The Effect of Folate and Vitamin B6 on Endothelial Function in Children with Type 1 Diabetes

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Abstract

Introduction

Endothelial dysfunction is a precursor of vascular disease. Children at high risk of vascular disease including children with type 1 diabetes (T1DM) have marked endothelial dysfunction. Endothelial dysfunction is reversible occurring early in the time-line of atherosclerosis. The detection of endothelial dysfunction in childhood allows the study of interventions at an early and potentially reversible stage of vascular damage.

We have previously shown that endothelial dysfunction is common in children with T1DM and relates to folate status (Wiltshire, Gent et al. 2002) despite higher serum and red cell folate levels and lower total plasma homocyst(e)ine (tHcy) than healthy controls (Wiltshire, Thomas et al. 2001; Wiltshire and Couper 2004). Even with these higher folate levels, in a pilot, cross-over study we have shown that folate supplementation improves endothelial function in children with T1DM (Pena, Wiltshire et al. 2004).

Beneficial effects of folate on endothelial function are being demonstrated in increasing numbers of studies (Verhaar, Wever et al. 1998; Woo, Chook et al. 1999; Doshi, McDowell et al. 2001; Thambyrajah, Landray et al. 2001; van Etten, de Koning et al. 2002; Woo, Chook et al. 2002). Improvement in endothelial function, has also been observed within hours of additional oral folate (Doshi, McDowell et al. 2002) and within minutes of intravenous 5-methyltetrahydrofolate (MTHF), the active form of folate (Verhaar, Wever et al. 1998; van Etten, de Koning et al. 2002).

Treatment with combination folate and vitamin B6 lowers markers of endothelial activation (Constans, Blann et al. 1999; Vermeulen, Stehouwer et al. 2000). However, there is limited literature examining the effect of B6 alone on the endothelium. Vitamin B6 improves endothelial function in cardiac transplant recipients (Miner, Cole et al. 2001). There is no data examining the effect of supplemental vitamin B6 in T1DM or children at risk of vascular disease.

Atherosclerosis is an inflammatory process and high-sensitivity C-reactive protein (Hs-CRP), a marker of inflammation, predicts cardiovascular events in adults. Elevated Hs-
CRP in otherwise healthy children is associated with impaired endothelial function. Similar studies in children with T1DM have not been performed.

We therefore aimed to determine the effects, acutely, of folate and vitamin B6 on endothelial function, and over eight weeks, of folate and vitamin B6, alone and in combination, on endothelial function. In addition, we sought to determine whether Hs-CRP, is associated with vascular endothelial and smooth muscle dysfunction, in children with T1DM and healthy control subjects.

**Methods**

A randomised, double-blind, placebo-controlled study of folate 5mg daily and vitamin B6 100mg daily in 124 children with T1DM determined the immediate and eight week effects of these vitamins, alone and in combination, on endothelial function. Endothelial function, assessed by flow mediated dilatation (FMD) and glyceryl-trinitrate (GTN)-induced dilatation using high resolution ultrasound of the brachial artery, was measured at baseline, at two and four hours after the first dose (n=35), and at four and eight weeks of treatment (n=122). Serum and red cell folate, serum vitamin B6, Hs-CRP, tHcy, HbA1c and blood glucose were measured at each assessment of endothelial function.

Hs-CRP and endothelial function, were measured at baseline, in 121 subjects with T1DM. 31 subjects with T1DM that were randomised to receive placebo treatment were studied at four and eight weeks and were included in the longitudinal analysis of Hs-CRP and endothelial function. Hs-CRP and endothelial function were also studied in 33 age-matched, healthy control subjects.

**Results**

FMD normalised in all treatment groups. At baseline and eight weeks FMD [mean(SD)] on folate improved from 2.6(4.3)% to 9.7(6.0)% (p<0.001), on vitamin B6 from 3.5(4.0)% to 8.3(4.2)% (p<0.001), and on folate/vitamin B6 from 2.8(3.5)% to 10.5(4.4)% (p<0.001) respectively. This improvement in FMD occurred within two hours and was maintained
over eight weeks for each treatment. FMD in the placebo group, and GTN-induced dilatation in all groups, did not change. Increase in serum folate, red cell folate, and vitamin B6 related to increase in FMD. Improvement in FMD was independent of change in tHcy, glucose, HbA1c and Hs-CRP. Baseline red cell folate and baseline diastolic blood pressure inversely related to improvement in FMD. Serum triglycerides and LDL-cholesterol inversely related to baseline FMD.

Hs-CRP did not differ between subjects with T1DM and healthy, age-matched controls. In both controls and subjects with T1DM, Hs-CRP did not relate to FMD or GTN-induced dilatation at baseline or at intervals over eight weeks in subjects with T1DM. Hs-CRP did not change over time. In T1DM, but not healthy controls, Hs-CRP related to BMI z-score(r=0.47,p<0.001), weight z-score(r=0.41,p<0.001) and female sex(p=0.008).

Conclusions

High dose folate and vitamin B6 rapidly normalise endothelial dysfunction in children with T1DM. This effect is maintained over eight weeks with ongoing supplementation. Combination treatment over eight weeks does not confer additional benefit.

Hs-CRP is not associated with early vascular dysfunction in children with T1DM. However, in children and adolescents with T1DM, Hs-CRP is associated with female sex and children with higher BMI suggesting these groups may be at greater cardiovascular risk.

In addition to optimising metabolic control, intervention with folate or vitamin B6, at an early stage in childhood, could have a major impact on long-term diabetic vascular complications, and requires further investigation. Maintenance of a healthy BMI may be important in the prevention of vascular disease of T1DM.
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Abbreviations

2D Two-dimensional
ACE Angiotensin converting enzyme
AER Albumin excretion rate
AGEs Advanced glycation end products
AST Aspartate aminotransferase
BH₂ Dihydrobiopterin
BH₄ Tetrahydrobiopterin
cGMP cyclic 3’5’ guanosine monophosphate
CHD Coronary heart disease
CRP C-reactive protein
CYWHS Children, Youth and Women’s Health Service
DAG Diacylglycerol
DCCT Diabetes Control and Complications Trial
DKA Diabetic ketoacidosis
EGC Electrocardiogram
EDIC Epidemiology of Diabetes Interventions and Complications
EDRF Endothelium derived relaxing factor
EDTA Ethylene-diamine-tetra acetic acid
eNOS Endothelial nitric oxide synthase
ET-1 Endothelin-1
FDA Food and Drug Administration
FMD Flow mediated dilatation
GFR Glomerular filtration rate
GTN Glyceryl trinitrate
HbA1c Haemoglobin A1c
HDL High density lipoprotein
Hs-CRP High sensitivity C- reactive protein
IDDM Insulin dependent diabetes mellitus
IDF International Diabetes Federation
Ig Immunoglobulin
IMT Intimal medial thickness
IMVS Institute of Medical and Veterinary Science
LDL Low density lipoprotein
LED Light emitting diode
MTHF Methyltetrahydrofolate
MTHFR Methylene tetrahydrofolate reductase
NADPH Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NF-κB Nuclear factor kappa B
NO Nitric oxide
ORPS Oxford Regional Prospective Study
PAI-1 Plasminogen activator inhibitor-1
PGA Pteroylmonoglutamate
PKC Protein kinase C
PLP Pyridoxal 5'-phosphate
RAGE Receptors for AGE
RCT Randomised Controlled Trial
ROS Reactive oxygen species
SAH S-adenosyl-L-homocysteine
T1DM Type 1 Diabetes Mellitus
TGF-β Transforming growth factor-β
tHcy Total plasma homocyst(e)ine
TSH Thyroid stimulating hormone
U.S. United States
VEGF Vascular endothelial growth factor
VD Vessel diameter
VLDL Very low density lipoprotein
vWF von Willebrand Factor
WCH Women’s and Children’s Hospital
# Units

- **µg/min**: micrograms per minute  
- **µmol/l**: micromoles per litre  
- **µg**: micrograms  
- **µg/l**: micrograms per litre  
- **℃**: degrees Celsius  
- **cm**: centimetres  
- **kg**: kilogram  
- **kg/m²**: kiligrams per square metre  
- **m/s**: metres per second  
- **mg/day**: milligrams per day  
- **mg/l**: milligram per litre  
- **mm**: millimetre  
- **mmHg**: millimeters mercury  
- **mmol/l**: millimoles per litre  
- **nmol/l**: nanomoles per litre  
- **units/kg**: units per kilogram
Chapter 1. Introduction

Problem Statement

Type 1 diabetes is caused by autoimmune destruction of the pancreatic islet cells and is one of the commonest chronic diseases of childhood.

The discovery of insulin more than 80 years ago has changed type 1 diabetes from an imminently fatal disease to a chronic condition. While insulin therapy enables normal growth and development and prevents the immediate life threatening complications of diabetes, people affected by diabetes have a shortened life expectancy complicated by vascular complications. Clinical care of diabetes now focuses on monitoring and preventing these complications.

Diabetic vascular disease involves both micro- and macrovascular disease. The microvascular disease of diabetes involves kidneys, eyes, peripheral nerves and leads to end stage renal failure, blindness, and both peripheral and autonomic neuropathy. The macrovascular disease, due to premature advanced atherosclerosis, leads to coronary artery disease, stroke and peripheral vascular disease. Vascular complications cause considerable mortality and morbidity in patients with type 1 diabetes.

Current evidence emphasises the role of intensive management to improve glycaemic control in patients with type 1 diabetes in order to prevent microvascular complications (1993) and macrovascular injury (Krolewski, Kosinski et al. 1987; 1993; Larsen, Brekke et al. 2002; Nathan, Lachin et al. 2003).

Despite the advances in insulin therapy and management of diabetes that have occurred, we are unable to prevent the long-term vascular complications of diabetes. Good metabolic control greatly reduces the risk of vascular complications but does not eliminate the risk completely suggesting other factors are also involved.

Clinical and epidemiological research emphasises that traditional vascular risk factors, including lipids and blood pressure, also play an important role in the natural history of microvascular complications in patients with type 1 diabetes. The focus on glycaemic control should not obscure the attention owed to other vascular risk factors. An
understanding of vascular risk factors and the contribution they make during the early stages of type 1 diabetes is critical in preventing the long-term vascular complications of diabetes (Wiltshire).

Homocyst(e)ine has been identified as being a significant risk factor for vascular disease given its detrimental effect on endothelial function and its association with advanced atherosclerosis (Clarke, Daly et al. 1991; Welch and Loscalzo 1998; Kuan, A et al. 2002; Wald, Law et al. 2002). In diabetes, homocyst(e)ine may also play a role in the pathogenesis of complications (Vaccaro, Ingrosso et al. 1997; Hofmann, Kohl et al. 1998; Okada, Oida et al. 1999; Chiarelli, Pomilio et al. 2000).

Recently, given the homocyst(e)ine lowering effect of folate, there has been significant work focusing on the effect of folate on endothelial function. Studies in adults have shown both a rapid response (Verhaar, Wever et al. 1998; Doshi, McDowell et al. 2002) and a maintained improvement in endothelial function with high doses of folate (Woo, Chook et al. 2002).

Vitamin B6 is the other essential cofactor in homocyst(e)ine metabolism and also important in homocyst(e)ine lowering. There is limited literature on the effects of vitamin B6 on the endothelium however one study has shown beneficial effects of vitamin B6 (Miner, Cole et al. 2001). Vitamin B6 requirements are higher in diabetes (Okada, Shibuya et al. 1999) and children with type 1 diabetes have low vitamin B6 levels (Wilson and Davis 1977). Low vitamin B6 levels confer an independent risk of vascular disease (Robinson, Arheart et al. 1998; Kelly, Shih et al. 2003; Wilmink, Welch et al. 2004).

The process leading to the vascular complications in type 1 diabetes begins early, decades before the first clinical evidence of vascular disease becomes apparent. Interventions at this stage may have the greatest long-term benefit in reducing the burden of diabetic vasculopathy.

It is well recognised that one of the hallmarks of diabetic vascular disease is endothelial dysfunction and there is considerable evidence that endothelial dysfunction occurs early in the pathogenesis of diabetic vascular disease.

In our unit we have focused on endothelial function in children with type 1 diabetes. Previous work from our unit has shown that despite children with type 1 diabetes having
lower total homocyst(e)ine (tHcy) than age matched controls (Wiltshire, Thomas et al. 2001) endothelial dysfunction is common and relates to folate status (Wiltshire, Gent et al. 2002; Wiltshire and Couper 2004). In children with type 1 diabetes and normal folate status, short term, high dose folate improves endothelial function independently of tHcy (Pena, Wiltshire et al. 2004).

The aim of our research has been to concentrate on potential strategies that intervene at an early stage of the diabetic disease process and may reduce the risk of the vascular complications and their subsequent burden.

**Generation of Hypotheses**

In view of the above problem statement and the background discussed in Chapter 2, the following hypotheses were generated:

1. Endothelial function is impaired in children with type 1 diabetes.
2. Endothelial function is related to folate status.
3. Subjects with higher red blood cell folate at baseline have less response of the endothelium to supplementary folate.
4. Folate improves endothelial function acutely.
5. Folate maintains the improvement endothelial function over an eight week period.
6. Supplemental folate improves endothelial function in children with type 1 diabetes mellitus, independent of lowering homocyst(e)ine.
7. Vitamin B6 improves endothelial function acutely.
8. Vitamin B6 maintains the improvement endothelial function over an eight week period.
9. Vitamin B6 is additive to the effect of folate on endothelial function in children with type 1 diabetes.
10. High sensitivity C-reactive protein relates to endothelial function in children with type 1 diabetes.
Aims

1. To determine the immediate effect of folate on endothelial function in children with type 1 diabetes
2. To determine the immediate effect of vitamin B6 on endothelial function in children with type 1 diabetes
3. To investigate the effect of folate and vitamin B6 alone and in combination on endothelial function in children with type 1 diabetes over eight weeks.
4. To determine if there is an additive effect of vitamin B6 on the effect of folate on endothelial function.
5. To determine whether high sensitivity C-reactive protein relates to endothelial function in children with type 1 diabetes.
Research Strategy

As a result of the significant burden of vascular complications of diabetes, I have focused on a strategy using vitamin supplementation to reduce and possibly prevent these complications. Endothelial function is the earliest marker of vascular disease; we have therefore sought interventions to improve endothelial function in children before any clinical vascular disease can be detected. While folate and vitamin B6 are recently trialled interventions in adults with diabetes and established vascular disease with outcomes released after the work of this thesis (Lonn, Yusuf et al. 2006), less is known about the effects of folate and vitamin B6 on in children with type 1 diabetes without established vascular disease but with early endothelial dysfunction. Folate is proving to be an increasingly promising intervention in adults with endothelial dysfunction and vitamin B6 is a yet unknown entity.

I sought to examine the effects of folate and vitamin B6 both alone and in combination on endothelial function in children with type 1 diabetes.

Chapter 2 provides background information on type 1 diabetes including the interesting history of diabetes. Chapter 2 also explores the current understanding of the pathogenesis of diabetes complications. It reviews rationale for choosing folate and vitamin B6 as interventions for this study by initially reviewing the metabolism of homocyst(e)ine and its regulation. The evidence for the role of homocyst(e)ine in endothelial function and atherosclerosis is detailed. The determinants of homocyst(e)ine metabolism are discussed, providing the initial reasoning behind the use of folate and vitamin B6 in studies of vascular function. Since the first trials of homocyst(e)ine lowering with folate, the understanding of the effect of folate on the endothelium has grown and new hypotheses regarding the mechanism of the beneficial effect of folate on the endothelium have developed. There are substantive trials supporting the beneficial effect of folate on the endothelium, these trials are discussed as well as the current understanding of the effect of folate on the endothelium, from both the laboratory and clinical trials. In as much as folate is increasingly well recognised in having an important role in vascular health, vitamin B6 is very much a new player, there are limited numbers of trials. These trials are small and in the same way as the understanding effect of folate has developed, new theories regarding the mechanism of action of vitamin B6 are developing. The evidence on vitamin B6 is discussed and the current understanding of
vitamin B6 is reviewed. Finally a potentially useful marker of vascular disease, high sensitivity C-reactive protein (Hs-CRP), is discussed. Hs-CRP is a tool proving to be a useful marker of vascular disease in large epidemiological studies. The current evidence on Hs-CRP is discussed as are its potential benefits to clinicians caring for people with type 1 diabetes.

Chapter 3 describes the methods used in the clinical trial. The laboratory methods are detailed as well as the method of determining endothelial function, flow mediated dilatation (FMD) and glyceryl trinitrate (GTN)-induced dilatation.

Chapter 4 is the manuscript as published in the journal ‘Pediatrics’ (MacKenzie, Wiltshire et al. 2006). Impact factor 2007, 4.473. This is an overall summary of the study.

In Chapter 5, I discuss in more depth, the immediate effects study that looks at the acute effect of folate and vitamin B6 on endothelial function. This study reviews baseline flow mediated dilatation as well as the correlates of baseline and improvement in flow mediated dilatation. This chapter predominantly relates to hypothesis 3, 4, 6 and 7. As the study numbers are smaller in this study, the other hypotheses are better addressed in chapter 6. Aims 1 and 2, discussed above, are addressed in this chapter.

Chapter 6 discusses in more depth, the eight week study of the effect of folate, vitamin B6 and folate and vitamin B6 in combination on endothelial function. I have examined the factors that relate to endothelial dysfunction measured by the baseline flow mediated dilatation and also the factors that relate to an improvement in endothelial function. This chapter relates to hypothesis 6 and 8, and aim 3 as discussed above. As this study includes the entire cohort of subjects, i.e. the number of subjects in this study is greater than in the immediate effects study, hypotheses 1 to 3 and 5 are more adequately addressed in this chapter. Later in Chapter 6 the results of the combination of vitamin B6 and folate over the eight week period is presented. These results determine whether there is an additive effect of the combination of vitamin B6 and folate on endothelial function and specifically address hypothesis 9 and aim 4, as described above.

Chapter 7 presents the data from the 35 subjects that participated in both the immediate effects study and the eight week study. Their data from both studies is combined and presented. This chapter addresses the question: Does the early response of the endothelium to folate or vitamin B6 predict the response at eight weeks?
Chapter 8 is the manuscript ‘High Sensitivity CRP is associated with BMI, weight and female sex but not with endothelial function in children with type 1 diabetes’ as published in the journal ‘Pediatric Diabetes’. Impact factor 2007, 2.314. This publication is the result of additional analysis of the data collected during the study. This is an area with very little previously published data.

Chapter 9 reviews the results of high sensitivity C-reactive protein as it relates to endothelial function measured by flow mediated dilatation and other baseline attributes in the children with type 1 diabetes. This chapter addresses the hypothesis 10 and aim 5 as described above. It includes the additional analysis of data required for the manuscript ‘High Sensitivity CRP is associated with BMI, weight and female sex but not with endothelial function in children with type 1 diabetes’.

Chapter 10 reviews GTN-induced vasodilatation in children and adolescents with type 1 diabetes used as an endothelium independent control of vasodilatation and also as a marker of vascular smooth muscle dysfunction, another early risk factor for cardiovascular disease. This is another area with very little previously published data in children.

In Chapter 11, I discuss the unexpected findings and the limitations of the study especially the differences seen in the placebo group and the additional analysis involved. Other unexpected findings including the smokers and the Hs-CRP results are discussed. The dose GTN is raised as a limitation of the study.

Finally, in Chapter 12, I discuss the results of these studies and the implications of this work for children with type 1 diabetes. In addition I suggest strategies for future research and derive overall conclusions.
Chapter 2. Background

Type 1 Diabetes

Type 1 Diabetes- Definition and Incidence

Type 1 diabetes is an autoimmune disorder caused by cell-mediated destruction of the insulin producing β-cells in the pancreatic islet cells. The trigger for the cell-mediated destruction appears to be a combination of genetic susceptibility and environmental factors.

In the 1980s, Eisenbarth proposed the current model for the development of the immune form of type 1 diabetes (Eisenbarth 1986) and although our understanding has progressed significantly since then, the basic aspects of this model remain pertinent. This model postulates that everyone is born with a degree of susceptibility to develop type 1 diabetes: for some this susceptibility is high, for others very low. Susceptibility is largely inherited, residing predominantly in the HLA genotypes DR and DQ, and to a lesser extent in a host of other genetic loci termed IDDM (insulin-dependent diabetes mellitus) susceptibility genes. The HLA locus is thought to confer about 50% of the genetic susceptibility, roughly 15% from two specific IDDM genes—insulin-VNTR (IDDM2) and CTLA-4 (IDDM12)—with minor contributions from the other IDDM genes. The next step in the development of type 1 diabetes requires exposure to one or more environmental triggers that alter immune function, thereby initiating β-cell destruction. Putative triggers include viruses (e.g., enteroviruses, coxsackie, congenital rubella), environmental toxins (e.g., nitrosamines), or foods (e.g., early exposure to cow's milk proteins, cereals, or gluten).

Type 1 diabetes is characterised by elevated blood glucose levels. The resulting chronic hyperglycaemia and other metabolic abnormalities are associated with damage to and subsequent dysfunction of various organs including eyes, kidneys, nervous, heart and blood vessels.

Type 1 diabetes is one the commonest chronic disorders in childhood. In Australia the incidence in children less than 15 years is high with a mean annual incidence of 18.9
cases per 100,000 per year (Craig, Howard et al. 2003). In New Zealand, the incidence of type 1 diabetes in children is very high with a mean annual incidence of 21.9 cases per 100,000 per year (Karvonen, Viik-Kajander et al. 2000).

The incidence of type 1 diabetes is increasing in all populations worldwide. In New South Wales, Australia from 1990-2002, the increase in incidence was 2.1% per year (Craig, Howard et al. 2003). More recent Australian wide data shows the trend in type 1 diabetes incidence in Australia has continued to increase, from 1999-2005, the incidence in all States of Australia increased; in Western Australia type 1 diabetes incidence increased by 3.1%, New South Wales 2.8%, South Australia 1.2%, Australian Capital Territory 5.2%, Tasmania 13.1% (Bulsara, Lloyd et al. 2008). Data for Victoria, Northern Territory and Queensland for this time period were not available at the time of writing this thesis. Currently Australia is ranked seventh and New Zealand fourth in the worldwide incidence of type 1 diabetes in children less than 15 years of age (Karvonen, Viik-Kajander et al. 2000). In Europe, the average annual increase in incidence in children aged 0-14 years is 3.4%, rising up to 6.3% in the 0-5 year age group (2000). This increased in incidence, highest in the under 5 age group, has been confirmed in large epidemiological studies (Green and Patterson 2001).
Hypotheses for the Aetiology and Increasing Incidence of T1DM

Current hypotheses for the increase in incidence since the 1950s, particularly in younger children, include the hygiene hypothesis, the accelerator hypothesis also known as the overload hypothesis, and the effect of vitamin D deficiency (Mathieu and Badenhoop 2005; Mathieu, Gysemans et al. 2005).

The Hygiene Hypothesis

The ‘Hygiene Hypothesis’ links the autoimmune spectrum of diseases including allergies, asthma and type 1 diabetes. The rise in the incidence of type 1 diabetes has until recently, paralleled the rise in childhood asthma. Many attempts have been made to link the rise in asthma and the rise in type 1 diabetes to environmental changes that have occurred over the Twentieth and early Twenty-first Century. The possibility that something protective has been lost from the childhood environment has given rise to the concept known as the Hygiene Hypothesis. There is no single seminal statement of the Hygiene Hypothesis. Instead it has evolved from epidemiological observations that atopic disorders are more common in affluent societies and that there has been a steady rise in their prevalence in parallel with the adoption of a western lifestyle (Gale 2002). The Hygiene Hypothesis suggests that these disorders may be related to reduced exposure to infections and other immune challenges in early life. Within Europe, there is a wide variation of childhood type 1 diabetes incidence rates which can partially be explained by national prosperity indicators. These indicators may reflect differences in environmental risk factors such as nutrition or lifestyle (Patterson, Dahlquist et al. 2001).

The biological basis for the Hygiene Hypothesis is derived from studies of the immune system. Therefore a brief understanding of the immune system and the function of T cells is required to appreciate the Hygiene Hypothesis.

T cells regulate the activities of B cells, T cells, and other cells participating in immune responses. Mature T cells are small lymphocytes that recirculate continually from blood to lymphoid tissues to lymph, awaiting contact with antigen presenting cells that bear a
complementary peptide + MHC complex. Two principal categories of T cells have been defined, effector cells mediating cellular immune responses (cytotoxic cells), and regulatory cells (helper T cells). Helper T cells express CD4 and recognise antigen associated with self-MHC class II molecules. Subtypes of CD4+ T cells have been defined, including Th1, Th2. Th1 cells predominantly generate delayed hypersensitivity reactions, although they also provide B cell help. Th2 are prominent helpers for antibody production, especially IgE responses, and promote eosinophil development and activity.

Early environmental factors stimulate the repertoire of the Th1 responses and when this stimulation is reduced, a dysfunctional Th2 bias persists and predisposes to atopic disorders.

Atopic disorders and type 1 diabetes represent opposing forms of immune deviation with atopy characterised by Th2 immune response and autoimmune disorders such as type 1 diabetes being characterised by a Th1 immune response. In epidemiological studies, a decreased prevalence of atopic diseases has been shown in children with type 1 diabetes (2000) consistent with this immunologic concept of Th1 (type 1 diabetes - autoimmunity) and Th2 (atopy) disorders being mutually exclusive.

However, the Hygiene Hypothesis and how it relates to the increased incidence of type 1 diabetes has fuelled much speculation (Anderson and Watson 2001) and the evidence for the Hygiene Hypothesis is more extensive and more convincing for atopic disorders than for type 1 diabetes (Gale 2002).

**The Accelerator Hypothesis**

The ‘Accelerator Hypothesis’ postulates that increased childhood weight gain accelerates the disease process of type 1 diabetes via obesity induced insulin resistance up-regulating the β- cells which then become susceptible to autoimmune attack (Bjork, Kampe et al. 1992). Overloading of the β- cell may sensitise it to immune damage and apoptosis and accelerate the ongoing autoimmune process leading to its destruction (Dahlquist 2006).
The Accelerator Hypothesis identifies three processes or ‘accelerators’ which variably accelerate the loss of beta cells through apoptosis. The first is the intrinsic potential for a high rate of β-cell apoptosis, an essential but insufficient step in the development of diabetes. The second accelerator is insulin resistance, resulting typically from weight gain and physical inactivity, and is central to the proposed link between the two types of diabetes, type 1 and type 2 diabetes. Insulin resistance puts pressure on a β-cell mass already at risk for accelerated apoptosis, contributing to the expression of clinical diabetes. The third accelerator is present only in those individuals with genetically determined predisposition to β-cell autoimmunity. The metabolically more active β cell, in insulin-resistant individuals who are genetically biased towards a high rate of apoptosis, is at greatest risk for rapid functional deterioration and expression of typical type 1 diabetes. In the absence of this immune accelerator, apoptosis is slower and progression is towards type 2 diabetes.

None of the accelerators leads to diabetes without excess weight gain, a trend which the 'Accelerator Hypothesis' deems central to the rising incidence of both types of diabetes in the industrially developed world. Weight gain causes an increase in insulin resistance, which results in the weakened glucose control. The rising blood glucose accelerates beta-cell apoptosis directly in all and, by inducing beta-cell immunogens, further accelerates it in a subset genetically predisposed to autoimmunity. Rather than overlap between two types of diabetes, type 1 and type 2, the Accelerator Hypothesis envisages an overlay. In the Accelerator Hypothesis, body mass is central to the development and rising incidence of all diabetes, only tempo distinguishes the 'types' (Wilkin 2001).

Advocates of the Accelerator Hypothesis claim that the rise in childhood obesity parallels the rise in both types of diabetes in childhood, and that the decreasing age of onset of type 1 diabetes in heavier children lends further support to their argument (Wilkin 2001). Opponents argue that there is sufficient evidence to support the Eisenbarth model for type 1 diabetes causation without having to implicate the other accelerators (Daneman 2005).
Vitamin D Hypothesis

Vitamin D is essential for pancreatic β-cell function and normal insulin secretion (d'Emden, Dunlop et al. 1989) with vitamin D deficiency shown to inhibit pancreatic secretion of insulin in rodent models (Norman, Frankel et al. 1980).

Recent evidence suggests a role for vitamin D in pathogenesis and prevention of type 1 diabetes. Epidemiological studies suggest a link between vitamin D deficiency in early life and later onset type 1 diabetes (1999; Hypponen, Laara et al. 2001; Stene and Joner 2003). A seasonal pattern of disease onset has been described for type 1 diabetes (Green and Patterson 2001). Vitamin D is a candidate as a mediator of this effect.

Genomic variations of vitamin D metabolism and target cell action predispose to type 1 diabetes. In some populations, type 1 diabetes is associated with certain polymorphisms within the vitamin D receptor gene (Chang, Lei et al. 2000; Pani, Knapp et al. 2000; Motohashi, Yamada et al. 2003; Skrabic, Zemunik et al. 2003).

In studies of non-obese diabetic mice, pharmacological doses of 1,α,25-dihydroxy-vitamin D3 \([1\alpha,25(OH)(2)D(3)]\) or its structural analogues have been shown to delay the onset of diabetes mainly through immune modulation (Zella, McCary et al. 2003).

High-dose vitamin D supplementation early in life protects against type 1 diabetes (1999; Hypponen, Laara et al. 2001; Stene and Joner 2003). Higher maternal intake of vitamin D in pregnancy is associated with a decreased incidence of islet cell auto-antibodies in off-spring suggesting that maternal intake of vitamin D during pregnancy may also have a protective effect on the appearance of islet cell auto-antibodies (Fronczak, Baron et al. 2003).

Pharmacotherapy with vitamin D analogues is postulated to help prevent and treat diabetes (Mathieu and Badenhoop 2005; Mathieu, Gysemans et al. 2005).
Impact of Type 1 diabetes

Diabetes has a huge impact on healthcare worldwide. Although type 2 diabetes has a far greater prevalence than type 1 diabetes, the impact of both is significant. With the global incidence of diabetes, both type 1 and type 2, on the increase, this impact is escalating. In the western world, healthcare expenditure treating diabetes and diabetes related complications is considerable. The International Diabetes Federation (IDF) has estimated the direct annual healthcare cost of diabetes, including both type 1 and type 2, for people aged 20 - 79 years to be 153 - 286 billion international dollars per year. With the increasing incidence of diabetes the direct healthcare expenditure in 2025 is estimated to be 213 - 396 billion international dollars per year. The direct healthcare cost estimate attributable to diabetes includes hospital admissions directly related to diabetes such as diabetic ketoacidosis (DKA) and hypoglycaemia, usually diabetic retinopathy, nephropathy and neuropathy but does not include cardiovascular or cerebrovascular related admissions (IDF 2005).

A substantial proportion of health care costs are borne by the individual and the family. Estimates of these indirect costs of diabetes are as high or even higher than the direct costs (IDF 2005).

A shift towards earlier onset of type 1 diabetes has been noted in all countries and will undoubtedly result in an increased burden upon patients, their families and with earlier onset of complications of the disease will affect the whole community. The incidence trends highlighted indicate that the burden of diabetes is likely to increase.
Type 1 Diabetes- a Historical Perspective

Diabetes has a long and interesting history. It has been known to man for thousands of years.

In ancient Egyptian times, a polyuric state resembling diabetes mellitus was described and there are remedies written in the Papyrus Ebers. The Papyrus Ebers is one of the oldest of all medical documents dating from approximately 1500BC contains what is thought to be the first reference to diabetes mellitus. The following translation is from a version translated in 1890: (Major 1965)

A medicine to drive away the passing of too much urine:

**Prescription:** Cakes
- Wheat grain 1/8
- Fresh grits 1/8
- Green lead earth 1/32
- Water 1/3

Let stand moist; strain it; take it for 4 days.

