ensure subjects were consuming a reasonable amount of carbohydrate prior

to the glucose tolerance test. Meal standardisation was also administrated to minimize variation in the composition of meals consumed by subjects on the evening before measurement. It is known that the composition of meal consumed the day prior to the test can affect the lipid profile determined on the clinical day as well as resting and postprandial energy expenditure measurements (Granata and Brandon 2002; Karpe 1997). The evening meal provided was Vegetable Korma with rice (Crafty Chef Pty. Ltd., NSW, Australia), containing about 1720 kJ energy with a macronutrient composition of 68% energy as carbohydrate, 10% energy as protein and 21% energy as fat [Table 11].

4.3.4 Experimental protocol

Before each clinical visit, subjects were required to fast overnight following the standard evening meal (10-12 hours). They were also asked to refrain from alcohol and strenuous exercise for the 24 h before visits.

On the baseline (week 0) and week 6 clinical days, subjects attended at the out-patient clinic, School of Public Health, Curtin University in the morning (by 9.00 am) in fasting condition and brought their completed 24- hour urine collection. The 24-h urine volume was measured and recorded. A 30 mL sample of 24-h urine collection was then stored in separate 5 mL plain plastic vials at -80°C until analysis for albumin.

Upon arrival, participants were asked to empty their bladder and the time of urine passed was recorded. Height, weight and waist & hip circumferences were then taken with subjects dressed in a gown with no shoes and empty bladder as described in Section 3.3.3. BMI was calculated according to the standard equation (kilograms per meters squared). Subsequently, participants were asked to rest 30 minutes prior to measurements of blood pressure and energy expenditure, as well as taking fasting blood samples (see Sections 4.3.5, 4.3.6 & 4.3.7).

Following the fasting measurements, subjects were instructed to void and collect their urine (baseline urine collection). They then consumed the full bottle of a standard glucose tolerance test (GTT) drink (Glucaid: Fronine Pty. Ltd., NSW,

Australia), containing 75 g of glucose (75 g/300 mL) over a minutes. After consuming the glucose load, participants were only allowed very lightly activity, such as listening to music or reading. They were allowed to drink water either before or during the test.

Postprandial energy expenditure was measured for a period of 20 minutes before the second blood sample taken 2 hours after ingestion of the glucose solution. At the end of the GTT, participants were asked to void and collect their urine for the second time (postprandial urine collection). The times and volumes of baseline and postprandial urines passed were recorded. A 15 mL sample of each baseline and postprandial urine collection was stored in separate 5mL plain plastic vials at -80°C until being analysed for urinary urea and creatinine. Fasting and postprandial energy expenditures were calculated from measurements of oxygen consumption, carbon dioxide production and nitrogen excretion using the equation of Ferrannini (Ferrannini 1988) (see Section 4.3.6).

On the clinical visit in week 3, subjects were asked to come to the outpatient clinic in fasting condition. Height, weight and waist & hip circumferences were measured, with subjects dressed in a gown with no shoes and empty bladder. After a mandatory 30- minute resting period, fasting blood pressure and fat oxidation were measured, and a blood sample was taken as described for weeks 0 and 6.

Before completing clinical visits in weeks 0 and 3, subjects were supplied with bottles of thiamin/placebo capsules as well as the standard frozen meal for the evening prior to the next visit. Subjects were instructed to take two of the capsules, one hour before their breakfast, lunch and dinner (a total of 6 capsules per day) and return the bottles containing left-over capsules when they come back for the next clinical visit to assess subjects' compliance (see Section 4.3.9).

Additionally, at week 3, participants were given the light-resistant urine container to collect their urine for 24 hour, starting the day before the clinical visit at week 6.

Following completion of the first part and a 14-week wash out period, subjects came back to receive alternative capsules for another six weeks (second part).

Subsequently, clinical measurements were repeated on another three visits at weeks 0, 3 and 6, according to the protocol described for the first part.

4.3.5 Blood pressure measurement

Systolic and diastolic blood pressures were measured on each subject's right arm in supine position, using a vital signs patient monitor (Cardiocap II, Datex, Helsinki, Finland) with a standard cuff for adults. Three blood pressure measurements, separated with 2-minute intervals, were recorded in the fasting state and the average value was reported.

4.3.6 Energy expenditure determination

The effect of thiamin supplementation on each subject's energy expenditure was assessed by indirect open-circuit calorimetry using a Deltatrac II machine (Datex, Helsinki, Finland). The principal of operation is illustrated in Figure 25.

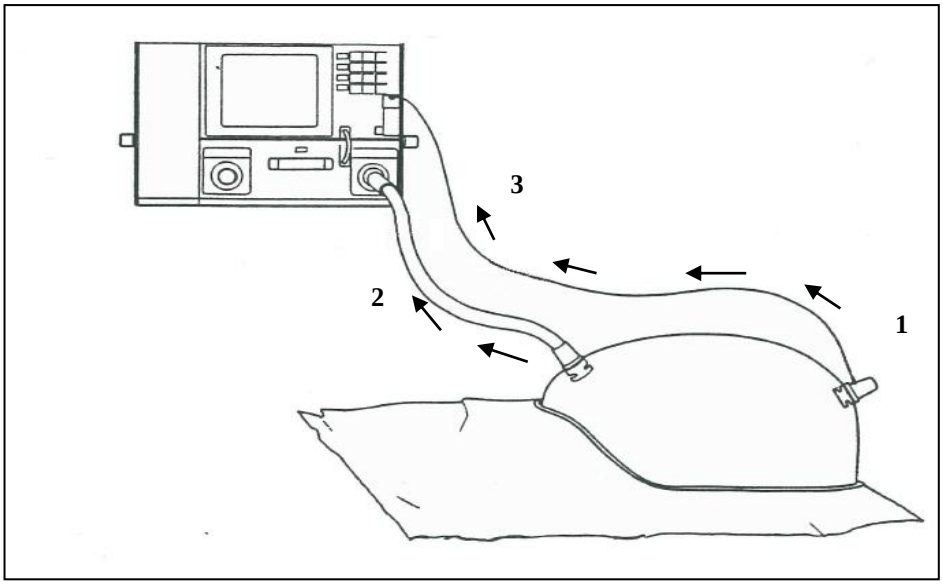


Figure 25. Deltatrac II machine used for indirect calorimetry. *The subject inhales the atmospheric air entering the canopy via tube 1. The expired gases are then transferred to the monitor by the collecting tube 2. The system also takes a sample from the atmospheric air via a separate sampling line (tube 3).*

The Detatrac Metabolic Monitor is attached to a ventilator canopy with a constant flow rate of 46.5 L/min (determined for adult patients). The subject inhales the atmospheric air entering the canopy via tube1. The expired gases are then transferred to the mixing chamber of the monitor through the canopy hose (tube 2), to be analysed for O_2 and CO_2 concentrations by a paramagnetic sensor and an infra-red analyser, respectively. The system also takes a sample from the atmospheric air via a separate inspiratory sampling line (tube 3). Following analysis of gas samples, data collected minute by minute is displayed on the monitor, showing rates of carbon dioxide production (VCO_2) and oxygen consumption (VO_2), and respiratory quotient ($RQ = VCO_2/ VO_2$).

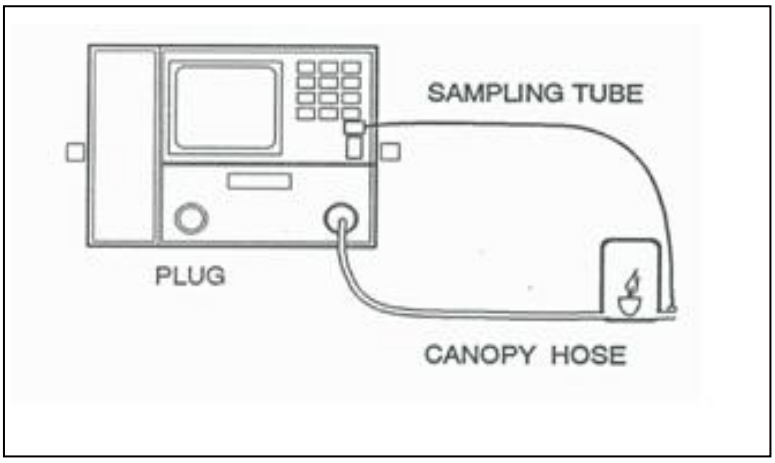


Figure 26. Alcohol burning test set up

The performance of the Deltatrac II Metabolic Monitor can be checked by analysing the gases produced from the burning of pure ethanol (Alcohol burning test) [Figure 26]. According to the manufacturer's instructions, the test should continue for at least 30 minutes. Accordingly, four alcohol burning tests were carried out intermittently during the period of the study, with the mean (±SD) RQ value of 0.66 (± 0.02) for the last 15 minutes and a CV of 3.03 %, being in the range recommended by the Datex company (0.64-0.69).

After warming up of the Deltatrac II monitor on the morning of each clinical visit, gas calibration was manually performed, using a calibration gas mixture of 95% oxygen and 5% carbon dioxide (GE Healthcare Finland Oy, Finland). The system was also calibrated for the ambient pressure using a standard barometer (Regen

Veranderlich Schon Barometer, Germany). Energy expenditure measurements were then conducted under standardised conditions (Henry 2005; Piers et al. 1992).

As previously mentioned, total energy expenditure consists of three main components: basal metabolic rate (BMR), diet-induced thermogenesis (DIT) and energy expenditure physical activity. BMR represents the minimal rate of energy required for normal body function in a resting and awake state, accounting for approximately 50% to 70% of total energy expenditure (Ravussin and Bogardus 1992). The individual's BMR is best measured just after waking in the morning, when he/she is in a post absorptive state. Where overnight facilities are not available, resting metabolic rate (RMR) is usually determined instead of BMR (Gropper, Smith, and Groff 2009). RMR is termed resting energy expenditure (REE) when it is expressed in units of kcal/24 h (Food and Nutrition Board 2002).

In the present study, RMR was measured following the initial mandatory rest and measurement of blood pressure. The Deltatrac canopy was placed over the head of the subject lying in supine position in a temperature-controlled room (22±2°C). The edges of the canopy's plastic flaps were then tucked under the pillow. The fasting measurement was continued for 30 minutes and the average for the last 25 minutes was recorded. Each subject then collected the urine before drinking the test load (baseline urine collection).

In this study, the Deltatrac II metabolic monitor was also used to assess the impact of chronic thiamin supplementation on the thermogenic effect of the glucose solution. During the clinical visits at weeks 0 and 6, postprandial energy expenditure was determined for a period of 20 minutes just before taking the second blood sample; the average for the last 15 minutes was recorded. At the end of the measurement, participants were asked to collect their urines for the second time (postprandial urine collection). The times and volumes of baseline and postprandial urines passed were recorded. A 15 mL of each baseline and postprandial urine sample was stored in separate 5 mL plain plastic vials at -80°C until analysis for urinary urea and creatinine.

REE (kcal/24 h) was predicted from measurements of oxygen consumption, carbon dioxide production and nitrogen excretion (derived from urea analysis) using the equation of Ferranni:

[(3.91× rate of O_2 consumption in L/min) + (1.1× rate of CO_2 production in L/min) - (3.34 × rate of nitrogen excretion in g/min)] × 60 (Ferrannini 1988).

In the present study, total urinary nitrogn was estimated using urinary urea nitrogen and urinary creatinine nitrogen. Urinary urea nitrogen alone accounts for 86-90% of total urinary nitrogen and the combination of urinary urea nitrogn plus urinary creatinine nitrogn accounts for 90-94.5% of total urinary nitrogen (Gibson 2005; Manore et al. 2009).

Diet-induced termogenesis (DIT) was calculated by dividing increase in energy expenditure relative to fasting value by the energy content of ingested glucose load (Westerterp 2004):

$$\frac{\text{Post glucose load EE - Fasting EE}}{\text{kcal in glucose load}} \times 100$$

4.3.7 Biomedical assessments

Fasting blood samples were collected via venipuncture into serum, heparin and EDTA vacutainer tubes (BD Vacutainer®, NJ, USA). The plasma vacutainers were centrifuged at 1100 g for 10 minutes at 4°C immediately (within minutes), while the serum tube was left at room temperature for 20-30 minutes to clot before centrifugation. Subsequently, the isolated plasma and serum samples were stored in separate 1.5 mL microcentrifuge tubes at -70 °C until analysis. The same storage protocol was administrated to the blood sample collected 2 hours after ingestion of glucose beverage for measuring plasma glucose.

After separating plasma and removing buffy coat layer, extracted RBCs were washed three times using the protocol described for the cross-sectional study phase 1 (Talwar et al. 2000) (see Section 3.3.5). The samples prepared were then aliquoted and frozen at -80 °C until analysed.

Aliquotes of plasma (separated from the blood collected into EDTA tube), serum, washed RBC and urine samples were sent to Royal Perth Hospital for analysing of thiamin, glucose, insulin, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride, hs-CRP, creatinine, and urinary albumin, creatinine and urea (see Section 3.3.6).

The results of fasting plasma glucose and insulin were also used to estimate the HOMA score, as an indix of insulin sensitivity as described for the cross-sectional study (see Section 3.3.6).

In addition to measuring glucose in plasma, fasting and postprandial glucose levels were determined in whole blood by using a blood glucose meter (Accu-Chek Active, Roche Diagnostics, NSW, Australia) as well. After taking fasting blood samples, one drop of fresh whole blood left-over in the used blood collection set was placed on the glucose meter strip. This test was repeated immediately. The average of two readings was reported.

In this study, albumin excretion was determined in the sample of 24-h urine collection. Detection of albumin in a timed urine collection and determining protein/creatinine ratio in random urine are known as acceptable methods for screening of microalbuminuria. However, measuring albumin excretion in a 24-h urine collection is still the gold standard method when more precision is required (Weber 2001). Microalbuminuria was recognised in the presence of a urinary albumin excretion $\geq$ 30 and $<$ 299 mg/24h. An albumin excretion $\geq$ 300 mg/24h represents clinical albuminuria (Molitch et al. 2004). Renal function was also assessed by estimating GFR (glomerular filtration rate), using Cockroft- Gault formula adjusted for BSA (body surface area), as described in Section 3.3.6.

4.3.8 Physical activity & dietary intake assessments

Over the course of the intervention, subjects were asked to maintain their usual diet and level of physical activity. To monitor participants' dietary habits, they were instructed to record all food and drinks consumed over three consecutive days, including two week-days and one weekend day, before baseline and week six

clinical visits [Appendix 11]. The completed 3-days food & drink diaries were then analysed using FoodWorks - Premium version 2009 [Xyris Software (Australia) Pty Ltd].

The international physical activity questionnaire (IPAQ) - short version was used to evaluate participants' physical activity levels (Booth 2000), being completed for clinical visits at baseline and week six [Appendix 12]. Data collected from IPAQ were then analysed based on both categorical and continuous scoring protocol available on the IPAQ website.

4.3.9 Compliance evaluation

As a part of the intervention protocol, on clinical visits at week 0 and 3, subjects were supplied with containers of prepared capsules for three weeks. They were instructed to take two capsules [thiamin (50 mg/capsule) or placebo], one hour before their breakfast, lunch and dinner (a total of 6 capsules per day), and return the bottles containing left- over capsules when they come back for the next clinical visit. Compliance for each part of the intervention was then evaluated using the formula below:

$$\frac{\text{Total known number of capsules provided for six weeks - Total number of left over capsules}}{\text{Total known number of capsules provided for six weeks}} \times \mathbf{100}$$

Moreover, participants were asked to tick the box related to each meal on the provided sheet [Appendix 13 - a sample of the sheet], just after taking the capsules and bring back the completed sheets on the clinical visit at week six in each part. Subsequently, subject compliances was also assessed by checking the sheets and calculated by the formula:

$$\frac{\text{The number of capsules taken}}{\text{The number of capsules expected to be taken for the certain period of six weeks}} \times \mathbf{100}$$

If there was any discrepancy between the subjects' compliances calculated though the two described ways, the lesser value was reported as the participant's minimum compliance.

4.4 Statistical analysis

The main endpoint of this study was to investigate any significant changes in 2-h plasma glucose following consumption of thiamin supplement for 6 weeks relative to control. Given the cross-over design of this study, the effects of treatments (supplement and placebo) on cardiovascular risk factors were assessed at weeks 0, 3 and 6 using a linear mixed-model analysis, with treatment, week and treatment*week interaction as fixed effects. The metabolic characteristics of each group A&B at the baseline of each treatment were compared using a paired t-test, to assess the order effect. All statistical analyses were performed using SPSS for windows (version 16, SPSS Inc., USA). Data are presented as mean ± standard deviation (SD) (in the text and tables) and mean ± standard error of mean (SEM) (in charts). All tests were two-tailed and a $p<0.05$ was considered as statistically significant.

4.5 Results

For the intervention study (cross-over design), 17 hyperglycemic subjects (7 men and 10 women) were recruited initially. Of these total numbers, four subjects (2 females and 2 males) dropped out after completing the first part (2 subjects from each group A&B), because of the time involved or starting the medication for treatment of hyperglycemia as advised by their general practitioner. Data of another subject (1 female) were excluded later, due to the lack of compliance during the study. Thus, data of 12 subjects (5 men and 7 women) were used for the final analysis, except for fasting measurements at week 3 in the supplement arm that include data of 11 subjects.

These 12 subjects consisted of 10 cases of impaired glucose tolerance (IGT) and 2 new cases of diabetes. Mean (± SD) age was 57.16 ± 12.88 years and BMI 28.85± 4.43 kg/m^2. The subjects had a mean of total cholesterol, triglyceride, HDL-chol and LDL-chol within the normal ranges, based on the National Heart Foundation (National Heart Foundation and Australian Institute of Health 1990) and the Australian Diabetes Society (Australian Diabetes Society 1994) recommendations.

Table 16. Metabolic characteristics of subjects at the baseline of treatment 1 and treatment 2 in groups A & B

	Group A			Group B		
Parameters	**Week 0. Treatment 1 Mean(SD)**	**Week 0. Treatment 2 Mean(SD)**	**P-value**	**Week 0. Treatment 2 Mean(SD)**	**Week 0. Treatment 1 Mean (SD)**	**P - value**
Weight (kg)	80.30(11.44)	81.06(11.20)	0.50	85.25(17.09)	85.15(17.89)	0.89
RBC thiamin (nmol/gHb)	0.97(0.14)	0.99(0.17)	0.53	0.89(0.16)	0.94(0.18)	0.22
FPG (mmol/L)	6.18(0.55)	6.38(0.74)	0.30	5.77(0.56)	5.66(0.63)	0.14
2-h PG (mmol/L)	9.92(2.45)	10.86(2.94)	0.18	9.20(2.10)	9.11(1.77)	0.90
SBP (mmHg)	122.80(10.43)	120.60(13.41)	0.32	120.71(17.62)	113.57(10.31)	0.10
DBP (mmHg)	71.00(7.38)	71.20(5.54)	0.94	71.57(8.96)	68.43(9.18)	0.24
Fasting insulin (uIU/mL)	5.48(3.42)	7.62(4.64)	0.03	7.17(4.59)	7.47(3.48)	0.62
2-h insulin (uIU/mL)	40.36(34.42)	62.04(58.67)	0.14	54.24(29.29)	76.34(46.28)	0.14
Total cholesterol (mmol/L)	4.92(1.02)	5.29(1.00)	0.009	4.50(0.85)	4.64(0.60)	0.35
Triglyceride (mmol/L)	1.03(0.20)	1.26(0.68)	0.37	1.17(0.72)	1.43(1.32)	0.36
Serum HDL (mmol/L)	1.24(0.22)	1.31(0.23)	0.09	1.11(0.25)	1.11(0.27)	0.97
Serum LDL (mmol/L)	3.20(1.03)	3.40(0.80)	0.35	2.83(0.63)	2.85(0.55)	0.91
hs-CRP (mg/L)	1.50(1.21)	1.98(1.54)	0.13	2.96(2.11)	6.17(6.36)	0.19
eGFR* (ml/min/1.73 m^2)	89.93(14.23)	90.94(13.58)	0.79	99.58(32.64)	103.03(40.35)	0.34
Urine albumin (mg/d)	9.01(5.60)	6.34(4.31)	0.20	11.23(13.46)	40.68(70.65)	0.33
Dietary thiamin (mg/d)	2.22(0.51)	2.11(0.89)	0.85	1.41(0.83)	1.67(1.64)	0.54

* Estimated by using Cockroft - Gaulat formula adjusted for body surface area

Table 17. Comparison between metabolic characteristics of subjects in placebo and supplement arms at the baseline (week 0)

Parameters	Placebo arm Mean (SD)	Supplement arm Mean (SD)	P-value
Weight (kg)	83.13(15.12)	83.51(14.48)	0.53
RBC thiamin (nmol/g Hb)	0.95(0.16)	0.93(0.17)	0.43
Fasting plasma glucose (mmol/L)	5.87(0.63)	6.02(0.69)	0.07
2-h plasma glucose (mmol/L)	9.45(2.01)	9.89(2.50)	0.35
Systolic blood pressure (mmHg)	117.42(10.96)	120.67(15.32)	0.24
Diastolic blood pressure (mmHg)	69.50(8.21)	71.42(7.42)	0.31
Fasting insulin (uIU/mL)	6.64(3.45)	7.35(4.40)	0.22
2-h insulin (uIU/mL)	61.35(44.07)	57.49(41.66)	0.73
HOMA score	1.75(1.01)	2.00(1.32)	0.12
Serum cholesterol (mmol/L)	4.75(0.77)	4.83(0.96)	0.44
Serum triglyceride (mmol/L)	1.27(1.00)	1.21(0.67)	0.77
Serum HDL (mmol/L)	1.17(0.25)	1.20(0.26)	0.32
Serum LDL (mmol/L)	3.00(0.77)	3.07(0.73)	0.62
hs-CRP (mg/L)	4.22(5.33)	2.55(1.88)	0.27
eGFR (ml/min/1.73 m^2)	97.53(31.72)	95.98(25.85)	0.54
Urine albumin (mg/d)	27.48(54.77)	9.19(10.58)	0.27
Dietary intake of thiamin (mg/d)	1.84(1.28)	1.69(0.93)	0.66

The subjects were randomised to two groups (group A & group B) to receive either treatment 1 (placebo) for 6 weeks followed by a 14-week washout period and treatment 2 (supplement) for 6 weeks (Group A, n=5); or treatment 2 (thiamin supplement) for 6 weeks, a 14-week washout period and treatment 1 (placebo) for 6 weeks (Group B, n=7) [Figure 23]. Accordingly, all 12 subjects received both treatment 1 (placebo arm) and treatment 2 (supplement arm) in a cross-over manner.

To assess the order effect, metabolic characteristics of subjects at visit week 0 of the first and second treatments in each group were compared, using a paired samples t-test. The results showed that except for fasting insulin (group A) and total cholesterol (group A), subjects of each group had similar metabolic characteristics at the baseline of each treatment [Table 16].

In addition, there was no significant difference between characteristics of subjects in placebo and supplement arms at the baseline [Table 17].

No adverse effect was reported following consumption of 300 mg/d thiamin supplement or prepared placebo during the intervention period. All subjects had a compliance rate of at least 88% for the offered treatments, evaluated as indicated in Section 4.3.9.

Changes in anthropometric measurements and physical activity score

As presented in the Table 18, the mean values of subjects' weight, BMI, WC and physical activity score observed at week 0 were not significantly different from those at week 6 in both supplement and placebo arms.

Changes in dietary thiamin intake and RBC thiamin level

The dietary intake of thiamin assessed at week 6 did not change significantly from that observed at week 0 in placebo (1.89±1.20 vs. 1.84±1.28 mg/d, p=0.89) and supplement (1.19±0.32 vs. 1.69±0.93 mg/d, p=0.12) arms. Following supplementation with 300 mg/d thiamin, the subjects' RBC thiamin increased from 0.93 (±0.17) nmol/g Hb at week 0 to 1.56 (±0.31) nmol/g Hb at week 6 ($p<0.001$), while there was no significant change in RBC thiamin of subjects consuming placebo (p=0.94) [Table 18].

Table 18. Anthropometric measurements, physical activity score and thiamin status assessed at week 0, 3 and 6 in placebo and supplement arms

Parameters	Placebo					Supplement					P3 [c]
	Week 0 Mean (SD)	Week 3 Mean (SD)	Week 6 Mean (SD)	P1 [a]	P2 [b]	Week 0 Mean (SD)	Week 3 Mean (SD)	Week 6 Mean (SD)	P1 [a]	P2 [b]	
Weight (kg)	83.13 (15.11)	83.55 (14.77)	83.49 (14.78)	0.38	0.88	83.51 (14.48)	84.64 (14.62)	83.31 (14.58)	0.62	0.90	0.22
BMI (kg/m^2)	28.74 (4.39)	28.89 (4.35)	28.87 (4.35)	0.35	0.89	28.88 (4.25)	29.31 (4.11)	28.80 (4.23)	0.59	0.89	0.18
WC (cm)	97.62 (10.58)	97.83 (9.86)	97.67 (10.00)	0.91	0.63	98.04 (10.39)	99.41 (9.64)	97.92 (10.53)	0.72	0.61	0.65
Physical activity score	2295.3 (1713.1)	-	2427 (1878.8)	0.67	-	2636.9 (1617.3)	-	2167.8 (1872.3)	0.13	-	0.10
Dietary thiamin intake (mg/d)	1.84 (1.28)	-	1.89 (1.20)	0.86	-	1.69 (0.93)	-	1.19 (0.32)	0.12	-	0.66
RBC thiamin (nmol/g Hb)	0.95 (0.17)	-	0.95 (0.16)	0.94	-	0.93 (0.17)	-	1.56 (0.31)	<0.001	-	<0.001

a. p value - comparison between week 0 and week 6
b. p value - comparison between week 3 and week 6
c. p value - comparison between placebo and supplement arms

Changes in plasma glucose

In the placebo arm, the mean (±SD) of fasting plasma glucose observed at week 6 was found to be significantly higher than that at week 0 (6.11±0.70 vs. 5.87±0.63 mmol/L, p=0.003) and week 3 (6.11±0.70 vs. 5.87±0.77 mmol/L, p=0.003). However, there was no significant difference between fasting plasma glucose measured at week 0 and week 6 (6.02±0.68 vs. 6.01±0.79 mmol/L, p=0.83) or between week 3 and week 6 (6.04±0.70 vs. 6.01±0.79 mmol/L, p=0.78) in supplement arm [Figure 27]. There was also a significant difference between supplement and placebo arms for the changes in the fasting plasma glucose during the 6-week intervention period (-0.02± 0.25 vs. 0.23 ±0.15 mmol/L, p=0.009).

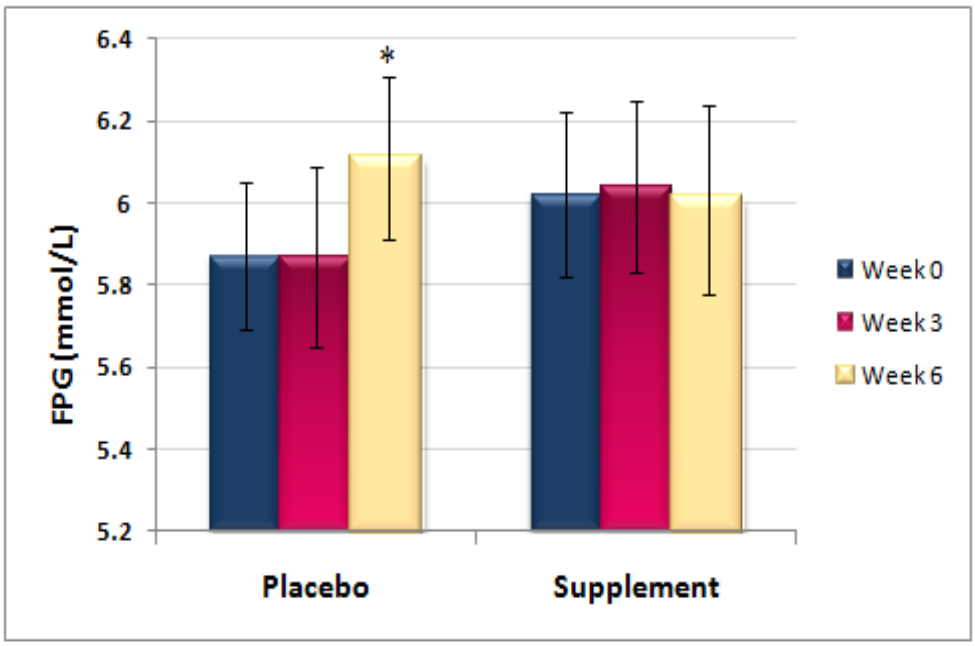


Figure 27. Fasting plasma glucose at week 0, week 3 and week 6 in placebo and supplement arms. *In subjects receiving placebo, fasting plasma glucose at week 6 was significantly higher than at week 0 (p=0.003) and at week 3 (p*=0.003). *Data are presented as mean ± SEM.*

In the present study, glucose tolerance was assessed at week 0 and week 6. Our results showed that in placebo arm, 2-h plasma glucose at week 6 was higher than at week 0 (9.62±2.25 vs. 9.45±2.01 mmol/L, p=0.65), although it was not significant statistically. However, in the supplement arm, 2-h plasma glucose measured at the baseline decreased significantly after 6 weeks (9.89±2.50 vs. 8.78±2.20 mmol/L, p=0.004) [Figure 28]. Two arms were also significantly different when they were compared for the changes in 2-h plasma glucose (placebo arm: 0.17±0.85 mmol/L; supplement arm: -1.11±0.92 mmol/L, p=0.004).