**Prescription:** Branches of Qadet plant 1/4
- Grapes 1/8
- Honey 1/4
- Berries from üan tree 1/32
- Sweet beer 1/6

Cook: filter and take for 2 days
Aretaeus of Cappadocia (2AD) provides a vivid description of the disease. He first used the term diabetes, from a Greek word meaning to pass through. His chapter “de diabetes” is considered the first accurate account of diabetes that has come from ancient times. He described:

“a wonderful affection, not frequent among men, being a melting down of flesh and limbs into urine. The course is a common one, namely, the kidneys and bladder; for the patients never stop making water, but the flow is incessant, as if from the opening of aqueducts. The nature of the disease, then, is chronic, and it takes a long period to form; but the patient is short lived, if the constitution of the disease be completely established; for the melting is rapid, the death speedy. Moreover, life is disgusting and painful; thirst unquenchable; excessive drinking, which, is disproportionate to the large quantity of urine, for more urine is passed; and one cannot stop them either from drinking or making water. Or if they abstain from drinking, their mouth becomes parched and their body dry; the viscera seem as if scorched up; they are affected with nausea, restlessness, and a burning thirst; and at no distant term they expire. Thirst, as if scorched up with

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Filter and take 1 day
fire. But by what method could they be restrained from making water? ……..Hence the disease appears to me to have got the name diabetes, as if from the Greek word διαβήτης (which signifies a siphon), because the fluid does not remain in the body, but uses the man’s body as a ladder (διαβαθρη), whereby to leave it. (Major 1965)

In the ancient Sanskrit literature of the 5th - 6th centuries, the sweetness of the urine was recorded as sweet urine attracting ants.

It was in the 17th century, that the term ‘mellitus’ derived from the Latin word for honey was applied to distinguish this disease from polyuric states in which the urine is tasteless - ‘insipidus’.

Thomas Willis (1621-1675), physician to Charles I, again observed that this polyuric state was characterised with sweet urine “as if imbued with sugar or honey”. It was this observation that differentiated diabetes mellitus from diabetes insipidus and introduced the modern era in the study of diabetes. In 1776, Matthew Dobson extended the findings of Willis and proved the sweetish taste was sugar.

One of the most significant clinical signs seen in severe diabetic ketoacidosis is the characteristic “air hunger” first described by Adolf Kussmaul in 1874.

“Since I have seen three diabetics in the course of a year die, with remarkably similar symptoms in which there was a peculiar comatose condition preceded and accompanied by dyspnoea, I believe it is not merely by chance, but am of the opinion that it has to do with a form of death in diabetes which is rarely observed and bears the closest relationship to the disturbance of the metabolism in diabetes.” (Major 1965).
Experimental physiology by Oskar Minkowski and Von Mering, in the 19th century implicated dysfunction of the pancreas as the possible cause of diabetes. Patients with diabetes still faced a short life and death usually resulted from ketoacidosis. A starvation diet could briefly normalise blood glucose levels but malnutrition was inevitable and in those who opted for such a diet, life was prolonged for only a few months.

Eugene Opie (1873-1971), while working at the Johns Hopkins University School of Medicine, described hyaline degeneration of the Islands of Langerhans in cases of diabetes mellitus. This was an important discovery that led Sir Edward Sharpey-Schafer (1850-1935), an English physiologist, to postulate that diabetes mellitus was due to the lack of a hypothetical internal secretion. He coined the word "insulin" after theorising that a single substance from the pancreas was responsible for diabetes mellitus.

One of the major landmarks in the history of diabetes was the discovery of insulin by Banting and Best. In 1921, a surgeon Frederick Banting (1891 –1941), working at the University of Toronto, in Ontario, Canada working with medical student Charles Best (1899-1978) were the first to extract insulin from the pancreas. They showed that extracts of whole pancreas lowered the blood glucose of pancreatectomised dogs.

Professor J. J. R. MacLeod (1876-1935) of the University of Toronto's Department of Physiology was overseeing the work of Frederick Banting and Charles Best in their search for a treatment for diabetes. In December, when Banting and Best were having difficulties in refining the pancreatic extract, MacLeod freed Collip, a Professor of Biochemistry on sabbatical from the University of Alberta, from his other research to enable him to join the research team.

James Collip (1892–1965), then, in 1922, isolated insulin using an extraction and purification process. The first injection of insulin was given on January 1, 1922 to a 14 year old boy. The first injection was ineffective but a further injection three weeks later normalised blood glucose levels and abolished the ketonuria.

Banting and Macleod were jointly awarded the Nobel Prize for Medicine in 1923 for the discovery of insulin to treat diabetes.
In collaboration with Eli Lilly, insulin was then produced commercially and was widely available in Europe and North America in 1923. The impact on mortality and morbidity from diabetes was immediate. Insulin was a life saving remedy.

Historically, type 1 diabetes was a fatal disorder. Children with type 1 diabetes in this pre-insulin era died from ‘diabetic coma’ or infection and had a life expectancy of less than a year (Belchetz and Hammond 2003). The discovery of insulin was one the greatest medical advances of the twentieth century and led to a dramatic change in the management of type 1 diabetes (Belchetz and Hammond 2003).

Over the remainder of the 20th century, improvements have been made in the delivery and manufacture of insulin and in our understanding of the physiology and pathophysiology of diabetes. Advances in insulin therapy and medical management have made it possible to control the acute diabetic metabolic complications and manage diabetes on a daily basis. However, as people survived with type 1 diabetes it become apparent that even with insulin therapy, life expectancy was still reduced.

The long term complications of diabetes are now well recognised. The predominant features of diabetes complications are microvascular; diabetic nephropathy, diabetic neuropathy, retinopathy and macrovascular; coronary artery disease, cerebrovascular disease, and peripheral vascular disease.

As a consequence of its microvascular pathology, diabetes is a leading cause of blindness, end-stage renal disease and a variety of debilitating neuropathies. Diabetes is also associated with accelerated atherosclerotic macrovascular disease and people with diabetes have a higher risk of myocardial infarction, stroke and limb amputation. These long-term complications are the major sources of long-term morbidity and premature death in diabetes.

As a reflection of the understanding of diabetes in the mid 20th century, in 1965, Sir Derrick Dunlop, in his opening address of a diabetes mellitus symposium in Edinburgh, posed this question: “is the occurrence of these complications to be regarded fatalistically as something inherent in the diabetic process…or can they be postponed by care and trouble directed to the control of the metabolic disturbance?"
There is now irrefutable epidemiological evidence from the North American 10 year prospective Diabetes Control and Complications Trial (DCCT) showing that glycaemia is a major contributor to the risk and progression of diabetic microvascular complications (1993). The DCCT and the succeeding observational Epidemiology of Diabetes Interventions and Complications (EDIC) (1999) study are now the landmark studies of the development of diabetes complications in Type 1 diabetes Mellitus (T1DM).

The DCCT was a multi-centre prospective, randomised clinical trial to compare intensive insulin therapy to achieve near-normoglycaemia with conventional therapy with regard to their effects on the development and progression of early vascular and neurological complications of type 1 diabetes.

The trial ran between 1983 and 1993 and involved 1441 subjects, aged 13-39 years. It confirmed conclusively that intensive therapy, with the goal of achieving glycaemic levels as close to the non-diabetic range as possible, effectively delayed the onset and slowed the progression of diabetic retinopathy, nephropathy and neuropathy. The complications are seldom seen before puberty even in long standing diabetes. Therefore the effects of the intensive therapy on the adolescent sub cohort of the DCCT (n=195, aged 13-17 years) were examined (1994). The results were similar, although Haemoglobin A1c (HbA1c) levels achieved remained higher in the adolescents compared with the adult subjects. This study concluded that, when initiated during the adolescent years, intensive diabetes therapy for type 1 diabetes was effective in substantially lowering glycaemia and beneficial in reducing long-term diabetic complications (1994).

After completion of the DCCT, this adolescent subgroup of the DCCT was invited to participate in the Epidemiology of Diabetes Interventions and Complications (EDIC) study, a long-term observational study. The follow-up of this adolescent subgroup of the DCCT confirmed that the effects of suboptimal control in adolescence could not be reversed with improved control in adult hood (White, Cleary et al. 2001).

From this work, it is now well recognised that the focus on young people is particularly crucial because improved diabetic control in the early years after diagnosis has a disproportionately high impact on vascular outcomes.
Our current recommendations for the practice of diabetes management are now based upon these findings presented by the DCCT and EDIC research groups (1993; 1994; White, Cleary et al. 2001).

Even with the advances in insulin therapy and management of diabetes that have occurred, we are unable to prevent the long-term complications of diabetes. Despite optimal therapy, vascular complications have still developed in the intensively managed group and some patients with poorer control do not develop the microvascular complications. Data from the EDIC study showed that after four years of follow-up, although 65% of the conventional group showed a progression of retinopathy from the original baseline, so did 32% of the intensively treated group. The prevalence of worsening nephropathy, measured by microalbuminuria, was 13.6% in the conventional group compared with 8.1% of the intensive group. This suggests factors other than glycaemic control are involved in the pathogenesis of diabetic vascular disease.

Family history is an important contributing factor for microvascular complications, suggesting genetic factors are also involved in the pathogenesis of diabetic vascular disease. Subsequent analysis from the DCCT showed a significant family clustering of both nephropathy and retinopathy (1997) and provided the first evidence that the severity of diabetic retinopathy is influenced by familial (possibly genetic) factors and confirmatory evidence that such factors also influence the development diabetic nephropathy.

Diabetic vascular disease has a complex pathogenesis and although it is now well recognised that both glycaemic control and genetic factors are contributory, other factors are also involved. Without doubt, total glycaemic exposure is the principal determinant associated with the progression of vascular complications. Apart from glycaemic control and duration of diabetes, the other factors that contribute to risk of diabetic vascular disease include the well established vascular risk factors of cigarette smoking, hypertension, as well as family history of hypertension, unfavourable lipid profile and age (Ebeling and Koivisto 1997). Duration of diabetes, hyperglycaemia, HbA1c, hypertension and triglycerides have all been shown to be predictive of diabetic complications (Ebeling and Koivisto 1997). Interestingly, risk factors for macrovascular complications have shown international variance with triglycerides and hypertension predictive of cardiovascular disease in North American men (Pittsburg Epidemiology of Diabetes Complications) and age and HDL-cholesterol, more predictive for cardiovascular disease.
in European men (EURODIAB IDDM Complications Study) (Orchard, Stevens et al. 1998).

A worldwide variation in micro- and macrovascular complications of childhood onset type 1 diabetes has also been shown (Walsh, Zgibor et al. 2006), with Central European centres exhibiting higher rates of retinopathy, laser treatment and neuropathy that are unexplained by measured healthcare practice variables. In this analysis, hypertension and duration of diabetes were consistently strong predictors of all complications worldwide (Walsh, Zgibor et al. 2006).

Working towards a better understanding the factors that contribute to the pathogenesis of the vascular complications and exploring additional protective strategies that could be implemented during the early stages of type 1 diabetes may be critical to preventing the long-term complications of diabetes.

The focus of our research has been on potential strategies that intervene at an early stage of the disease process to reduce the risk of these complications and their subsequent burden.
Endothelium and Atherosclerosis

Anatomy of the Blood Vessel

The blood vessel wall consists of three concentric layers intima, media, and adventitia. The intima is adjacent to the blood vessel lumen and in normal arteries, is composed of a monolayer of endothelial cells. It is this layer, the endothelium, which lines the luminal surface of blood vessels, which is the focus of this work. The media contains predominantly muscle cells and elastic fibres in the elastic arteries and is separated from the adventitia and intima by an internal and external elastic lamina. The adventitia is the outermost connective tissue covering of the blood vessel.

For many decades the endothelium was viewed simply as a semi-permeable barrier between the blood and interstitium, facilitating the exchange of water and small molecules. Experiments have demonstrated that the endothelium has an enormous range of vital functions and the normal endothelium maintains vascular homeostasis ensuring adequate blood flow while preventing thrombosis.

Normal Endothelium

The endothelium is an active, dynamic structure that lies between the blood vessel lumen and the vascular smooth muscle. It is strategically situated to act as a direct interface between the components of circulating blood and local tissue.

Because of this strategic anatomic position between circulating blood and the vessel wall, the endothelium regulates numerous local blood vessel functions including vascular tone and maintenance of blood circulation, cell adhesiveness, coagulation, inflammation and permeability (Kharbanda and Deanfield 2001).
The endothelium maintains both the vessel tone and the non-thrombotic nature of its surface by regulation of platelet activity and secretion of anticoagulant and fibrinolytic factors. The endothelium releases prostacyclin, nitric oxide (NO) (the endothelium derived relaxing factor [EDRF]), endothelins, endothelial growth factors, interleukins, plasminogen inhibitors and von Willebrand Factor (vWF) (Celermajer 1997). Interestingly, the endothelium produces both vasodilatory factors and vasoconstricting factors.

The endothelium is able to balance vasodilatation and vasoconstriction and regulate resistance of the vasculature to maintain steady tissue perfusion. As mentioned above, it does this by is producing and reacting to several locally active mediators, the most important of which is nitric oxide.

The Endothelium and Nitric Oxide

Nitric oxide (NO) is one of the most important endothelium derived molecules and the availability of NO represents a key marker in vascular health (Creager, Luscher et al. 2003).

NO is synthesised by the vascular endothelium by the enzyme endothelial nitric oxide synthase (eNOS) in response to stimuli that act on the endothelial cell surface. eNOS utilises L-arginine as substrate, the reaction is stereospecific, and L-arginine is converted to NO and L-citrulline. L-arginine can be synthesised from L-citrulline in endothelial and other cell types, thereby providing a recycling pathway for the conversion of L-citrulline to NO via L-arginine (Flam, Eichler et al. 2007).

eNOS can be activated both by physical stimuli such as shear stress and by calcium-mobilising agonists that bind to cell surface receptors in vascular endothelial cells. NO causes vasodilatation via activating guanylate cyclase on sub-adjacent vascular smooth muscle cells generating greater concentrations of cyclic 3’5’ guanosine monophosphate (cGMP). cGMP reduces intracellular calcium within the smooth muscle causing smooth muscle relaxation (Arnold, Mittal et al. 1977).
NO also protects the blood vessel from endogenous injury, atherosclerosis, by preventing platelet and leucocyte interaction with the vascular wall and inhibiting smooth muscle cell proliferation and migration. The loss of endothelium-derived NO permits increased activity of the pro-inflammatory transcription factor nuclear factor kappa B (NF-κB), resulting in expression of leukocyte adhesion molecules and production of chemokines and cytokines. These actions promote monocyte and vascular smooth muscle cell migration into the intima and formation of macrophage foam cells, characterising the initial morphological changes of atherosclerosis (Creager, Luscher et al. 2003). This development of atherosclerosis is described in detail in the section: ‘Endothelial Dysfunction and the Development of Atherosclerosis’.

The availability of NO reflects a balance between its production via eNOS and its degradation. Many of the metabolic derangements known to occur in diabetes, including hyperglycaemia, excess free fatty acid liberation, and insulin resistance, mediate abnormalities in endothelial cell function by affecting the synthesis or degradation of NO. Hyperglycaemia induces a series of cellular events that increase the production of reactive oxygen species, discussed in depth in the later section: ‘Mechanisms underlying Vascular Disease in Diabetes’. The production of reactive oxygen species (such as superoxide anion) inactivate NO to form peroxynitrite. Peroxynitrite oxidises the eNOS co-factor, tetrahydrobiopterin, this uncouples the enzyme, which then preferentially increases superoxide anion production over NO production (Creager, Luscher et al. 2003). In addition, eNOS appears to be glycosylated in endothelial cells exposed to high glucose.

Clinical studies have found endothelium dependent vasodilatation is abnormal in diabetes, thus, decreased levels of NO in diabetes may underlie its atherogenic predisposition (Creager, Luscher et al. 2003). This process is discussed in more detail in the section of this chapter: ‘Diabetes and Vascular disease’.
Endothelial Dysfunction

The endothelium is a key regulator of vascular function (Furchgott and Zawadzki 1980). Alterations in one or more of the physiological roles of the endothelium constitutes endothelial dysfunction. Endothelial dysfunction may also be defined as the failure of the vascular endothelium to execute its normal role in vasodilatation and/or vascular haemostasis.

Endothelial dysfunction is accompanied by decreased local availability of nitric oxide. Because NO is a local vasodilator and also inhibits platelet adherence and aggregation, smooth muscle proliferation and endothelial cell leucocyte interactions, reduced NO activity may contribute to the initiation and progression of atherogenesis (Celermajer 1997).

It is well established that the endothelium becomes dysfunctional in arteries chronically exposed to cardiovascular risk factors. Hyperglycaemia (Brownlee 2001; Sheetz and King 2002), hypercholesterolaemia (Sorensen, Celermajer et al. 1994), hypertension (Panza, Quyyumi et al. 1990) and smoking (Zeiher, Schachinger et al. 1995; Celermajer, Adams et al. 1996) are the most common risk factors associated with endothelial dysfunction (Celermajer, Sorensen et al. 1992).

Endothelial Dysfunction and the Development of Atherosclerosis

Atherosclerosis is a chronic, systemic, and diffuse disease with focal complications in different vascular beds. It is well recognised that endothelial dysfunction is an important initial event in the development of atherosclerosis. Over time endothelial dysfunction leads to thickening of the intima and media and the eventual development of plaque in the medium and large arteries. Persistence of severe endothelial dysfunction leads to instability of the endothelial surface, erosion of the fibrous cap, subclinical plaque rupture and vessel thrombosis. This atherosclerosis time-line is summarised in Figure 1, a PowerPoint slide produced by Professor D. Celermajer and courtesy of Pfizer Pharmaceuticals.
The process of the formation of these atherothrombotic lesions in atherosclerosis has been thoroughly described in the review article by R. Ross in the New England Journal of Medicine and his work is summarised in the following paragraphs (Ross 1999).

Atherosclerosis is an inflammatory disease. The earliest type of lesion, the so-called fatty streak is present in children including neonates at post-mortem (Stary 1989) and is a pure inflammatory lesion consisting of macrophages and T-lymphocytes.

The changes that precede the formation of the lesions of atherosclerosis take place in the endothelium. They include the increased endothelial permeability which is mediated by nitric oxide and other endothelium derived molecules, the up-regulation of leucocyte adhesion molecules and endothelial adhesion molecules and the migration of leucocytes into the endothelial wall (Figure 2).
The fatty streaks initially consist of lipid-laden monocytes and macrophages (foam cells) together with T-lymphocytes. The next process is the migration of smooth muscle cells, T-cell activation and platelet adherence and aggregation (Figure 3).

NOTE:
This figure is included on page 49 of the print copy of the thesis held in the University of Adelaide Library.
The next phase in the progression of atherosclerosis is formation of an advanced, complicated lesion. As the fatty streaks progress to intermediate and advanced lesions, they tend to form a fibrous cap that walls off the lesion from the lumen. This represents a type of healing or fibrous response to the injury. The fibrous cap covers a mixture of leucocytes, lipid and debris which may form a necrotic core. These lesions expand at their shoulders by means of continued leucocyte adhesion and entry mediated by numerous endothelium derived molecules (Figure 4).

**Figure 4. Atherosclerosis (Ross 1999).**

NOTE:
This figure is included on page 50 of the print copy of the thesis held in the University of Adelaide Library.

Finally, unstable plaques are formed. Rupture of the fibrous cap or ulceration of the fibrous plaque can rapidly lead to thrombosis and usually occurs at sites of thinning of the fibrous cap that covers the advanced lesion. Thinning of the fibrous cap is apparently due to the continuing influx and activation of macrophages which release proteolytic enzymes at these sites. These enzymes cause the degradation of the matrix which can lead to haemorrhage from the vasa vasorum or from the lumen of the artery and can result in thrombus formation and occlusion of the artery (Figure 5).
Atherosclerosis in Children

The development of atherosclerosis begins in childhood. Autopsy findings have shown fatty streaks, the precursor of atherosclerosis, in the aortas of children from age three and in the coronary arteries from adolescence (Stary 1989). Endothelial dysfunction occurs in the earliest stages of atherosclerosis and continues to deteriorate over time as atherosclerosis develops (Figure 1). Atherosclerosis is a disease that has its origins in early life, therefore, interventions to prevent atherosclerosis and its consequences will need to begin early to be effective.
Measurement of Endothelial Dysfunction

Endothelial dysfunction is an early central phase in the evolution of atherosclerosis and independently predicts future cardiovascular events. Although endothelial function assessment has not yet gained routine use in clinical practice, there is a substantial body of evidence supporting its predictive value for cardiovascular events.

In clinical practice, endothelial function is measured indirectly by examining the ability of an artery to vasodilate in response to a pharmacological stimulus and/or a physiological stimulus that stimulates NO release in a healthy blood vessel.

Clinically, endothelial function can be measured in a variety of ways using either intracoronary or peripheral artery testing.

Intra-coronary testing is considered the gold standard and endothelial dysfunction of the coronary arteries has been shown to predict long term atherosclerotic disease progression and cardiovascular events (Schachinger, Britten et al. 2000). Intra-coronary testing however is invasive and testing is limited by its expense and relative inaccessibility. Quantitative angiography involves cardiac catheterisation and an injection dye into the coronary arteries to study the changes in coronary arterial luminal diameter of the coronary arteries in response to pharmacological or physiological vasomotor stimuli. Intra-coronary doppler techniques evaluate endothelial function of the coronary vasculature using coronary blood flow velocity again in response to pharmacological or physiological stimuli. Intra-coronary testing is unsuitable for use in children and adults with no clinical signs of disease.

Non-invasive detection endothelial dysfunction of the brachial artery was first described in 1992 using a technique called flow mediated dilatation (FMD) (Celermajer, Sorensen et al. 1992). FMD is now becoming a commonly used method of testing endothelial function and measures the vasodilator response of the blood vessel to increased blood flow. The method is described in Chapter 3. FMD is technically demanding but can be standardised to yield reproducible results (Deanfield, Halcox et al. 2007). A high degree of investigator expertise is required to determine brachial artery vasodilatation using ultrasonography. Intraobserver and interobserver variability in image acquisition and analysis need to be established (Corretti, Anderson et al. 2002). This weakness of FMD
becomes particularly problematic when FMD is used in multicenter trials. Despite this, FMD is the most reproducible method of non-invasive assessment of endothelial function (Donald, Charakida et al. 2006) and is now considered the gold standard for clinical research on conduit artery endothelial function (Deanfield, Halcox et al. 2007).

Brachial artery FMD is both a sensitive and specific test of vascular function (Schroeder, Enderle et al. 1999) and provides a surrogate measure of coronary endothelial function. FMD has been shown to independently predict cardiovascular events (Schachinger, Britten et al. 2000; Gokce, Keaney et al. 2003). It is a well tolerated procedure which correlates well with invasive testing of the coronary endothelial function (Anderson, Uehata et al. 1995; Neunteufl, Katzenschlager et al. 1997; Takase, Uehata et al. 1998) and also carotid artery intimal medial thickness (Ravikumar, Deepa et al. 2002).

Carotid artery intimal medial thickness (IMT) measures the early structural atherosclerotic changes in the carotid arteries by measuring the thickness of the intima media wall of the carotid artery using with high resolution B-mode ultrasonography. Carotid intima media thickness is a well established index of atherosclerosis that correlates with prevalent and incident coronary heart disease and stroke (Nathan, Cleary et al. 2005). Carotid artery IMT also requires a high level of technical expertise.

Carotid IMT and the progression of IMT correlate well with cardiovascular and cerebrovascular end-points. Extensive data supports the use of carotid IMT as a predictor of cardiovascular risk, however, endothelial dysfunction measured by FMD may be an earlier predictor of coronary artery disease, with increased carotid IMT being evident at a later stage in the process of atherogenesis (Furumoto, Fujii et al. 2002).

Endothelial function can be measured in the peripheral circulation by other non-invasive techniques. Some of examples of these techniques include forearm plethysmography, photoplethysmography and pulse wave analysis and pulse wave velocity.

Forearm plethysmography examines the change in forearm blood flow in response to direct intra-arterial (brachial artery) administration of vasodilator stimuli. This technique is invasive and the reproducibility appears limited.

Photoplethysmography involves measuring the digital volume pulse via infrared light transmission through the finger. The digital volume pulse resembles the carotid pressure
wave and reacts in a similar way in response to vasoactive stimuli. Photoplethysmography is another method of assessing vascular ‘stiffening’ in which both endothelial-dependent and independent responses can be determined. This method is not universally accepted and reproducibility is not well established.

Arterial compliance can be measured by analysis of waveform morphology also known as pulse wave analysis and pulse wave velocity. Pulse wave analysis and pulse wave velocity are both simple techniques that use arterial tonometry to establish arterial wall ‘stiffness’ and can be useful in determining cardiovascular risk. These techniques may be influenced by factors that confound the data such as pulse rate and height. The validity of pulse wave analysis and pulse wave velocity to measure endothelial dysfunction in children has not been determined.

As FMD is a widely used and accepted, non-invasive measure of endothelial function and suitable for use in children, in this study, we measured endothelial function using FMD. FMD has been extensively performed in our unit and the operator expertise is well established.

**Endothelial Dysfunction and Diabetes**

One of the hallmarks of diabetic vascular disease is endothelial dysfunction. In patients with diabetes, brachial artery FMD has been shown to correlate well with carotid intimal medial thickness (Ravikumar, Deepa *et al.* 2002). Endothelial dysfunction occurs early in type 1 diabetes in the absence of clinically detectable atherosclerotic disease (Clarkson, Celermajer *et al.* 1996), it is critical to the pathogenesis of microvascular and macrovascular complications of diabetes (Cohen 1993).

The microvascular and macrovascular complications of diabetes have a complex pathogenesis involving dysfunction and damage of vascular endothelial cell (Vane, Anggard *et al.* 1990; Stehouwer, Nauta *et al.* 1992) as well as vascular smooth muscle cell and platelet function. The metabolic abnormalities that characterise diabetes provoke
the molecular abnormalities that contribute to vascular dysfunction and cause atherosclerosis. These effects of diabetes on vascular function will be discussed in depth in the next chapter.

**Endothelial Dysfunction in Children with Type 1 Diabetes**

Brachial artery endothelial function is impaired in children and adults at risk of atherosclerosis (Celermajer, Sorensen *et al.* 1992). It is impaired in children with type 1 diabetes (Jarvisalo, Raitakari *et al.* 2004).

In our unit we have also shown that FMD is impaired in children with type 1 diabetes and relates to folate status (Wiltshire, Gent *et al.* 2002) despite higher serum and red cell folate levels and lower tHcy than healthy controls (Wiltshire, Thomas *et al.* 2001; Wiltshire and Couper 2004). In a subsequent, cross-over design, pilot study have shown that even with these higher folate levels, folate supplementation improves endothelial function in children with type 1 diabetes (Pena, Wiltshire *et al.* 2004). These studies are discussed in more detail in the section in this chapter: Folate.
Recognised Contributory Factors to Endothelial Dysfunction.

**Diet**

Postprandial endothelial function is transiently impaired after a high fat meal, probably as a consequence of the formation of potentially atherogenic triglyceride-rich lipoproteins (Clarkson, Celermajer *et al.* 1996; Vogel, Corretti *et al.* 1997; Wilmink, Stroes *et al.* 2000; Zhao, Liu *et al.* 2001). As endothelial function is influenced by the fat content of a meal, the testing of endothelial function is ideally performed in the fasting state or after a low fat meal, as in the immediate effects study presented in Chapter 6.

**Exercise**

Regular aerobic exercise training improves endothelial function in healthy, young adults (Clarkson, Montgomery *et al.* 1999). Exercise training also improves endothelial function in adults with type 1 diabetes however this benefit is not maintained after training is discontinued (Fuchsjager-Mayrl, Pleiner *et al.* 2002).

**Puberty**

Puberty is recognised to play a detrimental role in the development as well as the progression of diabetic complications. Assessment of serum markers of endothelial function in subjects with type 1 diabetes suggests that puberty modulates endothelial function (Elhadd, Khan *et al.* 1998).

Hormonal variation during the menstrual cycle also affects endothelial function (Williams, Westerman *et al.* 2001; Slyper 2004). Therefore, measuring flow mediated dilatation at the same time of the menstrual cycle is likely to provide the most consistent results.

In this study, FMD was performed four weeks apart and although a menstrual history was not obtained, the studies are likely to have been performed at a similar phase of the cycle in girls who had regular ovulatory cycles.
Smoking

Cigarette smoking including passive smoking is associated with endothelial dysfunction in otherwise healthy young adults (Zeiher, Schachinger et al. 1995; Celermajer, Adams et al. 1996; Lekakis, Papamichael et al. 1997). For this reason we sought to exclude children who admitted to active smoking. Children were screened on entry into the study with serum cotinine, a by-product of cigarette smoking. Folic acid however does significantly improve endothelial function in otherwise healthy cigarette smokers (Mangoni, Sherwood et al. 2002; O'Grady, Leahy et al. 2002).

Insulin

Insulin has been shown to directly affect the endothelium. In healthy subjects, insulin increases endothelium dependent (NO-mediated) vasodilatation. Insulin induces vasodilatation by causing an endothelium dependent increase in blood flow, this occurs independently from glucose and is mediated via nitric oxide (Steinberg, Brechtel et al. 1994).

In type 1 diabetes, it is unknown as to whether subcutaneously provided insulin has direct effects on the endothelium however it has been hypothesised that the insulin deficiency of type 1 diabetes may reduce NO release and therefore affect endothelial function (Chan, Vallance et al. 2000). Other hypotheses raised by these authors to the aetiology of endothelial dysfunction of type 1 diabetes include reduced bioavailability of endothelial derived NO or defective smooth muscle response to NO release (Chan, Vallance et al. 2000). The later hypotheses appear more robust and are discussed in depth later in this chapter.
Reversibility of Endothelial Dysfunction

Several studies have shown endothelial dysfunction to be reversible (Doshi, McDowell et al. 2001; Woo, Chook et al. 2002; Dinckal, Aksoy et al. 2003). Current research is examining strategies that might improve arterial endothelial function (Celermajer 1997). Flow mediated dilatation is a useful marker to assess the effectiveness of such strategies. Strategies to protect the endothelium early in the atherogenic process are important and intuitively, interventions in children at the earliest stages of arterial wall damage are likely to have the most significant long-term impact.
Diabetes and Vascular Disease

Vascular disease is the major cause of morbidity and mortality in patients with diabetes.

The vascular complications of diabetes are divided into micro- and macrovascular complications. As a consequence of the microvascular disease, diabetes is the leading cause of blindness, end-stage renal disease as well as disabling neuropathies. The accelerated macrovascular disease associated with diabetes substantially increases the risk of developing coronary artery disease, cerebrovascular disease and peripheral vascular disease. Both the macrovascular disease and microvascular disease are more prevalent in diabetic than non-diabetic populations.

Macrovascular Complications

Diabetes Mellitus is associated with accelerated atherosclerosis. Vascular inflammation contributes critically to the initiation and progression of atherosclerosis and these processes are accelerated in hyperglycaemia. The processes and the resultant accelerated atherosclerosis play key roles in the increased prevalence and severity of vascular disease in diabetes of which cardiovascular disease causes the greatest morbidity and mortality.

Cardiovascular Disease

With increasing duration of diabetes, cardiovascular disease is responsible for most of the long term mortality with cardiovascular disease accounting for two-thirds of deaths in the third decade after diagnosis (Laing, Swerdlow et al. 1999; Laing, Swerdlow et al. 2003). From the age of 30 onwards, in the British Diabetic Association Cohort Study, cardiovascular disease was the greatest single cause of death in both sexes (Laing, Swerdlow et al. 1999).

Although cardiovascular disease is not specific to diabetes, diabetes is an independent risk factor for coronary heart disease (CHD). Type 1 diabetes specifically, is associated
with an up to 10 fold greater risk of death from coronary heart disease than aged matched non-diabetic individuals (Dorman, Laporte et al. 1984; Krolewski, Kosinski et al. 1987; Laing, Swerdlow et al. 2003). The sex difference in CHD seen in the general population is demolished (Krolewski, Kosinski et al. 1987).

Myocardial ischaemia due to coronary atherosclerosis commonly occurs without symptoms in patients with type 1 diabetes. This so-called silent coronary atheromatosis has been shown to be highly prevalent in adults with type 1 diabetes (Larsen, Brekke et al. 2002).

Long term glycaemic control is a significant predictor of coronary atherosclerosis and patients with type 1 diabetes have a more rapidly developing form of coronary artery disease than non-diabetic subjects (Larsen, Brekke et al. 2002).