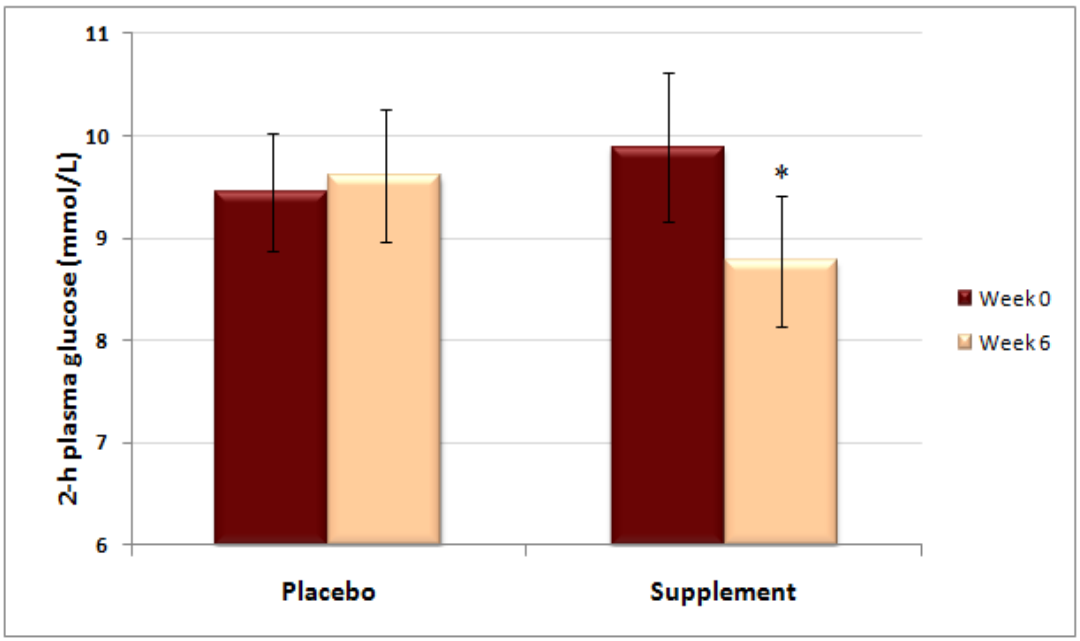
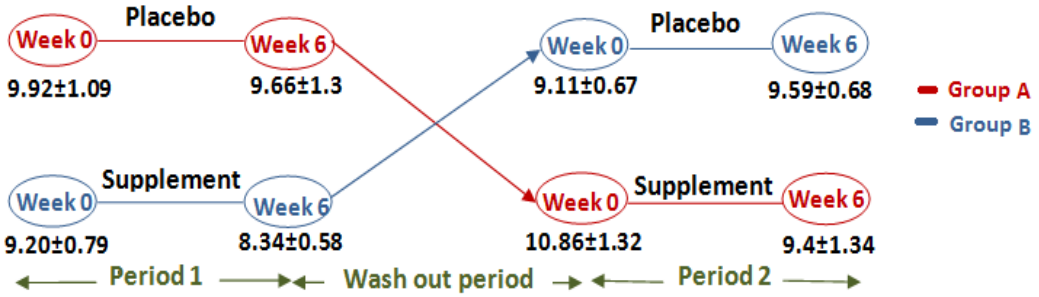


Figure 28. Concentrations of 2-h plasma glucose measured at week 0 and week 6 in placebo and supplement arms. *A significant difference between 2-h plasma glucose at week 6 and those at week 0 was observed in the supplement arm (p=0.004), but not in subjects receiving placebo (p=0.65). Data are presented as mean ± SEM.*

a



b

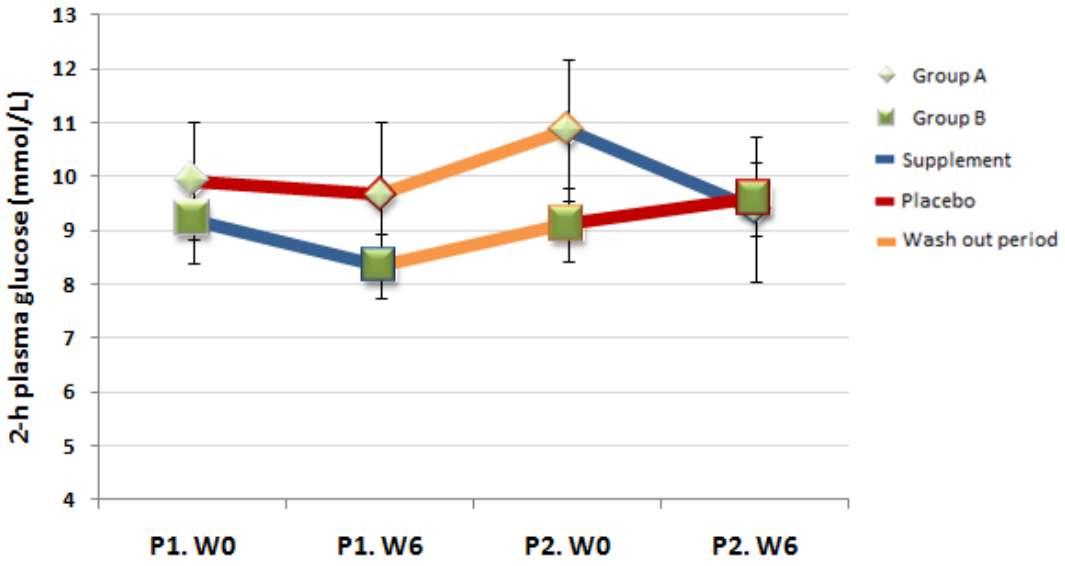


Figure 29 (a& b). Changes in 2-h plasma glucose of groups A and B, during the period that they received supplement or placebo (Periods 1& 2), with a cross-cover design (see Sections 4.1&4.3). *P1.W0, Period 1.Week 0; P1.W6, Period 1.Week 6; P2.W0, Period 2.Week 0; P2.W6, Period 2.Week 6. Data are presented as mean ± SEM.*

Changes in plasma insulin and HOMA score

In the placebo arm, fasting plasma insulin measured at week 6 was significantly higher than when measured at week 0 (7.67± 4.39 vs. 6.64± 3.45 µIU/mL, p=0.04), but there was no significant difference between fasting plasma insulin at week 3 and week 6 (7.17±3.50 vs. 7.67± 4.39 µIU/mL, p=0.30). In the subjects receiving supplement, there was no significant difference between fasting insulin measured at week 6 and week 3 or week 0 (7.46 ±4.67, 7.53±4.22 and 7.36± 4.40 µIU/mL, respectively, p> 0.05) [Figure 30]. These results were not changed considerably after adjusting for order effect.

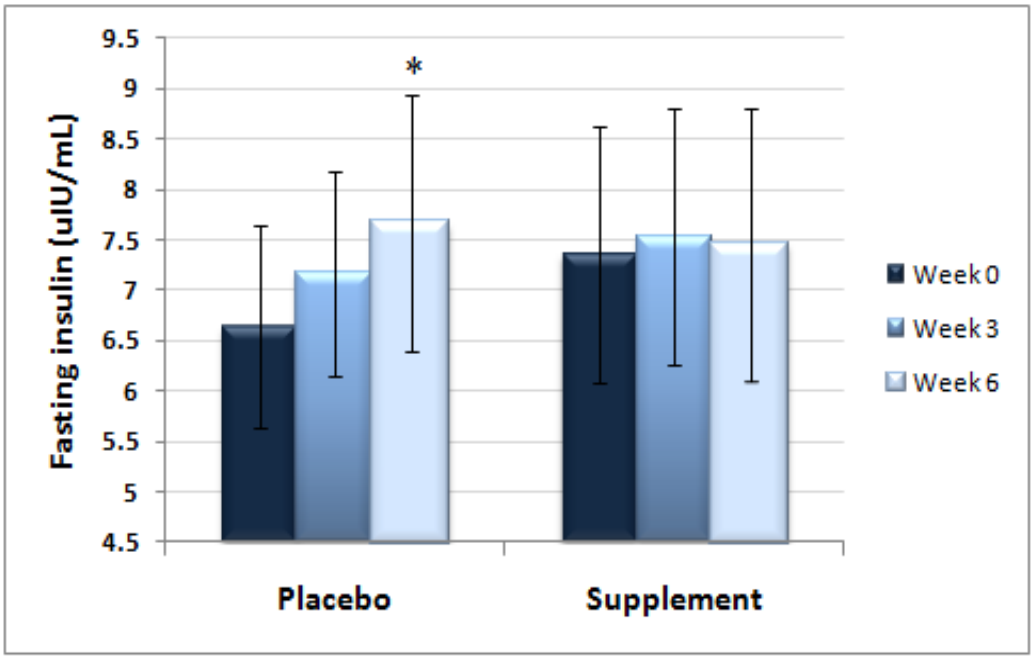


Figure 30. Fasting plasma insulin measured at week 0, week 3 and week 6 in placebo and supplement arms. *In subjects receiving placebo, fasting plasma insulin measured at week 6 was significantly higher than week 0 (p=0.04), but there was no significant change in fasting insulin levels in supplement arm (p> 0.05). Data are presented as mean ± SEM.*

As to be expected, the significant changes in fasting plasma insulin and glucose in placebo arm was accompanied by a significant increase in HOMA score (week 0: 1.75±1.01 vs. week 6: 2.10±1.32, p= 0.02). The change was not significant when HOMA score at week 6 was compared with week 3 (1.89±1.07, p= 0.17). In the supplement arm, there was no significant difference between the HOMA score determined at week 0 and week 6 (2.00±1.32 vs. 2.05±1.51, p=0.75) or between week 3 and week 6 (2.04±1.27 vs. 2.05±1.51, p=0.60) [Figure 31].

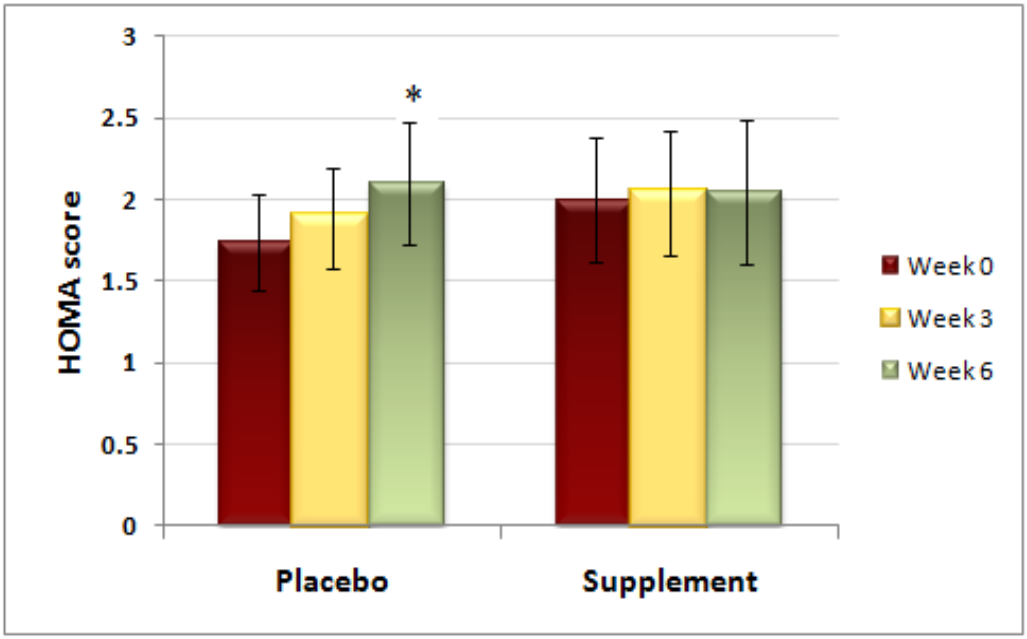


Figure 31. HOMA score determined at week 0, week 3 and week 6 in placebo and supplement arms. *In subjects receiving placebo, a significant increase in HOMA score was observed after six weeks from baseline (p= 0.02). However, HOMA score did not differ significantly in supplement arm. Data are presented as mean ± SEM.*

The mean 2-h insulin measured at week 0 and week 6 were not significantly different in either placebo (61.35±44.07 vs. 68.34±52.58 µIU/mL, p=0.37) or supplement (57.49±41.66 vs. 64.88±49.92 µIU/mL, p=0.35) arms [Figure 32]. There was also no significant difference between supplement and placebo arms, when they were compared for the changes in 2-h insulin during the 6-week intervention period (7.39 ±32.85 vs. 6.99 ±18.57 µIU/mL, p=0.97).

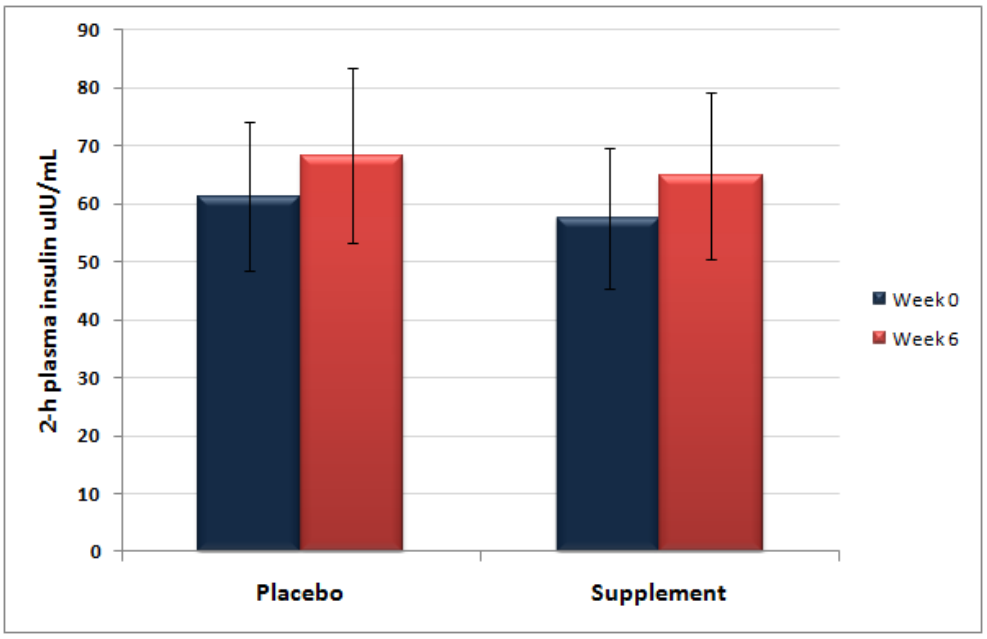


Figure 32. Plasma insulin measured 2 hours after ingestion of glucose load, at weeks 0 and 6 in supplement and placebo arms. *The mean of 2-h plasma insulin measured at week 0 and week 6 were not significantly different in placebo (p= 0.37) or supplement (p=0.35) arms. Data are presented as mean ± SEM.*

Changes in SBP and DBP

The mean systolic blood pressure measured at week 6 did not vary significantly compared to what measured at week 0 in the placebo arm (116.50±11.70 vs. 117.42±10.95 mm Hg, p= 0.68). In the supplement arm, there was a tendency toward a lower SBP at week 6 relative to week 0, although it was not statistically significant (116.50±11.04 vs. 120.67±15.32 mm Hg, p=0.06) [Figure 33]. Diastolic blood pressure of subjects consuming thiamin supplement decreased significantly in week 6 (67.92± 5.84 mm Hg) when compared with those at week 0 (71.42 ±7.41 mm Hg, p=0.005) and week 3 (70.90.±5.84 mm Hg, p=0.02) [Figure 34]. There was no significant change in the diastolic blood pressure of subjects in the placebo arm during the 6-week intervention period (69.50±8.22 (week 0) vs. 70.75±6.68 (week 6) mm Hg, p= 0.30). Also, there was no significant correlation between the changes in the blood pressure (systolic& diastolic) and hs-CRP.

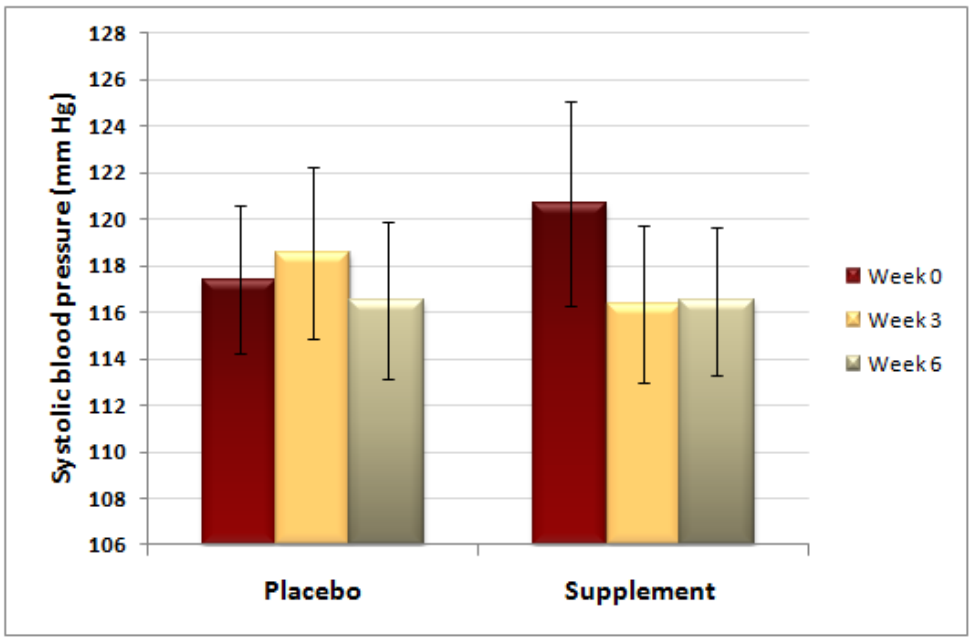


Figure 33. Systolic blood pressure measured in supplement and placebo arms. *In supplement arm, there was a tendency toward a lower SBP at week 6 relative to those at week 0, although it was not statistically significant (p=0.06). Data are presented as mean ± SEM.*

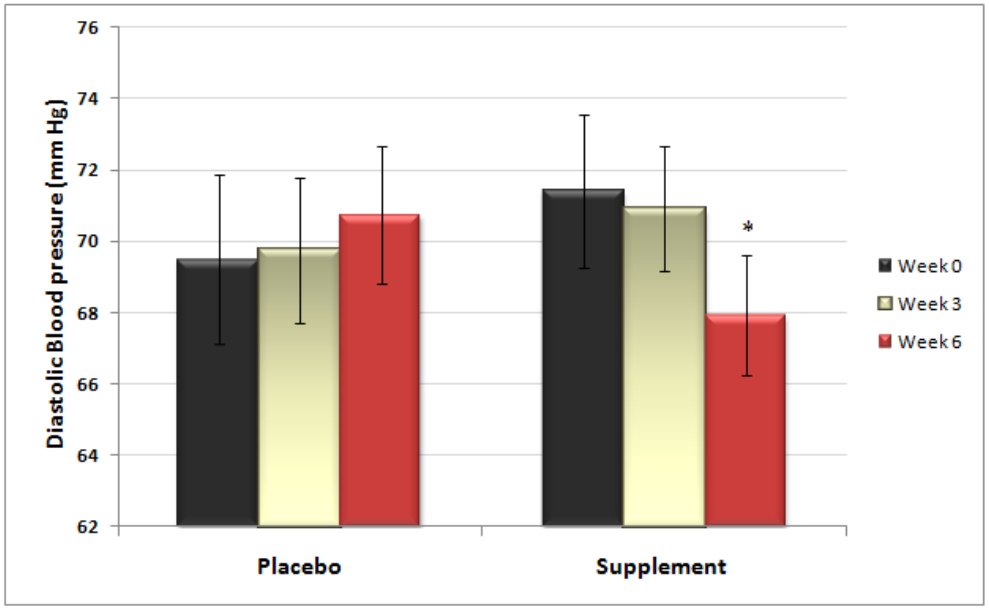


Figure 34. Diastolic blood pressure measured in supplement and placebo arms. *Diastolic blood pressure of subjects consuming thiamin supplement decreased significantly in week 6 compared to week 0 (p= 0.005) and week 3 (p= 0.02). There was no significant change in the diastolic blood pressure of subjects in the placebo arm during the 6-week intervention period (p= 0.30) Data are presented as mean ± SEM.*

Changes in lipid profile, hs-CRP and renal function tests

As presented in the Table 19, no statistically significant change was detected in the supplement or placebo arms when they were assessed for fasting total cholesterol, triglyceride, HDL, LDL, hs-CRP, urine albumin and eGFR. There was also no significant difference in the change between two arms for these variables.

Table 19. Lipid profile, hs-CRP, blood pressure and renal function tests measured in different visits in placebo and supplement arms

Parameters	Placebo					Supplement					P3 [c]
	Week 0 Mean (SD)	Week 3 Mean (SD)	Week 6 Mean (SD)	P1 [a]	P2 [b]	Week 0 Mean (SD)	Week 3 Mean (SD)	Week 6 Mean (SD)	P1 [a]	P2 [b]	
Serum cholesterol (mmol/L)	4.75 (0.77)	4.85 (0.84)	5.11 (0.84)	0.05	0.15	4.83 (0.96)	4.84 (0.85)	5.00 (0.83)	0.34	0.28	0.51
Serum triglyceride (mmol/L)	1.26 (1.00)	1.40 (0.98)	1.37 (0.85)	0.43	0.85	1.21 (0.68)	1.18 (0.69)	1.40 (0.94)	0.18	0.07	0.70
Serum HDL (mmol/L)	1.16 (0.25)	1.20 (0.22)	1.21 (0.24)	0.16	0.67	1.20 (0.26)	1.21 (0.27)	1.19 (0.23)	0.93	0.40	0.27
Serum LDL (mmol/L)	3.00 (0.77)	3.00 (0.63)	3.27 (0.76)	0.07	0.08	3.07 (0.73)	3.08 (0.72)	3.17 (0.65)	0.49	0.47	0.47
hs-CRP (mg/L)	4.22 (5.33)	2.54 (2.05)	3.22 (4.39)	0.33	0.50	2.55 (1.88)	3.14 (3.80)	2.46 (2.74)	0.93	0.55	0.28
Urine albumin (mg/d)	27.49 (54.77)	-	8.63 (14.5)	0.11	-	9.19 (10.5)	-	9.60 (14.3)	0.97	-	0.25
eGFR (ml/min/1.73 m2)	97.53 (31.7)	98.95 (28.4)	98.28 (28.3)	0.69	0.72	95.98 (25.8)	97.91(29.2)	95.14 (25.1)	0.66	0.24	0.49
SBP (mmHg)	117.42 (10.96)	118.58 (12.78)	116.50 (11.70)	0.68	0.35	120.67 (15.32)	116.36 (11.34)	116.50 (11.04)	0.06	0.78	0.37
DBP (mmHg)	69.50 (8.22)	69.75 (7.09)	70.75 (6.67)	0.31	0.41	71.42 (7.42)	70.90 (5.84)	67.92 (5.84)	0.005	0.02	0.003

a. p value - comparison between week 0 and week 6
b. p value - comparison between week 3 and week 6
c. p value - comparison between placebo and supplement arms

Changes in resting and postprandial energy expenditure, and diet-induced thermogenesis rate (DIT)

In the supplement arm, resting energy expenditure assessed at week 6 was significantly lower than that at week 0 (59.78±11.63 vs. 61.78±11.47 kcal/24 h, p=0.02). This change remained significant after adjusting for the subjects' weight and gender as potential confounders (p=0.03). There was no significant difference between resting energy expenditure of subjects receiving placebo at week 6 and week 0 (60.42±10.73 vs. 60.22±10.82 kcal/24 h, p=0.81) [Figure 35]. No significant change was observed in the supplement or placebo arms for the postprandial energy expenditure and diet-induced thermogenesis [Table 20].

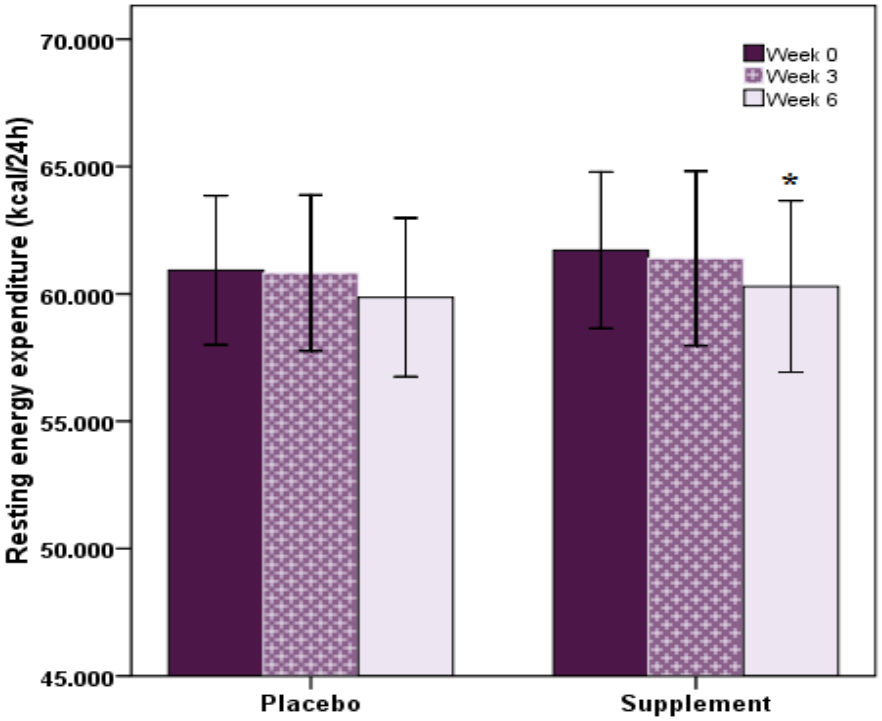


Figure 35. Resting energy expenditure determined in supplement and placebo arms. *In the supplement arm, resting energy expenditure assessed at week 6 was significantly lower than that at week 0 (p=0.02). There was no significant change in resting energy expenditure of subjects receiving placebo. Data are presented as mean ± SEM.*

Table 20. Resting and postprandial energy expenditure measured in different visits in placebo and supplement arms

	Placebo					Supplement				
	Week 0 Mean (SD)	Week 3 Mean (SD)	Week 6 Mean (SD)	P1 [a]	P2 [b]	Week 0 Mean (SD)	Week 3 Mean (SD)	Week 6 Mean (SD)	P1 [a]	P2 [b]
REE (kcal/24h)	60.22 (10.82)	61.14 (11.64)	60.42 (10.73)	0.81	0.40	61.78 (11.47)	61.12 (11.94)	59.78 (11.63)	0.02	0.12
REE (kcal/24h/kg)	0.74 (0.10)	0.73 (0.08)	0.73 (0.08)	0.17	0.55	0.74 (0.08)	0.73 (0.08)	0.72 (0.08)	<0.05	0.24
Fasting RQ [c]	0.89 (0.05)	0.89 (0.06)	0.88 (0.06)	0.40	0.62	0.83 (0.08)	0.87 (0.09)	0.87 (0.04)	0.23	0.85
Δ post prandial EE [d] (kcal/h)	5.66 (3.17)	-	7.63 (6.22)	0.34	-	2.21 (6.44)	-	4.88 (3.05)	0.20	-
2-h RQ [e]	0.96 (0.09)	-	0.91 (0.06)	0.07	-	0.88 (0.07)	-	0.90 (0.06)	0.45	-

a. p value - comparison between week 0 and week 6
b. p value - comparison between week 3 and week 6
c. Fasting respiratory quotient (f VCO_2/f VO_2)
d. Change in post prandial energy expenditure (2-h energy expenditure minus fasting energy expenditure)
e. Respiratory quotient assessed 2 hours after glucose load (2-h VCO_2/ 2-h VCO_2)

4.6 Discussion

The present intervention study was designed to investigate whether in hyperglycemic adults, chronic consumption of a thiamin supplement improves cardiovascular disease risk factors relative to control.

Our results showed that in subjects receiving placebo, fasting plasma glucose increased significantly from baseline after six weeks. This significant change was accompanied with concomitant increases in fasting plasma insulin and HOMA score, which is an indicator of insulin resistance. However, in the supplement arm there was no significant change in fasting plasma glucose, fasting insulin or HOMA score, indicating that supplementation with high dose thiamin may have prevented the natural progression of hyperglycemia toward diabetes mellitus in individuals with impaired glucose metabolism at early stages. Recently, supplementation with 150 mg thiamin (once daily) for 1 month was shown to decrease the fasting plasma glucose of 12 diabetic patients with HbA_{1C} level < 8%, who were not treated with medications (Gonzalez-Ortiz et al. 2011); however, it did not change significantly in the placebo group (n=12). No data about the duration of diabetes since diagnosis in these patients were provided. These findings are inconsistent with the results of Rabbani et al. (2009) who showed no significant change in the fasting glucose of 40 diabetic subjects receiving either placebo or thiamin (3×100 mg/d) treatment for 3 months. In the study conducted by Rabbani and her colleagues subjects had persistent microalbuminuria, and had been diagnosed with diabetes for at least 5 years. Also, except one subject who achieved glycemic control with diet only, all other patients were on treatment with antihyperglycemic medications. These results suggest that thiamin therapy may be less effective on the fasting plasma glucose of patients with long-term diabetes mellitus.

It is known that, in addition to thiamin, some other dietary factors (see Sections 2.3 & 2.5.5) may influence the metabolism of glucose or thiamin. In the present study, we attempted to control for them by asking the subjects not to change their usual diets during the study. It was confirmed by the assessment of the subjects' dietary habits (see Section 4.3.8) that did not reveal any significant changes.

This was particularly important for magnesium which, like thiamin, is a co-factor for transketolase in the pentose phosphate pathway (Fattal-Valevski 2011; Lonsdale, Shamberger, and Obrenovich 2011). It has also a role in the phosphorylation of thiamin to its active form (Voskoboyev and Ostrovsky 1982). As a result, co-existing magnesium deficiency may limit the response to thiamin therapy in the patients with thiamin deficiency (Traviesa 1974). There has been no evidence indicating hypomagnesemia in individuals with pre-diabetic hyperglycemia (Simmons, Joshi, and Shaw 2010), however both type 1 and type 2 diabetic patients are at risk of magnesium deficiency due to the increased excretion (Tosiello 1996). This may be a reason why in the study conducted by Rabbani et al. (2009), thiamin supplementation had no effect on the glucose levels of diabetic patients with nephropathy.