Data recently published from the Diabetes Control and Complications Trial (DCCT) has confirmed the benefit of intensive therapy on the long term risk of cardiovascular disease in patients with type 1 diabetes (Nathan, Cleary et al. 2005). Between 1983 and 1993 the DCCT randomly assigned 1441 subjects with type 1 diabetes to intensive or conventional therapy. 93% of subjects were followed until February 2005, during the observational Epidemiology of Diabetes Interventions and Complications (EDIC) study. In the New England Journal of Medicine article, Nathan et al. report cardiovascular outcomes of these subjects. 46 cardiovascular disease events occurred in 31 patients in the intensive treatment arm of the DCCT compared with 98 events in 52 patients who received conventional treatment. Intensive treatment reduced the risk of any cardiovascular disease event by 42%. The decrease in glycosylated haemoglobin values during the DCCT was significantly associated with most of the positive effects of intensive treatment on the risk of cardiovascular disease (Nathan, Cleary et al. 2005).

The DCCT/EDIC cohort has convincingly shown that those initially treated intensively, and achieving reduced HbA1c concentrations, continue to have greater protection against development or progression of complications than those initially receiving conventional therapy. This protection arises despite similar levels of glycaemic control in the 6–10 years after completion of the intervention part of the study. This metabolic or hyperglycaemic memory demands that intensive management be instituted as soon as diabetes is diagnosed.
The DCCT/EDIC study are unique in its long-term objective documentation of glycaemic control and the status of both micro- and macrovascular complications. The virtually complete follow-up for more than two decades of the DCCT/EDIC cohort facilitate landmark documentation of T1DM outcomes and provide compelling evidence for the benefits of intensive therapy for diabetes.

**Cerebrovascular Disease**

Cerebrovascular disease causes significant increased mortality in subjects with diabetes. The incidence and severity of ischaemic stroke are increased in diabetes, and outcome from stroke is poorer. The mortality from cerebrovascular disease is at least three fold greater in patients with type 1 diabetes than in the general population and the risk is especially high in young adults (Laing, Swerdlow *et al.* 2003).

Recently an association between glycaemia and carotid intima-media thickness which is a sensitive marker for both coronary and cerebral vascular disease, in patients with type 1 diabetes has been documented as part of the EDIC study (Nathan, Lachin *et al.* 2003). 1229 subjects participating in the EDIC study underwent ultrasonography of the carotid arteries in 1994-1996 and again in 1998-2000. Carotid artery intima medial thickness was determined in 611 subjects who had been randomised to receive conventional treatment during the DCCT and 618 who had been randomised to receive intensive treatment. At one year of the EDIC study, the carotid artery intima media thickness was similar to that in an age- and sex- matched population. After six years, the intima-media thickness was significantly greater in diabetic subjects. The progression of the intima-media thickness was significantly less in the group who received intensive therapy during the DCCT than in the group that received conventional therapy (Nathan, Lachin *et al.* 2003).
Microvascular Complications

The DCCT and the EDIC study, the DCCT’s long term follow-up study, have also shown that glycaemia is a major contributor to the risk and progression of diabetic microvascular complications; retinopathy, nephropathy and neuropathy (1993; 1999; 2000).

Family history is an important contributing factor for microvascular complications, suggesting genetic factors are also involved in the pathogenesis of diabetic vascular disease. Subsequent analysis from the DCCT have shown a significant family clustering of both nephropathy and retinopathy (1997).

Retinopathy

Diabetic retinopathy remains the most common cause of acquired blindness in people over 16 years. Five years after diagnosis, about 20% of patients with type 1 diabetes have detectable retinopathy and by 20 years after diagnosis, over 90% will be affected. 50% of those affected with diabetic retinopathy will have proliferative retinopathy. In 20%, this proliferative retinopathy will be eventually sight threatening and approximately 2% of patients with type 1 diabetes will become blind (Belchetz and Hammond 2003).

Prevention or delaying of diabetic retinopathy can be achieved by good glycaemic control (1994; 2000) and additional analysis from the DCCT has provided evidence that the severity of diabetic retinopathy is influenced by familial (possibly genetic) factors (1997).

Nephropathy

Diabetic nephropathy is the commonest cause of end-stage renal failure worldwide. Renal disease is one of the common causes of death in patients with type 1 diabetes. In the Pittsburgh insulin-dependent diabetes mellitus morbidity and mortality study, after age 20, the annual mortality risk was approximately 2%, which was more than 20 times
greater than for the U.S. population and renal disease was responsible for the majority of these deaths (Dorman, Laporte et al. 1984).

The earliest clinical feature of nephropathy is microalbuminuria, which has a prevalence of about 60% in patients with type 1 diabetes of 30 years’ duration (Belchetz and Hammond 2003). Microalbuminuria is a recognised risk factor for increased mortality and renal failure in type 1 diabetes and in patients with long-standing type 1 diabetes the presence of microalbuminuria remains a powerful predictor of increased mortality (Allen and Walker 2003).

Microalbuminuria identifies both patients at risk of progression to nephropathy and patients without overt nephropathy who are at risk of developing cardiovascular disease. Microalbuminuria and albuminuria are each strongly associated with an increased risk of cardiovascular disease (Nathan, Lachin et al. 2003; Nathan, Cleary et al. 2005) and an increase in coronary heart disease mortality (Torffvit, Lovestam-Adrian et al. 2005).

There is clear evidence that angiotensin converting enzyme (ACE) inhibitors reduces albumin excretion in adults with persistent microalbuminuria when blood pressure is normal (Rudberg, Osterby et al. 1999). Treatment of microalbuminuria with ACE inhibitors not only delays the progression of microalbuminuria to diabetic nephropathy but also has been shown to benefit in preventing cardiovascular disease in type 1 diabetes (2000). In the paediatric population, there is a high rate of transient microalbuminuria (Shield, Hunt et al. 1995). The Oxford Regional Prospective Study (ORPS) of childhood diabetes is the only study providing accurate data concerning the prevalence of microalbuminuria in a cohort of patients with T1DM recruited at diagnosis (Schultz, Konopelska-Bahu et al. 1999). Despite this large cohort study, there is a lack of clear definition of the natural history of microalbuminuria in the paediatric population, and, as a result, there is no current consensus as to who to treat and when treatment with renoprotective drugs should begin (Chiarelli, Trotta et al. 2002).
Neuropathy

Diabetic neuropathies are as a group the most common chronic complication but probably the least understood (Sugimoto, Murakawa et al. 2000).

Diabetic neuropathies include several distinct syndromes among which distal sensory polyneuropathy, often associated with autonomic neuropathy, is the most common. Classically polyneuropathies are characterised by loss of peripheral sensation and when accompanied by diabetic micro- and macrovascular disease predispose to non-healing ulcers that are the leading cause of non-traumatic limb amputation. The autonomic neuropathy of diabetes predisposes to among other things, silent cardiovascular disease as discussed above.

Diabetic neuropathy may occur from direct hyperglycaemia induced damage to the nerve parenchyma via the hyperglycaemia activated mechanisms discussed below and from neuronal ischaemia caused by impaired blood flow to the neurovasculature. Impairment of nerve blood flow may result from a reduction in endothelial dependent dilatation in the endoneurium (Sugimoto, Murakawa et al. 2000).

Evidence also exists that the aetiology of diabetic autonomic neuropathy may have an autoimmune basis. Recent work has shown that autoantibodies to autonomic nerve structures (ANabs) are associated with the development and progression of cardiac and peripheral autonomic neuropathy in type 1 diabetes (Granberg, Ejskjaer et al. 2005).
Mechanisms underlying Vascular Disease in Diabetes

There is accumulating evidence that common pathogenic mechanisms underlie the development of the diabetic microvascular and macrovascular complications. Endothelial dysfunction is an early event in diabetic vascular disease. The pathogenesis is complex and involves abnormalities in both the endothelium and vascular smooth muscle cell. Many of the metabolic abnormalities that characterise diabetes contribute to endothelial dysfunction, including hyperglycaemia and insulin resistance. Hyperglycaemia however, is the major causal factor in the development of diabetic vascular complications and exerts its adverse effects on the endothelium through multiple mechanisms.

These mechanisms through which excess glucose results in tissue damage remain incompletely understood, however, many of the biochemical pathways associated with chronic hyperglycaemia are recognised to contribute to endothelial dysfunction.

There are several pathways that are implicated in the glucose mediated process of endothelial damage. Until recently there was focus on four specific pathways thought to contribute to the pathogenesis of diabetic vascular disease. These four pathways include the activation of the polyol pathway, the increased formation of advanced glycosylation end products (AGEs), the increased hexosamine pathway flux and the stimulation of the diacetylglcerol (DAG)- protein kinase C (PKC) pathway (Brownlee 2001) (Figure 6).

These pathways and their contribution to diabetic vasculopathy are reviewed by Brownlee (Brownlee 2001) and Sheetz (Sheetz and King 2002) in two reviews of the molecular mechanisms of diabetic vascular disease and are discussed in the subsequent paragraphs.
Figure 6. A Summary of the Four Hyperglycaemia Induced Pathways (Brownlee 2001)

NOTE:
This figure is included on page 66 of the print copy of the thesis held in the University of Adelaide Library.
Polyol Pathway and Aldose Reductase

The development of the concept that aldose reductase is involved in diabetic complications stems from its early beginning when Dr Van Heyningen first found polyols in sugar cataracts in 1959 (Kinoshita 1990).

In tissues that do not require insulin for cellular glucose uptake such as kidney, retina, nerves and blood vessels, hyperglycaemia activates the polyol pathway resulting in the formation of sorbitol. There is a well-established link between the increased polyol pathway and the occurrence of diabetic complications. Aldose reductase activity and gene expression are increased in patients with complications of diabetes (Hamada, Kitoh et al. 1993; Shah, Dorin et al. 1997).

Aldose reductase is the first enzyme in the polyol pathway. In the hyperglycaemic environment, increased intracellular glucose activates aldose reductase which uses NADPH (reduced Nicotinamide Adenine Dinucleotide Phosphate) as a cofactor to reduce glucose to the polyalcohol, sorbitol. Sorbitol is then oxidised to fructose via sorbitol dehydrogenase using NAD⁺ as a cofactor. As mentioned above, this pathway is site and tissue dependent.

A number of mechanisms have been proposed to explain the potential detrimental effects of hyperglycaemia-induced increases in the polyol pathway. Among these include the accumulation of sorbitol which may cause changes in osmotic gradients. Due to the high level of expression of aldose reductase in the lens compared with other tissues, an increase level of sorbitol may contribute to the development of cataracts (Kinoshita 1974). In the microvasculature, it was originally suggested that sorbitol may again cause osmotic damage (Gabbay 1975), however sorbitol concentrations measured in diabetic vessels are too low to cause osmotic damage (Brownlee 2001).

The polyol pathway affects energy metabolism, the reduction of glucose to sorbitol by NADPH consumes NADPH. The polyol pathway increases cytosolic NADH/NAD⁺ and decreases cytosolic NADPH. As NADPH is required for regenerating glutathione, reduced NADPH could exacerbate intracellular oxidative stress.

Aldose reductase is present in vascular endothelial cells and more recent evidence suggests that the decline in cellular NADPH, caused by the increase in aldose reductase
activity, may decrease the generation of nitric oxide in endothelial cells (Tesfamariam 1994). NADPH is an essential cofactor for eNOS for the synthesis of NO, its depletion as a result of chronic hyperglycaemia leads to a reduction in NO production.

Aldose reductase inhibitors showed early promise in reversing glucose-induced changes in sorbitol metabolism and preventing some of the pathological changes in rodent models of retinopathy, neuropathy and nephropathy (Cameron, Leonard et al. 1986; Mauer, Steffes et al. 1989; Robison, Nagata et al. 1989). However studies of the inhibition of the polyol pathway have yielded inconsistent results in humans (1990; 1993).

**Advanced Glycation End (AGE) Products**

During the normal course of aging, proteins, lipids, nucleotides etc. become irreversibly modified by sugars in a process known as the Maillard reaction in which glucose reacts with protein groups to form Schiff bases which then convert *in vivo* into more stable Amadori products. Over a long period, a small proportion of Amadori products are then irreversibly transformed into advanced glycation end products (AGEs).

In the presence of high plasma glucose concentrations, this process is accelerated. Glucose binds non-enzymatically to free amino acid groups on proteins or to lipids causing cross-linking of proteins or lipids by glucose. This glycation of amino groups underlies the formation of HbA1c (an Amadori product), a well recognised marker of glycaemic control in diabetes. As mentioned, a series of further irreversible reactions results in the formation of AGEs. These do not reduce with the resolution of hyperglycaemia but accumulate over the lifetime of the protein.

The generation of AGEs was first described as an epiphenomenon of aging and subsequently the AGE theory has developed as an attempt to explain diabetic complications. AGEs accumulate during the progression of diabetes and it has been demonstrated that advanced glycation end products are more abundant in diabetics than in age-matched controls and are elevated early in the course of type 1 diabetes (Tsukahara, Sekine et al. 2003).
The formation of advanced glycation end products (AGEs) and their precursors damage cells by impairing the function of a wide range of proteins including both extracellular structural proteins and intracellular proteins (Brownlee, Vlassara et al. 1984; Brownlee 1995; Brownlee 2001) and alter cell function by the activation and up-regulation of receptors for AGE (RAGE) (Schmidt, Hofmann et al. 2000). The activation of RAGE in turn activates the transcription factor NF-κB, causing pathological changes in gene expression (Brownlee 2001).

Advanced glycation end products interfere with the function of blood vessels and contribute to endothelial dysfunction. They alter vessel wall homeostasis in a pro-atherogenic fashion through multiple mechanisms, these include alterations in endothelial permeability, release of inflammatory cytokines and growth factors, alterations of anti-thrombotic properties of the endothelium and interference with the ability of the endothelium to modulate vascular tone (Basta, Schmidt et al. 2004). Accumulation of AGEs in the sub-endothelial collagen ‘quench’ NO released by the endothelial cells (Bucala, Tracey et al. 1991). AGEs also enhance oxidative stress (Tsukahara, Sekine et al. 2003) and promote atherogenesis by increasing oxidation of low density lipoproteins (Basta, Schmidt et al. 2004). Oxidised LDL inactivates endothelial nitric oxide after its release from endothelial cells (Chin, Azhar et al. 1992) and hence contributes to endothelial dysfunction.

Sustained expression of RAGE in the endothelium and smooth muscle cells, propagates cellular dysfunction (Schmidt, Hofmann et al. 2000) and contributes to endothelial dysfunction. In addition to increased expression of RAGE, evidence suggests that the engagement of RAGE, a multi-ligand member of the immunoglobulin superfamily, by its signal transduction ligands (i.e. AGEs), evoke inflammatory cell infiltration and activation in the vessel wall thereby have an important role in both early development and progression of atherosclerosis and vascular inflammation. In diabetes, when fuelled by oxidant stress and hyperglycaemia, the ligand-RAGE amplifies vascular stress and accelerates atherosclerosis and neo-intimal expansion. In rodent models, studies support this premise that RAGE is an amplification step in vascular inflammation and acceleration of atherosclerosis (Naka, Bucciarelli et al. 2004). Studies in animal models of vascular injury highlight the potent impact of RAGE blockade. Administration of ligand-binding decoys of RAGE or antibodies to the receptor reduced the consequences of diabetes, hyperlipidaemia, and physical injury to the vessel wall (Yan, Naka et al. 2006).
The Hexosamine Pathway.

Hyperglycaemia causes increased activation of the Hexosamine pathway in endothelial cells. In this pathway, fructose-6-phosphate is diverted from glycolysis to provide substrates for reactions such as proteoglycan synthesis and the formation of O-linked glycoproteins. This is of relevance to the pathogenesis of diabetic vascular complications as eNOS activity is inhibited by hyperglycaemia induced O-acetyl glucosaminylation of the eNOS protein (Du, Edelstein et al. 2001). The increased activation of the hexosamine pathway by hyperglycaemia may also result in changes in gene expression and protein function, which also may contribute to the pathogenesis of diabetic vascular complications.

Protein Kinase C and Diacylglycerol

Diacylglycerol and Protein Kinase C (PKC) are critical intracellular signalling molecules that can regulate many vascular functions including vascular permeability and vasodilator release.

Hyperglycaemia causes the de novo synthesis of the lipid second messenger diacylglycerol which activates PKC. Diacylglycerol and PKC can also be activated by reactive oxygen species, increased activity of the polyol pathway and AGEs (Sheetz and King 2002). This pathological activation of PKC has been suggested as another mechanism contributing to endothelial dysfunction and the vascular complications of diabetes (Wolf, Williamson et al. 1991; Park, Ha et al. 1999).

The PKC family comprises of at least twelve isoforms. Activation of PKC, in particular the β and δ isoforms, has a number of pathogenic consequences by affecting the expression of endothelial nitric oxide synthase (eNOS) as well as endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β) and plasminogen activator inhibitor-1 (PAI-1) and by activating nuclear factor kappa B (NFκB) and NAD(P)H oxidases (Brownlee 2001). A summary of the effects of PKC pathway activation is illustrated (Figure 7).
PKC activation affects endothelial function by limiting the activity of pathways leading to the phosphorylation of eNOS. This reduces the activity of eNOS resulting in less NO production.

Activation of the PKC pathway, and particularly the PKCβ isoform, has been shown extensively to cause diabetic vascular dysfunction in rodent models. Thus, PKC inhibitors have been developed and in models of diabetes, endothelial dysfunction has been shown to be corrected with these PKC inhibitors (Tesfamariam, Brown et al. 1991; He and King 2005). The success of animal studies with PKC inhibitors has led to clinical studies which again are showing promise of PKC inhibition as a new therapeutic agent (He and King 2005). The PKCβ isoform-selective inhibitor, ruboxistaurin, has shown promising effects on delaying the progression of clinical parameters of diabetic nephropathy in type 2 diabetic patients (Tuttle, Bakris et al. 2005) and in a more recent review ruboxistaurin shows promise as an oral treatment for diabetic retinopathy (Clarke and Dodson 2007).
Reactive Oxygen Species and Oxidative Stress

One of the oldest theories of diabetic complications is that hyperglycaemia can increase oxidative stress. Oxidative stress continues to be a focus of current work on the pathogenesis of diabetic complications and many studies have shown that diabetes and hyperglycaemia increase oxidative stress (Giugliano, Ceriello et al. 1996).

Hyperglycaemia induces a cascade of reactions that increase the production of reactive oxygen species and there is evidence that elevations in glucose also depress natural antioxidant defences (Giugliano, Ceriello et al. 1996) both contributing to increased oxidative stress. Each of the mechanisms described above, the polyol pathway activation, PKC activation and advanced glycation end (AGE) product formation result in hyperglycaemia mediated overproduction of reactive oxygen species (Brownlee 2001).

The generic term reactive oxygen species (ROS) used in the subsequent paragraphs refers to free radicals (molecules with one or more unpaired electrons in their atomic structure that are highly reactive) such as superoxide anions, hydroxyl radicals and hydrogen peroxide that are generated in the hyperglycaemic milieu.

Reactive oxygen species cause the oxidative stress that underlies the development of the vascular complications (Brownlee 2001). Once formed ROS deplete antioxidant defences rendering affected tissues more susceptible to oxidative damage. The formation of reactive oxygen species cause increased glycation of proteins and accelerate AGE formation.

Exposure of the endothelial cells to high glucose results in augmented production of the reactive oxygen species, this in turn inactivates endothelial derived nitric oxide, interferes with endothelium dependent vasodilatation and causes endothelial dysfunction (Creager, Luscher et al. 2003). Specifically, hyperglycaemia increases the production of ROS that inactivate NO to form peroxynitrite. Peroxynitrite once formed, then oxidises the endothelial nitric oxide synthase cofactor tetrahydrobiopterin which results in uncoupling of the enzyme and preferential production of ROS over NO. The uncoupling of enzymes, such as eNOS, alters (and typically disrupts) of the activity of a physically or functionally coordinated biological system. With the uncoupling of eNOS, the cascade effect results in increased production of ROS and inactivation of NO (Creager, Luscher et al. 2003).
Nitric Oxide

Endothelial nitric oxide (NO), which has been alluded to in all of these theories, appears to be a common element.

NO is among the important molecules synthesised by the normal endothelium and is produced by endothelial nitric oxide synthase (eNOS). The bioavailability of nitric oxide is critical to vascular health and reflects a balance between its production by eNOS and its degradation. NO modulates vascular tone regulating basal blood flow and protects the blood vessel from endogenous injury by preventing platelet aggregation and leukocyte interaction with the vascular wall and inhibiting smooth muscle cell proliferation and migration. eNOS can be activated in complex ways both by haemodynamic shear stress and by a variety of calcium-mobilising agonists that bind to cell surface receptors in vascular endothelial cells. The complexity of eNOS regulation and the labile nature of its product permit the existence of numerous pathophysiological pathways by which diabetes impinges upon NO signalling (Igarashi and Michel 2001).

NO signalling may be affected by alterations in substrate availability, changes in eNOS expression, by derangement of the signalling pathways following eNOS activation, by changes in tetrahydrobiopterin (an essential cofactor) or NADPH levels, or by quenching of bioactive NO by glucose, lipoproteins, or reactive oxygen species among others (Igarashi and Michel 2001) as discussed in the previous paragraphs.

The loss of endothelial derived NO permits increased activity of the pro-inflammatory transcription factor nuclear factor kappa B (NF-κB), resulting in expression of leukocyte adhesion molecules and production of chemokines and cytokines. These actions promote monocyte and vascular smooth muscle cell migration into the intima and formation of macrophage foam cells characterising the initial changes of atherosclerosis (Creager, Luscher et al. 2003).

In diabetes, there may be either reduced NO release or reduced NO availability (Chan, Vallance et al. 2000). In either case, vascular NO activity is reduced, leading to impaired endothelium dependent vasodilatation and platelet aggregation (Giugliano, Ceriello et al. 1996). These decreased levels of NO in diabetes may underlie its atherogenic predisposition (Creager, Luscher et al. 2003).
In this study, by looking at flow mediated dilatation, which is an endothelial response to sheer stress causing nitric oxide release and vasodilatation in a healthy vessel, I have studied a nitric oxide dependent pathway.

**Free Fatty Acids**

Circulating free fatty acids are elevated in diabetes and are also contributory in the pathogenesis of diabetic vascular disease. Free fatty acids impair endothelial function through several mechanisms, including production of reactive oxygen species and activation of PKC which decreases NOS activity as discussed.

The liver responds to free fatty acids by increasing low density lipoprotein (LDL) production and cholesterol ester synthesis. This results in elevated triglyceride concentrations which is observed in diabetes and which also has been associated with endothelial dysfunction (Creager, Luscher et al. 2003).

**Summary**

Many of the metabolic derangements known to occur in diabetes, but in particular hyperglycaemia, result in activation of pathways that cause increased oxidative stress and result in decreased bioavailability of nitric oxide. In addition, excess free fatty acid liberation mediates abnormalities in endothelial cell function by also affecting synthesis and degradation of NO. Diminished NO and enhanced oxidative stress play a central role in the cellular events that lead to endothelial dysfunction and result in the development of diabetic vascular disease. Other contributory factors include abnormal platelet function and increased production of prothrombotic factors. Together, these abnormalities contribute to the atherogenic propensity of diabetes and ultimately result in the microvascular and macrovascular manifestations that occur in patients with diabetes and cause significant morbidity and increased mortality.
The Effect of Folate and Vitamin B6 on Endothelial Function in Children with Type 1 Diabetes
Dr Karen E MacKenzie

Homocyst(e)ine

Hyperhomocyst(e)inaemia is well known to be a important risk factor for advanced atherosclerosis. Homocysteine may play a role in the development of advanced diabetic vasculopathy (Stehouwer, Gall et al. 1999). For this reason, researchers from our unit originally looked at total plasma homocyst(e)ine (tHcy) in children with type 1 diabetes and found low levels of tHcy that related to their relatively high folate status and their high glomerular filtration rate (GFR) (Wiltshire, Thomas et al. 2001). These findings lead us to continue to examine tHcy and folate in our subsequent research. Despite low tHcy levels in children with type 1 diabetes, folate status independently related to endothelial function (Wiltshire, Gent et al. 2002). In this research, I have studied tHcy and the two essential cofactors of homocyst(e)ine metabolism, folate and vitamin B6 and the effect on endothelial function.

An understanding of the homocyst(e)ine pathway, and particularly the essential cofactors of homocysteine metabolism, has been essential in generating the hypothesis and choosing the interventions for this trial. I have measured total plasma homocyst(e)ine (tHcy) and its determinants and examined the possible effect of tHcy lowering therapy on endothelial function in children with type 1 diabetes in this research. For this reason this chapter has been dedicated to discussing the metabolism of homocyst(e)ine.

The term homocyst(e)ine or total plasma homocyst(e)ine (tHcy) is used to describe the combined pool of homocysteine (the amino acid), homocystine (the disulphide made from covalent bonding of two homocysteine molecules through their sulphydryl groups), mixed disulfides involving homocysteine (and a compound with another free sulphydryl group) and homocyst(e)ine thiolactone found in patients with hyperhomocyst(e)inaemia.

Homocyst(e)ine: The Background

For the last three decades, homocyst(e)ine has been the focus of interest in research involving the pathogenesis of vascular disease.
In 1969 McCully made the clinical observation linking elevated homocyst(e)ine concentrations with vascular disease (McCully 1969). He reported autopsy evidence of extensive arterial thrombosis and arteriosclerosis in two children with elevated homocyst(e)ine concentrations and homocystinuria. On the basis of this observation, he proposed that elevated homocyst(e)ine can cause atherosclerotic vascular disease.

Abundant epidemiological evidence has since confirmed McCully’s original hypothesis with homocyst(e)ine levels conferring an independent increased risk of coronary, cerebrovascular and peripheral vascular atherosclerotic disease (Wald, Law et al. 2002). Homocyst(e)ine is now a well recognised independent risk factor for atherosclerotic disease.

**Metabolism of Homocysteine**

Homocysteine is a sulphur containing amino acid formed during the metabolism of methionine, an essential amino acid. Homocysteine is metabolised through one of two pathways, the remethylation pathway or the trans-sulphuration pathway (Loscalzo 1996; Welch and Loscalzo 1998).

In the remethylation pathway, homocysteine undergoes remethylation to methionine in a reaction catalysed by methylenetetrahydrofolate homocysteine methyltransferase (methionine synthetase) methylenetetrahydrofolate (derived from folate) as the methyl donor, and vitamin B12 as an essential cofactor.

When cysteine synthesis is required or in the presence of excess methionine homocysteine enters the trans-sulphuration pathway. In this pathway, homocysteine first condenses with serine to form cystathionine in a rate limiting reaction catalysed by cystathionine-β-synthase, which is a B6-dependent enzyme, as it uses pyridoxal 5'-phosphate as the cofactor. Cystathionine-γ-lyase then catalyses the hydrolysis of cystathionine to yield α-ketobutyrate and cysteine in another reaction also requiring pyridoxal 5'-phosphate (Figure 8). The cysteine that is formed from homocysteine is ultimately converted to the sulphate and is excreted in the urine.
Figure 8. Homocysteine Metabolism (Welch and Loscalzo 1998)

NOTE:
This figure is included on page 77 of the print copy of the thesis held in the University of Adelaide Library.

tHcy is an important reflection of the status of methionine metabolism and its metabolism is influenced by alterations of folate, vitamin B6 and vitamin B12.

Elevated tHcy is classically caused by genetic defects in the enzymes involved in homocysteine metabolism. Deficiencies of the vitamin cofactors required for
homocysteine metabolism, folate, vitamin B6 and vitamin B12 can contribute to elevated tHcy levels.

Pathogenic Mechanism of Hyperhomocyst(e)inaemia

The atherogenic propensity of homocyst(e)ine results from endothelial dysfunction, followed by characteristic substantial platelet accumulation and subsequent platelet rich thrombus formation in areas of endothelial injury. Homocyst(e)ine causes both acute and chronic endothelial dysfunction (Celermajer, Sorensen et al. 1993; Woo, Chook et al. 1997; Chambers, McGregor et al. 1998). Although the exact mechanism of endothelial dysfunction is not known, there is growing evidence that homocyst(e)ine exerts its effects by promoting oxidative damage. Reactive oxygen species are generated in the auto-oxidation of homocyst(e)ine. The generation of reactive oxygen species promotes the activation of several atherogenic pathways (Figure 9). The mechanisms by which homocyst(e)ine is involved in these pathways are reviewed in two recent publications (Welch and Loscalzo 1998; Mangoni and Jackson 2002) and are summarised in the subsequent paragraphs.

Homocyst(e)ine is rapidly auto-oxidised when added to plasma, leading to the formation of homocystine, mixed disulfides and homocyst(e)ine thiolactone. Reactive oxygen species, including superoxide and hydrogen peroxide are produced during the auto-oxidation of homocysteine. There is extensive evidence that homocyst(e)ine induced endothelial cell injury in vitro is largely due to generation of hydrogen peroxide and the hydroxyl radical. These reactive oxygen species damage the endothelium and expose the underlying matrix and smooth muscle cells that in turn proliferate and promote the activation of platelets and leucocytes.

The reactive oxygen species produced during the auto-oxidation of homocyst(e)ine, in particular, the superoxide anion radical and the hydroxyl radical, have also been shown to initiate both lipid peroxidation and oxidation of low density lipoproteins (LDL). Oxidative modification of LDL promotes the formation of foam cells which in turn yields another source of reactive oxygen species (Figure 9).
The normal anti-thrombotic properties of the endothelium are altered in the presence of homocysteine. Homocysteine enhances the activity of factor XII and factor V and depresses the activation of protein C. It also suppresses anti-coagulant effects by suppressing anti-thrombin-III binding activity of endothelial heparin sulphate and inhibiting the expression of thrombomodulin. These effects facilitate the formation of thrombin and create a prothrombotic environment.

NOTE:
This figure is included on page 79 of the print copy of the thesis held in the University of Adelaide Library.
The vasodilator properties of the normal endothelial cell in particular, endothelial nitric oxide (NO) are adversely affected by homocysteine. Normal endothelial cells release nitric oxide that combines with homocysteine in the presence of oxygen to form S-nitroso-homocyst(e)ine. Nitrosation of the sulphhydryl group of homocysteine inhibits the sulphhydryl-dependent generation of hydrogen peroxide. This protective effect of nitric oxide is eventually compromised by long-term exposure to high levels of homocysteine which damages the endothelium sufficiently to limit nitric oxide production.

Impaired endothelial production of nitric oxide leaves the endothelium vulnerable to unopposed homocysteine mediated oxidative injury. Homocysteine decreases the bioavailability of nitric oxide by impairing its synthesis. Homocysteine promotes lipid peroxidation which in turn decreases the expression of endothelial nitric oxide synthase and directly degrades nitric oxide. Homocysteine suppresses the expression of cellular glutathione peroxidase by endothelial cells this promotes lipid peroxidation by the reactive oxygen species generated by oxidation of homocysteine.

**Homocyst(e)ine and Vascular Disease**

Homocyst(e)ine is well recognised to be implicated in the pathogenesis of vascular disease and as such has been the focus of much research and review over the past three decades. Elevated tHcy is an accepted and potentially modifiable risk factor for cardiovascular disease and death and is independent of other conventional risk factors (Nygard, Nordrehaug *et al.* 1997; Anderson, Muhlestein *et al.* 2000).

A recent meta-analysis of studies of cardiovascular disease, cerebrovascular disease, deep vein thrombosis and homocyst(e)ine lowering interventions, demonstrated the causal effects of tHcy and suggested beneficial effects of homocysteine lowering on outcome (Wald, Law *et al.* 2002).

Folate and homocyst(e)ine metabolism are closely linked such that administration of folate in doses as low as 0.4mg/day are enough to lower tHcy by up to 25% (1998; Doshi, McDowell *et al.* 2003). Folate alone and in combination with vitamin B6 and vitamin B12 have all been shown to reduce tHcy concentrations (1998).
As mentioned, homocyst(e)ine causes both acute and chronic endothelial dysfunction (Celermajer, Sorensen et al. 1993; Woo, Chook et al. 1997; Chambers, McGregor et al. 1998) and endothelial dysfunction has even been demonstrated in children with hyperhomocyst(e)inaemia (Celermajer, Sorensen et al. 1993).

tHcy lowering with folate appears to have a significant positive effect on endothelial function (Title, Cummings et al. 2000; Thambyrajah, Landray et al. 2001; Woo, Chook et al. 2002). These studies used large doses of folate (5-10mg per day) and studies using smaller doses (0.4mg/day) have not resulted in the same improvement in endothelial function (Pullin, Ashfield-Watt et al. 2001; Moat, Madhavan et al. 2006).