We also found that in subjects with impaired glucose metabolism, supplementation with 300mg/day thiamin for six weeks could significantly decrease 2-h plasma glucose, relative to control. This finding supports a part of our second hypothesis that the chronic consumption of high doses of thiamin can decrease the risk of hyperglycemic complications by enhancing glucose tolerance.

This finding is important from a clinical prospective. In a prospective cohort study involving 19,019 men followed for 38 years (Batty et al. 2008), a 1 mmol/L elevation in 2-h capillary whole blood glucose above the value 4.6 mmol/L was associated with a significant increase in the risk of stroke-related death (27%) and all-cause mortality (16%). In that study, the GTT was carried out using a glucose preparation containing 50 g carbohydrate.

The potential association between thiamin and glucose tolerance in humans was studied by Bakker et al. (1998) who showed that thiamin intakes of 2196 men and women, 50-75 years old without diabetes were inversely correlated to 2-h plasma glucose concentrations, suggesting that supplementation with thiamin may have a role in improving glucose tolerance (see Section 2.5.10). However, there have been no published studies to test this relationship in individuals with impaired glucose tolerance or diabetes. Only in one publication, thiamin supplementation (50 mg/d for 30 days) was shown to improve glucose tolerance in 25 patients with hepatic cirrhosis (Hassan, Qureshi, and Zuberi 1991).

As discussed before (see Section 2.5.6), patients with hyperglycemia have been reported to be prone to thiamin deficiency, because of an increased excretion of thiamin in the urine (Thornalley et al. 2007). Thiamin is known to play a critical regulatory role as a co-enzyme in metabolic pathways involved in glucose metabolism (see Section 2.5.7). Animal studies indicate that thiamin deficiency can reduce overall glucose utilisation (Hakim, Carpenter, and Pappius 1983; Hakim and Pappius 1981) and induce glucose intolerance (Oishi et al. 2002). Thus, additional thiamin may improve glycemic control in hyperglycemic patients.

Under hyperglycemic conditions, high dose thiamin has been also suggested to increase glucose utilisation via increasing transketolase activity (Babaei-Jadidi et al. 2003; Thornalley, Jahan, and Ng 2001), which leads to a shift in the excess levels of metabolic intermediates from the glycolytic pathway toward the reductive pentose shunt (see Section 2.5.9) (Hammes et al. 2003). This results in alleviating pressure on several pathways involved in hyperglycemia-induced complications, including the hexosamine pathway. In the liver of transgenic mice, overexpression of glutamine: fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme in the hexosamine pathway, was associated with impaired glucose tolerance (Veerababu et al. 2000). This suggests that in the presence of hyperglycemia, high dose thiamin may also influence some metabolic pathways which are specifically implicated in the pathogenesis of glucose intolerance.

It is also possible that thiamin has an influence on insulin levels. Basal secretion of insulin and insulin secretion in response to glucose and tolbutamide were shown to decrease in isolated pancreatic islets of thiamin deficient rats (Rathanaswami, Pourany, and Sundaresan 1991; Rathanaswami and Sundaresan 1991). Also, thiamin therapy for six weeks was shown to resolve glucose intolerance in a mouse model of TRMA in which developed diabetes mellitus was associated with decreased insulin secretion (Oishi et al. 2002). These findings suggest that apart from the activation of thiamin-dependent enzymes and increased glucose utilisation, improvement of β-cell function and insulin secretion may contribute to the beneficial effect of thiamin on glycemic control (Thornalley 2005). This would help explain why thiamin supplement appears to be more effective in those who still have insulin secretory

capacity (Hassan, Qureshi, and Zuberi 1991; Oishi et al. 2002) compared with those pancreatic functions are destroyed (Babaei-Jadidi et al. 2004).

It has been shown that 2-h plasma glucose is mainly determined by the postprandial insulin concentration at 30 minutes (Mitrakou et al. 1992), reflecting the response of beta-cells to the ingested glucose load. Unfortunately, this measurement was not undertaken in the present study. Therefore, although in our study no significant change was observed in 2-h plasma insulin in subjects treated with thiamin or placebo, it is not possible to judge that thiamin had no effect on insulin secretion. A further research focusing on the effect of high dose thiamin on insulin secretion by measuring postprandial plasma insulin every 30 minutes intermittently as well as C-peptide concentration would be informative.

While, the pathogenesis of hyperlipidemia associated with impaired glucose metabolism is not fully understood, a switch from lipid oxidation to lipogenesis in the liver has been considered as a possible mechanism (Taskinen 2003). The hexosamine pathway has been implicated in the glucose-mediated de novo lipogenesis in the liver and adipocytes by increasing mRNA levels of the lipogenic enzymes acetyl-CoA carboxylase, glycerophosphate dehydrogenese and fatty acid synthase (Rumberger et al. 2003; Veerababu et al. 2000). High dose thiamin has been suggested to inhibit these effects by diverting the excess glycolytic intermediate of fructose 6-phosphate away from the hexosamine pathway to the pentose phosphate shunt (Thornalley 2005). With regard to the importance of the hepatic hexosamine pathway in lipogenesis associated with hyperglycemia, it was proposed that supplementation with thiamin may alter this effect (Thornalley 2005).

Babaei-Jadidi et al. (2004) showed that thiamin therapy prevents diabetes-induced increases in plasma triglyceride and cholesterol in diabetic rats. This was associated with a significant reduction in hepatic UDP-N-acetylglucosamine and fatty acid synthase activities. However, observations in humans are in contrast to results reported for rats.

The present study has shown that chronic consumption of high dose thiamin had no significant effect on serum triglyceride, cholesterol, HDL-cholesterol and LDL-

cholesterol. This confirms the results of previous studies showing no significant change on lipid profile of diabetic patients receiving thiamin supplement (Gonzalez-Ortiz et al. 2011).

In the study conducted by Babaei-Jadidi et al., the beneficial effects of thiamin were achieved with a high dose of 70 mg/kg/d, and there was no improvement in plasma cholesterol or triglyceride with a lower dose (7 mg/kg/d). Extrapolation of the effective dose of thiamin in rats (70 mg/kg/d) to human shows that about 5 g/day of thiamin might be required for an average person, which is not feasible. This may be why in the previous studies in diabetic patients (Gonzalez-Ortiz et al. 2011; Rabbani et al. 2009) and also our study no effect was observed on the lipid profile of hyperglycemic subjects who received 150 or 300 mg/d thiamin supplement. It could be also due to a species difference.

There has been no published study so far to evaluate the effect of thiamin on the energy expenditure in humans. BMR is mainly dependent on the body size and composition. Accordingly, BMR reduces with age due to the loss of lean body mass (Tzankoff and Norris 1977). Furthermore, existing evidence indicates that body composition and subsequently energy expenditure can be influenced by the change in macro and micronutrient intake. For instance, some studies have shown that administration of thiamin increases muscle mass and energy expenditure in experimental animals (Hamano, Okada, and Tanaka 1999; Huang et al. 2010). The findings of human studies investigating the effect of thiamin on the body weight have been controversial: in a clinical trial conducted by Wilkinson et al. (1997), thiamin supplementation (10 mg/d) for 3 months resulted in a considerable decrease in the weight of subjects with subclinical thiamin deficiency. By contrast, Smidt et al. (1991) found that the weight of subjects with marginal thiamin deficiency taking thiamin supplement (10mg/d) increased significantly after 6 weeks. In another study, dietary intake of thiamin was significantly correlated with the body mass index, based on a gender difference (Chen and Vieira 2007).

In the present study, we have found a significant decrease in the RMR of subjects in the supplement arm which was not associated with measurable changes in the body weight or waist circumference Moreover, in spite of a significant decrease in 2-h

plasma glucose of subjects receiving thiamin, suggesting an improvement in the post prandial glucose utilisation, there was not a significant change in their post prandial energy expenditure and RQ. We are unable to offer an explanation for these findings, since this study did not include the measurement lean body mass. Also, we could only assess the fasting and postprandial energy expenditure 2 hours after glucose load. It would be interesting to have measurements at additional time points. The inconsistency between our results for the body weight and the findings of Wilkinson et al. and Smidt et al. may be partly because in those studies subjects were subclinical/marginal thiamin deficient, assessed by ETKA. While, subjects participating in our study had normal thiamin status, based on their erythrocyte thiamin levels.

According to our results, high dose thiamin had also no significant effect on urinary albumin and eGFR. This was inconsistent with the results of some previous in vitro and in vivo studies, investigating the effect of thiamin on hyperglycemia-induced nephropathy (Babaei-Jadidi et al. 2003; La Selva et al. 1996; Rabbani et al. 2009).

It is suggested that high cytosolic glucose concentration in renal endothelial cells and pericytes can result in the dysfunction of protein kinase C, activation of hexosamine and polyol pathways; metabolic pseudohypoxia; increase in oxidative stress; and formation of advanced glycation end products (AGEs) (Brownlee 2001). Accumulating evidence has demonstrated the suppressive effect of thiamin on the multiple key pathogenic pathways involved in diabetic nephropathy in vitro (La Selva et al. 1996). Additionally, it has been shown that high doses of thiamin inhibit the development of incipient nephropathy (the initial stage of development of nephropathy) in the streptozotocin (STZ)-induced diabetic rats, and increase the thiamin-dependent enzyme transketolase activity in renal glomeruli (Babaei-Jadidi et al. 2003). Recently, the positive effect of high dose thiamin (300 mg/d) on microalbuminuria was confirmed in human subjects with type 2 diabetes mellitus (Rabbani et al. 2009). In that study, volunteers were included if they had diabetes mellitus for a duration of at least five years, and persistent microalbuminuria (AER 30-299 mg/24 h). However, the present study consisted of subjects with pre-diabetic ranges of hyperglycemia (IGT) and new cases of diabetes, with a mean albumin excretion rate at the normal range (<30 mg/24 h). This is a probable reason for the

observed discrepancy between our findings and the results of previous study in diabetic patients. A further trial in subjects with hyperglycemia at early stages and constant microalbuminuria may provide more information in this regard.

We have found a significant decrease in diastolic blood pressure of subjects receiving thiamin supplement, but no significant change was observed in the placebo arm. It is of interest to note that in this study all subjects had diastolic blood pressure below the cut-off point for hypertension (90 mmHg). It was in contrast to a previous study reporting no substantial change in diastolic blood pressure of normotensive diabetic patients treated with thiamin supplement (Gonzalez-Ortiz et al. 2011; Rabbani et al. 2009). In the present study, a tendency toward a decrease in systolic blood pressure was also observed in the supplement arm, although it was not statistically significant. This is in line with a double - blind trial reporting thiamin (10 mg/d for 3 months) reduced the systolic blood pressure of elderly people with subclinical thiamin deficiency (Wilkinson et al. 1997). In that study, the subjects receiving thiamin supplement had a mean systolic blood pressure > 140 mm Hg at the baseline, while in our study subjects had a mean systolic blood pressure below the level considered as hypertension (140 mmHg). Thus, the results may be more significant in subjects with hypertension. In addition, based on thiamin status determined in RBC, there is no evidence that our subjects were thiamin deficient. In the study conducted in elderly people (Wilkinson et al. 1997), no data regarding the possible effect of thiamin on diastolic blood pressure were provided.

Potential mechanisms by which thiamin may affect the blood pressure was studied by Tanaka et al. (2007) who showed that thiamin repletion attenuates the hypertension in CD36 - defective spontaneous hypertensive rats (SHR) which exhibit metabolic abnormalities similar to the human metabolic syndrome. In that experimental study, reduced blood pressure was accompanied with the correction of the uncoupling of glucose oxidation to its cellular entry. In epididymal adipose tissue of SHR, thiamin repletion down - regulated the expression of mRNAs implicated in the hexosamine biosynthetic pathway and renin - angiotensin system.

4.7 Conclusion

This study has found that, in spite of significant increases in the fasting plasma glucose, fasting insulin and HOMA score in the control arm; these variables did not change in hyperglycemic subjects receiving high dose thiamin for six weeks. These results suggest that supplementation with high dose thiamin may prevent the natural progression of hyperglycemia toward diabetes mellitus as well as insulin resistance in individuals with pre-diabetic range of hyperglycemia. The next major finding was that high dose thiamin therapy can enhance glucose tolerance in patients with impaired glucose metabolism. The findings of this study indicate that thiamin therapy may be more effective in patients with hyperglycemia at early stages than individuals with developed diabetes mellitus. In addition, it was shown that supplemental thiamin can have positive effect on diastolic blood pressure. This has been accompanied with a tendency toward a decrease in the systolic blood pressure. Given that in the current study subjects had a mean systolic and diastolic blood pressure below the levels considered as hypertension, thiamin therapy may be more effective in patients with higher levels of blood pressure. Limited literature is available now and further studies are required to verify the positive effects of thiamin found in this study.

5. Overall conclusion

It was shown that diabetic patients are at higher risk of thiamin deficiency; however there has been no published study to investigate thiamin status in individuals with pre-diabetic range of hyperglycemia.

Our cross-sectional study has shown no significant difference in the levels of RBC thiamin in hyperglycemics relative to those in the normoglycemic subjects. The mean intakes of thiamin in both were greater than the Australian RDI for thiamin. Concentration of thiamin in erythrocytes is known as a useful index of thiamin status; however it may be less sensitive to detect thiamin deficiency in individuals with hyperglycemia, because of being affected by changes in the levels of thiamin transporter proteins. Due to technical limitations, we were unable to analyse thiamin urinary excretion and plasma status of our participants in this study. Further research could provide more definite evidence.

In the first study, participants were also assessed for different cardiovascular risk factors, including arterial stiffness. Arterial stiffness can be easily evaluated by the Pulse Trace system which produces a digital volume pulse waveform. Our findings indicate that higher levels of DVP parameters (SI & RI) in the fasting state, together with a blunted response to ingested glucose load, may be a means of detecting vascular dysfunction in patients with impaired glucose metabolism.

Previous studies examining the potential effects of thiamin under hyperglycemic condition have mainly been undertaken in animals. Limited literature investigated the effects of thiamin on metabolic outcomes in humans, particularly among individuals with hyperglycemia at early stages. In addition, the effect of supplemental thiamin on blood glucose of individuals with glucose intolerance has not been researched before.

Our intervention study has shown that high dose thiamin therapy may control fasting blood glucose, and delay the progression of insulin resistance toward diabetes mellitus in patients with hyperglycemia at pre-diabetic ranges. The findings of this study also indicate that supplementation with thiamin can improve glucose tolerance, and reduce diastolic blood pressure in patients with impaired glucose metabolism.

The current clinical study indicates that individuals with hyperglycemia can benefit from thiamin supplementation. The findings of this research have the potential to inform food formulations and dietary recommendations for people who are at risk of developing diabetes mellitus, and may have a role in the prevention of hyperglycemic complications.

One limitation of our study was that the number of subjects was relatively small. Further research with larger sample sizes is needed to confirm the above – mentioned findings. Our results serve as a base for further studies investigating the effects of supplemental thiamin on arterial stiffness and other hyperglycemic complications as well as the effectiveness of different doses of thiamin on cardiovascular risk factors.