The United States (U.S.) Food and Drug Administration (FDA) made the fortification of grains mandatory in January 1998, providing an additional 70-120 µg folate per day to middle aged and older adults in the U.S. Since this time, data from the Framingham study has shown that tHcy levels have fallen and plasma folate concentrations are higher in this population (Jacques, Selhub et al. 1999). Despite this, no reduction in incidence of cardiovascular mortality has yet been reported in the U.S. (Moat, Doshi et al. 2004) some questioning the benefit of low dose folate on cardiovascular outcome. However, it may just be too early to see any change in cardiovascular outcomes.

**Homocyst(e)ine and Diabetes**

It is well recognised that both hyperhomocyst(e)inaemia and diabetes are associated with severe vascular disease and advanced atherosclerosis. Many of the mechanisms by which homocysteine causes damage to the endothelial cell are similar to the pathogenic mechanisms by which hyperglycaemia causes damage, such as oxidative stress and endothelial dysfunction. It appears that homocyst(e)ine and hyperglycaemia have a synergistic negative effect on the vasculature.

tHcy levels are usually normal in diabetes but elevated tHcy in diabetes may be contributory to the pathogenesis of the advanced microvascular and macrovascular complications. tHcy levels in diabetes are modulated by glomerular hyperfiltration and renal dysfunction. Hyperhomocyst(e)inaemia is common in diabetic nephropathy (Hultberg, Agardh et al. 1991; Chico, Perez et al. 1998; Hofmann, Kohl et al. 1998) and
may contribute to the enhanced morbidity and mortality from cardiovascular disease observed in patients with diabetic nephropathy. Elevated tHcy is also a risk factor for retinopathy (Vaccaro, Ingrosso et al. 1997).

In a study of adolescents and young adults with type 1 diabetes of greater than seven years duration, those with microvascular complications, retinopathy and nephropathy had higher tHcy levels than those without complications. In this study, there was no difference in tHcy between these subjects with T1DM without complications and age matched, healthy, control subjects (Chiarelli, Pomilio et al. 2000). Other studies, including work from our unit, have also shown low/normal tHcy levels in adolescents with type 1 diabetes without microvascular complications (Pavia, Ferrer et al. 2000; Wiltshire, Thomas et al. 2001).

In the research from our unit, tHcy values were significantly lower in children and adolescents with type 1 diabetes than in aged matched healthy control subjects. The lower tHcy was associated with higher levels of serum folate, red cell folate and vitamin B12 but not with HbA1c or duration of diabetes. In the diabetes group, plasma creatinine concentration was lower and calculated creatinine clearance was higher in the control group. Food questionnaires, performed as part of the study, revealed that dietary folate intake over the previous twelve months correlated with serum folate levels in both children with type 1 diabetes and control subjects. Differences in dietary intake between children with type 1 diabetes and the control group may be one explanation for the differences in serum folate and the lower tHcy levels (Wiltshire, Thomas et al. 2001).

tHcy levels increase with albumin excretion rate (AER), and adolescents with an AER >70 µg/min have consistently elevated tHcy concentrations (Chiarelli, Pomilio et al. 2000). AER has been shown to be the strongest independent association with tHcy in adults with diabetes (Chico, Perez et al. 1998).

The evidence examining the other determinants of elevated tHcy in T1DM is conflicting. In some studies, elevated tHcy correlates with an earlier age of onset of diabetes and higher HbA1c (Hultberg, Agardh et al. 1997; Chiarelli, Pomilio et al. 2000). This is contrary to the findings both from the previous work from our unit where lower tHcy in children and adolescents did not correlate with either HbA1c or duration of diabetes (Wiltshire, Thomas et al. 2001) and the work from Spain by Pavia et al. where no significant differences were found in tHcy values in relation to the metabolic control of the
disease assessed by glycated haemoglobin or the duration of disease (Pavia, Ferrer et al. 2000). Lower folate levels have been associated with elevated tHcy in adults with T1DM (Hultberg, Agardh et al. 1997). This supports the work from our unit, described above, which showed lower tHcy was associated with higher levels of serum folate and red cell folate in children and adolescents (Wiltshire, Thomas et al. 2001) and the work by Pavia et al in which there was a negative correlation between tHcy, serum folate and vitamin B12 but not with vitamin B6 (Pavia, Ferrer et al. 2000). The relationship between tHcy and folate and vitamin B12 however was not shown in the study of adolescents with type 1 diabetes by Chiarelli et al. in which there was no correlation (Chiarelli, Pomilio et al. 2000). No association between tHcy and lipid parameters has been shown in one study (Pavia, Ferrer et al. 2000) but not in another where higher LDL-cholesterol was associated with higher tHcy levels (Chiarelli, Pomilio et al. 2000).

An association between tHcy levels and the macrovascular complication of coronary artery disease has been clearly demonstrated in type 2 diabetes (Okada, Oida et al. 1999). tHcy concentration has been shown a significant predictor of mortality patients with type 2 diabetes with or without albuminuria (Stehouwer, Gall et al. 1999) and the five year mortality in type 2 diabetes is related to high tHcy levels independent of other major risk factors. Elevated tHcy levels are a stronger risk factor for mortality in adults with type 2 diabetes than in non-diabetic subjects (Hoogeveen, Kostense et al. 2000). Because of this association, patients with diabetes are candidates for screening for elevated tHcy levels and treatment with folate.

The Controversies

Vitamin supplementation has been shown to reduce or normalise elevated tHcy levels although the benefits with respect to clinical endpoints are yet to be thoroughly and prospectively evaluated. Such studies are currently underway.

There is recent disappointing lack of benefit of high dose folate, vitamin B6, and vitamin B12 on vascular outcomes in adults with established vascular disease. A large, randomised control trial of combination B vitamin supplementation [25 mg vitamin B6, 0.4 mg cobalamin (vitamin B12), and 2.5 mg folate vs. 200 µg vitamin B6, 6 µg cobalamin and 20 µg folate], after non-disabling cerebral infarction, resulted in a moderate reduction
of total plasma homocyst(e)ine however had no effect on vascular outcomes during the
two years of follow-up (Toole, Malinow et al. 2004). In another large randomised, double-
blind, placebo controlled trial evaluating whether homocysteine lowering with B vitamin
therapy reduces the risk of major vascular events in a high risk population, the
combination of folate, vitamin B6, and vitamin B12 lowered tHcy significantly but did not
reduce the incidence of death from cardiovascular causes, myocardial infarction, and
stroke, during a mean follow-up period of five years (Lonn, Yusuf et al. 2006). A
retrospective meta-analysis of twelve randomised trials assessing the effects of lowering
homocysteine with B vitamin supplements on risk of cardiovascular disease, involving
approximately 52,000 subjects with established vascular disease, has also concluded
that the strength of association of homocysteine with risk of cardiovascular disease may
be weaker than had previously been believed (2006). In spite of this, children, at high
risk of vascular disease in later life, but without clinically evident disease, should be
amenable to long term benefits of tHcy lowering treatment, if in fact homocysteine is
contributory to vascular disease, and this warrants further investigation.

It has become controversial as to whether the increased risk of cardiovascular disease is
mediated directly by tHcy, whether tHcy may be contributory, or whether tHcy is a marker
of other causative factors. As tHcy levels are related to renal dysfunction, smoking,
elevated blood pressure, and other cardiovascular risk factors and are higher in people
with atherosclerosis than in those without, tHcy could be a marker, but not a cause, of
vascular disease, and the data implicating homocysteine in vascular disease could
possibly be the result of confounding.

Folate therapy, used in intervention trials for tHcy lowering, has been shown to have
beneficial effects on the endothelium. Evidence is revealing the main effect of folate on
the endothelium may be independent of tHcy, as high dose folate improves endothelial
function prior to any measurable change in plasma tHcy (Doshi, McDowell et al. 2002).
This suggests an independent action of folate on the endothelium. The potential
mechanisms of folate action on the endothelium are discussed further in the following
section of this chapter.
Folate

Folate is a water-soluble B vitamin that occurs naturally in a wide variety of foods. Leafy green vegetables (like spinach and turnip greens), fruits (like citrus fruits and juices), and dried beans and peas are all natural sources of folate. With fortification folate, in the form of folic acid, is present in foods including cereals and grains including breads.

Folate in the Prevention of Disease.

Lucy Wills' key observations in 1931 in Bombay (Mumbai), India led to the identification of folate being effective against the macrocytic anaemia of late pregnancy. Dr. Wills demonstrated that the anaemia could be corrected by a yeast extract and this was initially called 'The Wills Factor'. This description is the first record of folate being used for prevention of disease.

Folate however was not isolated as the critical factor involved at this time. It was identified as the key factor in brewer's yeast in the late 1930’s and was extracted from spinach leaves in 1941.

The significance of folate in preventive medicine was again shown in a series of research, culminating in the Medical Research Council Vitamin Study Group report in 1991, which documented the reduction of neural tube defects with peri-conceptional folate (1991). Supplemental folate prior to conception and for the first three months of pregnancy is now widely routinely recommended peri-conceptually to reduce the risk of neural tube defects. The overwhelming evidence showing folate fortification reduces neural tube defects led to mandatory enrichment of flour through fortification of grain with folate at source in the U.S. The U.S. Food and Drug Administration (FDA) authorised the addition of folic acid to enriched grain products in March 1996 and made compliance mandatory by January 1998, in an effort to reduce the occurrence of folate-preventable neural tube defects.

Interest in folate over the past decade has rocketed in comparison with other nutrients, not only because of the effect on preventing neural tube defects, but also because of the benefit folate has in treating a range of disorders.
Studies in the area of vascular disease, showing tHcy is an independent risk factor for atherosclerotic disease, raised the possibility that folate may also be beneficial in prevention of vascular disease. Dietary folate lowers tHcy through de novo biosynthesis of methionine and this is discussed in the previous chapter. Given the tHcy lowering effects of folate, folate has been the focus of considerable cardiovascular research.

Beneficial effects of folate on endothelial function are being demonstrated in increasing numbers of studies (Verhaar, Wever et al. 1998; Woo, Chook et al. 1999; Doshi, McDowell et al. 2001; Thambyrajah, Landray et al. 2001; van Etten, de Koning et al. 2002; Woo, Chook et al. 2002). The mechanism underlying the improvement in endothelial function remains controversial and until recently, had been attributed to reductions in tHcy that occur with folate or other vitamin B therapy. Recently, additional benefits of folate have been proposed. Folate appears to interact with nitric oxide metabolism, enhance the bioavailability of tetrahydrobiopterin (BH4), reduce superoxide anion generation and hence improve endothelial function.

**Folate and Homocyst(e)ine**

Inadequate intake of folate and to a lesser extent vitamin B6 and B12 increase tHcy levels. As described in the previous chapter, elevated tHcy promotes atherosclerosis through increased oxidant stress, impaired endothelial dysfunction and induction of thrombosis.

Treatment with folate significantly reduces tHcy levels even when plasma levels of folate and tHcy are in the normal range (1998). The homocyst(e)ine lowering effect of folate can be explained by its actions as a substrate in the remethylation of homocysteine to methionine. Increasing the intake of folate leads to a reduction in tHcy of up to 25% (1998). This led to the proposal that folate treatment may lower cardiovascular risk by reducing tHcy.

Recently several studies have questioned the tHcy lowering effect of folate as the main mechanism leading to improved vascular function. Improvement in endothelial function, has been observed within hours of additional oral folate (Doshi, McDowell et al. 2002) and within minutes of intravenous 5-methyltetrahydrofolate (MTHF), the active form of
folic acid (Verhaar, Weyer et al. 1998; van Etten, de Koning et al. 2002), before any significant reduction in tHcy providing evidence that homocyst(e)ine reduction is unlikely to account for the acute and possibly chronic improvement in endothelial function observed with folic acid.

Plausible mechanisms exist to explain how folic acid may acutely enhance endothelial function independent of homocyst(e)ine. These include direct interactions with endothelial nitric oxide and endothelial nitric oxide synthase and antioxidant effects.

**Folate Status, Homocyst(e)ine and Endothelial Function in Type 1 Diabetes**

Work from our unit has examined folic acid status, tHcy levels and endothelial function in children with type 1 diabetes.

The initial studies from our unit as discussed above demonstrated that children with type 1 diabetes have significantly lower tHcy concentrations and significantly higher vitamin B12, serum folic acid and red cell folic acid values than a group of healthy age and sex-matched control children (Wiltshire, Thomas et al. 2001).

The difference in tHcy concentrations found between children with type 1 diabetes and group of healthy control subjects in this study was predominantly explained by higher vitamin B12 and folic acid status in children with diabetes compared with that in members of the control group. Food frequency questionnaires were completed by both subjects with type 1 diabetes and the members of the control group. The food frequency data indicated that the higher serum folic acid levels were a reflection of dietary intake, but this was not demonstrated for vitamin B12. There was a suggestion of a difference in vitamin B6 intake between children with type 1 diabetes and the control subjects, however vitamin B6 levels are less likely to affect fasting tHcy levels. Differences between subjects in the diabetes group and the control group were largely related to higher intakes of foods containing complex carbohydrates, particular fruits, vegetables, and cereals in the diabetes group, and lower intake of foods containing refined sugar (Wiltshire, Thomas et al. 2001).
The relevance of this finding of lower tHcy and higher folate status in children with type 1 diabetes was then studied in relation to endothelial function. In this study, despite normal folate status and low tHcy levels children with type 1 diabetes had significant endothelial dysfunction (Wiltshire, Gent et al. 2002).

Endothelial dysfunction was common in children and adolescents with type 1 diabetes of short duration. Folate status was an important determinant of endothelial function in these children, both when measured by FMD and biochemical markers of endothelial activation (vWF and thrombomodulin). There was an independent association between FMD and red cell folate. Red cell folate likely reflects endothelial cell folate status. The ratio of FMD: GTN-induced dilatation (the best measure of endothelial function per se) was also independently associated with red cell folate, again suggesting that folate status is an important factor protecting against endothelial dysfunction. Resting vessel diameter correlated negatively with tHcy. This result is the reverse of that expected from the increase in both vessel diameter and tHcy with age (Wiltshire, Gent et al. 2002).

A pilot intervention trial to assess whether folate improves endothelial function in children and adolescents with type 1 diabetes was therefore initiated in our unit. A randomised, double-blind, placebo-controlled cross over trial compared 5mg oral daily folate with placebo. Subjects were randomised to receive either folate or placebo for eight weeks. There was an eight week washout period, and the subjects then crossed over to placebo or folate, respectively, for a further eight weeks. This pilot study showed that despite normal folate status and low tHcy levels an improvement in endothelial function occurred with high dose supplemental folate. Mean FMD of children and adolescents with type 1 diabetes after folate treatment reached the lower normal range of our paediatric age and sex matched control subjects (Figure 10). This effect of folate on FMD was independent of any change in tHcy. The biochemical marker of endothelial activation, vWF, did not change with folate therapy. In this study, there was a strong carry-over effect for RCF and a small carryover effect for serum folate (Figure 11), without a carry-over effect for FMD, which may suggest the need for ongoing treatment with high-dose folate to maintain an effect on FMD (Pena, Wiltshire et al. 2004).
A longitudinal study was also conducted through our unit examining folate status in South Australian children and adolescents, including a cohort of children and adolescents with type 1 diabetes, over a mean of twelve months following the introduction of folate fortification (Wiltshire and Couper 2004).
Changes in folate status in children and adolescents with type 1 diabetes occurred over time with the introduction of fortification without folate supplements. These results are contrary to the expected age-related decline in both serum and red cell folate. The timing of these samples during a period of increasing folate fortification of food suggests that fortification has improved the folate status of children and adolescents in South Australia. The improvement in folate status was more obvious in children with diabetes. While this may in part have reflected the size of the two groups, it is also consistent with fortification being the explanation for the observed change, as the diabetic diet contains significant quantities of fortified foods (particularly complex carbohydrates), and our baseline study showed these children and adolescents with diabetes to have significantly higher intakes of complex carbohydrate than controls, although their intake of folate at baseline was not significantly higher. There were strong correlations between each of the variables (tHcy, serum folate, red cell folate, B12, creatinine) at the two time points, suggesting these values ‘track’ at the same approximate centile over time. tHcy tended to increase with time, as expected with increased age. However there was considerable variation between individuals in the degree to which it changed, in both diabetes subjects and controls. The regression analysis suggested change in serum folate was a major determinant of change in tHcy.

**Folate and Nitric Oxide**

Endothelial dysfunction measured by FMD is a nitric oxide (NO) mediated process (Joannides, Haefeli et al. 1995). Impaired endothelial NO activity causes endothelial dysfunction.

In diabetes, hyperglycaemia has direct effects on NO availability. The reduced NO availability results from increased degradation of NO by reactive oxygen species and decreased NO formation due to hyperglycaemia mediated uncoupling of endothelial nitric oxide synthase (eNOS) (Cosentino, Hishikawa et al. 1997; Vasquez-Vivar, Kalyanaraman et al. 1998). The mechanisms by which hyperglycaemia affects NO are discussed fully in the Chapter: Diabetes and Vascular Disease.

Folate interacts with NO metabolism. It restores the function of eNOS, enhances the availability of tetrahydrobiopterin (BH4), an essential cofactor in NO synthesis, reduces
the generation of reactive oxygen species and as a result enhances the endothelial cell production of NO (Stroes, van Faassen et al. 2000).

The formation of NO is critically dependent on the availability of the cofactor tetrahydrobiopterin (BH4). BH4 stimulates the conversion, by eNOS, of L-arginine to NO and L-citrulline. BH4 acts as a coenzyme by providing electrons and in the process becomes oxidised to the inactive quinonoid dihydrobiopterin (BH2). Improvement in endothelial function with oral BH4 has been described (Tiefenbacher, Bleeke et al. 2000). One mechanism by which folate restores NO formation is by stimulating the endogenous BH4 regeneration from the oxidised form BH2. Folate is essential in the redox recycling of BH2 back to the active form BH4 (Kaufman 1991).

It has been suggested that it is MTHF, the active form of folate, that facilitates the 1-electron oxidation of BH4 to the BH4 radical which is crucial in the catalysis of NO formation by eNOS (Stroes, van Faassen et al. 2000).

**Folate and Antioxidant Actions**

Folate has antioxidant potential and has been shown to abolish the homocyst(e)ine induced increases in endothelial superoxide (Doshi, McDowell et al. 2001). Subsequent studies have confirmed the active form of folate, 5-MTHF, is an anti-oxidant and has the potential *in vitro* to scavenge superoxide. The scavenging capacity of folate is less than vitamin C, a well known antioxidant, therefore it is suggested that *in vivo* the main beneficial effects are through modulation of the enzymatic activity of eNOS (Stroes, van Faassen et al. 2000) and this is discussed above.

**Folate Dose**

Over the years folate intake recommendations have changed. Previously, adequate folate intake was defined as an absence of macrocytic anaemia. However, as discussed, over the past decade, low folate intake has been consistently associated with birth defects and in particular, neural tube defects. More recently, folate is not only being recommended peri-conception to reduce these birth defects but it is also postulated as a
therapy which may reduce cardiovascular risk. The clinical benefits of this intervention are as yet unproven and the choice of dose remains unclear. Lower doses of folate, 0.4 mg/day have not been shown to improve endothelial function in healthy subjects (Pullin, Ashfield-Watt et al. 2001) and in subjects with coronary artery disease (Moat, Madhavan et al. 2006). It is only high dose folate (5-10 mg/day) that have resulted in a measured improvement in endothelial function (Title, Cummings et al. 2000; Doshi, McDowell et al. 2002; Woo, Chook et al. 2002; Moat, Madhavan et al. 2006).

As discussed above, studies from our unit have shown that children with type 1 diabetes, despite normal folate levels, have significant endothelial dysfunction (Wiltshire, Gent et al. 2002). In these children, the pilot, cross-over design study showed that high dose folate (5 mg/day) reverses the endothelial dysfunction (Pena, Wiltshire et al. 2004). We therefore used the same dose of folate (5 mg/day) in this current study.

**Safety Considerations**

The well recognised concern about folate therapy is that in people with subclinical vitamin B12 deficiency, folate therapy may mask the haematological manifestations of vitamin B12 deficiency and allow progression of the neurological damage. This effect can be avoided if B12 deficiency is determined prior to initiating folate therapy.

Folate is generally regarded as safe and as discussed above, has long been presumed to be purely beneficial and an ideal supplement for disease prevention. At the time of the clinical trial, there were no known side effects of folate at a dose of 5 mg daily.

Despite the clear benefits of folate supplementation, recent concerns have been raised regarding the mass use of folate in fortified foods (Lucock 2004; Eichholzer, Tonz et al. 2006; Hubner, Houlston et al. 2007) and the long term effects of folate supplementation in pregnancy (Charles, Ness et al. 2004). These concerns were published following the completion of the clinical research component of this trial.

The report by Ludlock (Lucock 2004), discusses that the form of folate in supplements and in fortified foods is pteroylmonoglutamate (PGA), a form that does not occur in nature. PGA is both cheap and stable unlike most native forms of the vitamin. Folate
metabolism is extraordinarily complex. The body metabolises PGA to methyl folate, the normal form of the vitamin transported in plasma. However, it has been shown that the absorption and biotransformation process is saturated at doses in the region of 400 µg PGA or less. Therefore at doses at or just below 400 µg PGA, the synthetic form of folate is converted into biologically active methyl folate during absorption. At higher doses synthetic PGA is also transported into the blood in a manner that is directly proportional to dose. This raises the possibility of extended exposure to unmetabolised PGA where mandatory fortification is undertaken. It is unknown whether such exposure may present a health risk.

The discussion by Hubner and colleagues (Hubner, Houlston et al. 2007) presents the case against mandatory folate supplementation. The authors cite data suggesting cancer promoting effects of folate. Although some epidemiological studies have found that high folate intake is associated with a reduced risk of cancers of the breast, lung, pancreas, oesophagus, stomach, cervix, and in particular of the colorectum, more recent studies have challenged this widely accepted notion, and doubts over the validity of the epidemiological evidence have emerged.

The effects of long term exposure to high concentrations of the synthetic form of folate are unknown, although as yet there is no evidence of toxicity. However, with documented concerns, further investigation and long term follow-up studies are warranted to determine any potential long term effects of folate supplementation.
Vitamin B6

Vitamin B6 has attracted renewed interest recently because of its role in homocysteine metabolism and its possible relation to cardiovascular risk.

Vitamin B6 therapy is routinely medically recommended for genetic disorders such as sideroblastic anaemia, cystathioninuria and homocystinuria as well as pyridoxine dependent seizures of infancy. It is also recommended to prevent the neuropathy associated with isoniazid therapy. It is marketed in general for stress, depression associated with pre-menstrual syndrome and for carpal tunnel syndrome although it is doubtful that it has any more than a placebo effect in these conditions. The number of people self medicating with vitamin B6 is enormous - an estimated three million users in the UK alone (Editorial 1998).

Vitamin B6 is present in a wide variety of foods, such as fruit and vegetables including potatoes and bananas, as well as beans, nuts, chicken, beef, fish and fortified cereals, therefore primary dietary deficiency of vitamin B6 is considered rare in developed countries. Recently however, low vitamin B6 concentrations have been reported in some populations these low concentrations have been related to increased risk of vascular diseases. This is discussed subsequently.

Vitamin B6 Metabolism

Vitamin B6 is an essential, water-soluble vitamin that functions as a coenzyme in numerous enzymatic reactions involved in the metabolism of amino acids, carbohydrates, neurotransmitters, and lipids.

Pyridoxine, pyridoxal, pyridoxamine, their phosphorylated derivatives [pyridoxal 5’-phosphate (PLP) and pyridoxamine 5’-phosphate], and the end product of vitamin B6 metabolism, 4-pyridoxic acid are the major forms of vitamin B6 found in human tissues and body fluids.

In the diet, vitamin B6 is predominantly present in three forms, pyridoxine, pyridoxamine and pyridoxal. After passive intestinal absorption these major forms of the vitamin are
delivered to the liver and transported into hepatic cells by facilitated diffusion where they are converted to pyridoxal 5'-phosphate.

Pyridoxal 5'-phosphate either remains in the hepatocyte, or it is released into the serum, where it is tightly bound to albumin. Pyridoxal 5'-phosphate is available for other cells only after being hydrolyzed to pyridoxal by alkaline phosphatase, but most cells have pyridoxal kinase activity and are therefore able to re-phosphorylate pyridoxal to pyridoxal 5'-phosphate. Free pyridoxal is degraded by alkaline phosphatase, hepatic and renal aldehyde oxidases, and pyridoxal dehydrogenase. Excess pyridoxal is oxidized to 4-pyridoxic acid by the liver and excreted in the urine (Bor, Refsum et al. 2003).

Pyridoxine 5'-phosphate is the active form of vitamin B6 and an essential cofactor in the hydrolysis of glycogen and various transamination, decarboxylation and synthesis pathways involving carbohydrate, sphingolipid, amino acid, heme and neurotransmitter metabolism (Bor, Refsum et al. 2003).

**Measurement of Vitamin B6**

Currently there is no agreement as to which form of vitamin B6 should be assayed to routinely assess vitamin B6 status (Bor, Refsum et al. 2003). In Adelaide, we had access to measurement of erythrocyte aspartate aminotransferase discussed in the methods chapter. Erythrocyte aspartate aminotransferase are long term indicators of functional pyridoxine status due to the 120 day life span of erythrocytes.

**Vitamin B6 and Homocyst(e)ine**

Both folate and vitamin B6 (pyridoxine) are important cofactors in distinct aspects of homocysteine metabolism. Vitamin B6 is the coenzyme in the transulphuration pathway that irreversibly converts homocysteine to cysteine. This step is important in postprandial homocysteine metabolism and responsible for postprandial tHcy levels whereas folate is responsible for the basal tHcy levels via the remethylation pathway.

Homocysteine metabolism is discussed thoroughly in the earlier section of this chapter on homocyst(e)ine.
Vitamin B6 and Vascular Disease

Vitamin B6 has attracted renewed interest because of its role in homocysteine metabolism and its possible relation to cardiovascular risk. Recent work has focused on B vitamin supplementation, both with folate, vitamin B6 as well as vitamin B12, with the hypothesis that improved vascular endothelial function is mediated through reduced concentrations of tHcy.

The Homocysteine Lowering Trialists’ Collaboration performed a meta-analysis of data from 12 trials to determine homocysteine lowering effect that was achieved with different doses of folic acid and with the addition of vitamin B12 or vitamin B6 (1998). This meta-analysis showed that folate had the dominant effect on homocyst(e)ine lowering, that additional doses of B12 had a small additional effect and that vitamin B6 did not have an additional effect, however the trials did not assess effects on blood homocyst(e)ine after methionine loading which is determined by the transulphuration pathway, of which B6 is an essential cofactor.

Since this meta-analysis, in the era of folate fortification, B6 is proving to be an independent predictor of vascular disease (Kelly, Shih et al. 2003). With this and other evidence documented, I decided to pursue the effects of vitamin B6 on endothelial function.

There are a number of studies looking at combination of folate and vitamin B6 supplementation in tHcy lowering and in the treatment in vascular disease. Reviewing these trials of vascular function, patients with coronary heart disease and hyperhomocysteinaemia have improved endothelium dependent vasodilatation, exercise performance and exercise-induced myocardial ischaemia measured with exercise treadmill testing when given tHcy lowering therapy of combination folate, vitamin B6 and vitamin B12 (Dinckal, Aksoy et al. 2003). In combination these vitamins, folate, vitamin B6 and vitamin B12, have also been shown to ameliorate the endothelial dysfunction caused by post methionine load hyperhomocysteinaemia (Chao, Chien et al. 1999). Folate and vitamin B6 in combination have been shown to improve biological markers of endothelial function, von Willebrand factor (vWF) and thrombomodulin over a three month period (Constans, Blann et al. 1999). Subclinical atherosclerosis measured by exercise electrocardiography, was improved with treatment with folate and vitamin B6 (Vermeulen, Stehouwer et al. 2000).
There is limited literature however, examining the effect of vitamin B6 alone on the endothelium. In the only published study comparing vitamin B6 with folate, in cardiac transplant recipients there was a significant improvement in endothelial function, measured by flow mediated dilatation, with vitamin B6 supplementation but not folate or placebo. In this study, pyridoxine supplementation was associated with significant improvement in endothelial function and this effect was independent of homocyst(e)ine (Miner, Cole et al. 2001).

Interestingly, low pyridoxal concentrations have been shown to confer an increased and independent risk for premature coronary artery disease, peripheral vascular disease and cerebrovascular disease in a number of independent studies (Robinson, Mayer et al. 1995; Robinson, Arheart et al. 1998; Kelly, Shih et al. 2003; Wilmink, Welch et al. 2004).

**Vitamin B6 and Diabetes**

Vitamin B6 levels have been shown to be low in subjects with diabetes. In a series of 518 subjects with diabetes, serum pyridoxal levels were significantly lower compared to healthy controls suggesting that there may have an increased demand for vitamin B6 in diabetes (Davis, Calder et al. 1976). In a subsequent a large case control series, children with type 1 diabetes were also shown to have lower serum pyridoxal concentrations than healthy children (Wilson and Davis 1977).

In experimental work, vitamin B6 utilisation and requirements of vitamin B6 have been shown to be higher in diabetic compared with non-diabetic rats (Okada, Shibuya et al. 1999).

**Vitamin B6 and Inflammation**

There is evidence to suggest that low B6 status may be related to acute and chronic inflammation. Low vitamin B6 levels have been associated with elevated C-reactive protein levels in healthy subjects (Friso, Jacques et al. 2001) and in subjects with type 2 diabetes (Friedman, Hunsicker et al. 2004). C-reactive protein is also a strong marker of
risk of coronary heart disease and has been used to predict the risk of cardiovascular events (Ridker, Rifai et al. 2002; Danesh, Wheeler et al. 2004).

In the study by Friso et al., there was a strong association with low pyridoxal 5'-phosphate (PLP) and higher CRP levels, they hypothesized that low levels of PLP may reflect a higher utilisation of the coenzyme in an underlying inflammatory process rather than defective intake or excessive vitamin B6 catabolism (Friso, Jacques et al. 2001).

C-reactive protein levels were measured in this study and, the relationship between C-reactive protein levels and diabetes is discussed in the next section of this chapter.

**Vitamin B6 and Safety Considerations:**

**Adverse Effects of Vitamin B6 and Neuropathy.**

Adverse events of vitamin B6 therapy include, headache, skin sensitivities, gastrointestinal symptoms, particularly nausea however the adverse effect of most concern and controversy is sensory neuropathy.

The original published report of sensory neuropathy in humans described seven adults presenting with ataxia and ‘sensory-nervous-system dysfunction’ associated with daily doses of between two and six grams of pyridoxine for a duration of four to forty months. There was resolution of the symptoms after ceasing the pyridoxine supplementation in these cases (Schaumburg, Kaplan et al. 1983).

The first reports of pyridoxine toxicity were in the beagle dog (Phillips, Mills et al. 1978; Schaeppi and Krinke 1982). The studies documented neurological examination, electrophysiological testing and microscopic post-mortem examination to study the neuropathy induced in the beagle dog by administration of excessive amounts of vitamin B6. In this work by Schaeppi et al., two female dogs received repeated oral doses of three grams of vitamin B6 daily. The treatment was ceased when the dogs developed severe general morbidity, including uncoordinated gait and abnormal neurologic symptoms. The symptoms were most severe during and early after cessation of treatment, and in general they regressed during the subsequent two months of treatment-
free observation. Sensory central and peripheral maximum nerve conduction velocity started to decrease after a considerable delay; the decrease progressed until late after termination of treatment and failed to fully regress. Morphologic lesions were confined to large, first order sensory neurons. The neurologic examination revealed the early changes, while electrodiagnostics and microscopic neuropathology were indicators of more advanced stages of toxic neuropathy (Schaeppi and Krinke 1982).

Concerns of peripheral neuropathy with vitamin B6 therapy are controversial (Beckett, Dalton et al. 1998; Editorial 1998). The concerns raised of neurotoxicity have occurred at doses of grams per day rather than the milligram per day doses that are more commonly used. In a review of the literature on adverse effects of vitamin B6, doses of less than 500 mg per day appeared to be safe per periods of up to six years (Cohen and Bendich 1986).

**Vitamin B6 Dose**

The Australian Medicines Handbook gives no recommended daily dose for children. Doses for pyridoxine range depending on the condition: for pyridoxine-dependent seizures doses are up to 100 mg per day, for homocystinuria the dose recommended is 25 mg per day and for sideroblastic anaemia 100-200 mg per day is recommended.

For metabolic indications in children up to 12 years, a dose range of 50-250 mg either once or twice daily is recommended by the British Paediatric Formulary produced by the Royal College of Paediatrics and Child Health (Royal College of Paediatrics and Child Health 2003).

The US Food and Nutrition Board of the Institute of Medicine established an upper tolerable intake level for vitamin B6 of 100 mg per day (1998).

Tablets are available through the Women’s and Children’s Hospital pharmacy department in doses of 25 mg and 100 mg. We chose to use 100 mg per day in this study in order to gain maximal effect over the study period, without risk of toxicity.
High Sensitivity C-Reactive Protein

C-reactive protein (CRP) is a non-specific acute phase reactant. As with other acute phase reactants, it increases during times of inflammation and remains elevated in chronic inflammatory states. It is one of the most sensitive acute phase reactants and can increase several hundred fold during an acute inflammation. CRP is synthesised in the liver in response to pro-inflammatory cytokines, particularly IL6.

Atherosclerosis is now widely considered to represent a chronic inflammatory process (Ross 1999). As inflammation plays a major role in atherosclerosis, slight elevations, within the normal range, of inflammatory markers such as CRP are proving to be an indicator of such inflammation.

The standard assay for C-reactive protein lacks the sensitivity to determine the levels of inflammation within the normal range, therefore highly sensitive assay systems are now commercially available that are able detect the subtle elevations in CRP that occur with the low grade inflammation associated with atherosclerosis and increased vascular risk. Measurement of high sensitivity C-reactive protein (Hs-CRP) is proving to be a useful predictor of cardiovascular outcome as discussed subsequently.

Increased CRP has been considered an epiphenomenon in atherosclerosis. However recent evidence suggests that CRP could directly participate in atherogenesis. CRP has been shown to mediate the uptake of biochemically intact LDL into macrophages, a mechanism of foam cell formation, which suggests CRP may have direct pathogenic role promoting the formation of early atherosclerotic lesions (Zwaka, Hombach et al. 2001).

Hs-CRP in Cardiovascular Disease

In large epidemiological studies elevated CRP levels predict not only the long term prognosis in patients with documented cardiovascular disease but also cardiovascular disease in asymptomatic subjects (Ridker, Cushman et al. 1997; Koenig, Sund et al. 1999; Danesh, Wheeler et al. 2004). In acute coronary disease, elevated inflammatory markers are associated with an unfavourable prognosis (Liuzzo, Biasucci et al. 1994).
Elevated Hs-CRP is associated with endothelial dysfunction, the earliest detectable event in the atherosclerotic process (Fichtlscherer, Rosenberger et al. 2000), and also with the development of carotid atherosclerosis (Hashimoto, Kitagawa et al. 2001). Importantly, normalisation of CRP levels over time has been associated with an improvement in endothelial dysfunction (Fichtlscherer, Rosenberger et al. 2000).

**Hs-CRP and Diabetes**

It is well recognised that diabetes is an independent risk factor for the development of atherosclerosis. Adults with type 1 diabetes have been shown to have elevated Hs-CRP levels compared with healthy controls in several studies (Schalkwijk, Poland et al. 1999; Hayaishi-Okano, Yamasaki et al. 2002; Schram, Chaturvedi et al. 2003). Although, the mechanisms which trigger the activation of subclinical inflammation in diabetes are not clear, it is thought to be part due to chronic exposure to glucose (Schalkwijk, Poland et al. 1999; Stehouwer, Gall et al. 2002).

In the EURODIAB prospective complications study of 543 individuals with type 1 diabetes, elevated Hs-CRP was associated with female sex, diabetes duration, glycaemic control, advanced glycation end products, BMI, HDL- cholesterol, triglycerides and systolic blood pressure (Schram, Chaturvedi et al. 2003). In this series, as well as the series from Schalkwijk (Schalkwijk, Poland et al. 1999) of 40 non-smoking subjects with type 1 diabetes without evidence of macrovascular disease, measures of inflammation were strongly associated with serum markers of endothelial dysfunction suggesting a relationship between activation of the endothelium and chronic inflammation.

In the series from Hayaishi-Okano (Hayaishi-Okano, Yamasaki et al. 2002), young adults with type 1 diabetes had significantly higher CRP levels than age matched controls. CRP levels were significantly correlated with the mean and maximal intimal medial thickness (IMT) of patients with type 1 diabetes and with the maximal IMT of the non-diabetic patients. In this work, the multivariate regression analyses showed that Hs-CRP levels were independently correlated with intimal medial thickness as well as with diastolic blood pressure, sex, and duration of diabetes. Hs-CRP levels were elevated even in young patients with type 1 diabetes.
A study by Colhoun et al., found that Hs-CRP was elevated in women but not men with type 1 diabetes. This was not explained by measures of obesity or other correlates of Hs-CRP. These authors explained the possible differences may be related to coronary heart disease risk in the diabetic population as the relative risk of CHD is higher in type 1 diabetic women than in type 1 diabetic men (Colhoun, Schalkwijk et al. 2002).

In type 1 diabetes, there is subclinical, chronic inflammation which is, at least in part, independent of clinically manifest micro- and macrovascular complications. Targher and colleagues, have recently shown Hs-CRP is elevated in type 1 diabetes and is not associated with the traditional cardiovascular risk factors i.e. smoking, hypertension, dyslipidaemia and obesity (Targher, Bertolini et al. 2005). This inflammation has been shown to related to the magnitude and duration of hyperglycaemia (Hayaishi-Okano, Yamasaki et al. 2002; Schram, Chaturvedi et al. 2003; Targher, Bertolini et al. 2005).

**Hs-CRP in Children**

In healthy children without diabetes, elevated C-reactive protein is associated with decreased endothelial vasodilatation (endothelial function) measured by flow mediated dilatation and increased carotid artery IMT (Jarvisalo, Harmoinen et al. 2002). In these children, Hs-CRP is also associated with BMI, which confirmed previous observations, however after correcting for BMI, Hs-CRP was still significantly associated with endothelial function and carotid IMT.

In children with type 1 diabetes, raised endothelial activation markers including serum vWF and tissue plasminogen activator, correlate with inflammatory markers including elevated Hs-CRP (Romano, Pomilio et al. 2001).

Elevated CRP levels are related with early functional and structural atherosclerotic vascular changes in adults with T1DM and in healthy children. The association between Hs-CRP and endothelial function, in children with type 1 diabetes, has not yet been studied.
CRP and Vitamin B6

Low vitamin B6 levels are also associated with an adverse prognosis in vascular disease including coronary artery disease, cerebral vascular disease and peripheral vascular disease (Robinson, Mayer et al. 1995; Robinson, Arheart et al. 1998; Kelly, Shih et al. 2003; Wilmink, Welch et al. 2004).

There is evidence to suggest that low vitamin B6 status may be related to acute and chronic inflammation. Recent studies have elicited an association low vitamin B6 levels and elevated C-reactive protein levels in both healthy subjects (Friso, Jacques et al. 2001) and in subjects with type 2 diabetes (Friedman, Hunsicker et al. 2004). The aetiology of the inverse relationship however is unclear.

Pyridoxal 5'-phosphate (PLP), the active form of vitamin B6, is an important coenzyme in maintaining the balance between protein synthesis and degradation. In the study by Friso et al., there was a strong association with low PLP and higher CRP levels, they hypothesised that low levels of PLP may reflect a higher utilisation of the coenzyme in an underlying inflammatory process rather than defective intake or excessive vitamin B6 catabolism. In other words, increased vitamin B6 utilisation in the presence of an underlying inflammatory process may explain the decrease B6 levels seen in vascular disease. In contrast to this work, a study by Folsom et al. found that CRP was not associated inversely with PLP in healthy middle aged adults (Folsom, Desvarieux et al. 2003). Another recently published study of supplemental folate (5mg) and vitamin B6 (250mg) showed no change in CRP with treatment over a two year treatment period (Vermeulen, Rauwerda et al. 2003).

The hypothesis linking increased utilisation of vitamin to the inflammatory process is controversial and requires further verification.
Summary

In this chapter, I have provided the background to this research.

There is an overview of type 1 diabetes including the fascinating history of type 1 diabetes and a discussion of the considerable impact T1DM has both at an individual and a global level. The micro- and macrovascular complications of type 1 diabetes mellitus are discussed. The aetiology of vascular disease is reviewed in some detail with a review of the multiple mechanisms by which hyperglycaemia plays a role in the pathogenesis of the vascular disease of diabetes. These mechanisms lead to endothelial dysfunction which in turn leads to development of diabetic vascular disease. The measurement of endothelial function in clinical practice is discussed.

Homocysteine well known to be involved in endothelial damage and lead to advanced atherosclerosis is reviewed. The controversial hypothesis that homocysteine may play a role in the development of advanced diabetic vasculopathy is discussed as is the metabolism of homocysteine including the essential cofactors of homocysteine metabolism, folate and vitamin B6.

In clinical trials, an improvement in endothelial function occurs with folate and vitamin B6 treatment. I have discussed in detail the evidence for the use of folate and vitamin B6 in vascular disease and the potential pathways for the effect of folate on endothelial function as well as the potential benefit of folate and vitamin B6 treatment on diabetic vascular disease.

In this current research, this understanding of homocysteine metabolism and tHcy lowering interventions were considered in generating the hypothesis and choosing the interventions for this trial. In addition to folate, we hypothesised that vitamin B6 would be a likely effective independent factor and that these interventions alone and in combination would improve endothelial function in children with type 1 diabetes.

Within these discussions, I have presented the previous work from our unit, which has examined folate status, tHcy levels and endothelial function in children with type 1 diabetes and which preceded this research.
The initial studies from our unit, demonstrated that children with type 1 diabetes have significantly lower tHcy concentrations and significantly higher serum folate and red cell folate values than a group of healthy age and sex matched control children. The relevance of this finding of lower tHcy and higher folate status in children with type 1 diabetes was then studied in relation to endothelial function. Despite normal folate status and low tHcy levels children with type 1 diabetes had significant endothelial dysfunction. Endothelial dysfunction in children with type 1 diabetes relates to folate status and is independent of tHcy levels. A pilot intervention trial to assess whether folate improves endothelial function in children and adolescents with type 1 diabetes was initiated in our unit and the results are discussed.

The final area discussed in this chapter, is the marker of vascular disease, high sensitivity CRP. This marker is associated with poor cardiovascular outcome. Elevated CRP levels have been shown to be related with early functional and structural atherosclerotic vascular changes in adults with type 1 diabetes and in healthy children however the association between Hs-CRP and endothelial function has not previously been studied in children with type 1 diabetes.
Chapter 3. Methods

Clinical Methods:

Subjects

Subjects with type 1 diabetes were recruited consecutively from the diabetes clinic at Women’s and Children’s Hospital (WCH) Adelaide, Australia, between November 2002 and November 2003. A power calculation, based on results from our previous study (Pena, Wiltshire et al. 2004), indicated 30 subjects in each treatment group would have 74% power to detect a difference in FMD with treatment of $2.0 \pm 3\%$. To allow for drop outs, 124 subjects were recruited.

Subjects were excluded before recruitment if they were known to have celiac disease (which may reduce absorption of folate and/or vitamin B6), were active smokers or were taking supplemental vitamins, including multivitamins. All subjects were screened for coeliac antibodies (anti-gliadin, endomysial, total IgA), thyroid function and vitamin B12 deficiency which precludes folate treatment.

All parents and subjects received a verbal explanation and appropriate written information and participated in the study following informed and written consent. The study was approved by the Children, Youth and Women’s Health Service (CYWHS) Human Ethics Committee.

Contact was maintained with the subjects to ensure early reporting of adverse events that would have resulted in that subject being withdrawn from the study. No adverse events were reported. To aid adherence to the study and daily tablet taking, phone calls to subjects were made at weeks three and seven.

124 of 162 subjects with T1DM, who were approached consecutively accepted to enter the study. Subjects had had diabetes for at least one year, were normotensive and had no clinically detectable microvascular disease assessed by overnight urinary albumin excretion and direct fundoscopy performed through dilated pupils by experienced Paediatric Ophthalmologists. None had clinical evidence of infection or any chronic inflammatory disease such as connective tissue disease, inflammatory bowel disease or
arthriti s. One subject withdrew after the immediate effects study, one subject withdrew after the 4 week study, therefore data is reported on 122 subjects with T1DM. Baseline biochemical data was unable to be obtained on one further subject, therefore Hs-CRP data was available on 121 subjects with T1DM (Figure 12).

At time of testing, these subjects were well, without history of, or clinical evidence of intercurrent infection, fever, ketosis or hypoglycaemia during the previous 24 hours.

Clinical measurements were performed on entry to the study. Height was measured on a Harpenden Stadiometer and weight was measured on the same standardised scale at the diabetes clinic, WCH. Blood pressure was taken using the subjects the right arm, on each day of the measure of endothelial function, using a Dinimap® blood pressure monitor (Johnson and Johnson Medical Inc.).

Recruitment, consent, clinical measurements, venesection and follow up phone calls were all performed by KEM.

**Control Subjects**

Thirty-three age and sex-matched control subjects, used in the analysis of the Hs-CRP data, had been previously studied in our unit. Additional blood, taken from these control subjects at the time of their vascular studies, had been stored. Hs-CRP was determined and the results used in this analysis. The control subjects had been recruited from two sources: healthy siblings or school friends of the participating subjects with T1DM and relatives of staff members. Control subjects had been studied under identical conditions, with the same ultrasonographers performing the vascular studies. At time of testing, control subjects were well, without clinical evidence of intercurrent infection, with no clinical evidence of any chronic inflammatory disease such as connective tissue disease, inflammatory bowel disease or arthritis.
Ethical approval

The study was approved by the Women’s and Children’s Hospitals’ Human Research Ethics Committee, Trial number REC1318, and the Drug and Therapeutic Committee.

The study was notified under the Clinical Trials Notification Scheme [(CTN) scheme] under the Therapeutic Goods Act 1989. Protocol 1318, Trial number 2002/532.
Figure 12. Flow chart of recruitment

162 approached
- 124 recruited
  - 124 Baseline Data Obtained
    - 35 Acute study
      - 9 Placebo
      - 14 Folate
      - 12 Vitamin B6
      - 1 withdrew
      - 34
    - 89
  - 123 8 week study
    - 31 Placebo
    - 31 Folate and Placebo
    - 31 Vitamin B6 and Placebo
    - 30 Vitamin B6 and Folate
      - 123 subjects completed 4 week protocol and assessment
        - 31 Placebo
        - 31 Folate and Placebo
        - 31 Vitamin B6 and Placebo
        - 30 Vitamin B6 and Folate
        - 1 withdrew
      - 122 subjects completed the 8 week protocol and assessment
        - 30 Placebo
        - 31 Folate and Placebo
        - 31 Vitamin B6 and Placebo
        - 30 Vitamin B6 and Folate
Laboratory Methods:

Total Plasma Homocyst(e)ine (tHcy)

tHcy was measured using the Abbott AxSYM® system for homocyst(e)ine determination. The assay is a Fluorescence Polarisation Immunoassay. It involves the reduction of all forms of Hcy, homocystine, mixed disulphide and protein bound forms, to free Hcy using dithiothretol and then conversion of homocysteine to S-adenosyl-L-homocysteine (SAH). Free Hcy is converted to SAH by the use of SAH hydrolase and excess adenosine. SAH is measured using fluorescent polarisation immunoassay.

Samples for tHcy measurement were collected into tubes containing tri-potassium ethylene-diamine-tetra acetic acid (EDTA) and immediately processed. Plasma was separated within 20 minutes by centrifugation to prevent the accumulation of homocyst(e)ine from cells following collection. Plasma was frozen at -70°C and analysed in batches. The inter-assay coefficient of variation in the measurement of tHcy in our laboratory is 2.1%.

The methionine load or challenge test, using an oral dose of methionine, has been used to measure tHcy levels in people with suspected hyperhomocyst(e)inaemia who have normal homocyst(e)ine concentrations during fasting. This involves the testing of tHcy levels before the methionine challenge and four to eight hours afterwards. The methionine load test stresses the trans-sulphuration pathway and provides evidence of hyperhomocysteinaemia. This method has been recently criticised as a measure of homocysteinaemia because it reflects the action of the transulphuration pathway alone. The trans-sulphuration pathway is responsible for reversing the transient postprandial increases in tHcy concentration rather than reflecting basal tHcy levels. Fasting tHcy has been shown to be the most important determinant of vascular dysfunction (Welch and Loscalzo 1998).

In our studies, fasting tHcy was measured.
Serum and Red Cell Folate

Serum and red cell folate were measured by the Abbott AxSYM® immunoassay system using an ion capture reaction which measures all forms of folate.

In this method, a high molecular weight quaternary ammonium compound, ion capture solution, is dispensed on a glass fibre matrix of the matrix cell. This imparts a positive charge to the matrix which enables the capture of negatively charged analyte complexes. During the assay, negatively charged polyanion-analyte complexes are formed. These complexes are captured through electrostatic interaction with the positively charged glass fibre matrix.

The AxSYM Folate assay utilises a soluble affinity reagent comprised of folate binding protein affinity coupled to monoclonal antibodies, which in turn covalently coupled to carboxymethylamylose. Negatively charged analyte complexes are formed during the folate assay through the binding reaction between folate and the soluble affinity reagent. The negatively charged analytes are then captured through electrostatic interaction with the positively charged glass fibre matrix. Concentration of folate is calculated by measuring the amount of unoccupied folate binding protein.

The sample for red cell folate and serum folate was collected in an EDTA tube.

Vitamin B12

Serum vitamin B12 was collected in a plain tube and measured using a micro-particle assay with a commercial analyser, Abbott AxSYM®.

Vitamin B6

Vitamin B6 is the cofactor for cystathione-β-synthase and γ-cystathionase and is responsible for postprandial tHcy. Currently there is no agreement as to which form of vitamin B6 should be assayed to routinely assess of vitamin B6 status.
In our work, vitamin B6 status was measured using red blood cell aspartate aminotransferase (AST) activation by pyridoxal phosphate. Red cell aspartate aminotransferase is a long-term indicator of functional pyridoxine status due to the 120 day life span of erythrocytes.

Activation of the red cell transaminases including aspartate aminotransferase requires pyridoxal phosphate to be active. Addition of pyridoxal phosphate to red cell AST causes an increase in activity. The extent of the increase is a reflection of the deficiency of pyridoxal phosphate, therefore the lower the value the higher the subjects’ vitamin B6 status. If the activity of the enzyme is increased by more than 63% by the addition of pyridoxal phosphate, this suggests a deficiency state. The method used is an in house method at the Institute of Medical and Veterinary Science (IMVS), Adelaide, South Australia. Whole blood for red cell aspartate aminotransferase was collected into a non-gel lithium heparin tube. The sample was stored and analysed in batches weekly. Red cells were separated from the whole blood by centrifugation and then lysed. Pyridoxal 5-phosphate (Sigma), α-ketoglutarate (Sigma) and an aspartate substrate were added to the sample, placed in a Cobas reagent and analysed using a Cobas Bio analyser.

**Haemoglobin A1c (HbA1c)**

Haemoglobin A1c was measured using latex immuno-agglutination inhibition methodology (DCA 2000, Hemoglobin A1c Reagent Kit, Bayer, Toronto, Canada). The non-diabetic normal range is 4.0 - 6.0 %. This method has been correlated with high performance liquid chromatography in our laboratory ($r= 0.97$). The high performance liquid chromatography method is standardised with Diabetes Control and Complications Trial control sera.

**Lipids**

Lipids were measured because of the potential effect of lipids on endothelial function (Clarkson, Celermajer *et al.* 1996). Samples were collected after an overnight fast.
The Effect of Folate and Vitamin B6 on Endothelial Function in Children with Type 1 Diabetes

Dr Karen E MacKenzie

Triglycerides

Triglyceride concentration in plasma was measured using a commercial, timed end-point method on the Beckman Synchron CX5 Pro analyser. In this reaction, triglyceride is hydrolysed to glycerol and free fatty acids activated by lipase. A sequence of three coupled enzymatic steps using glycerol kinase, glycerophosphate oxidase and horse radish peroxidase causes the oxidative coupling of 3, 5-dichloro-2-hydroxykenzosulphuric acid with 4-aminoantipyrine to form a red guinoeimine dye. This dye is measured by spectrophotometry. The Synchron CX5 system monitors the change in absorbance at 520nm at a fixed time interval. The change in absorbance is directly proportional to the concentration in the plasma.

Total Cholesterol

Total cholesterol is also measured by a commercial, timed endpoint method on the Beckman Synchron CX5E analyser. The cholesterol ester is hydrolysed by cholesterol esterase to free cholesterol and fatty acid. The free cholesterol is oxidised to cholesteol-3-one and hydrogen peroxide by cholesterol oxidase. Peroxidase catalyses the reaction of hydrogen peroxide with 4-aminoantipyrine and phenol to produce a coloured quinoneimine product. The change in absorbancy at 520 nm is directly proportional to the concentration of total cholesterol in the sample.

The sample was collected by venepuncture into lithium heparin containing tubes, separated and assayed after collection.

HDL Cholesterol

HDL cholesterol was measure using a commercial assay on the Beckman Synchron CX5 analyser.

Low density lipoprotein (LDL) and very low density lipoprotein (VLDL) in plasma are precipitated by polyethylene glycol. The LDL and VLDL portions are then removed by centrifugation. The HDL cholesterol fraction remains in the supernatant and is assayed by a timed endpoint method on the Beckman Synchron CX5CE analyser. In the reaction,
the cholesterol esterase hydrolysates cholesterol ester to free cholesterol and fatty acids. The free cholesterol is oxidised to cholesten-3-one and hydrogen peroxide by cholesterol oxidase. Peroxidase catalyses the reaction hydrogen peroxide with 4-aminoantipyrine and phenol to produce a coloured quinoeimine product. The Synchron CX5E system monitors the change in absorbance at 520 nm at a fixed time interval. The change in absorbance is directly proportional to the concentration of cholesterol in the sample.

The sample was collected by venepuncture into a lithium heparin tube.

**High Sensitivity C-Reactive Protein**

High sensitivity C-reactive protein was measured by rate nephelometry and rate turbidimetry using the Beckman Coulter IMMAGE immunochemistry system.

A CRP reagent is used to react with the sample. Antibody-antigen, immunoprecipitin complexes are formed which cause an increase in light scatter and a decrease in light intensity as complexes are formed.

Rate nephelometry measures the increase in the intensity of the light scattered by the suspended particles. The light source for the rate nephelometer is a 670nm laser. The detector is placed at 90° from the laser beam to measure light scatter.

Rate turbidimetry measures the decrease in the intensity of the light as it passes through a solution of light scattering particles. The light source for the rate turbidimeter is a light emitting diode (LED) at a wavelength of 940 nm. Turbidimetric measurements are made at 0° from the incident beam.

**Cotinine**

This method used for the determination of cotinine is a competitive micro-plate immunoassay. The test relies on the competition between free cotinine in the sample and cotinine bound to enzyme for antibody fixed on a polystyrene plate. Excess enzyme
is washed away, substrate is added and the measured absorbance is inversely proportional to the amount of cotinine present in the sample.

Serum was used in preference to saliva for measurement of cotinine.

**Thyroid Function**

Thyroid function was assessed in all subjects. Thyroid stimulating hormone (TSH) was measured using a commercially available kit (ADVLA Centaur TSH assay, Bayer Corporation) by a two-site sandwich immunoassay. This method uses chemiluminometric technology. Two antibodies are used, the first antibody is a monoclonal mouse anti-TSH antibody labelled with an acridinium ester. The second is a polyclonal sheep anti-TSH antibody which is covalently coupled to paramagnetic particles.

Free thyroxine was measured by competitive immunoassay, using a commercially available kit (ADVLA Centaur FrT4 assay, Bayer Corporation). This is a direct chemiluminescent immunoassay in which the patient sample competes with acridinium ester-labelled T4 in the reagent for a limited amount of polyclonal rabbit anti T4 antibody, which is covalently coupled to paramagnetic particles in the solid phase.

**Coeliac Screen**

Anti-endomysial antibodies, total IgA (Immunoglobulin A) and anti-gliadin antibodies were measured.

The test for gliadin specific IgA and IgG is an indirect solid phase enzyme immunoassay. A particular wheat gliadin fraction is reacted with specific IgA and IgG in patient serum forming a gliadin/ anti-gliadin-IgA or gliadin/ anti-gliadin -IgG complex. Anti-human IgA or anti-human IgG (as appropriate) is conjugated with the enzyme horseradish peroxidase which binds to the immobilised IgA and IgG. This complex is then reacted with a specific substrate to yield a green colour. Following termination of colour development absorbance is measured at 405-420nm and related to the level of gliadin specific IgA and IgG respectively.
Assessment of Endothelial Function.

Flow Mediated Dilatation

Endothelial function was measured using flow mediated dilatation as described by Professor Celermajer (Celermajer, Sorensen et al. 1992) and as per the guidelines for ultrasound assessment of endothelium dependent Flow Mediated Dilatation of the brachial artery published by the American College of Cardiology (Corretti, Anderson et al. 2002).

This technique has been used in this unit over several years and the coefficient of variation has previously been 3.9% (Wiltshire, Gent et al. 2002). The ultrasound scans were performed with Mr Roger Gent, Chief Sonographer and Mr Lino Piotto, Senior Paediatric Sonographer, Paediatric Ultrasound WCH.

Equipment

Ultrasound equipped with vascular software for two-dimensional (2D) imaging, colour and spectral Doppler, an internal electrocardiogram (ECG) monitor and a high frequency vascular transducer was used. The target artery was measured by high resolution, two dimensional external vascular ultrasound using a 10.0 MHz linear ray transducer (Advanced Technology Laboratories, Bothel, Washington, USA) using an ATI HDI 3000 system.

Subject Preparation

Numerous factors affect flow mediated vascular reactivity including temperature, food and drugs (Corretti, Anderson et al. 2002). Therefore the baseline, four week and eight week studies were taken in the fasting state, prior to the administration of insulin, at the same time of day and in a quiet, temperature controlled room.
Subjects had fasted at least eight to twelve hours before the study. In addition, as the studies were performed early in the morning, the subjects had not exercised prior to the study.

The two and four hour studies were taken after administration of subcutaneous insulin and after a low protein, low fat diet, carbohydrate based meal as advised by our dietician. Dietary fats in particular are known to interfere with the studies with FMD significantly decreasing after oral fat intake (Wilmink, Stroes et al. 2000). Participants of the acute study were required to remain recumbent in a temperature-controlled room for the duration of the study.

**Image Acquisition**

The right brachial artery was the target artery used in this study, measured 2-15 cm above the antecubital fossa in longitudinal section. The transmit (focus) zone was set to the depth of the near wall, in view of the greater difficulty of evaluating the near than the far wall ‘m’ line. The ‘m’ line is an echodense ultrasonic line generated from the interface between the vessel media and adventitia. Depth and gain settings were set to optimise images of the lumen/arterial wall interface. All images were obtained at the minimum field of viewing setting to maximise the size of the vessel in the image. Machine operating parameters were not changed during any study.

A suitable vessel was for imaging was first selected with clear anterior and posterior intimal interfaces between the lumen and vessel wall. The vessel selected preferably had reproducible ultrasonographic markers or anatomic landmarks, such as vessel bifurcations, veins or characteristic tissue planes to maintain the same image of the artery throughout the study and to ensure the measurement occurred at the same place for each scan. When a satisfactory transducer position was found, the position was marked on the skin and the arm remained in the same position throughout the study.

An ECG was recorded simultaneously and continuously with the ultrasonographic images. The ECG was recorded on the ultrasound video monitor.
In each study the scans were taken at rest (Figure 13), during reactive hyperaemia, at rest and after sublingual GTN spray.

**Figure 13. Ultrasound of the Brachial artery: Subject at Rest**

The subjects were positioned supine with the arm in a comfortable position for imaging the brachial artery. The subject lay at rest for 15 minutes before a first resting scan was recorded (Figure 13). The first scan was rerecorded (Figure 14) and then arterial flow velocity measured (Figure 15). Arterial flow velocity was measured by means of a pulsed Doppler velocity signal at a 70° angle to the vessel, with the range gate in the middle of the artery.
Figure 14. Ultrasound of the Brachial artery: Subject at Rest

![Ultrasound of the Brachial artery: Subject at Rest](image1)

Figure 15. Brachial Arterial Flow Velocity: At Rest

![Brachial Arterial Flow Velocity: At Rest](image2)
**Endothelium-dependent Flow Mediated Dilatation**

Reactive hyperaemia was then induced by occluding arterial blood flow using a sphygmomanometer inflated to 250 mmHg for four minutes followed by rapid release. The cuff was inflated to a supra-systolic pressure to occlude arterial flow to cause ischaemia and consequent dilation of downstream resistance of vessels via auto-regulatory mechanisms. The subsequent cuff deflation induces a brief high flow state through the brachial artery (reactive hyperaemia) to accommodate the dilated resistance vessels. The resulting increase in shear stress causes the brachial artery to dilate (Figure 16).

**Figure 16. Endothelium-dependent Flow Mediated Dilatation**

We used an adult size cuff in children as previous experience (Jarvisalo, Ronnemaa et al. 2002) has shown the use of a narrower cuff causes more discomfort to paediatric patients.

The second scan of the longitudinal image of the artery was recorded continuously during this reactive hyperaemia for 30 seconds before and 180 seconds after cuff deflation.
Repeat arterial flow velocity using a mid-artery pulsed Doppler signal was obtained upon immediate cuff release and measured for the first 15 seconds after cuff deflation to assess hyperaemic velocity.

This technique of upper arm occlusion is technically challenging for data acquisition as the image is distorted by collapse of the brachial artery and shift of the soft tissue. Because this and the shift in brachial artery position that frequently occurs when the cuff is released, great care was taken to find and image the largest lumen diameter (the centre of the artery) during the measurement post occlusion.

The change in brachial artery diameter, after cuff release, increases as the duration of cuff inflation increases from 30 seconds to five minutes. The change in diameter is similar after five and ten minutes of occlusion; therefore the more easily tolerated five minutes is typically used in adults, in this study and in previous work from our unit, we elected for four minutes of cuff occlusion. Maximal dilation occurs 45 to 120 seconds after cuff deflation in children (Jarvisalo, Ronnemaa et al. 2002), therefore scans were recorded for 180 seconds.

**Figure 17. Brachial Arterial Flow Velocity: During Cuff Inflation**
Figure 18. Ultrasound of the Brachial Artery: During Cuff Inflation

![Ultrasound Image]

Figure 19. Arterial Flow Velocity: During Reactive Hyperaemia

![Arterial Flow Velocity Image]
Endothelium-independent Vasodilatation with Glyceryl Trinitrate

Ten minutes was allowed for vessel recovery and a third, resting scan was performed to reflect the re-established baseline conditions (Figure 20). Glyceryl trinitrate spray, GTN (400 µg, Nitro lingual Spray, Rhone-Poulenc Rorer) was administered sublingually and the last scan was recorded for four minutes (Figure 21). GTN is given as an exogenous Nitric Oxide donor to determine the maximum obtainable vasodilator response and to serve as a measure of endothelium-independent vasodilatation reflecting vascular smooth muscle function. Peak vasodilatation occurs three to four minutes after GTN administration therefore images were recorded continuously during this time. Response to GTN reflects both smooth muscle function and arterial compliance that may play a role in any observed changes in Flow Mediated Dilatation.

Figure 20. Ultrasound of the Brachial Artery: Third resting scan
Figure 21. Ultrasound of the Brachial Artery: Post GTN
Analysis

All images were recorded onto high quality super VHS (Video Home Systems) videotapes for later, off-line analysis.

The diameter of the brachial artery was measured from the longitudinal images in which the lumen-intima interface was clearly visualised on the near (anterior) and far (posterior) walls. Visualisation of both the near and far wall lumen-intima boundaries indicates that the imaging plane is bisecting the vessel in the longitudinal direction and diameters from these images are more likely to reflect the true diameter. Once the image for analysis was chosen, the boundaries for diameter measurements were identified manually.

Vessel diameters were measured with ultrasonic callipers. The arterial diameter was measured at a fixed distance from an anatomic marker (e.g. a fascial plane). Measurements were taken from the anterior to the posterior 'm' line (media-adventitia interface) at end diastole, incident with the R-wave on the electrocardiograph. For each scan, the measurements were taken over four cardiac cycles and the measurements averaged.