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Appendices

Appendix 1.A

Part of the Latest Movement for Prevention of Diabetes and Cardiovascular Diseases

Approximately 25% of Australian adults have blood glucose higher than normal (hyperglycemia), while most of them are not aware of this. Studies indicate that hyperglycemia in both diabetic and pre-diabetic ranges is directly linked to a significant accelerated risk of cardiovascular diseases and other health problems being major causes of death and disability in Australia.

Existing evidence shows that thiamin (vitamin B1) may have an effective role in prevention of vascular problems associated with hyperglycemia, such as cardiovascular problems and stroke as well as kidney, nerve and retina damages.

However, according to recent findings, thiamin status may not be optimum in subjects with hyperglycemia, due to an increased excretion of the vitamin. It is envisaged that supplementation with thiamin may minimise the risk of complications in hyperglycemic people.

Given the importance of pre- diabetic stage of hyperglycemia in preventing of diabetes and cardiovascular diseases, for the first time a research team from Curtin University is now conducting a key study to investigate the thiamin status in people with hyperglycemia in pre-diabetic range. We invite adult volunteers who:

- Are aware of being Impaired Glucose Tolerance (according to a previous glucose tolerance test),
- Have had a fasting blood glucose level higher than normal, however they have not done a glucose tolerance test already, or
- Overweight and obese people who have a family history of diabetes or a history of gestational diabetes.

Volunteers must not smoke, must not be diagnosed as diabetic or any serious diseases and must not be on chronic medications for systemic illness.

The findings of this study may have a critical role in prevention of diabetes and cardiovascular complications, for those are at the risk of developing chronic disease. So, allocation up to 4 hours of your time for participating in this study will be very valuable in this service to science and humanity. Additionally, participants in this study will receive copies of all blood analyses results at the end of study, with a description of what these results indicate.

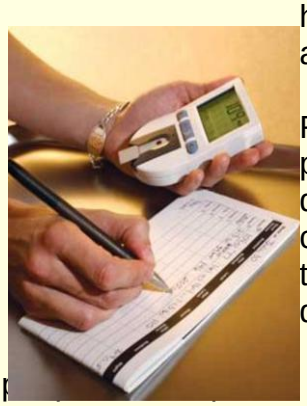
For further details regarding this exciting study, please visit the web site of Curtin University at the following link:

http://www.publichealth.curtin.edu.au/projects/thiamin/thiaminstudy.cfm

Or contact: : Phone no. (08) 9266 7452
Email: fariba.alaeishahmiri@postgrad.curtin.edu.au

Appendix 1.B

Part of the Latest Movement for Prevention of Diabetes and Cardiovascular Diseases



Approximately 25% of Australian adults have blood glucose higher than normal (hyperglycemia) and most of them are not aware of this.

Recent evidence has shown that a pre-diabetic degree of postprandial hyperglycemia (impaired glucose tolerance) is directly linked to a significant accelerated risk of cardiovascular diseases. In addition, impaired glucose tolerance is known as a major risk factor for developing diabetes mellitus in the future.

In p[illegible] glucose tolerance (IGT), blood glucose level after consuming a standard glucose drink (glucose tolerance test) is higher than normal, but the fasting glucose level is normal or moderately raised. As a result, people who just have a fasting blood glucose test may remain undiagnosed.

Existing evidence shows that thiamin (Vitamin B1) may have role in prevention of vascular problems associated with hyperglycemia. However, according to recent findings, thiamin status may not be optimum in subjects with hyperglycemia, due to an increased excretion of the vitamin.

The aim of study:

Given the importance of pre-diabetic stage of hyperglycemia, researchers from Curtin University of Technology are now conducting a key study to investigate the role of supplementation with thiamin in preventing diabetes and associated vascular problems in people with impaired glucose tolerance.

We invite adult volunteers who:

- Are aware of being impaired glucose tolerance (according to a previous glucose tolerance test), or
- Have had a fasting blood glucose level higher than normal, however they have not done a glucose tolerance test already, or
- Overweight and obese people who have a family history of diabetes or a history of gestational diabetes.

Volunteers must not smoke, must not be diagnosed as diabetic or any serious diseases and must not be on chronic medications for systemic illness.

What does the study involve?

In this study, eligible volunteers will be tested for glucose tolerance. Also, thiamin status will be determined in individuals with impaired glucose tolerance relative to healthy people. For participating in the first phase of this study, there will be a total of two visits.

The initial visit will include a short screening session to determine volunteer suitability, which requires 1/2-hour time commitment.

Once the screening criteria are met, there will be just one clinical visit that will commence in the morning and require up to 3-hour time commitment for body measurements, blood samples and answering a questionnaire.

On the clinical visit, volunteers will be assessed for cardiovascular risk factors, glucose tolerance and blood and urine thiamin status, and they will be required to fast for 10-12 hours before arriving in the morning.

Significance of the study:

This clinical study will be fundamental to confirming the beneficial effects of thiamin on cardiovascular risk factors in individuals with a blood glucose level higher than normal. The findings of this study may have the potential to help develop new functional foods, food formulations and dietary recommendations for those who are at risk of developing these chronic diseases. Also, they may have an essential role in prevention of diabetes and vascular complications associated with hyperglycemia. So, volunteering in this study will be very valuable to science and humanity.

Benefits to the volunteers:

Volunteers in the study will receive copies of all blood analyses and arterial compliance results with a description of what these results indicate.

This study has been approved by the Curtin University Human Research Ethics Committee.

If you would like to take part in this important study please fill the screening form available on-line at the web site of Curtin University at the link below:

http://publichealth.curtin.edu.au/research/projects/thiaminstudy.cfm

Or contact Dr. Fariba Alaei on (08) 9266 7452

Email: fariba.alaeishahmiri@postgrad.curtin.edu.au

School of Public Health, Curtin University of Technology

Appendix 2

Screening Checklist for Studies 1&2

Name:…… ID: Sex: Male☐ Female☐

Address: ……

Phone No. (Circle best contact)
H:…… W:…… Mobile:……

EMAIL:…… DOB:…… Age:……

Weight:…….. Hight:…….. W/H circumference:…….

Medical History	**Yes**	**No**	**Details**
Are you a Smoker			
Females: Pregnancy, lactating, post-menopausal, gestational diabetes			
Have you had your glucose or insulin, cholesterol, TG, measured recently? Can you remember the results?			
Have you had, or have, a history of diabetes in your family?			
Medications			
Supplements			
Major operations			
Major illnesses/diseases: Do you have • Diabetes &High BP • Kidney disease (renal problems) • Liver disease &Hepatitis • HIV& Blood diseases • Heart disease& GI problems			
Did you have faints or blackouts?			
Do you have any allergies?			
Would you be able to have the following food/ingredients? • Thiamin supplement& Gluten • Herbs& spices • Ginger • Coconut milk • Dairy product (Lactose)			
What type of alcoholic drink do you usually drink?			
How many standard alcoholic drinks do you drink per day? How many days per week?			
Do you exercise regularly?			
Can we keep your contact details for future reference in clinical research?			

Appendix 3

WRITTEN INFORMED CONSENT FORM

Project Title: Thiamin status in individuals with hyperglycemia

I, _____ hereby consent to be a volunteer for the study "Thiamin status in individuals with hyperglycemia". I understand that the screening and intervention process will involve: weight, height, waist/hip measurements, blood pressure, vascular stiffness measurement, filling food frequency questionnaire and screening questionnaire, and blood samples for assessments. I consent to having blood samples drawn by a trained phlebotomist in fasting condition and after 2 hours of drinking a beverage containing glucose on the assessment day, which will be assessed for various biochemical assessments. I consent to collect my urine for 24 hours prior to assessment day and bring it on the assessment day. The potential risks of all the procedures have been explained to me. I understand that my participation in this project is voluntary and I can withdraw from the project at any time.

Signature:_____

Name:_____

Witness:_____

Date:___/___/___

Any questions or misunderstandings may be answered or clarified by contacting Dr. Fariba Alaei on telephone 9266 7452 or email: fariba.alaeishahmiri@postgrad.curtin.edu.au , or Dr. Jill Sherriff on telephone 9266 7948 or email: J.Sherriff@curtin.edu.au

Appendix 4

Curtin University of Technology

Participation Information sheet

Project title: *Thiamin status in individuals with hyperglycemia*

You have been accepted as a volunteer in this study . The following document must be read carefully and fully understood prior to signing the written consent form. Do not sign the consent form if there is any section in the following Participation Information Sheet that is not understood. Please feel free to ask any questions at any time.

Background Information:

Approximately 25% of Australian adults have a blood glucose higher than normal (hyperglycemia) and this rate is dramatically higher among overweight people. The findings of recent studies have shown that hyperglycemia is directly linked to accelerated risks of cardiovascular diseases and other health problems being major causes of death and disability in Australia. Accordingly, as a part of prevention strategies for chronic diseases, studies over the last decades have investigated the impact of macro and micronutrients on hyperglycemia.

There are some suggestions that the thiamin status may not be optimum in subjects with hyperglicemia. This present study aims to evaluate thiamin status in people with a blood glucose level higher than normal relative to healthy people.

What does the study involve?

There will be a total of 2 visits at the School of Public Health, Curtin University. The initial visit will include a short screening session to determine volunteer suitability (see below), which requires 1/2-hour time commitment. Once the screening criteria are met, there will be just 1 clinical day. The clinical day will commence in the morning and require up to 3-hour time

commitment. Before the intervention day, subjects will be required to fast an overnight fast for about 12-14 hours.

Screening visit:

The screening visit will comprise a 15 to 30 minute session at the School of Public Health, Curtin University, where the study's procedure and protocol will be fully explained and the written consent of each volunteer will be obtained.

Main study:

On the day before the clinical day you will be required to follow a diet provided by the investigators and collect your urine for a 24-hour period. On the clinical day, you will be assessed for cardiovascular risk factors as well as your blood and urine thiamin status. This visit will be for up to a 3-hour time period commencing in the morning and you will be required to fast for 10 -12 hours before coming in on the morning of clinical day.

Upon arrival to the School of Public Health, Curtin University, the following procedures will take place: 1) body weight, height, waist circumference, blood pressure and vascular stiffness will be measured 2) questionnaire pertaining to your dietary thiamin intake will be completed; 3) blood samples will be taken by a trained phlebotomist. After these measurements have been taken, you will be required to consume a beverage containing glucose. 2 hours after consuming the beverage, another blood sample will be taken for measuring your glucose level (oral glucose tolerance test) also vascular stiffness will be measured intermittently every 30 minutes during the period of 2 hours after drinking the beverage .

Possible adverse effects:

The blood samples may cause minor discomfort and possible bruising for some subjects. This bruising however is minor and will dissipate after a few days.

Benefits to the volunteers:

This clinical study will be fundamental to evaluate thiamin status in individuals with blood glucose higher than normal. The findings of this study may have a critical role in prevention of cardiovascular complications, for those are at the risk of developing chronic disease. So, your participation in this study will be very valuable in this service to science and humanity.
For your services as a volunteer in the study, you will receive copies of all blood analyses results with a description of what these results indicate.

Please note that your participation in this study is completely voluntary and you are free to withdrawal at any time. All results and individual information are subject to strict confidentiality and are only accessible by members of the research group.

Any questions or misunderstandings may be answered or clarified by contacting:

Dr. Fariba Alaei on telephone no: 9266 7452
Email: fariba.alaeishahmiri@postgrad.curtin.edu.au or
Associate Prof. Jill Sherriff on telephone no: 9266 7948
Email: J.Sherriff@curtin.edu.au

This study has been approved by the Curtin University Human Research Ethics Committee (Approval Number HR 118/2008). If needed, verification of approval can be obtained either by writing to the Curtin University Human Research Ethics Committee, c/- Office of Research and Development, Curtin University of Technology, GPO Box U1987, Perth, 6845 or by telephoning 9266 2784 or by emailing hrec@curtin.edu.au.

Appendix 5

Curtin University of Technology

Participant Test Instruction Sheet

Glucose Tolerance Test

- You should be on a normal carbohydrate diet of at least 150-200 g of carbohydrate daily, during three days prior to the test. Fruits, breads, cereals, grains, rice, crackers, and starchy vegetables such as potatoes, beans, and corn could be considered as good sources of carbohydrate.
- On the evening before the clinical day you will be required to consume a standard meal provided by the investigators.
- Following consuming standard evening meal, you are required to fast overnight (10-12 hours) and have nothing to eat, including chewing gum, lollies, tea and coffee. You are permitted to drink water.
- Upon arrival to the out-patient clinic, you should rest for 30 minutes before commencement of the test.
- At the commencement of the test a blood sample will be taken and the fasting glucose checked.
- After taking fasting blood sample, a glucose load in the form of a single bottle of glucose beverage will be given and you should drink the entire bottle within five minutes.
- You are to stay in out-patient clinic for the duration of the test. So, please bring reading materials along with you if you like.
- No smoking or physical activity is permitted during the test. It also includes walking around during the test.
- One more blood sample will be taken at 2 hour after ingestion of glucose drink.

24 Hour Urine Collection

- EAT AND DRINK NORMALLY prior and during to the collection, as the results will be affected.
- It is recommended that you commence with your first morning urine on the day before clinical day.
- For the first passing of urine, completely empty the bladder by urinating into the toilet – DO NOT SAVE IT INTO CONTAINER – but note the time and date on the container label.
- After passing the first urine of the day, commence collection and collect all urine for the next 24 hours. DO NOT DISCARD ANY URINE DURING THIS COLLECTION TIME.
- Exactly 24 hours after the start time, empty bladder and COLLECT URINE INTO THE CONTAINER.
- Collection is now completed, you are to note this completion time on the label of the container – which should be the same time as the commencement time.
- Store the container in a cool, dark and dry place.
- Bring the completed 24 hour urine collection to the out-patient clinic, School of Public Health, Curtin University on the scheduled clinical day.

Important

- Given that an Acid preservative has been added to the container, please consider the following precautions:
- Keep out of reach of children
- Do not discard the preservative prior to starting collection.
- Do not void directly into the container. Void into another clean container first and then transfer it completely into the 24-h urine collection container.

Appendix 6

Protocol of the oral glucose tolerance test provided by WHO (Alberti and Zimmet 1998)

The Oral Glucose Tolerance Test

The oral glucose tolerance test (OGTT) is principally used for diagnosis when blood glucose levels are equivocal, during pregnancy, or in epidemiological studies.

The OGTT should be administered in the morning after at least three days of unrestricted diet (greater than 150 g of carbohydrate daily) and usual physical activity. Recent evidence suggests that a reasonable (30–50g) carbohydrate containing meal should be consumed on the evening before the test. The test should be preceded by an overnight fast of 8–14 hours, during which water may be drunk. Smoking is not permitted during the test. The presence of factors that influence interpretation of the results of the test must be recorded (e.g. medications, inactivity, infection, etc.).

After collection of the fasting blood sample, the subject should drink 75 g of anhydrous glucose or 82.5 g of glucose monohydrate (or partial hydrolysates of starch of the equivalent carbohydrate content) in 250–300 ml of water over the course of 5 minutes. For children, the test load should be 1.75 g of glucose per kg body weight up to a total of 75 g of glucose. Timing of the test is from the beginning of the drink. Blood samples must be collected 2 hours after the test load.