The timing of the measurement of the brachial artery diameter during the cardiac cycle is essential. The onset of the R-wave is used to identify end diastole, and the peak T wave reproducibly identifies end systole. Peak systolic diameter is larger than end diastolic diameter because the vessel expands during systole to accommodate the increase in pressure and volume generated by left ventricular contraction. The magnitude of systolic expansion is affected by the vessel compliance and may be reduced by factors that cause reduced bioavailability of Nitric Oxide.

Timing of FMD

Flow mediated dilatation is an endothelium dependent process that reflects the relaxation of the conduit artery when exposed to shear stress. Increased flow and thereby increased shear stress through the brachial artery occurs during post-occlusive reactive hyperaemia. Maximal dilation dilatation occurs 45 to 120 seconds after cuff deflation in children (Jarvisalo, Ronnemaa et al. 2002). The measurement during reactive
hyperaemia was taken between 45-180 seconds after cuff deflation and was the maximal vessel diameter reached.

Flow mediated dilatation is typically expressed as the change in post stimulus diameter as a percentage of the baseline diameter (Celermajer, Sorensen et al. 1992). The baseline diameter influences percent change in two ways. For a given absolute change in the post flow diameter, a larger baseline diameter yields a smaller measure of percent change and smaller arteries appear to dilate relatively more than larger arteries.

Vessel diameters in scans after reactive hyperaemia, the second baseline and after GTN were measured and expressed as a percentage of the first control, resting scan.

Scans in which the diameter of the artery differed by more than 3% between the first resting control and third resting, re-control, were excluded from analysis.

Vessel response to reactive hyperaemia is dependent on the release of nitric oxide. Vessel response to GTN occurs independently of nitric oxide derived from the endothelium. Comparison of the two measures therefore provides the best assessment of function of the endothelium per se.

Application of Flow Mediated Dilatation in Clinical Trials

The use of brachial artery flow mediated dilatation as a measure of systemic endothelial function has been well characterised and FMD is a recognised surrogate marker of vascular disease. Abnormalities in brachial artery flow mediated dilatation reflect generalised endothelial dysfunction.

Endothelial dysfunction is a key event in the development of atherosclerosis and can be measured in a variety of ways. Endothelial dysfunction of the coronary arteries is the 'gold standard' and has been shown to predict long term atherosclerotic disease progression and cardiovascular events (Schachinger, Britten et al. 2000). Intra-coronary testing is invasive and unsuitable for use in children and adults with no clinical signs of disease, for this reason, non invasive detection endothelial dysfunction of the brachial artery was first described in 1992 using FMD (Celermajer, Sorensen et al. 1992). FMD
correlates well with invasive testing of the coronary endothelial function (Anderson, Uehata et al. 1995; Neunteufl, Katzenschlager et al. 1997). It is both a sensitive and specific screening test to predict the presence of coronary artery disease (Schroeder, Enderle et al. 1999) and independently predicts long term cardiovascular events in patients with peripheral arterial disease (Gokce, Keaney et al. 2003). In patients with diabetes, brachial artery FMD has been shown to correlate well with carotid artery intimal thickness (Ravikumar, Deepa et al. 2002).

FMD therefore provides a surrogate measure of coronary endothelial function and is an important tool in assessing the reversibility of endothelial dysfunction in asymptomatic subjects at high risk of arterial disease. Endothelial dysfunction has been shown to be reversible (Doshi, McDowell et al. 2001; Woo, Chook et al. 2002; Dinckal, Aksoy et al. 2003). Numerous studies demonstrate that brachial artery reactivity improves both with risk factor modification and with treatment with medication to reduce cardiovascular risk.

FMD is a safe, well-tolerated and non-invasive measure of peripheral arterial endothelial function that is suitable for use in children. FMD is impaired in children and adults at risk of atherosclerosis (Celermajer, Sorensen et al. 1992). Work in our unit has shown that FMD is impaired in children with type 1 diabetes (Wiltshire, Gent et al. 2002).

The vasodilator response to glyceryl trinitrate may also be impaired with cardiovascular risk factors (Corretti, Anderson et al. 2002). This is consistent with experimental studies demonstrating that inactivation of NO by reactive oxygen species is an important mechanism of vascular dysfunction. In type 1 diabetes, reactive oxygen species are thought to be central to the development of complications (Brownlee 2001) as discussed in the previous chapter.

### Statistical Methods

Specific statistical methods are discussed in each individual chapter.
Chapter 4. Publication

Folate and Vitamin B6 rapidly normalize endothelial dysfunction in children with Type 1 Diabetes.

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Chapter 5. The immediate effect of folate and vitamin B6 on endothelial function in children and adolescents with type 1 diabetes

Introduction

This chapter presents the results of the immediate effect study, conducted over four hours, to determine the two and four hour effect of folate and vitamin B6 on endothelial function in children and adolescents with type 1 diabetes. This chapter expands on the results described in the publication in ‘Pediatrics’ presented in Chapter 4 and contains the extended statistical data analysis.

Background

Work from our unit has examined folate status, tHcy levels and endothelial function in children with type 1 diabetes.

The initial studies demonstrated that children with type 1 diabetes have significantly lower tHcy concentrations and significantly higher serum folate, red cell folate as well as higher vitamin B12 values than a group of healthy age and sex matched control children (Wiltshire, Thomas et al. 2001).

The relevance of this finding of lower tHcy and higher folate status in children with type 1 diabetes was then studied in relation to endothelial function. In this study, despite normal folate status and low tHcy levels children with type 1 diabetes had significant endothelial dysfunction (Wiltshire, Gent et al. 2002). Folate status was found to be an important determinant of endothelial function in children with type 1 diabetes, both when measured by FMD and biochemical markers of endothelial activation (vWF and thrombomodulin). The ratio of FMD: GTN-induced dilatation (the best measure of endothelial function per se) was also independently associated with red cell folate, which suggested that folate status is an important factor protecting against endothelial dysfunction. (Wiltshire, Gent et al. 2002).
A pilot intervention trial to assess whether folate improved endothelial function in children and adolescents with type 1 diabetes, was then performed in our unit. This trial was a randomised, double-blind, placebo-controlled cross-over trial which compared 5 mg oral daily folate with placebo. Subjects were randomised to receive either folate or placebo for eight weeks. There was an eight week washout period, and the subjects then crossed over to placebo or folate, respectively, for a further eight weeks. This pilot study showed that despite normal folate status and low tHcy levels an improvement in endothelial function occurred with high dose supplemental folate (Pena, Wiltshire et al. 2004).

In other units improvement in endothelial function has been observed within hours of additional oral folate (Doshi, McDowell et al. 2002) and within minutes of intravenous 5-MTHF, the active form of folate (Verhaar, Wever et al. 1998; van Etten, de Koning et al. 2002). Folate and vitamin B6 in combination have also been shown to improve biological markers of endothelial function (Constans, Blann et al. 1999). Vitamin B6 alone improves endothelial function over ten weeks (Miner, Cole et al. 2001) however the immediate effect of vitamin B6 on endothelial function has not been established.

Therefore in this study, we sought to determine the immediate effects of both folate and vitamin B6 on endothelial function in children with type 1 diabetes.

**Hypothesis**

The following hypotheses were generated. These are discussed fully in chapter 1.

1. Endothelial function is impaired in children with type 1 diabetes.
2. Endothelial function is related to folate status.
3. Subjects with higher red blood cell folate at baseline have less response of the endothelium to supplementary folate.
4. Folate improves endothelial function acutely.
5. Supplemental folate improves endothelial function in children with type 1 diabetes mellitus, independent of lowering tHcy.
6. Vitamin B6 improves endothelial function acutely.
The Effect of Folate and Vitamin B6 on Endothelial Function in Children with Type 1 Diabetes

Dr Karen E MacKenzie

Aims

1. To determine the immediate effect of folate on endothelial function in children with type 1 diabetes
2. To determine the immediate effect of vitamin B6 on endothelial function in children with type 1 diabetes
3. To determine whether high sensitivity C-reactive protein relates to endothelial function in children with type 1 diabetes.

Outcome Measures

Primary

The main outcome measures of the study were endothelial function and smooth muscle function measured by function FMD, GTN-induced dilatation and the ratio FMD: GTN.

Secondary

The secondary outcome measures were the change in folate status, vitamin B6 status, tHcy and Hs-CRP.

Study Design

35 subjects with type 1 diabetes were studied over four hours to determine the immediate effect of folate or vitamin B6 on endothelial function.

The study had a randomised, double-blind, placebo controlled design. There were three study groups that received:

(i) folate 5mg (Sigma Pharmaceuticals)
(ii) vitamin B6 100mg (Rhone-Poulenc-Rorer Pharmaceuticals)
(iii) one placebo tablet (Sigma Pharmaceuticals).
After recruitment, subjects were assigned a unique identifying number. Randomisation, using this identifying number, was then performed using a Fischer table by the Pharmacy Department, WCH. Two-tablet randomisation was performed as most subjects, all but one, who participated in this immediate effects study continued into the eight weeks study that is described in the next chapter. The study tablets were provided in two identical bottles labeled ‘A’ or ‘B’ by the Pharmacy Department, WCH. The two bottles were supplied in a paper bag labeled only with the subjects’ unique identifying number. Tablet A was the tablet used in this study of the immediate effects. The numbers of subjects in each group in the immediate effects study were monitored by the randomising pharmacist. As subjects participating in the short effects study were not consecutively studied, to ensure the numbers in each group were kept relatively even, the pharmacist pre-assigned a study number for the subjects participating in this immediate effects study.

For the randomisation, Fischer Tables page 134, table XXXIII random numbers (l) were used. Randomisation was from top to bottom, right to left, balanced blocks of twelve, with the first three subjects receiving A (folate and placebo; with tablet A, folate), second group of three receiving B (placebo and placebo; with tablet A, placebo), third group of three receiving C (vitamin B6 and placebo; with tablet A, vitamin B6) and the fourth group of three receiving D (vitamin B6 and folate; with tablet A, vitamin B6).

Subjects attended for four hours and underwent three assessments commencing between 0730-0830hrs: the first assessment at baseline, the second and third assessment at two and four hours after the study tablet. At the baseline assessment, subjects were fasted and had withheld morning insulin. Clinical data were collected, brachial artery response to flow mediated dilatation (FMD) and GTN-induced dilatation were assessed and venous blood collected at each assessment. Blood was collected at baseline, two and four hours as shown in Table 1.

Baseline studies were performed, subjects were instructed to take tablet ‘A’, as per the randomisation and assessments were repeated at two and four hours after the tablet. Subjects were recumbent during the four hours and remained within a temperature-controlled environment (22-24 °C). A carbohydrate based, low-fat, low-protein, caffeine-free breakfast was provided and subcutaneous insulin given immediately after the baseline assessment. Breakfast consisted of bread/toast or pickets with diabetic jam. The meal was completed within 30 minutes of the baseline assessment.
Subjects had been instructed not to take any additional vitamin supplements prior to the study.

**Subjects**

Subjects with type 1 diabetes were recruited from the diabetes clinic at Women’s and Children’s Hospital (WCH) Adelaide as described in the methods section. 35 subjects participated in this study of the immediate effects of folate and vitamin B6 on the endothelium.

**Ethical approval**

As discussed in the Methods Chapter, Chapter 3, the study was approved by the Women’s and Children’s Hospitals’ Human Research Ethics Committee and the Drug and Therapeutic Committee. The study was notified under the Clinical Trials Notification Scheme [(CTN) scheme] under the Therapeutic Goods Act 1989.
Study Protocol and Laboratory Methods

Study Protocol

FMD was measured at baseline, before the first tablet and at two and four hours after the study tablet. Biochemical parameters were measured after each measurement of FMD (Table 1).

Table 1. Study Protocol for the Immediate Effects Study

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>2 hours</th>
<th>4 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum cotinine</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting lipids</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma glucose</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Serum Folate</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Vitamin B6 pyridoxine phosphate % activation</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Serum Vitamin B12</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tHcy</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Flow Mediated dilatation</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>GTN- induced dilatation</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Hs-CRP</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Biochemical Parameters

Lipids, blood glucose, HbA1c, serum and red cell folate, vitamin B12 and Hs-CRP were analysed on the day of sampling, other samples were separated and stored until analysis.
Biochemical Assays

Biochemistry including lipids, glucose, serum and red cell folate, vitamin B12, pyridoxine phosphate activation, total plasma homocyst(e)ine, Hs-CRP and serum cotinine were measured using methods as described in the methods chapter.

Non-invasive Measure of Endothelial Function

Endothelial function was assessed by flow mediated dilatation (FMD) and glyceryl trinitrate (GTN)- induced vasodilatation using high resolution ultrasound and vessel wall tracking as originally reported (Celermajer, Sorensen et al. 1992) and described fully in the methods chapter. Vascular studies were performed by two senior experienced paediatric vascular ultrasonographers.

Our previously determined age-matched control range was determined by studying twenty healthy, age-matched children under identical conditions, with FMD performed by the same ultrasonographer, on the same equipment and read by the same blinded observer (Wiltshire, Gent et al. 2002).

Statistics

The data was analysed using SPSS software version 10.0.7.

Comparisons of clinical characteristics, biochemical parameters and endothelial function at baseline across the three treatment groups (placebo, folate and vitamin B6) were made using one-way analysis of variances (ANOVA). Least significant difference post-hoc tests were used to undertake pair-wise post-hoc comparisons of endothelial function variables at baseline between two treatment groups. The linear association of FMD at baseline with other variables of interest at baseline were assessed using Spearman’s rank correlations.
The magnitude of the change in each variable of interest over time at baseline, two hours and four hours separately within the placebo, folate and vitamin B6 treatment groups were tested using linear mixed models. A linear mixed model can account for the within subject correlations (subject effect) from repeated measurements on the same individual over the three periods. The linear mixed model used repeated contrasts to test the change in the expected mean of variables between successive time periods, i.e., mean change from baseline to two hours and mean change from two hours to four hours.

The change from baseline to four hours for each variable of interest was calculated for each individual with measurements at baseline and at four hours. Comparisons of the mean change in variables from baseline to four hours between two treatment groups treatment groups was made using one-way ANOVAs and pair-wise post-hoc comparisons using least significant differences post-hoc tests.

The linear association of the change in FMD from baseline to four hours with other variables of interest at baseline and their change from baseline to four hours were assessed using Spearman’s rank correlations.

The distribution of Hs-CRP showed a strong right skew. The skewed distribution may influence the results of the Hs-CRP analysis by overestimating the central location of Hs-CRP. Nonparametric tests were used for the uni-variable analysis of Hs-CRP. A $\log_{10}$ transformation was applied to the Hs-CRP data and the $\log_{10}$ transformed data was used in the linear mixed models of Hs-CRP. A $\log_{10}$ transformation is consistent with previous studies (Kilpatrick, Keevil et al. 2000).

Statistical significance was inferred with a p value less than 0.05
Results

Subjects

35 subjects with type 1 diabetes completed the immediate effects protocol with no adverse effects.

Baseline Characteristics

Baseline clinical characteristics of the subjects are shown in Table 2. There were no significant differences between the groups in baseline characteristics assessed. Results are expressed as the mean (with standard deviation).

Table 2. Baseline Clinical Characteristics, Immediate Effects Study

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Folate</th>
<th>Vitamin B6</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>14</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>15.5 (2.28)</td>
<td>14.7 (2.59)</td>
<td>15.3 (2.73)</td>
<td>0.73</td>
</tr>
<tr>
<td>Age range (years)</td>
<td>11.5-18.9</td>
<td>9.1-18.1</td>
<td>11.0-18.0</td>
<td></td>
</tr>
<tr>
<td>Sex M:F</td>
<td>5:4</td>
<td>9:5</td>
<td>6:6</td>
<td>0.14*</td>
</tr>
<tr>
<td>Insulin dose (Units/kg)</td>
<td>0.98 (0.33)</td>
<td>1.3 (0.35)</td>
<td>1.2 (0.31)</td>
<td>0.14</td>
</tr>
<tr>
<td>Duration of diabetes (yrs)</td>
<td>4.4 (3.75)</td>
<td>5.6 (4.37)</td>
<td>4.2 (3.92)</td>
<td>0.64</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.1 (1.3)</td>
<td>8.2 (0.91)</td>
<td>8.7 (1.59)</td>
<td>0.51</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>164.2 (7.30)</td>
<td>166.1 (14.78)</td>
<td>166.2 (11.80)</td>
<td>0.92</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>57.8 (10.61)</td>
<td>59.0 (13.75)</td>
<td>64.6 (16.15)</td>
<td>0.47</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.3 (2.75)</td>
<td>21.2 (3.08)</td>
<td>23.0 (3.40)</td>
<td>0.28</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>121 (17.6)</td>
<td>119 (16.0)</td>
<td>120 (12.4)</td>
<td>0.97</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>68 (7.3)</td>
<td>65 (6.9)</td>
<td>63 (8.3)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

* Chi-square test used for sex. One-way ANOVA used for other comparisons.
On baseline testing, one subject was coeliac antibody positive and was later confirmed to have coeliac disease by small bowel biopsy. This subject was included in this study as the subject had completed the study protocol including the eight week study by the time the small bowel biopsy confirmed the diagnosis of coeliac disease. Exclusion did not alter the final results.

None gave a history of smoking however three subjects had an elevated serum cotinine (>28 nmol/l) at baseline. All three subjects had levels within the smoking range (115-5700 nmol/l) at 131, 216 and 392 nmol/l. One subject was randomised to the placebo group, one to the B6 group and the subject with the highest baseline cotinine of 392 nmol/l was randomised to the folate group. These subjects were included in the analysis. Exclusion did not alter the final results.

**Baseline Biochemical Parameters**

Baseline biochemical parameters are shown in Table 3. There are no significant differences in biochemical parameters between the groups. All subjects had serum folate, red cell folate and vitamin B6 levels within the reference range.
Table 3. Baseline Biochemical Parameters, Immediate Effects Study

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Folate</th>
<th>Vitamin B6</th>
<th>Significance (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>14</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>3.7 (0.87)</td>
<td>4.5 (1.63)</td>
<td>4.2 (0.80)</td>
<td>0.33</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/l)</td>
<td>1.5 (0.211)</td>
<td>1.4 (0.378)</td>
<td>1.59 (0.36)</td>
<td>0.48</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/l)</td>
<td>2.03 (0.624)</td>
<td>2.38 (0.733)</td>
<td>2.35 (0.542)</td>
<td>0.45</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.40 (0.197)</td>
<td>0.43 (0.294)</td>
<td>0.44 (0.258)</td>
<td>0.73</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>16.1 (4.68)</td>
<td>11.9 (6.03)</td>
<td>15.2 (4.40)</td>
<td>0.13</td>
</tr>
<tr>
<td>Vitamin B12 (pmol/l)</td>
<td>344 (138.3)</td>
<td>320 (104.6)</td>
<td>288 (59.7)</td>
<td>0.46</td>
</tr>
<tr>
<td>Red cell folate (ug/l)</td>
<td>451.3 (154.86)</td>
<td>549.6 (143.67)</td>
<td>538.6 (141.84)</td>
<td>0.21</td>
</tr>
<tr>
<td>Serum folate (ug/l)</td>
<td>13.2 (2.43)</td>
<td>10.7 (4.55)</td>
<td>12.4 (2.05)</td>
<td>0.19</td>
</tr>
<tr>
<td>Pyridoxine phosphate activation (%) (Vitamin B6)</td>
<td>53.2 (5.8)</td>
<td>47.27 (9.7)</td>
<td>48.0 (4.8)</td>
<td>0.17</td>
</tr>
<tr>
<td>tHcy (µmol/l)</td>
<td>6.4 (1.20)</td>
<td>6.7 (1.72)</td>
<td>6.5 (1.58)</td>
<td>0.86</td>
</tr>
<tr>
<td>Hs-CRP (mg/l)*</td>
<td>0.8 (0.25 - 2.8)</td>
<td>0.6 (0.25 - 20)</td>
<td>1.0 (.25 -18)</td>
<td>0.29</td>
</tr>
</tbody>
</table>

* Geometric mean and range shown and Kruskal-Wallis test used for comparison

Baseline Endothelial Function

There was a significant difference in FMD between the groups at baseline (p= 0.04) with the folate group having lower FMD. There was one clear outlier in the folate group which skewed the results of the FMD. This subject had a markedly elevated cotinine indicating he was a moderate to heavy smoker (392 nmol/l).

The mean difference (standard error) in baseline FMD between the folate and placebo groups was -4.281 (1.658) % (p= 0.02), between the folate and vitamin B6 groups was -
2.829 (1.527) % (p= 0.07), there was no significant difference in baseline FMD between the vitamin B6 and placebo groups, -1.453 (1.711) % (p= 0.40).

There was no significant difference in baseline GTN-induced dilatation and the baseline FMD: GTN ratio (Table 4). However, between individual treatment groups, there was a significant difference in the baseline FMD: GTN between the folate and placebo group. The mean difference (standard error) in the baseline FMD: GTN ratio between the folate and placebo group was 0.150 (0.07) % (p= 0.04). There was no significant difference between the folate and vitamin B6 groups or the vitamin B6 and placebo groups. The mean difference (standard error) in the baseline FMD: GTN ratio between folate and vitamin B6 was 0.129 (0.07) % (p= 0.06) and between vitamin B6 and placebo was 0.021 (0.07) % (p= 0.77).

Results of the baseline FMD, GTN-induced dilatation and the ratio FMD: GTN are shown in Table 4.

Table 4. Baseline Endothelial Function, Immediate Effects Study

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Folate</th>
<th>Vitamin B6</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>14</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>FMD (%)</td>
<td>6.2 (3.56)</td>
<td>1.9 (3.93)</td>
<td>4.7 (4.04)</td>
<td>0.036</td>
</tr>
<tr>
<td>GTN-Induced dilatation (%)</td>
<td>22.6 (7.25)</td>
<td>20.2 (7.74)</td>
<td>18.9 (5.4)</td>
<td>0.497</td>
</tr>
<tr>
<td>FMD: GTN ratio</td>
<td>0.26 (0.150)</td>
<td>-0.11 (0.172)</td>
<td>0.24 (0.160)</td>
<td>0.068</td>
</tr>
</tbody>
</table>
Correlates of Baseline FMD

The data presented here are the correlates of baseline FMD of the 35 subjects that participated in the immediate effects study. This group is included in the 124 subjects participating in the eight week study and the correlates of the baseline FMD for the entire cohort are presented in the next chapter.

In this study, baseline FMD only correlated with Hs-CRP (r = 0.38, p = 0.03) (Table 5).

Table 5. Correlates of Baseline FMD, Immediate Effects Study

<table>
<thead>
<tr>
<th>Baseline variable</th>
<th>Correlation coefficient</th>
<th>Significance (2 tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>r = -0.061</td>
<td>p = 0.727</td>
</tr>
<tr>
<td>Insulin dose: Units/kg</td>
<td>r = -0.061</td>
<td>p = 0.728</td>
</tr>
<tr>
<td>Duration of Diabetes (years)</td>
<td>r = -0.222</td>
<td>p = 0.200</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>r = 0.080</td>
<td>p = 0.648</td>
</tr>
<tr>
<td>Blood pressure, systolic (mmHg)</td>
<td>r = -0.205</td>
<td>p = 0.237</td>
</tr>
<tr>
<td>Blood pressure, diastolic (mmHg)</td>
<td>r = 0.000</td>
<td>p = 0.998</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>r = -0.047</td>
<td>p = 0.793</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/l)</td>
<td>r = 0.020</td>
<td>p = 0.911</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/l)</td>
<td>r = -0.191</td>
<td>p = 0.296</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>r = -0.108</td>
<td>p = 0.551</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>r = -0.237</td>
<td>p = 0.171</td>
</tr>
<tr>
<td>Red cell Folate (µg/l)</td>
<td>r = 0.223</td>
<td>p = 0.197</td>
</tr>
<tr>
<td>Serum Folate (µg/l)</td>
<td>r = 0.248</td>
<td>p = 0.151</td>
</tr>
<tr>
<td>Pyridoxine phosphate % activation (B6)</td>
<td>r = 0.212</td>
<td>p = 0.236</td>
</tr>
<tr>
<td>tHcy (µmol/l)</td>
<td>r = -0.279</td>
<td>p = 0.876</td>
</tr>
<tr>
<td>Hs-CRP (mg/l)</td>
<td>r = 0.377</td>
<td>p = 0.026*</td>
</tr>
</tbody>
</table>

* Correlation is significant at the 0.05 level (2-tailed).
Change in Flow Mediated Dilatation (FMD)

The main outcome measure of the study was change in flow mediated dilatation at two and four hours.

The change in FMD from baseline to four hours was significant in the folate group and the vitamin B6 group. There was no significant change in FMD in the placebo group over the four hours (Figure 22) (Table 6).

The significant increase in FMD occurred in the first two hours in the folate group (p<0.001) and in the vitamin B6 group (p=0.007). There was no further significant change in FMD from two to four hours in the folate group (p=0.78) or the vitamin B6 group (p=0.59) (Figure 22) (Table 6).

In all subjects receiving folate, FMD increased to above the 10th centile of our previously determined age-matched control range, in nine of the fourteen subjects, FMD improved to above the mean of the control range and in five of the fourteen subjects receiving folate, FMD increased to above the 90th centile of the control range. In all subjects receiving vitamin B6, FMD increased above the 10th centile of the control range, in five of the twelve subjects, FMD improved above the mean.

Table 6. FMD over Four Hours

<table>
<thead>
<tr>
<th>FMD</th>
<th>Baseline</th>
<th>2 hours</th>
<th>4 hours</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>6.2 (3.56)</td>
<td>7.07 (4.31)</td>
<td>5.90 (4.33)</td>
<td>0.411</td>
</tr>
<tr>
<td>Folate</td>
<td>1.9 (3.93)</td>
<td>10.5 (5.12)</td>
<td>10.1 (4.67)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>4.7 (4.04)</td>
<td>8.4 (4.3)</td>
<td>9.0 (4.92)</td>
<td>0.004</td>
</tr>
</tbody>
</table>
Figure 22. FMD over Four Hours
Change in GTN-Induced Dilatation

As seen in Figure 23, a decrease in GTN-induced vasodilatation occurred in all groups over the four hours. The change in GTN-induced dilatation over the four hours was not significant in the folate and the vitamin B6 group. There was a significant change in GTN-induced dilatation in the placebo group over the four hours (Table 7). There was no significant difference in GTN-induced vasodilatation from baseline to two hours or from two to four hours in any one group.

Table 7. GTN-Induced Dilatation over Four Hours

<table>
<thead>
<tr>
<th>GTN</th>
<th>Baseline</th>
<th>2 hours</th>
<th>4 hours</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>22.5 (7.2)</td>
<td>19.4 (7.5)</td>
<td>17.0 (6.07)</td>
<td>0.027</td>
</tr>
<tr>
<td>Folate</td>
<td>20.2 (7.74)</td>
<td>20.5 (7.19)</td>
<td>18.9 (8.84)</td>
<td>0.411</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>18.9 (5.42)</td>
<td>18.5 (6.36)</td>
<td>16.7 (6.49)</td>
<td>0.105</td>
</tr>
</tbody>
</table>

Figure 23. GTN-Induced Dilatation over Four Hours
Change in FMD: GTN

There was a significant increase in the FMD: GTN ratio in the folate group (p< 0.001) and vitamin B6 group (p= 0.001) over the four hours (Table 8) (Figure 24). The significant increase in FMD: GTN occurred in the first two hours in the folate group (p< 0.001) and vitamin B6 group (p= 0.006). There was no further significant change in FMD: GTN at four hours. There was no significant difference in the FMD: GTN ratio between the folate group and the vitamin B6 group at four hours (p= 0.34). The FMD: GTN ratio in the placebo group also increased over the four hours, however this change was not significant (Table 8). There was no significant change in FMD: GTN in the placebo group at four weeks (p= 0.16) and at eight weeks (p= 0.85).

Table 8. FMD: GTN over Four Hours

<table>
<thead>
<tr>
<th>FMD: GTN</th>
<th>Baseline</th>
<th>2 hours</th>
<th>4 hours</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>0.26 (0.150)</td>
<td>0.33 (0.177)</td>
<td>0.34 (0.203)</td>
<td>0.215</td>
</tr>
<tr>
<td>Folate</td>
<td>0.11 (1.110)</td>
<td>0.55 (0.280)</td>
<td>0.56 (0.174)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>0.23 (0.160)</td>
<td>0.46 (0.267)</td>
<td>0.54 (0.190)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Figure 24. FMD: GTN over 4 Hours
Serum Folate

There was no significant difference in serum folate (p= 0.19) between the groups at baseline (Table 3). There was a rapid rise in serum folate in the folate group. In all subjects in the folate group, serum folate increased to above the normal range (3.3-15.0 µg/l) within the four hours (Figure 25). Five subjects in the vitamin B6 group and three subjects in the placebo group increased serum folate above the normal range.

There were seven subjects in the folate group whose serum folate values rose to supraphysiologic values following their oral folate dose. The serum folate levels in these seven subjects ranged from 57.6 to 465.0 µg/l. In one subject in the vitamin B6 group, the serum folate level rose to 146.0 µg/l.

Figure 25. Serum Folate over 4 hours

For the purposes of statistical analysis, serum folate levels above 20 µg/l were adjusted to the upper level of 20 µg/l. There was a significant change in adjusted serum folate in
the folate group over the four hours. There was no significant change in adjusted serum folate in the placebo group and the vitamin B6 group over the four hours (Table 9).

**Table 9. Adjusted Serum Folate over Four Hours**

<table>
<thead>
<tr>
<th>Adjusted Serum Folate</th>
<th>Baseline</th>
<th>2 hours</th>
<th>4 hours</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>13.2 (2.43)</td>
<td>14.7 (2.36)</td>
<td>15.1 (2.42)</td>
<td>0.056</td>
</tr>
<tr>
<td>Folate</td>
<td>10.7 (4.55)</td>
<td>19.5 (0.22)</td>
<td>19.2 (0.57)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>12.4 (2.05)</td>
<td>13.5 (2.54)</td>
<td>13.9 (3.09)</td>
<td>0.108</td>
</tr>
</tbody>
</table>

The significant change in adjusted serum folate in the folate group, occurred at two hours (p<0.001). There was no further significant change from two to four hours (p= 0.44).
**Red Cell Folate**

There was no significant difference in red cell folate (RCF) between the groups at baseline (p= 0.21) (Table 3). There was no significant change in RCF in the placebo group and the vitamin B6 group over the four hours. RCF in the folate group increased significantly over the four hours (Table 10).

**Table 10. Change in RCF over Four Hours**

<table>
<thead>
<tr>
<th>Red Cell Folate</th>
<th>Baseline</th>
<th>2 hours</th>
<th>4 hours</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>549.6 (143.7)</td>
<td>556.3 (153.65)</td>
<td>558.8 (147.70)</td>
<td>0.869</td>
</tr>
<tr>
<td>Folate</td>
<td>451.3 (154.86)</td>
<td>692.6 (178.90)</td>
<td>623.4 (179.06)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>538.6 (141.85)</td>
<td>550.6 (138.11)</td>
<td>548.5 (139.65)</td>
<td>0.638</td>
</tr>
</tbody>
</table>

The significant change in RCF in the folate group, occurred at two hours, with an increase in RCF (p< 0.001) and also at four hours, with a decrease in RCF (p= 0.009) (Figure 26).

**Figure 26. Red Cell Folate over Four Hours**

![Figure 26. Red Cell Folate over Four Hours](image-url)
Vitamin B6: Pyridoxine Phosphate Activation

There was no significant difference in pyridoxine phosphate activation (%) between the groups at baseline (p= 0.17) (Table 3). Pyridoxine phosphate activation in the vitamin B6 group decreased significantly over the four hours, indicating improved vitamin B6 status. There was no significant change in pyridoxine phosphate activation (%) in the placebo group and the folate group over the four hours (Table 11) (Figure 27).

Table 11. Change in Pyridoxine Phosphate Activation over Four Hours

<table>
<thead>
<tr>
<th>Vitamin B6</th>
<th>Baseline</th>
<th>2 hours</th>
<th>4 hours</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>53.2 (5.81)</td>
<td>53.9 (6.07)</td>
<td>52.0 (6.24)</td>
<td>0.321</td>
</tr>
<tr>
<td>Folate</td>
<td>47.1 (10.23)</td>
<td>47.9 (9.55)</td>
<td>47.9 (9.55)</td>
<td>0.658</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>48.0 (4.85)</td>
<td>42.6 (6.26)</td>
<td>43.6 (4.53)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

The significant change in % pyridoxine phosphate activation, in the vitamin B6 group, occurred at two hours (p= 0.002). There was no further significant change at four hours (p= 0.44) (Figure 27).

Figure 27. Pyridoxine Phosphate Activation over Four Hours
Total Plasma Homocyst(e)ine (tHcy)

There was no significant difference in total plasma homocyst(e)ine (tHcy) between the groups at baseline (p = 0.86) (Table 3) (Figure 28). tHcy levels decreased over time and the change in tHcy over the four hours was significant in all groups (Table 12).

<table>
<thead>
<tr>
<th>tHcy</th>
<th>Baseline</th>
<th>2 hours</th>
<th>4 hours</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>6.7 (1.72)</td>
<td>5.4 (1.27)</td>
<td>5.3 (0.99)</td>
<td>0.027</td>
</tr>
<tr>
<td>Folate</td>
<td>6.4 (1.20)</td>
<td>5.6 (1.28)</td>
<td>5.7 (1.04)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>6.5 (1.58)</td>
<td>5.9 (1.86)</td>
<td>5.4 (1.62)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Figure 28. tHcy over Four Hours
Glucose

There was no significant difference in blood glucose between the groups at baseline (p=0.13) (Table 3) and there was no significant change in blood glucose over the four hours (Table 13) (Figure 29).

Table 13. Change in Glucose over Four Hours

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Baseline</th>
<th>2 hours</th>
<th>4 hours</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>16.1 (4.68)</td>
<td>18.2 (6.58)</td>
<td>13.3 (3.96)</td>
<td>0.116</td>
</tr>
<tr>
<td>Folate</td>
<td>11.9 (6.04)</td>
<td>12.3 (7.47)</td>
<td>13.6 (5.79)</td>
<td>0.566</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>15.2 (4.40)</td>
<td>19.3 (8.66)</td>
<td>15.9 (6.94)</td>
<td>0.134</td>
</tr>
</tbody>
</table>

Figure 29. Glucose over Four Hours
High Sensitivity C-Reactive Protein (Hs-CRP)

Analysis of Hs-CRP data is presented in Chapters 8 and 9.

Correlates of Change in FMD

Change in FMD over the four hours was correlated with baseline FMD, baseline vitamin B6 status (% pyridoxine phosphate activation) and insulin dose (units per kg). Change in FMD weakly correlated with baseline triglycerides and baseline LDL-cholesterol although these did not meet statistical significance (Table 14).

Table 14. Correlates of change in FMD with Baseline Variables

<table>
<thead>
<tr>
<th>Baseline variable</th>
<th>Correlation coefficient</th>
<th>Significance (2 tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>r = -0.053</td>
<td>p = 0.761</td>
</tr>
<tr>
<td>Insulin dose (Units/kg)</td>
<td>r = 0.364</td>
<td>p = 0.032*</td>
</tr>
<tr>
<td>Duration of Diabetes (yrs)</td>
<td>r = 0.150</td>
<td>p = 0.389</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>r = 0.036</td>
<td>p = 0.836</td>
</tr>
<tr>
<td>Blood pressure, systolic (mmHg)</td>
<td>r = 0.005</td>
<td>p = 0.976</td>
</tr>
<tr>
<td>Blood pressure, diastolic (mmHg)</td>
<td>r = -0.022</td>
<td>p = 0.900</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>r = 0.315</td>
<td>p = 0.070</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/l)</td>
<td>r = 0.063</td>
<td>p = 0.729</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/l)</td>
<td>r = 0.304</td>
<td>p = 0.091</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>r = 0.336</td>
<td>p = 0.056</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>r = -0.023</td>
<td>p = 0.894</td>
</tr>
<tr>
<td>Red cell Folate (µg/l)</td>
<td>r = 0.102</td>
<td>p = 0.561</td>
</tr>
<tr>
<td>Serum Folate (µg/l)</td>
<td>r = 0.046</td>
<td>p = 0.793</td>
</tr>
<tr>
<td>Pyridoxine phosphate % activation (vitamin B6)</td>
<td>r = -0.382</td>
<td>p = 0.028*</td>
</tr>
</tbody>
</table>
The Effect of Folate and Vitamin B6 on Endothelial Function in Children with Type 1 Diabetes
Dr Karen E MacKenzie

<table>
<thead>
<tr>
<th>tHcy (µmol/l)</th>
<th>r = 0.027</th>
<th>p = 0.876</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs-CRP (mg/l)</td>
<td>r = -0.208</td>
<td>p = 0.230</td>
</tr>
<tr>
<td>Baseline Flow mediated dilatation (%)</td>
<td>r = -0.430</td>
<td>p = 0.010**</td>
</tr>
</tbody>
</table>

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

There was a significant association in the change in FMD over the four hours with an increase in RCF and improvement in vitamin B6 status (pyridoxine phosphate activation) and was independent of change in tHcy, blood glucose, HbA1c or Hs-CRP (Table 15).

**Table 15. Correlates of Change in FMD with Change in Other Variables**

<table>
<thead>
<tr>
<th>Changing variable</th>
<th>Correlation coefficient</th>
<th>Significance (2 tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>r = 0.015</td>
<td>p = 0.931</td>
</tr>
<tr>
<td>Red cell Folate (µg/l)</td>
<td>r = 0.423</td>
<td>p = 0.011*</td>
</tr>
<tr>
<td>Serum Folate (µg/l)</td>
<td>r = 0.257</td>
<td>p = 0.178</td>
</tr>
<tr>
<td>Pyridoxine phosphate % activation (vitamin B6)</td>
<td>r = 0.350</td>
<td>p = 0.049*</td>
</tr>
<tr>
<td>tHcy (µmol/l)</td>
<td>r = -0.248</td>
<td>p = 0.150</td>
</tr>
<tr>
<td>Hs-CRP (mg/l)</td>
<td>r = 0.075</td>
<td>p = 0.673</td>
</tr>
</tbody>
</table>

* Correlation is significant at the 0.05 level (2-tailed).
**Discussion**

This study of the immediate effects of folate and vitamin B6 on endothelial function in children with type 1 diabetes showed that both single high dose folate and single high dose vitamin B6 normalise FMD within four hours.

Improvement in endothelial function was observed within two hours in subjects receiving 5 mg oral folate and in subjects receiving 100 mg oral vitamin B6. FMD was normalised in these subjects at two hours and this effect was maintained at four hours. There was no further significant change in FMD between two and four hours.

In all subjects receiving folate, FMD increased to above the 10\textsuperscript{th} centile of our previously determined control range (Wiltshire, Gent *et al.* 2002). In nine of the fourteen subjects, FMD improved to above the mean, and in five of the fourteen subjects receiving folate, FMD increased to above the 90\textsuperscript{th} centile of our control range. In all subjects receiving vitamin B6, FMD increased above the 10\textsuperscript{th} centile of our control range, in five of the twelve subjects receiving vitamin B6, FMD improved above the mean.

Levels of serum folate were increased to above the normal range in all subjects receiving folate and to supra-physiological levels in seven of the fourteen of the children randomised to folate within two hours and remained elevated thereafter. In one subject in the vitamin B6 group the serum folate also rose to above physiological levels, this subject was allocated to the folate/vitamin B6 group and we were suspicious that the tablet for the effects immediate study had been taken from the wrong bottle. Removal of this subject from analysis did not alter the final result and as we could not confirm our suspicions, we did not exclude this subject from the analysis. As with the serum folate, RCF increased significantly at two hours in the folate but not in the placebo or the vitamin B6 groups. This change in folate status is a reflection of the high dose of folate given. The folate levels observed in this study cannot be achieved by dietary fortification with folate or low dose (0.4 mg) folate supplementation (Doshi, McDowell *et al.* 2002).

Baseline FMD did not correlate with either baseline serum folate or baseline RCF. Change in FMD related to increase in RCF but not to increase in serum folate. This is the first study to show normalisation of FMD with administration of folate in children with type 1 diabetes. Our results showing the immediate improvement in FMD with folate
administration are supported by previous studies that have also observed an improvement in endothelial function within hours of additional folate (Verhaar, Wever et al. 1998; Doshi, McDowell et al. 2002; van Etten, de Koning et al. 2002), before any significant reduction in homocyst(e)ine.

It is likely that the majority of improvement in endothelial function observed in this study is due to direct pharmacological actions of folate rather than reductions in tHcy. As discussed later, the reduction in tHcy was not associated with improvement in FMD. Mechanisms exist to explain how folate may acutely enhance endothelial function independent of homocysteine. Under normal conditions, the majority (>80 %) of circulating folate exists as the metabolically active form, 5-MTHF with the enzyme dihydrofolate reductase converting folate to 5-MTHF. In vitro, 5-MTHF has intrinsic antioxidant actions, can increase nitric oxide production by endothelial nitric oxide synthase, and can reduce superoxide production by endothelial nitric oxide synthase in the setting of reduced cofactor tetrahydrobiopterin (BH₄) bioavailability. In vivo, the intra-arterial infusion of 5-MTHF will acutely improve endothelial function in familial hypercholesterolaemia (Verhaar, Wever et al. 1998), CAD (Doshi, McDowell et al. 2001) and type 2 diabetes (van Etten, de Koning et al. 2002) without lowering homocysteine.

Improvement in FMD correlated with baseline vitamin B6 and the improvement in vitamin B6 status. This is the first study demonstrating the immediate effect of vitamin B6 on endothelial function. The mechanism of action is unclear. To date, there is only one published work determining the effects of vitamin B6 on the endothelium, this showed an improvement in endothelial function with vitamin B6 over ten weeks in cardiac transplant subjects (Miner, Cole et al. 2001).

tHcy decreased in all three groups over the four hours. This reduction was significant in all groups: folate, vitamin B6 and placebo. Despite the decrease in tHcy, this was not associated with the change in the endothelial function that was observed. This observation strengthens the hypothesis that improvement in endothelial function was independent of tHcy in the acute phase. No correlation was found between FMD improvement and tHcy reduction at any time point.

As tHcy decreased in all groups, it is unlikely that metabolism of tHcy by folate and vitamin B6 were the sole mechanisms responsible for the reduction in tHcy seen. Other mechanisms therefore may explain the fall in tHcy over the four hours. A decrease in
tHcy has been documented in similar study of the acute effect of folate on endothelial function and was thought to be due to the reduction in plasma albumin induced by the supine posture (Doshi, McDowell et al. 2002). The posture of a person contributes significantly to the variation of within-person tHcy, with the horizontal position reducing tHcy levels. In this study, subjects were required to remain recumbent, as much as was physically possible, during the study period so that factors affecting FMD remained constant. By doing this we may have affected tHcy. The use of a tourniquet has not been shown to have a noticeable effect on tHcy levels (Rasmussen, Moller et al. 1999; Thirup and Ekelund 1999). A tourniquet was used to collect samples and its use should not have altered tHcy levels. Another factor contributing to the fall in tHcy over the study period may have been insulin administration with postprandial activation of cystathionine-\(\beta\)-synthase increasing homocysteine degradation. Consumption of tea and coffee can increase tHcy concentrations by up to 20% and a high-protein meal also increases tHcy transiently. The breakfast was low protein and caffeine free in order to avoid increases in tHcy.

In the 35 subjects, insulin dose (U/kg) was associated with improvement in FMD. In this study, unlike the eight week effects study described in the next chapter, FMD was studied after administration of subcutaneous insulin. Higher insulin dose (U/kg) was associated with a greater improvement in FMD, independently of treatment. Acute administration of insulin causes vasodilatation by increasing endothelium derived nitric oxide (Steinberg, Brechtel et al. 1994). Therefore this improvement in FMD may be a direct effect of a larger dose of insulin administered with breakfast.

Improvement in FMD was independent of blood pressure, glucose, HbA1c or Hs-CRP.

Blood glucose levels trended higher at two hours in all groups and decreased slightly in the placebo and vitamin B6 groups at four hours. The changes in glucose were not significant. The change in glucose at two hours is likely to be the postprandial rise in glucose following the low-protein, low-fat, carbohydrate based breakfast which followed the baseline assessment and demonstrates insulin mismatching. Insulin doses were not adjusted for the purpose of this study and subjects were advised to give their normal morning subcutaneous insulin with this meal. The finding that blood glucose did not correlate with either baseline FMD or change in FMD is consistent with our previous work (Wiltshire, Gent et al. 2002; Pena, Wiltshire et al. 2004). In contrast to this, in otherwise healthy subjects rather than subjects with type 1 diabetes, acute hyperglycaemia has
been shown to impair endothelium-dependent vasodilatation (Williams, Goldfine et al. 1998).

Chronic hyperglycaemia has been shown to impair endothelial function, with endothelial function lower in subjects with Insulin Dependent Diabetes Mellitus compared with healthy controls and lower in subjects with poorer glycaemic control (Makimattila, Virkamaki et al. 1996). Baseline FMD did not correlate with HbA1c in this study. This may be a reflection of the shorter duration of diabetes in our study group as our subjects were children and teenagers. However, the finding that HbA1c did not correlate with FMD, is consistent with our previous work (Wiltshire, Gent et al. 2002; Pena, Wiltshire et al. 2004).

Baseline FMD did correlate with baseline Hs-CRP in this study, however this association was not seen in the larger cohort that participated in the eight week study described in the following chapter. There was no significant change in Hs-CRP and change in Hs-CRP did not correlate with change in FMD. Hs-CRP is discussed fully in Chapter 9, Hs-CRP.
Summary

Baseline endothelial function was impaired in this group of children and adolescents with type 1 diabetes. Both high dose folate and high dose vitamin B6 normalised endothelial function at two hours, an effect that was maintained at four hours. This improvement in FMD was associated with the increase in RCF but not to baseline serum folate, baseline RCF or to the increase in serum folate. Improvement in FMD also correlated with baseline vitamin B6 and the improvement in vitamin B6 status. Improvement in FMD was independent of tHcy and Hs-CRP in this study.

This supports our initial hypotheses:

- Endothelial function is impaired in children with type 1 diabetes
- Folate improves endothelial function acutely
- Vitamin B6 improves endothelial function acutely
- Supplemental folate improves endothelial function in children with type 1 diabetes mellitus, independent of lowering tHcy.

The hypotheses that endothelial function is related to folate status and that high sensitivity C-reactive protein relates to endothelial function in children with type 1 diabetes were not supported by this study.
Chapter 6. The four and eight week effect of folate and vitamin B6 on endothelial function in children and adolescents with type 1 diabetes

Introduction

This chapter presents the results of the four and eight week study, to determine the effects of folate and vitamin B6 on endothelial function in children and adolescents with type 1 diabetes over this longer term. This chapter expands on the results described in the publication in ‘Pediatrics’ presented in Chapter 4 and contains the extended statistical data analysis.

Background

As presented in Chapter 5, work from our unit has examined folate status, homocyst(e)ine levels and endothelial function in children with type 1 diabetes.

Despite normal folate status and low tHcy levels children with type 1 diabetes have significant endothelial dysfunction (Wiltshire, Gent et al. 2002). Folate status was found to be an important determinant of endothelial function in children with type 1 diabetes (Wiltshire, Gent et al. 2002). A pilot intervention trial, in the form of a randomised, double-blind, placebo-controlled cross-over trial, showed that despite normal folate status and low tHcy levels an improvement in endothelial function occurred with high dose supplemental folate in children and adolescents with type 1 diabetes (Pena, Wiltshire et al. 2004).

Folate and vitamin B6 in combination have also been shown to improve biological markers of endothelial function (Constans, Blann et al. 1999). Vitamin B6 alone improves endothelial function over ten weeks (Miner, Cole et al. 2001).

We sought to confirm and extend the findings of the pilot study and examine the effects of both folate and vitamin B6, alone and in combination, on endothelial function in children with type 1 diabetes over eight weeks with assessments performed at four and eight weeks.

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Hypotheses

The following hypotheses were generated. These are discussed fully in chapter 1.

1. Endothelial function is impaired in children with type 1 diabetes.
2. Endothelial function is related to folate status.
3. Subjects with higher red blood cell folate at baseline have less response of the endothelium to supplementary folate.
4. Supplemental folate improves endothelial function in children with type 1 diabetes mellitus, independent of lowering tHcy.
5. Folate improves and maintains the improvement endothelial function over an eight week period.
6. Vitamin B6 improves and maintains the improvement endothelial function over an eight week period.
7. Vitamin B6 is additive to the effect of folate on endothelial function in children with type 1 diabetes.
8. High sensitivity C-reactive protein relates to endothelial function in children with type 1 diabetes.

Aims

1. To investigate the effect of folate and vitamin B6 alone and in combination on endothelial function in children with type 1 diabetes over eight weeks.
2. To determine if there is an additive effect of vitamin B6 on the effect of folate on endothelial function.
3. To determine whether high sensitivity C-reactive protein relates to endothelial function in children with type 1 diabetes.
Outcome Measures

Primary

The main outcome measures of the study were endothelial function and smooth muscle function measured by function FMD, GTN- induced dilatation and the ratio FMD: GTN.

Secondary

The secondary outcome measures were the change in folate status, vitamin B6 status, tHcy and Hs-CRP.

Study Design

124 subjects with type 1 diabetes were studied at baseline, four and eight weeks to determine the effects of folate and/or vitamin B6 on endothelial function.

The study had a randomised, double-blind, placebo controlled design. The four study groups received:

(i) folate 5 mg (Sigma Pharmaceuticals) and matched placebo (Sigma Pharmaceuticals),
(ii) vitamin B6 100 mg (Rhone-Poulenc-Rorer Pharmaceuticals) and matched placebo,
(iii) folate 5 mg and vitamin B6 100 mg and
(iv) two placebo tablets.

After recruitment, subjects were assigned a unique identifying number. Randomisation, using this identifying number, was then performed using a Fischer table by the Pharmacy Department, WCH as described in Chapter 5. According to randomisation, subjects were allocated to one of the four study groups above to receive two tablets daily for eight weeks. The tablets were provided in two identical bottles labeled ‘A’ or ‘B’ by the Pharmacy Department, WCH. The two bottles were supplied in a paper bag labeled only
with the subjects’ unique identifying number. Subjects were instructed to take one tablet from each bottle daily and to take both tablets at the same time each day, preferably in the morning. Subjects were also instructed not to take any additional vitamin supplements during the study period.

Subjects underwent three morning assessments (0730-0830hrs): baseline, four and eight weeks. At each assessment, subjects were fasted and had withheld morning insulin. Subjects had also been advised to not take the study tablets prior to the morning assessments. Clinical data were collected, brachial artery response to FMD and GTN were assessed and venous blood collected at each assessment. Blood was collected at baseline for vitamin B12, serum cotinine and lipids. Blood was collected at each assessment for glucose, HbA1c, high sensitivity C-reactive protein (Hs-CRP), red cell folate (RCF), serum folate, vitamin B6 status and total plasma homocyst(e)ine (tHcy).

Subjects

Subjects with type 1 diabetes were recruited from the diabetes clinic at Women’s and Children’s Hospital (WCH) Adelaide, as described in the methods section. 124 subjects participated.

Ethical Approval

As discussed in the Methods chapter, Chapter 3, the study was approved by the Women’s and Children’s Hospitals’ Human Research Ethics Committee and the Drug and Therapeutic Committee. The study was notified under the Clinical Trials Notification Scheme [(CTN) scheme] under the Therapeutic Goods Act 1989.
Study Protocol and Laboratory Methods

Study Protocol

FMD was measured at baseline, four and eight weeks. Biochemical parameters were also measured at each assessment (Table 16).

Table 16. Study Protocol for the Eight Week Study

<table>
<thead>
<tr>
<th></th>
<th>Baseline*</th>
<th>4 weeks</th>
<th>8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum cotinine</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting lipids</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Glucose</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Serum Folate and RCF</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>(pyridoxine phosphate %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>activation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Vitamin B12</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tHcy</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Flow Mediated dilatation</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>GTN-induced dilatation</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Hs-CRP</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

*as performed at baseline in immediate effects study.

Biochemical Parameters

Venous blood samples were collected at baseline for vitamin B12, serum cotinine and lipids after an overnight fast and before administration of insulin. Blood was obtained after each ultrasound measurement for glucose, serum and red cell folate (RCF), red cell transaminase pyridoxine phosphate activation, total plasma homocyst(e)ine (tHcy), and
high sensitivity C-reactive protein (Hs-CRP) (Table 16). Lipids, blood glucose, HbA1c, serum and red cell folate, vitamin B12, and Hs-CRP were analysed on the day of sampling, other samples were separated and stored until analysis.

**Biochemical Assays**

Biochemistry including lipids, glucose, serum and red cell folate, vitamin B12, red cell transaminase pyridoxine phosphate activation, tHcy, Hs-CRP and serum cotinine were measured using methods described in the methods chapter.

**Non-invasive Measure of Endothelial Function**

Endothelial function was assessed by flow mediated dilatation (FMD) and glycercyl trinitrate (GTN)-induced dilatation using high resolution ultrasound and vessel wall tracking as described in the methods chapter.

The previously determined control subjects were twenty age-matched healthy subjects and studied under identical conditions, using the same equipment and the studies were performed by the same sonographer and read by the same blinded observer (Wiltshire, Gent *et al.* 2002).
Statistics

The data was analysed using SPSS software version 10.0.7.

Comparisons of clinical characteristics, biochemical parameters and endothelial function at baseline across the four treatment groups were made using one-way analysis of variances (ANOVA). The linear association of FMD at baseline with other variables of interest at baseline were assessed using Spearman's rank correlations.

Multivariable ANOVA models for FMD and GTN-induced dilatation at baseline were developed to suggest the predictors with the strongest influence on FMD and GTN-induced dilatation at baseline. A variable given as ‘variable (ln)’ indicates that a natural log transformation was applied. Variables with a p-value < 0.1 for their association with FMD or GTN-induced dilatation were initially included in each multivariable ANOVA. For FMD, vessel diameter (VD), age (ln), total cholesterol, LDL-cholesterol, triglycerides (ln) and sex were included in the model, for GTN-induced dilatation, VD, age (ln), insulin/kg, BMI z-score, systolic blood pressure (ln), HDL-cholesterol (ln) and sex were included in the model. Natural log transformations were applied to some variables so satisfy the assumption of normality. A backwards selection method was used to obtain a final model for FMD and GTN-induced dilatation. In a backwards selection method all variables are initially entered into the model as predictors then the least significant variable is removed and the model is recalculated. The removal of the least significant predictor is repeated until all remaining predictors in the model have a p-value < 0.1, this is the final model.

In the final multivariable ANOVA model for FMD and GTN-induced dilatation, β (Greek letter beta) indicates the standardized correlation between a predictor and the outcome (FMD or GTN-induced dilatation) and has been adjusted to control for the influence of the other predictors in the model. R² indicates the proportion of variability in the outcome (FMD or GTN-induced dilatation) that can be explained by the model.

An independent samples t-test was used to compare baseline FMD and GTN-induced dilatation between sexes.

The magnitude of the change in each variable of interest over time at baseline, four weeks and eight weeks separately within the placebo, folate, vitamin B6 and combination of folate & vitamin B6 treatment groups were tested using linear mixed models. A linear
mixed model can account for the within subject correlations (subject effect) from repeated measurements on the same individual over the three periods. The linear mixed model used repeated contrasts to test the change in the expected mean of variables between successive time periods.

The distribution of Hs-CRP showed a strong right skew. The skewed distribution may influence the results of the Hs-CRP analysis by overestimating the central location of Hs-CRP. Nonparametric tests were used for the uni-variable analysis of Hs-CRP. A $\log_{10}$ transformation was applied to the Hs-CRP data and the $\log_{10}$ transformed data was used in the linear mixed models of Hs-CRP. A $\log_{10}$ transformation is consistent with previous studies (Kilpatrick, Keevil et al. 2000).

The change from baseline to eight weeks for each variable of interest was calculated for each individual with measurements at baseline and at eight weeks. The linear association of the change in FMD from baseline to eight weeks with other variables of interest at baseline and their change from baseline to eight weeks were assessed using Spearman’s rank correlations.

Pair-wise comparisons of FMD between treatment groups at week eight were made using independent samples t-tests.

Comparisons of individual adherence across the four treatment groups were made using one-way ANOVA.
Results

Subjects

123 subjects with type 1 diabetes entered the study.  122 subjects completed the eight week protocol with no adverse effects.  There was one elective withdrawal after the four week assessment.

Baseline Characteristics

Baseline clinical characteristics of the subjects are shown in Table 17, baseline biochemical characteristics are shown in Table 18.

On baseline testing, nine subjects were coeliac antibody positive and five were later confirmed to have coeliac disease by small bowel biopsy.  One subject was diagnosed with Grave’s disease.  Two subjects reported recent infection and had elevated Hs-CRP at baseline only.  These subjects were included in the analysis of this study, as exclusion did not alter the final results.

None gave a history of smoking however five subjects had an elevated serum cotinine (>28 nmol/l) at baseline.  Two subjects whose levels were consistent with passive smoking (<115 nmol/l) were randomised to the placebo group.  There were three subjects with cotinine levels within the smoking range (115-5700 nmol/l): 131, 216 and 392 nmol/l.  One subject was randomised to the placebo group, one to the vitamin B6 group and the subject with the highest baseline cotinine of 392 nmol/l was randomised to the folate group.

There were no significant differences in baseline clinical characteristics between the groups.  Results are expressed as the mean (with standard deviation).
The placebo group was younger and had a shorter duration of diabetes, however the differences in age (p= 0.72) and duration of diabetes (p= 0.19) were not significant between the treatment groups.

Table 17. Baseline Characteristics, Eight Week Study

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Folate</th>
<th>Vitamin B6</th>
<th>Folate/ B6</th>
<th>Significance (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>31</td>
<td>31</td>
<td>31</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>13.6 (2.81)</td>
<td>14.3 (2.62)</td>
<td>14.0 (2.65)</td>
<td>14.3 (3.44)</td>
<td>0.718</td>
</tr>
<tr>
<td>Age Range (years)</td>
<td>8.1-18.9</td>
<td>8.5-18.2</td>
<td>9.7-19.3</td>
<td>8.2-18.6</td>
<td></td>
</tr>
<tr>
<td>Sex M:F</td>
<td>18:14</td>
<td>23:8</td>
<td>16:15</td>
<td>14:16</td>
<td>0.132*</td>
</tr>
<tr>
<td>Insulin dose (Units/kg)</td>
<td>1.07 (0.366)</td>
<td>1.22 (0.320)</td>
<td>1.22 (0.351)</td>
<td>1.12 (0.379)</td>
<td>0.113</td>
</tr>
<tr>
<td>Duration of diabetes (yrs)</td>
<td>4.2 (2.84)</td>
<td>5.8 (3.65)</td>
<td>5.5 (4.46)</td>
<td>5.9 (4.06)</td>
<td>0.189</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.8 (1.19)</td>
<td>8.5 (1.27)</td>
<td>8.7 (1.45)</td>
<td>8.8 (1.47)</td>
<td>0.774</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>159.3(13.21)</td>
<td>164.1(14.96)</td>
<td>163.6(13.06)</td>
<td>160.2(17.50)</td>
<td>0.486</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>55.9 (15.47)</td>
<td>59.9 (16.07)</td>
<td>60.8 (20.67)</td>
<td>58.1 (18.48)</td>
<td>0.707</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.6 (3.46)</td>
<td>21.9 (3.42)</td>
<td>22.2 (5.09)</td>
<td>21.9 (3.95)</td>
<td>0.946</td>
</tr>
<tr>
<td>Systolic blood pressure(mmHg)</td>
<td>120.2(15.80)</td>
<td>117.1(15.50)</td>
<td>118.0(16.08)</td>
<td>118.0(15.57)</td>
<td>0.882</td>
</tr>
<tr>
<td>Diastolic blood pressure(mmHg)</td>
<td>65.2 (8.64)</td>
<td>63.7 (7.66)</td>
<td>61.8 (8.48)</td>
<td>62.1 (7.76)</td>
<td>0.338</td>
</tr>
</tbody>
</table>

* Chi-square test used for sex. One-way ANOVA used for other comparisons.
Baseline Biochemical Parameters

All subjects had normal folate status as determined by serum and red cell folate. Four subjects were deficient in vitamin B6 [median 71.4 (63.8 - 93.2) %, normal range <63%]. There was a significant difference in baseline blood glucose between the groups (p=0.04) with the folate/B6 group having the highest blood glucose at baseline. Baseline biochemical parameters are shown in Table 18.

Table 18. Baseline Biochemical Parameters, Eight Week Study

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Folate</th>
<th>Vitamin B6</th>
<th>Folate/B6</th>
<th>Significance (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>31</td>
<td>31</td>
<td>31</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td><strong>Total Cholesterol (mmol/l)</strong></td>
<td>4.14 (0.730)</td>
<td>4.43 (1.225)</td>
<td>4.52 (0.730)</td>
<td>4.36 (0.750)</td>
<td>0.396</td>
</tr>
<tr>
<td><strong>LDL Cholesterol (mmol/l)</strong></td>
<td>2.40 (0.594)</td>
<td>2.25 (0.720)</td>
<td>2.71 (0.624)</td>
<td>2.53 (0.682)</td>
<td>0.055</td>
</tr>
<tr>
<td><strong>Triglycerides (mmol/l)</strong></td>
<td>0.42 (0.273)</td>
<td>0.60 (0.491)</td>
<td>0.53 (0.447)</td>
<td>0.53 (0.280)</td>
<td>0.406</td>
</tr>
<tr>
<td><strong>Glucose (mmol/l)</strong></td>
<td>13.8 (4.48)</td>
<td>12.3 (5.41)</td>
<td>12.5 (4.36)</td>
<td>15.4 (3.97)</td>
<td>0.039*</td>
</tr>
<tr>
<td><strong>Vitamin B12 (pmol/l)</strong></td>
<td>396.6 (192.60)</td>
<td>345.3 (144.21)</td>
<td>352.0 (150.21)</td>
<td>332.9 (118.34)</td>
<td>0.396</td>
</tr>
<tr>
<td><strong>Red cell folate (µg/l)</strong></td>
<td>505.7 (141.88)</td>
<td>456.0 (129.10)</td>
<td>473.5 (158.92)</td>
<td>470.1 (130.63)</td>
<td>0.553</td>
</tr>
<tr>
<td><strong>Serum folate (µg/l)</strong></td>
<td>13.3 (2.91)</td>
<td>12.3 (4.40)</td>
<td>12.8 (3.11)</td>
<td>12.5 (2.67)</td>
<td>0.661</td>
</tr>
<tr>
<td><strong>Pyridoxine phosphate % activation (B6)</strong></td>
<td>48.8 (9.24)</td>
<td>46.3 (8.16)</td>
<td>48.3 (6.87)</td>
<td>48.3 (7.97)</td>
<td>0.626</td>
</tr>
<tr>
<td><strong>tHcy (µmol/l)</strong></td>
<td>6.1 (1.28)</td>
<td>6.5 (1.55)</td>
<td>6.5 (1.88)</td>
<td>6.2 (1.61)</td>
<td>0.554</td>
</tr>
<tr>
<td><strong>Hs-CRP (mg/l)</strong> †</td>
<td>0.65 (0.25-2.8)</td>
<td>0.64 (0.25 -20)</td>
<td>0.57 (0.25-3.7)</td>
<td>0.94 (0.25 -18)</td>
<td>0.343</td>
</tr>
</tbody>
</table>

* The mean difference is significant at the 0.05 level
† Geometric mean and range shown. Kruskal-Wallis test used for comparison.
Baseline Endothelial Function

There was a significant difference in baseline FMD between the groups. There were no significant differences in baseline GTN-induced dilatation and FMD: GTN between the groups. Results of the baseline FMD, GTN-induced dilatation and the FMD: GTN ratio are shown in Table 19.

Table 19. Baseline Endothelial Function, Eight Week Study

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Folate</th>
<th>Vitamin B6</th>
<th>Folate/B6</th>
<th>Significance (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>31</td>
<td>31</td>
<td>31</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>FMD (%)</td>
<td>6.16 (4.104)</td>
<td>2.6 (4.27)</td>
<td>3.5 (4.02)</td>
<td>2.8 (3.45)</td>
<td>0.001</td>
</tr>
<tr>
<td>GTN-induced dilatation (%)</td>
<td>23.8 (7.64)</td>
<td>20.6 (7.50)</td>
<td>20.6 (6.76)</td>
<td>20.5 (4.99)</td>
<td>0.117</td>
</tr>
<tr>
<td>FMD:GTN ratio</td>
<td>0.27 (0.211)</td>
<td>0.1 (0.762)</td>
<td>0.15 (0.244)</td>
<td>0.14 (0.156)</td>
<td>0.072</td>
</tr>
</tbody>
</table>

The difference in baseline FMD occurred between the folate and placebo groups, the vitamin B6 and placebo groups and the folate/B6 and placebo groups. The folate group had a lower baseline FMD and the placebo group had a higher baseline FMD. The mean difference (standard error) in baseline FMD between folate and placebo was -3.630 (1.000) % (p< 0.001), between the vitamin B6 and placebo groups was -2.731 (1.000) % (p= 0.007), between the folate/B6 and placebo groups was -3.436 (1.008) % (p= 0.001).