Appendix 7

WHO Clinical criteria for metabolic syndrome (Alberti and Zimmet 1998)

Insulin resistance, identified by 1 of the following:

- Type 2 diabetes
- Impaired fasting glucose
- Impaired glucose tolerance
  or for those with normal fasting glucose levels (_110 mg/dL),
  glucose uptake below the lowest quartile for background population
  under investigation under hyperinsulinemic, euglycemic conditions

Plus any 2 of the following:

- Antihypertensive medication and/or high blood pressure (_140 mm Hg systolic or _90 mm Hg diastolic)
- Plasma triglycerides _150 mg/dL (_1.7 mmol/L)
- HDL cholesterol _35 mg/dL (_0.9 mmol/L) in men or _39 mg/dL
- (1.0 mmol/L) in women
- BMI _30 kg/m2 and/or waist:hip ratio _0.9 in men, _0.85 in women
- Urinary albumin excretion rate _20 _g/min
  or albumin:creatinine ratio_30 mg/g

Appendix 8

Dietary Questionnaire

QUESTIONS ABOUT WHAT YOU USUALLY EAT AND DRINK

Please fill in the date you completed this questionnaire:

DAY		MTH	YEAR
		○ JAN	○ 2004
		○ FEB	○ 2005
0	0	○ MAR	○ 2006
1	1	○ APR	○ 2007
2	2	○ MAY	○ 2008
3	3	○ JUN	○ 2009
	4	○ JUL	○ 2010
	5	○ AUG	○ 2011
	6	○ SEP	○ 2012
	7	○ OCT	○ 2013
	8	○ NOV	○ 2014
	9	○ DEC	○ 2015

INSTRUCTIONS:

This questionnaire is about your **usual** eating habits **over the past 12 months**. Where possible give only **one answer per question** for the type of food you eat **most often.**
(If you can't decide which type you have most often, answer for the types you usually eat.)

- Use a soft pencil only, preferably 2B.
- Do not use **any** biro or felt tip pen.
- Erase mistakes fully.
- Make no stray marks.

Please MARK LIKE THIS: ○●○○

1. How many pieces of fresh fruit do you usually eat per day? *(Count 1/2 cup of diced fruit, berries or grapes as one piece.)*

- ○ I didn't eat fruit
- ○ less than 1 piece of fruit per day
- ○ 1 piece of fruit per day
- ○ 2 pieces of fruit per day
- ○ 3 pieces of fruit per day
- ○ 4 or more pieces of fruit per day

2. How many different vegetables do you usually eat per day? *(Count all types, fresh, frozen or tinned.)*

- ○ less than 1 vegetable per day
- ○ 1 vegetable per day
- ○ 2 vegetables per day
- ○ 3 vegetables per day
- ○ 4 vegetables per day
- ○ 5 vegetables per day
- ○ 6 or more vegetables per day

3. What type of milk do you usually use?

- ○ none
- ○ full cream milk
- ○ reduced fat milk
- ○ skim milk
- ○ soya milk

4. How much milk do you usually use per day? *(Include flavoured milk and milk added to tea, coffee, cereal, etc.)*

- ○ none
- ○ less than 250 ml *(1 large cup or mug)*
- ○ between 250 and 500 ml *(1-2 cups)*
- ○ between 500 and 750 ml *(2-3 cups)*
- ○ 750 ml *(3 cups)* or more

5. What type of bread do you usually eat?

- ○ I don't eat bread
- ○ high fibre white bread
- ○ white bread
- ○ wholemeal bread
- ○ rye bread
- ○ multi-grain bread

6. How many slices of bread do you usually eat per day? *(Include all types, fresh or toasted and count one bread roll as 2 slices.)*

- ○ less than 1 slice per day
- ○ 1 slice per day
- ○ 2 slices per day
- ○ 3 slices per day
- ○ 4 slices per day
- ○ 5-7 slices per day
- ○ 8 or more slices per day

7. Which spread do you usually put on bread?

- ○ I don't usually use any fat spread
- ○ margarine of any kind
- ○ polyunsaturated margarine
- ○ monounsaturated margarine
- ○ butter and margarine blends
- ○ butter

8. On average, how many teaspoons of sugar do you usually use per day? *(Include sugar taken with tea and coffee and on breakfast cereal, etc.)*

- ○ none
- ○ 1 to 4 teaspoons per day
- ○ 5 to 8 teaspoons per day
- ○ 9 to 12 teaspoons per day
- ○ more than 12 teaspoons per day

9. On average, how many eggs do you usually eat per week?

- ○ I don't eat eggs
- ○ less than 1 egg per week
- ○ 1 to 2 eggs per week
- ○ 3 to 5 eggs per week
- ○ 6 or more eggs per week

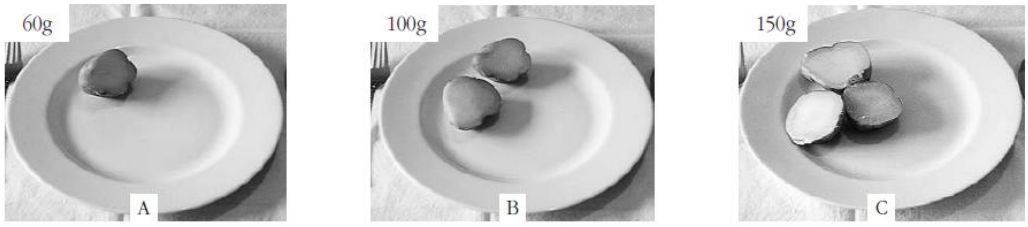
10. What types of cheese do you usually eat?

- ○ I don't eat cheese
- ○ hard cheeses, e.g. parmesan, romano
- ○ firm cheeses, e.g. cheddar, edam
- ○ soft cheeses, e.g. camembert, brie
- ○ ricotta or cottage cheese
- ○ cream cheese
- ○ low fat cheese

DO NOT WRITE IN THIS AREA.

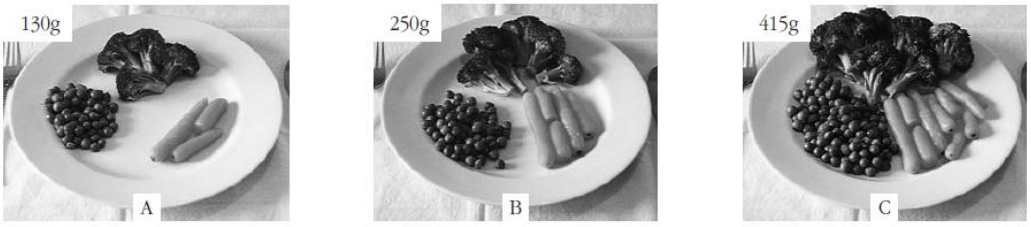
For each food shown on this page, indicate ***how much on average you would usually have eaten at main meals during the past 12 months.*** *When answering each question, think of the* ***amount*** *of that food you usually ate, even though you may rarely have eaten the food on its own.*
If you usually ate more than one helping, fill in the oval for the serving size closest to the ***total amount*** *you ate.*

11. When you ate potato, did you usually eat: ○ I never ate potato



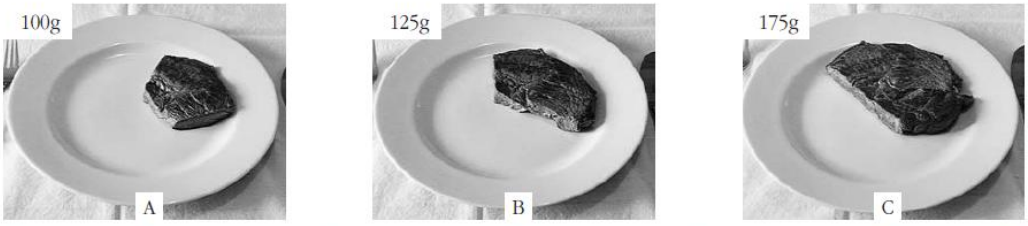
○ Less than A ○ A ○ Between A & B ○ B ○ Between B & C ○ C ○ More than C

12. When you ate vegetables, did you usually eat: ○ I never ate vegetables



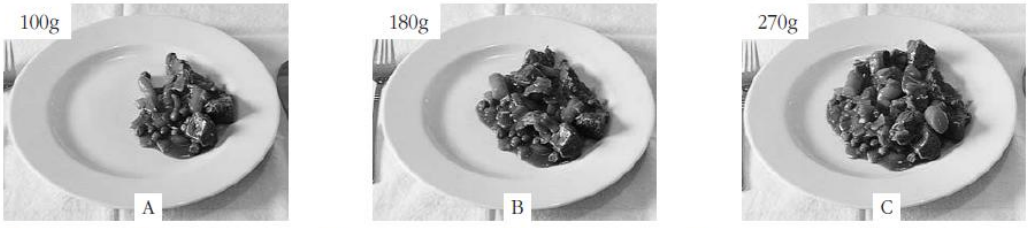
○ Less than A ○ A ○ Between A & B ○ B ○ Between B & C ○ C ○ More than C

13. When you ate steak, did you usually eat: ○ I never ate steak



○ Less than A ○ A ○ Between A & B ○ B ○ Between B & C ○ C ○ More than C

14. When you ate meat *or* vegetable casserole, did you usually eat: ○ I never ate casserole



○ Less than A ○ A ○ Between A & B ○ B ○ Between B & C ○ C ○ More than C

15. Over the last 12 months, on average, *how often* did you eat the following foods? *Please completely fill one oval in every line.*

Please MARK LIKE THIS: ○●○○ NOT LIKE THIS: ✔ ✘ ⊖ ◯

Times You Have Eaten		NEVER	less than once per month	1 to 3 times per month	1 time per week	2 times per week	3 to 4 times per week	5 to 6 times per week	1 time per day	2 times per day	3 or more times per day
CEREAL FOODS, SWEETS & SNACKS											
All Bran™	A1	○	○	○	○	○	○	○	○	○	○
Sultana Bran™, FibrePlus™, Branflakes™	A2	○	○	○	○	○	○	○	○	○	○
Weet Bix™, Vita Brits™, Weeties™	A3	○	○	○	○	○	○	○	○	○	○
Cornflakes, Nutrigrain™, Special K™	A4	○	○	○	○	○	○	○	○	○	○
Porridge	A5	○	○	○	○	○	○	○	○	○	○
Muesli	A6	○	○	○	○	○	○	○	○	○	○
Rice	A7	○	○	○	○	○	○	○	○	○	○
Pasta or noodles (include lasagne)	A8	○	○	○	○	○	○	○	○	○	○
Crackers, crispbreads, dry biscuits	A9	○	○	○	○	○	○	○	○	○	○
Sweet biscuits	A10	○	○	○	○	○	○	○	○	○	○
Cakes, sweet pies, tarts and other sweet pastries	A11	○	○	○	○	○	○	○	○	○	○
Meat pies, pasties, quiche and other savoury pastries	A12	○	○	○	○	○	○	○	○	○	○
Pizza	A13	○	○	○	○	○	○	○	○	○	○
Hamburger with a bun	A14	○	○	○	○	○	○	○	○	○	○
Chocolate	A15	○	○	○	○	○	○	○	○	○	○
Flavoured milk drink (cocoa, Milo™, etc.)	A16	○	○	○	○	○	○	○	○	○	○
Nuts	A17	○	○	○	○	○	○	○	○	○	○
Peanut butter or peanut paste	A18	○	○	○	○	○	○	○	○	○	○
Corn chips, potato crisps, Twisties™, etc.	A19	○	○	○	○	○	○	○	○	○	○
Jam, marmalade, honey or syrups	A20	○	○	○	○	○	○	○	○	○	○
Vegemite™, Marmite™ or Promite™	A21	○	○	○	○	○	○	○	○	○	○
DAIRY PRODUCTS, MEAT & FISH											
Cheese	B1	○	○	○	○	○	○	○	○	○	○
Ice-cream	B2	○	○	○	○	○	○	○	○	○	○
Yoghurt	B3	○	○	○	○	○	○	○	○	○	○
Beef	B4	○	○	○	○	○	○	○	○	○	○
Veal	B5	○	○	○	○	○	○	○	○	○	○
Chicken	B6	○	○	○	○	○	○	○	○	○	○
Lamb	B7	○	○	○	○	○	○	○	○	○	○
Pork	B8	○	○	○	○	○	○	○	○	○	○
Bacon	B9	○	○	○	○	○	○	○	○	○	○
Ham	B10	○	○	○	○	○	○	○	○	○	○
Corned beef, luncheon meats or salami	B11	○	○	○	○	○	○	○	○	○	○
Sausages or frankfurters	B12	○	○	○	○	○	○	○	○	○	○
Fish, steamed, grilled or baked	B13	○	○	○	○	○	○	○	○	○	○
Fish, fried (include take-away)	B14	○	○	○	○	○	○	○	○	○	○
Fish, tinned (salmon, tuna, sardines, etc.)	B15	○	○	○	○	○	○	○	○	○	○
FRUIT											
Tinned or frozen fruit (any kind)	C1	○	○	○	○	○	○	○	○	○	○
Fruit juice	C2	○	○	○	○	○	○	○	○	○	○
Oranges or other citrus fruit	C3	○	○	○	○	○	○	○	○	○	○
Apples	C4	○	○	○	○	○	○	○	○	○	○
Pears	C5	○	○	○	○	○	○	○	○	○	○
Bananas	C6	○	○	○	○	○	○	○	○	○	○
Watermelon, rockmelon (cantaloupe), honeydew, etc.	C7	○	○	○	○	○	○	○	○	○	○
Pineapple	C8	○	○	○	○	○	○	○	○	○	○
Strawberries	C9	○	○	○	○	○	○	○	○	○	○
Apricots	C10	○	○	○	○	○	○	○	○	○	○
Peaches or nectarines	C11	○	○	○	○	○	○	○	○	○	○
Mango or paw paw	C12	○	○	○	○	○	○	○	○	○	○
Avocado	C13	○	○	○	○	○	○	○	○	○	○

Times You Have Eaten CONTINUED

		NEVER	less than once per month	1 to 3 times per month	1 time per week	2 times per week	3 to 4 times per week	5 to 6 times per week	1 time per day	2 times per day	3 or more times per day
VEGETABLES (INCLUDING FRESH, FROZEN AND TINNED)											
Potatoes, roasted or fried (include hot chips)	D1	○	○	○	○	○	○	○	○	○	○
Potatoes cooked without fat	D2	○	○	○	○	○	○	○	○	○	○
Tomato sauce, tomato paste or dried tomatoes	D3	○	○	○	○	○	○	○	○	○	○
Fresh or tinned tomatoes	D4	○	○	○	○	○	○	○	○	○	○
Peppers (capsicum)	D5	○	○	○	○	○	○	○	○	○	○
Lettuce, endive, or other salad greens	D6	○	○	○	○	○	○	○	○	○	○
Cucumber	D7	○	○	○	○	○	○	○	○	○	○
Celery	D8	○	○	○	○	○	○	○	○	○	○
Beetroot	D9	○	○	○	○	○	○	○	○	○	○
Carrots	D10	○	○	○	○	○	○	○	○	○	○
Cabbage or Brussels sprouts	D11	○	○	○	○	○	○	○	○	○	○
Cauliflower	D12	○	○	○	○	○	○	○	○	○	○
Broccoli	D13	○	○	○	○	○	○	○	○	○	○
Silverbeet or spinach	D14	○	○	○	○	○	○	○	○	○	○
Peas	D15	○	○	○	○	○	○	○	○	○	○
Green beans	D16	○	○	○	○	○	○	○	○	○	○
Bean sprouts or alfalfa sprouts	D17	○	○	○	○	○	○	○	○	○	○
Baked beans	D18	○	○	○	○	○	○	○	○	○	○
Soy beans, soy bean curd or tofu	D19	○	○	○	○	○	○	○	○	○	○
Other beans (include chick peas, lentils, etc.)	D20	○	○	○	○	○	○	○	○	○	○
Pumpkin	D21	○	○	○	○	○	○	○	○	○	○
Onion or leeks	D22	○	○	○	○	○	○	○	○	○	○
Garlic (not garlic tablets)	D23	○	○	○	○	○	○	○	○	○	○
Mushrooms	D24	○	○	○	○	○	○	○	○	○	○
Zucchini	D25	○	○	○	○	○	○	○	○	○	○

16. Over the last 12 months, how often did you drink beer, wine and/or spirits?

Times That You Drank		NEVER	less than once a month	1-3 days per month	1 day per week	2 days per week	3 days per week	4 days per week	5 days per week	6 days per week	every day
Beer (low alcohol)	1	○	○	○	○	○	○	○	○	○	○
Beer (full strength)	2	○	○	○	○	○	○	○	○	○	○
Red wine	3	○	○	○	○	○	○	○	○	○	○
White wine (include sparkling wines)	4	○	○	○	○	○	○	○	○	○	○
Fortified wines, port, sherry, etc.	5	○	○	○	○	○	○	○	○	○	○
Spirits, liqueurs, etc.	6	○	○	○	○	○	○	○	○	○	○

When answering the next two questions, please convert the amounts you drank into glasses using the examples given below. For spirits, liqueurs, and mixed drinks containing spirits, please count each nip (30 ml) as one glass.

1 can or stubby of beer = 2 glasses
1 large bottle beer (750 ml) = 4 glasses
1 bottle wine (750 ml) = 6 glasses
1 bottle of port or sherry (750 ml) = 12 glasses

17. Over the last 12 months, on days when you were drinking, how many glasses of beer, wine and/or spirits altogether did you *usually* drink?

TOTAL NUMBER OF GLASSES PER DAY	1	2	3	4	5	6	7	8	9	10 or more
	○	○	○	○	○	○	○	○	○	○

18. Over the last 12 months, what was the *maximum* number of glasses of beer, wine and/or spirits that you drank in 24 hours?

MAXIMUM NUMBER OF GLASSES PER 24 HOURS	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19 or more
	○	○	○	○	○	○	○	○	○	○

Thank You for completing this questionnaire

DO NOT WRITE IN THIS AREA.

Appendix 9

WRITTEN INFORMED CONSENT FORM

Project Title: The chronic effects of thiamin on cardiovascular risk factors in individual with hyperglycemia

I, _____ hereby consent to be a volunteer for the study "the chronic effects of thiamin on cardiovascular risk factors in individuals with hyperglycemia". I will attend an initial screening session where measurements of weight, height, waist/hip, and blood pressure will be taken to determine my suitability as a volunteer. I understand that once admitted into the project, I will attend the outpatient clinic of School of Public Health, Curtin University on three assessment days. Clinical measurements will be taken at weeks 0, 3 and 6. I understand that in two of these assessment days (week 0 and week 6) following fasting measurements, I will consume a liquid preload, containing glucose. I consent to having blood samples drawn by a trained phlebotomist in fasting condition and after 2 hours of drinking a beverage containing glucose on the assessment day, which will be assessed for various biochemical assessments. I consent to having my blood pressure and arterial stiffness measured in fasting condition to assess cardiovascular risk factors and indirect calorimetry, a non-invasive and safe method for measuring fat oxidation. I agree to filling in the screening questionnaire, food diary and physical activity log. I consent to collect my urine for 24 hours prior to assessment day and bring it on the assessment day. The potential risks of all the procedures have been explained to me. I understand that my participation in this project is voluntary and the signing of this declaration does not prevent me from withdrawing from the study at any point in time, without prejudice.

Signature: _____

Name: _____

Witness: _____

Date: ____/____/_____

Any questions or misunderstandings may be answered or clarified by contacting Dr. Fariba Alaei on telephone 9266 7452 or email: fariba.alaeishahmiri@postgrad.curtin.edu.au , or Assoc. Prof. Jill Sherriff on telephone 92667948 or email: J.Sherriff@curtin.edu.au

Please see the comment mentioned for appendix 10.

Appendix 10

Curtin University of Technology

Participation Information Sheet

Project title: *The chronic effects of thiamin on cardiovascular risk factors*

You have been accepted as a volunteer in this study because you have blood glucose levels above the normal range. The following document must be read carefully and fully understood prior to signing the written consent form. Do not sign the consent form if there is any section in the following Participation Information Sheet that is not understood. Please feel free to ask any questions at any time.

Background Information:

Approximately 25% of Australian adults have a blood glucose higher than normal (hyperglycemia) and this rate is dramatically higher among overweight people. The findings of recent studies have shown that hyperglycemia is directly linked to accelerated risks of cardiovascular diseases and other health problems which are major causes of death and disability in Australia. Accordingly, as a part of prevention strategies for chronic diseases, studies over the last decades have investigated the impact of macro and micronutrients on hyperglycemia.

There are some studies showing thiamin supplement has many positive effects on cardiovascular risk factors under hyperglycemic condition. However, almost all of these previous studies have been done using animals. This present study aims to confirm the beneficial effects of thiamin on cardiovascular risk factors in humans, particularly in individuals with a blood glucose level higher than normal, but in the pre-diabetic range.

What does the study involve?

Required commitment to the study:

The amount of time that a subject will be involved in the study will be approximately 7 weeks from the initial to the final analysis. There will be a total of 4 visits to Curtin University. Before each of these visits, subjects will be required to fast overnight fast for about 12-14 hours. The initial visit will determine volunteer suitability and will include a screening session at the School of Public Health, Curtin University, which takes about 1/2 hour. Once the screening criteria are met there will be 3 clinical days, at weeks 0, 3 and 6, which will take place at the School of Public Health, Curtin University. The clinical days will commence in the morning and at weeks 0 and 6 require up to 3-hour time commitment. The clinical day at week 3 takes about 1 hour.

Screening visit:

The screening visit will comprise a 15 to 30 minute session at the Curtin University where measurements of weight, height, waist/hip, and blood pressure will be taken. The study's procedure and protocol will be fully explained and the written consent of each volunteer will be obtained. **Also, if you are allergic to any supplements containing thiamin, please let the researcher know during screening visit.**

Food diaries and Physical activity logs:

Physical activity logs and food diaries will be required to be completed 3 days before each day. Techniques for completing the food records and the activity logs will be explained to you in detail and any questions will be answered before the commencement of the study.

Main study:

After screening, the volunteers will be randomly allocated into two groups to consume either 100 mg of thiamin or placebo supplements three times a day (300 mg/d) for 6 weeks. Clinical measurements will be taken at weeks 0, 3 and 6. The night before each assessment period, you will be required to fast overnight following a standard evening meal for 10-12 hours before attending the School of Public Health, Curtin University.

On the day before the clinical day you will be required to eat an evening meal provided by the investigators and collect your urine for a 24-hour period. Over the course of the intervention, you should maintain your usual diet and level of physical activity, and to refrain from alcohol and strenuous exercise 24 h before visits.

On the clinical days at weeks 0 and 6, you will be required to visit the School of Public Health of Curtin University. These visits will be for up to a 3-hour time period commencing in the morning and you will be required to fast for 10 -12 hours before coming in on the morning of each clinical day.

Upon arrival to the outpatient clinic of School of Public Health, the following procedures will take place: 1) body weight, height, waist circumference, blood pressure, fat oxidation and vascular stiffness will be measured; 2) blood samples will be taken by a trained phlebotomist. 3) After these measurements have been taken, you will be required to consume a liquid preload, containing 75 g of glucose. 4) The second blood sample will be taken 2 hours after the test load.

On the clinical day at week 3, you will be required to fast overnight following a standard evening meal for 10-12 hours before attending the School of Public Health, Curtin University for approximately 1/2 hour. Measurements will be taken, including fasting blood samples, blood pressure, fat oxidation and vascular stiffness.

Possible adverse effects:

The blood samples may cause minor discomfort and possible bruising for some subjects. This bruising however is minor and will dissipate after a few days.

The symptoms of toxicity or allergic sensitivity to thiamin, following its oral consumption in humans is very rare and they are usually relieved following cessation of treatment or reduction of dose. However, in order to minimise any risk, please let investigators know immediately, if you have any symptoms of skin redness, itching, eczema, headache, nausea, irritability, insomnia, rapid pulse and weakness.

Benefits to the volunteers:

This clinical study will be fundamental to confirm the beneficial effects of thiamin on cardiovascular risk factors in individuals with a blood glucose level higher than normal. The findings of this study may have the potential to help develop new functional foods, food formulations and dietary recommendations for those are at the risk of developing chronic disease. Also, they may have an essential role in prevention of hyperglycemia complications. So, your volunteering in this study will be very valuable in this service to science and humanity.

As a volunteer in the study, you will receive copies of all blood analyses and arterial compliance results with a description of what these results indicate.
Please note that your participation in this study is completely voluntary and you are free to withdraw at any time. All results and individual information are subject to strict confidentiality and are only accessible by members of the research group.

Any questions or misunderstandings may be answered or clarified by contacting:

Dr. Fariba Alaei on telephone no: 9266 7452
Email: fariba.alaeishahmiri@postgrad.curtin.edu.au or:
Assoc. Prof. Jill Sherriff on telephone no: 9266 7948
Email: J.Sherriff@curtin.edu.au

This study has been approved by the Curtin University Human Research Ethics Committee (Approval Number HR 161/2008). The Committee is comprised of members of the public, academics, lawyers, doctors and pastoral carers. Its main role is to protect participations. If needed, verification of approval can be obtained either by writing to the Curtin University Human Research Ethics Committee, c/- Office of Research and Development, Curtin University of Technology, GPO Box U1987, Perth, 6845 or by telephoning 9266 2784 or by emailing hrec@curtin.edu.au.

Comment: *this study was initially planned to be carried out with a parallel design. Therefore, the original participants' information sheet and consent form were prepared for a six - week intervention study. However, due to the problems in recruiting the required number of eligible subjects, the study design was changed to a cross - over design. Accordingly, following completion of the first part, subjects were invited to pass a 14 -week wash out period and come back to receive alternative capsules for another six weeks.*

Appendix 11

Day and Date	Time	Food/Drink Eg, Bread	Type Eg, wholemeal	Brand Eg Helga	Cooking method Eg toasted	Amount/Weight Eg 40 g or 2 slices	Waste Eg crust, 20g

Appendix 12

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** 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. Think only about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

   _____ **days per week**

   ☐ No vigorous physical activities → **Skip to question 3**

2. How much time did you usually spend **doing vigorous** physical activities on one of those days?

   _____ **hours per day**
   _____ **minutes per day**

   ☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the last 7 days, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis?
   Do not include walking.

   _____ **days per week**

   ☐ No moderate physical activities → **Skip to question 5**

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ **hours per day**
_____ **minutes per day**

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ **days per week**

☐ No walking → **Skip to question 7**

6. How much time did you usually spend walking on one of those days?

_____ **hours per day**
_____ **minutes per day**

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent 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.

7. During the **last 7 days**, how much time did you spend **sitting** on **a week day?**

_____ **hours per day**
_____ **minutes per day**

☐ Don't know/Not sure

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

Appendix 13

ID: **March 2010**

S	M	T	W	T	F	S
	Visit Week 0	**2** ☐ Breakfast ☐ Lunch ☐ Dinner	**3** ☐ Breakfast ☐ Lunch ☐ Dinner	**4** ☐ Breakfast ☐ Lunch ☐ Dinner	**5** ☐ Breakfast ☐ Lunch ☐ Dinner	**6** ☐ Breakfast ☐ Lunch ☐ Dinner
7 ☐ Breakfast ☐ Lunch ☐ Dinner	**8** ☐ Breakfast ☐ Lunch ☐ Dinner	**9** ☐ Breakfast ☐ Lunch ☐ Dinner	**10** ☐ Breakfast ☐ Lunch ☐ Dinner	**11** ☐ Breakfast ☐ Lunch ☐ Dinner	**12** ☐ Breakfast ☐ Lunch ☐ Dinner	**13** ☐ Breakfast ☐ Lunch ☐ Dinner
14 ☐ Breakfast ☐ Lunch ☐ Dinner	**15** ☐ Breakfast ☐ Lunch ☐ Dinner	**16** ☐ Breakfast ☐ Lunch ☐ Dinner	**17** ☐ Breakfast ☐ Lunch ☐ Dinner	**18** ☐ Breakfast ☐ Lunch ☐ Dinner	**19** ☐ Breakfast ☐ Lunch ☐ Dinner	**20** ☐ Breakfast ☐ Lunch ☐ Dinner
21 ☐ Breakfast ☐ Lunch ☐ Dinner	**22** ☐ Breakfast ☐ Lunch ☐ Dinner	**23** ☐ Breakfast ☐ Lunch ☐ Dinner	**24** ☐ Breakfast ☐ Lunch ☐ Dinner	**25** ☐ Breakfast ☐ Lunch ☐ Dinner	**26** ☐ Breakfast ☐ Lunch ☐ Dinner	**27** ☐ Breakfast ☐ Lunch ☐ Dinner
28 ☐ Breakfast ☐ Lunch ☐ Dinner	**29** ☐ Breakfast ☐ Lunch ☐ Dinner	**30** ☐ Breakfast ☐ Lunch ☐ Dinner	**31** ☐ Breakfast ☐ Lunch ☐ Dinner			

ID: **April 2010**

S	M	T	W	T	F	S
				1 ☐ Breakfast ☐ Lunch ☐ Dinner	**2** ☐ Breakfast ☐ Lunch ☐ Dinner	**3** ☐ Breakfast ☐ Lunch ☐ Dinner
4 ☐ Breakfast ☐ Lunch ☐ Dinner	**5** ☐ Breakfast ☐ Lunch ☐ Dinner	**6** ☐ Breakfast ☐ Lunch ☐ Dinner	**7** ☐ Breakfast ☐ Lunch ☐ Dinner	**8** ☐ Breakfast ☐ Lunch ☐ Dinner	**9** ☐ Breakfast ☐ Lunch ☐ Dinner	**10** ☐ Breakfast ☐ Lunch ☐ Dinner
11 ☐ Breakfast ☐ Lunch ☐ Dinner	**Visit** **Week 6**					

1. Please note that you should take 2 capsules, 1 hour before your breakfast, lunch and dinner (a total of 6 capsules per day).

2. Please remember to bring these sheets, the remained capsules and both containers when you attend in clinical days .