There was no difference in baseline FMD between the vitamin B6 and folate/B6 groups (p= 0.49), between the folate and vitamin B6 groups (p= 0.37) and between the folate and folate/B6 groups (p= 0.85).
Correlates of Baseline Endothelial Function

Baseline FMD correlated with baseline vessel diameter, triglycerides and LDL-cholesterol. Baseline FMD did not correlate with HbA1c, blood glucose, tHcy or Hs-CRP. Baseline GTN-induced dilatation correlated with baseline vessel diameter, age, insulin dose, systolic blood pressure, BMI Z-score and HDL-cholesterol. Baseline GTN-induced dilatation did not correlate with HbA1c, tHcy or Hs-CRP (Table 20).

Table 20. Correlates of Baseline FMD and Baseline GTN-Induced Dilatation.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline FMD</th>
<th>Baseline GTN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>Baseline FMD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline GTN</td>
<td>0.352</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Baseline Vessel Diameter</td>
<td>-0.328</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Age (years)</td>
<td>-0.158</td>
<td>0.079</td>
</tr>
<tr>
<td>Age at diagnosis (years)</td>
<td>0.025</td>
<td>0.785</td>
</tr>
<tr>
<td>Insulin / kg</td>
<td>-0.102</td>
<td>0.261</td>
</tr>
<tr>
<td>BMI z-score</td>
<td>0.023</td>
<td>0.803</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>-0.130</td>
<td>0.165</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>0.101</td>
<td>0.282</td>
</tr>
<tr>
<td>Hs-CRP (mg/l)</td>
<td>0.061</td>
<td>0.506</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>-0.173</td>
<td>0.058</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>0.131</td>
<td>0.155</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>-0.181</td>
<td>0.050*</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>-0.250</td>
<td>0.007**</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>0.019</td>
<td>0.832</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>0.032</td>
<td>0.724</td>
</tr>
<tr>
<td>Red Cell Folate (µg/l)</td>
<td>-0.009</td>
<td>0.923</td>
</tr>
<tr>
<td>Serum Folate (µg/l)</td>
<td>0.027</td>
<td>0.771</td>
</tr>
<tr>
<td>Pyridoxine phosphate % activation (Vitamin B6)</td>
<td>0.032</td>
<td>0.727</td>
</tr>
<tr>
<td>tHcy ((µmol/l)</td>
<td>-0.151</td>
<td>0.096</td>
</tr>
</tbody>
</table>

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).
The main influences on baseline FMD were baseline vessel diameter and LDL-cholesterol (Table 21).

**Table 21. Final multivariable ANOVA for FMD**

<table>
<thead>
<tr>
<th></th>
<th>β</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline Vessel Diameter</td>
<td>-0.391</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>-0.205</td>
<td>0.017</td>
</tr>
</tbody>
</table>

R² = 0.181

The main influences on baseline GTN-induced dilatation were baseline vessel diameter, systolic blood pressure and insulin dose (U/kg) (Table 22).

**Table 22. Final multivariable ANOVA for GTN-Induced Dilatation.**

<table>
<thead>
<tr>
<th></th>
<th>β</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline Vessel Diameter</td>
<td>-0.518</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>-0.194</td>
<td>0.015</td>
</tr>
<tr>
<td>Insulin dose (Units/kg)</td>
<td>-0.156</td>
<td>0.031</td>
</tr>
</tbody>
</table>

R² = 0.448

**Baseline Endothelial Function and Sex**

An independent samples t-test was used to compare baseline FMD and GTN-induced dilatation between sexes. There was a significant gender difference in both GTN-induced dilatation and FMD (Table 23.).

**Table 23. The relationship between Baseline Endothelial Function and Sex.**

<table>
<thead>
<tr>
<th></th>
<th>Baseline FMD</th>
<th>Baseline GTN-induced Dilatation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>p</td>
</tr>
<tr>
<td>Male</td>
<td>3.1 (4.2)</td>
<td>0.026</td>
</tr>
<tr>
<td>Female</td>
<td>4.8 (4.0)</td>
<td></td>
</tr>
</tbody>
</table>
Flow Mediated Dilatation (FMD)

The main outcome measure of the study was change in FMD. FMD increased in all but the placebo group. The change in FMD from baseline to eight weeks was significant in the folate group, vitamin B6 group and the folate/B6 group. There was no significant change in FMD in the placebo group (Table 24). The significant increase in FMD occurred in the first four weeks in the folate group (p< 0.001), vitamin B6 group (p< 0.001) and folate/B6 group (p< 0.001). There was no further significant increase in FMD at eight weeks (Figure 30).

Table 24. Change in Flow Mediated Dilatation over Eight Weeks

<table>
<thead>
<tr>
<th>FMD</th>
<th>Baseline</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>6.16 (4.104)</td>
<td>4.69 (3.373)</td>
<td>5.44 (4.237)</td>
<td>0.092</td>
</tr>
<tr>
<td>Folate</td>
<td>2.60 (4.272)</td>
<td>9.66 (5.996)</td>
<td>9.10 (5.775)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>3.50 (4.020)</td>
<td>8.31 (4.207)</td>
<td>9.64 (4.367)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Folate / B6</td>
<td>2.79 (3.453)</td>
<td>10.48 (4.391)</td>
<td>10.23 (4.439)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Figure 30. FMD over Eight Weeks.
GTN-Induced Dilatation

There was no significant change in GTN-induced dilatation over the eight weeks in any group (Table 25) (Figure 31). There was no significant change in GTN-induced dilatation from baseline to four weeks or from four to eight weeks in any group.

Table 25. Change in GTN-Induced Dilatation over Eight Weeks

<table>
<thead>
<tr>
<th>GTN</th>
<th>Baseline</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>23.82 (7.641)</td>
<td>21.74 (6.897)</td>
<td>22.93 (7.677)</td>
<td>0.096</td>
</tr>
<tr>
<td>Folate</td>
<td>20.65 (7.499)</td>
<td>22.39 (6.854)</td>
<td>19.88 (9.174)</td>
<td>0.168</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>20.627 (6.760)</td>
<td>21.50 (6.091)</td>
<td>20.83 (6.115)</td>
<td>0.605</td>
</tr>
<tr>
<td>Folate/ B6</td>
<td>20.51 (4.993)</td>
<td>22.48 (7.257)</td>
<td>21.89 (7.636)</td>
<td>0.201</td>
</tr>
</tbody>
</table>

Figure 31. GTN-Induced Dilatation over Eight Weeks.
FMD: GTN

There was a significant increase in FMD: GTN in all but the placebo group over the eight week study (Table 26). The significant increase in FMD: GTN occurred in the first four weeks in the folate (p= 0.007), vitamin B6 (p< 0.001) and folate/B6 (p< 0.001) groups. There was no further significant increase in FMD: GTN at eight weeks. There was no significant change in FMD: GTN in the placebo group at four weeks (p= 0.25) and eight weeks (p= 0.50) (Figure 32).

Table 26. Change in FMD: GTN over Eight Weeks

<table>
<thead>
<tr>
<th>FMD:GTN</th>
<th>Baseline</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>0.27 (0.211)</td>
<td>0.23 (0.212)</td>
<td>0.24 (0.170)</td>
<td>0.502</td>
</tr>
<tr>
<td>Folate</td>
<td>-0.01 (0.762)</td>
<td>0.43 (0.271)</td>
<td>0.64 (0.662)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>0.15 (0.244)</td>
<td>0.40 (0.202)</td>
<td>0.48 (0.252)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Folate / B6</td>
<td>0.14 (0.156)</td>
<td>0.47 (0.204)</td>
<td>0.49 (0.199)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Figure 32. FMD: GTN-Induced Dilatation over Eight Weeks.
Serum Folate and Red Cell Folate

There were no significant differences in baseline serum folate between the groups (Table 18). At four and eight weeks, there were eight subjects that had markedly elevated serum folate levels above 20 µg/l (normal range 3.3-15.0 µg/l). These subjects were in the folate or the combined folate and vitamin B6 (folate/B6) group. For the purposes of statistical analysis, serum folate levels above 20 µg/l were adjusted to the upper level of 20 µg/l. As expected, adjusted serum folate increased significantly in the folate (p< 0.001) and folate/B6 (p< 0.001) groups. In the vitamin B6 group the adjusted serum folate fell significantly (p< 0.001). There was no significant change in the placebo group (p= 0.24) (Figure 33).

Figure 33. Serum Folate over Eight Weeks
There were no significant differences in baseline red cell folate between the groups (Table 18). At eight weeks, there were two subjects in the folate group that had elevated RCF levels above 1000 µg/l (normal range >168 µg/l, 16-388µg/l). For the purposes of statistical analysis, RCF levels >1000 µg/l were adjusted to the upper level of 1000 µg/l. RCF rose significantly over the study period in the folate group (p< 0.001) and the folate/B6 (p< 0.001) group. RCF fell in the vitamin B6 group (p= 0.01). There was no significant change in RCF in the placebo group (p= 0.86) (Figure 34).

Figure 34. Red Cell Folate over Eight Weeks.
Vitamin B6: Pyridoxine Phosphate Activation

There was no significant difference in pyridoxine phosphate activation (vitamin B6 status) between the groups at baseline (Table 18). Pyridoxine phosphate activation (%) improved significantly in the vitamin B6 group (p= 0.01) and the folate/B6 group (p< 0.001) at eight weeks. The placebo group showed a significant improvement in pyridoxine phosphate activation (p= 0.04). There was no significant change in pyridoxine phosphate activation in the folate group (p= 0.33) (Figure 35).

Figure 35. Pyridoxine Phosphate Activation over Eight Weeks
Total Plasma Homocyst(e)ine (tHcy)

Baseline tHcy showed no significant differences between the groups (Table 18). tHcy fell significantly in all groups, i.e. in the folate (p< 0.001), vitamin B6 (p< 0.001), folate/B6 (p= 0.001) and placebo (p= 0.001) groups, over the study period (Figure 36).

Figure 36. tHcy over Eight Weeks
High Sensitivity C-Reactive Protein (Hs-CRP)

Analysis of Hs-CRP data is presented in Chapters 8 and 9.

Glucose

There was a significant difference in baseline glucose between the groups (Table 18) with the folate/B6 group having a significantly higher baseline blood glucose. There was no significant difference in blood glucose from baseline to eight weeks in the placebo (p=0.48), vitamin B6 (p=0.29) and folate (p=0.59) groups. There was a significant reduction in glucose in the folate/B6 group at eight weeks (p=0.011).

Figure 37. Glucose over Eight Weeks
HbA1c

There were no significant differences in baseline HbA1c between the groups (Table 17). There were no significant changes from baseline to eight weeks in HbA1c in any group (Figure 38).

Figure 38. HbA1c over Eight Weeks
Correlates of Change in Flow Mediated Dilatation

The improvement in FMD was related to the baseline variables; red cell folate, diastolic blood pressure and baseline flow mediated dilatation. Improvement in FMD was not related to age, duration of diabetes, baseline Hs-CRP (Table 27).

Table 27. Correlates of the Change in FMD to Baseline Variables, Eight Week Study.

<table>
<thead>
<tr>
<th>Baseline Variable</th>
<th>Correlation coefficient</th>
<th>Significance (2 tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>r = -0.084</td>
<td>p = 0.357</td>
</tr>
<tr>
<td>Insulin dose: Units/kg</td>
<td>r = -0.014</td>
<td>p = 0.881</td>
</tr>
<tr>
<td>Duration of Diabetes (yrs)</td>
<td>r = 0.166</td>
<td>p = 0.067</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>r = -0.041</td>
<td>p = 0.652</td>
</tr>
<tr>
<td>Blood pressure, systolic</td>
<td>r = -0.118</td>
<td>p = 0.212</td>
</tr>
<tr>
<td>Blood pressure, diastolic</td>
<td>r = -0.220</td>
<td>p = 0.019*</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>r = 0.154</td>
<td>p = 0.094</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/l)</td>
<td>r = -0.003</td>
<td>p = 0.977</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/l)</td>
<td>r = 0.147</td>
<td>p = 0.115</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>r = 0.122</td>
<td>p = 0.199</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>r = 0.008</td>
<td>p = 0.931</td>
</tr>
<tr>
<td>Red cell Folate (µg/l)</td>
<td>r = -0.183</td>
<td>p = 0.044*</td>
</tr>
<tr>
<td>Serum Folate (µg/l)</td>
<td>r = -0.038</td>
<td>p = 0.677</td>
</tr>
<tr>
<td>Pyridoxine phosphate % activation (Vitamin B6)</td>
<td>r = 0.046</td>
<td>p = 0.619</td>
</tr>
<tr>
<td>tHcy (µmol/l)</td>
<td>r = 0.157</td>
<td>p = 0.085</td>
</tr>
<tr>
<td>Hs-CRP (mg/l)</td>
<td>r = 0.70</td>
<td>p = 0.447</td>
</tr>
<tr>
<td>Baseline Flow Mediated Dilatation (%)</td>
<td>r = -0.497</td>
<td>p &lt; 0.001**</td>
</tr>
</tbody>
</table>

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).
Change in FMD related to increase in serum folate, increase in RCF and improvement in vitamin B6 status (pyridoxine phosphate activation). Change in FMD was independent of change in tHcy, blood glucose, HbA1c or Hs-CRP (Table 28).

**Table 28. Correlates for Change in FMD and Change in Other Variables, Eight Week Study.**

<table>
<thead>
<tr>
<th>Changing variable</th>
<th>Correlation coefficient</th>
<th>Significance (2 tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c (%)</td>
<td>$r = -0.120$</td>
<td>$p = 0.189$</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>$r = -0.079$</td>
<td>$p = 0.393$</td>
</tr>
<tr>
<td>Red Cell Folate ($\mu g/l$)</td>
<td>$r = -0.449$</td>
<td>$p = 0.000^{**}$</td>
</tr>
<tr>
<td>Serum Folate ($\mu g/l$)</td>
<td>$r = -0.270$</td>
<td>$p = 0.004^{**}$</td>
</tr>
<tr>
<td>Pyridoxine phosphate activation (%) (Vitamin B6)</td>
<td>$r = -0.235$</td>
<td>$p = 0.010^*$</td>
</tr>
<tr>
<td>tHcy ($\mu mol/l$)</td>
<td>$r = -0.135$</td>
<td>$p = 0.143$</td>
</tr>
<tr>
<td>Hs-CRP (mg/l)</td>
<td>$r = -0.136$</td>
<td>$p = 0.145$</td>
</tr>
</tbody>
</table>

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).
Additive Effect of Vitamin B6

The effect of the combined treatment with folate and vitamin B6 (folate/B6) over treatment with folate alone showed no significant difference in FMD at eight weeks. The mean difference in FMD at eight weeks between the folate group and the folate/B6 group was 0.715 % (p= 0.23) (Figure 39).

There was no significant difference in FMD at eight weeks between subjects receiving vitamin B6 and those receiving the combination of both folate and vitamin B6 (folate/B6) daily for eight weeks (p= 0.17). There was also no significant difference in FMD at eight weeks between subjects receiving folate and those receiving vitamin B6 daily for eight weeks (p= 0.96).
Adherence

The subjects were given two bottles labelled tablet A and tablet B and were required to take one tablet from each bottle each day. Individual adherence was calculated as number of tablets taken over the number of days in the study. Results are expressed as mean with standard deviation. There was no statistical difference between the bottles: tablet A, $p = 0.63$; tablet B, $p = 0.63$ (Table 29).

Table 29. Adherence

<table>
<thead>
<tr>
<th>Group</th>
<th>Tablet A</th>
<th>Tablet B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo and placebo</td>
<td>0.917 (0.109)</td>
<td>0.928 (0.115)</td>
</tr>
<tr>
<td>Folate and placebo</td>
<td>0.924 (0.133)</td>
<td>0.890 (0.170)</td>
</tr>
<tr>
<td>Vitamin B6 and placebo</td>
<td>0.883 (0.141)</td>
<td>0.887 (0.141)</td>
</tr>
<tr>
<td>Folate and vitamin B6</td>
<td>0.906 (0.107)</td>
<td>0.918 (0.108)</td>
</tr>
</tbody>
</table>
Discussion

This study of the eight week effect of folate and/or vitamin B6 on endothelial function in children with type 1 diabetes showed that high dose folate, high dose vitamin B6 and the combination of folate and vitamin B6 improved endothelial function at four weeks and this was maintained at eight weeks.

In all 31 subjects receiving folate, FMD increased to above the 10th centile of our previously determined healthy control range (Wiltshire, Gent et al. 2002). In 21 of the 31 subjects, FMD improved to above the mean, and in five of the 31 subjects receiving folate, FMD increased to above the 90th centile of our control range. In all subjects receiving vitamin B6, FMD increased above the 10th centile of our control range, in 22 of the 31 subjects receiving vitamin B6, FMD improved above the mean, and in two of the 31 subjects receiving vitamin B6, FMD increased to above the 90th centile of our control range. In all subjects receiving the combination folate/B6, FMD increased above the 10th centile of our control range, in 26 of the 30 subjects receiving folate/B6, FMD improved above the mean and in four of the 30 subjects receiving folate/B6, FMD increased to above the 90th centile of our control range.

Serum and red cell folate levels increased significantly from baseline in all subjects randomised to the folate group and the combination folate/B6 group. Red cell folate also increased significantly in the folate group and folate/B6 group. Interestingly, serum folate and RCF decreased significantly from baseline to eight weeks in the vitamin B6 group. Other reports have also shown a decrease in plasma folate in response to vitamin B6 supplementation in doses from 10 to 120 mg per day however the mechanism and biological significance of this interaction is unknown (Ubbink, van der Merwe et al. 1996; Bosy-Westphal, Holzapfel et al. 2001). There was no significant change in serum and RCF in the placebo group.

Baseline FMD did not correlate with either baseline serum folate or baseline RCF. However, change in FMD related to the increase in serum folate and to the increase in RCF. The dose of folate used in this study was pharmacological, with plasma levels far in excess of the normal range, and it cannot be assumed that the same effects demonstrated on endothelial function will occur with low dose (400 μg/ day) or improved dietary folate intake.
Vitamin B6 status improved from baseline in all subjects randomised to the vitamin B6 group and the folate/B6 group. Vitamin B6 status did not change in the placebo or the folate groups. Baseline FMD did not correlate with baseline vitamin B6 status, however, change in FMD correlated with an improvement in vitamin B6 status. As discussed in Chapter 5, there is only one other published work determining the effects of vitamin B6 on the endothelium, this showed an improvement in endothelial function with vitamin B6 over ten weeks in cardiac transplant subjects (Miner, Cole et al. 2001).

The metabolism of folate, vitamin B6 and homocysteine are interrelated. Therefore, most studies assessing the effect of folate and B vitamins on endothelial function have investigated this through lowering total plasma homocyst(e)ine (tHcy) levels. In this study, although tHcy decreased over the eight weeks and this reduction was significant in all groups, the improvement in endothelial function was independent of both baseline tHcy levels and of change in tHcy.

Our findings of an effect of folate and/or vitamin B6 on endothelial function, independent of tHcy, as well as the immediate effect, within hours of a single oral dose of folate and a single oral dose of vitamin B6, also independent of tHcy, presented in Chapter 5, provide evidence for a direct effect of folate and vitamin B6 on the endothelium. Potential mechanisms exist for the effect of folate on the endothelium and include interactions with the endothelial nitric oxide synthase. Mechanisms for the effect of vitamin B6 on the endothelium are uncertain however appear to be independent of tHcy.

Hyperglycaemia has been shown to contribute to endothelial dysfunction in diabetes (Makimattila, Virkamaki et al. 1996; Brownlee 2001). However in this study, neither blood glucose nor HbA1c correlated with either baseline FMD or change in FMD, findings that are consistent with our previous work (Wiltshire, Gent et al. 2002; Pena, Wiltshire et al. 2004).

HbA1c did not change over the study period. This is contrary to the pilot study during which HbA1c decreased over the study period (Pena, Wiltshire et al. 2004). Additional advice regarding diabetes management was not actively given to study participants and insulin doses and regimes were not adjusted for the purpose of this study.

Hs-CRP did not change over the study period. Hs-CRP did not correlate with either baseline FMD or change in FMD. Hs-CRP is discussed fully in Chapter 8, Hs CRP.
Total cholesterol, elevated LDL-cholesterol, low HDL-cholesterol and elevated triglycerides are well established risk factors for cardiovascular disease. The association between LDL cholesterol and triglycerides with baseline endothelial dysfunction was demonstrated in this study, however, lipids were not associated with the change endothelial function seen over this study.

One limitation of the study was that the placebo group had a significantly higher baseline FMD. The placebo group also had a younger mean age, a shorter duration of diabetes and used less insulin/ kg. These factors have been shown to relate to FMD in other studies however these correlations were not significant in this study. Biochemically, the placebo group had a lower serum triglyceride and higher baseline serum and red cell folate. Although there were no significant differences in these biochemical parameters between the placebo group and the other groups, triglycerides did correlate with baseline FMD. Individually these differences in these known influences on FMD were not significant, however the additive effect may have contributed to the higher baseline FMD seen in the placebo group. The study was double blinded and subjects were randomised upon entry into the study. We did not randomise at the outset of the study on the basis of FMD, GTN-induced dilatation or on the characteristics of the group. The randomisation was completed correctly with double blinding. Therefore, the variation seen in baseline between the groups is likely to be a result of this randomisation process and therefore the difference in the baseline FMD occurred by chance. There was no one measured variable that explained the higher FMD in the placebo group.

Another limitation of this study was that menstrual histories were not specifically obtained from the participating adolescent females. Hormonal variation during the menstrual cycle is known to affect endothelial function (Williams, Westerman et al. 2001). In this study, FMD was performed four weeks apart and although a menstrual history was not obtained, the studies are likely to have been performed at a similar phase of the cycle in girls who had regular ovulatory cycles.
Summary

This study of the four and eight week effects of folate and vitamin B6 on endothelial function in children with type 1 diabetes showed that both high dose folate and high dose vitamin B6 normalised flow mediated dilatation at four weeks, and this effect was maintained at eight weeks. There was no additional improvement in FMD with combination treatment with folate and vitamin B6.

The study follows on from the immediate effects study (Chapter 5) and the results of this study support three of the initial hypotheses:

- Supplemental folate improves endothelial function in children with type 1 diabetes mellitus, independent of lowering homocyst(e)ine,
- Folate maintains the improvement endothelial function over an eight week period and
- Vitamin B6 maintains the improvement endothelial function over an eight week period.

Two hypotheses were negated by this study:

- Vitamin B6 is additive the effect of folate on endothelial function in children with type 1 diabetes and
- High sensitivity C-reactive protein is a marker of vascular disease in children with type 1 diabetes
Chapter 7. Subjects who Participated in Both the Immediate and Eight Week Studies.

Introduction

35 subjects completed both the immediate effects study and the eight week study. Additional analysis of these 35 subjects’ results was performed. The results of FMD for the five assessments for each subject, i.e. the assessments of the immediate and the eight week study from these 35 subjects are combined and presented in this chapter. Although not set out in the original hypothesis, this chapter addresses two additional questions:

1. Does the early response to folate, at two and four hours, predict the on-going response at eight weeks?
2. Is the mechanism for the acute response similar to or, different from the response at eight weeks?

Results

Fourteen subjects had folate (5 mg) in the immediate effects study followed by folate (5 mg) and placebo for the eight week study. Five subjects had vitamin B6 (100 mg) in the immediate effects study followed by vitamin B6 (100 mg) and placebo for the eight week study. Seven subjects had vitamin B6 (100 mg) in the immediate study followed by vitamin B6 (100 mg) and folate (5 mg) for the eight week study. Nine subjects had a placebo for the immediate effects study followed by two placebo tablets for the eight week study. The results of the FMD [mean (SD)] of these groups are presented in Table 30.
### Table 30. FMD Mean (SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Baseline</th>
<th>2 hours</th>
<th>4 hours</th>
<th>4 weeks</th>
<th>8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate then Folate/ Placebo</td>
<td>14</td>
<td>1.9 (3.9)</td>
<td>10.5 (5.1)</td>
<td>10.1 (4.7)</td>
<td>6.6 (6.9)</td>
<td>8.1 (7.2)</td>
</tr>
<tr>
<td>Vitamin B6 then B6 &amp; Placebo</td>
<td>5</td>
<td>4.8 (5.1)</td>
<td>8.4 (3.6)</td>
<td>10.0 (5.7)</td>
<td>10.8 (3.7)</td>
<td>9.7 (5.1)</td>
</tr>
<tr>
<td>Vitamin B6 then Folate/B6</td>
<td>7</td>
<td>4.6 (3.6)</td>
<td>8.3 (5.0)</td>
<td>8.3 (4.6)</td>
<td>11.3 (1.3)</td>
<td>9.3 (4.0)</td>
</tr>
<tr>
<td>Placebo then Placebo/Placebo</td>
<td>9</td>
<td>6.2 (3.6)</td>
<td>7.1 (4.3)</td>
<td>5.9 (4.3)</td>
<td>3.0 (3.3)</td>
<td>5.4 (4.5)</td>
</tr>
</tbody>
</table>

Using spearman’s correlations, in the folate followed by folate/placebo group, there was a positive association between FMD at baseline and FMD at two hours ($r = 0.51$, $p = 0.06$), between FMD at two hours and FMD at four hours ($r = 0.64$, $p = 0.02$), between FMD at two hours and FMD at four weeks ($r = 0.55$, $p = 0.04$), between FMD at four hours and FMD at four weeks ($r = 0.53$, $p = 0.05$), between FMD at four hours and FMD at eight weeks ($r = 0.6$, $p = 0.02$) and between FMD at four weeks and FMD at eight weeks ($r = 0.52$, $p = 0.06$).

Although the same trend occurred for the groups of subjects receiving vitamin B6 then vitamin B6/ placebo and vitamin B6 then folate/vitamin B6, there were insufficient numbers in these groups to present meaningful data.

FMD of the individuals from each group are presented in Figures 40-43.
The Effect of Folate and Vitamin B6 on Endothelial Function in Children with Type 1 Diabetes
Dr Karen E MacKenzie

Figure 40. FMD. Folate followed by Folate/Placebo Group (n= 14)

Figure 41. FMD. Vitamin B6 followed by Vitamin B6/Placebo Group (n= 5).
Figure 42. FMD. Vitamin B6 followed by Vitamin B6/Folate Group (n= 7)

Figure 43. FMD. Placebo followed by Placebo/Placebo Group (n= 9)
Further statistical analysis was performed on these 35 subjects that were involved in both the immediate effects study and the eight week study, to examine the relationship between the early response to folate or vitamin B6, at two and four hours and the ongoing response at four and eight weeks. There was an association between the FMD response at two and four hours and the response at four and eight weeks.

The change in FMD from baseline was calculated at two and four hours and at four and eight weeks. Pearson’s correlations were used to measure the association between the change in FMD from baseline in the immediate effects study, i.e. at two and four hours, with the change in FMD from baseline in the eight week study, i.e. at four and eight weeks. No analysis within the four treatment groups was undertaken due to low sample sizes (Table 31).

**Table 31. Pearson’s correlations for the change in baseline FMD between the Immediate effects study and eight week study**

<table>
<thead>
<tr>
<th>Change in FMD from baseline to 2 hours</th>
<th>Change in FMD from baseline to 4 weeks</th>
<th>r = 0.4, p = 0.018</th>
<th>Change in FMD from baseline to 4 weeks</th>
<th>Change in FMD from baseline to 8 weeks</th>
<th>r = 0.17, p = 0.33</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in FMD from baseline to 4 hours</td>
<td>r = 0.47, p = 0.005</td>
<td>r = 0.44, p = 0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Graphs illustrating these associations are shown in Figures 44-49.
Figure 44. Correlation between the change in FMD at two hours with the change in FMD at four hours.

Figure 45. Correlation between the change in FMD at two hours with the change in FMD at four weeks.
Figure 46. Correlation between the change in FMD at two hours with the change in FMD at eight weeks.

Figure 47. Correlation between the change in FMD at four hours with the change in FMD at four weeks.
Figure 48. Correlation between the change in FMD at four hours with the change in FMD at eight weeks.

Figure 49. Correlation between the change in FMD at four weeks with the change in FMD at eight weeks.
There was a significant correlation between the change in FMD at two hours with the change in FMD at four weeks (p = 0.018) but there was no significant correlation between change in FMD at two hours with the change in FMD at eight weeks. There were significant correlations between the change in FMD at four hours with the change in FMD at four weeks (p = 0.005) and the change in FMD at eight weeks (p = 0.01) (Table 31).

These results suggest that a greater early endothelial response may be associated with a greater later response at four or eight weeks. Although the most likely explanation for this is the response to treatment with either folate or vitamin B6, the small sample size of 35 children does not give enough power to test the association between an immediate response and a later response within treatment groups.

Summary

The results of the FMD for the subjects who participated in both the immediate effects study and the eight week studies are presented. The early endothelial response to folate or vitamin B6, i.e. at two and four hours, appeared to predict response at eight weeks. As the early response was predictive of the eight week response, it would suggest similar mechanisms may underlie the early and late responses. Potential mechanisms are discussed in Chapter 6.
Chapter 8. Publication:

High sensitivity-CRP is associated with weight, BMI and female sex but not with endothelial function in children with type 1 diabetes.

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NOTE:
This publication is included on pages 213-220 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

Chapter 9. Hs CRP

Introduction

This chapter presents data related to high sensitivity CRP (Hs-CRP) not published in the manuscript in Paediatric Diabetes: ‘High Sensitivity CRP is associated with BMI, weight and female sex but not with endothelial function in children with type 1 diabetes’ (MacKenzie, Wiltshire et al. 2009) presented in Chapter 8.

This chapter contains the extended statistical analysis of data pertaining to Hs-CRP and includes the additional analysis of data required for the manuscript.

Background

Atherosclerosis is a chronic inflammatory process, with inflammation playing a major role in its development and progression. Slight elevations, within the normal range, of inflammatory markers such as C-reactive protein (CRP) are proving to be indicators of such inflammation. High-sensitivity CRP (Hs-CRP) is a strong marker of risk of coronary heart disease and has been used to predict the risk of cardiovascular events in large epidemiological studies (Ridker, Cushman et al. 1997; Koenig, Sund et al. 1999; Ridker, Rifai et al. 2002; Danesh, Wheeler et al. 2004). In addition to being a risk marker for vascular disease, there is some evidence suggesting that CRP may have a direct pro-atherogenic role (Pasceri, Willerson et al. 2000; Pasceri, Cheng et al. 2001; Zwaka, Hombach et al. 2001).

Elevated Hs-CRP is associated with impaired endothelial function in both healthy children and adults. In children and adults with T1DM, inflammatory markers including Hs-CRP are associated with serum markers of endothelial function. However, the relationship between endothelial function and Hs-CRP has not been studied in children with T1DM.
Because atherosclerosis begins in childhood and can be detected by flow mediated dilatation, we sought to determine whether Hs-CRP was associated with endothelial dysfunction in children and adolescents with type 1 diabetes.

In this part of the study, using cross-sectional data in a group of healthy control children, and both cross-sectional and longitudinal data in a large group of children with T1DM, we sought to determine the correlates of Hs-CRP. Specifically, we sought to determine whether Hs-CRP was associated with the early functional atherosclerotic vascular changes in endothelial and smooth muscle function measured by FMD and GTN-induced dilatation and therefore a marker of vascular disease in children with T1DM.

**Statistical Analysis**

Hs-CRP data had a large number of < 0.25 mg/l measurements, such measurements were considered to be 0.25 mg/l in the Hs-CRP analysis. The distribution of Hs-CRP showed a strong right skew. The skewed distribution may influence the results of the Hs-CRP analysis by overestimating the central location of Hs-CRP. Non-parametric tests were used for the uni-variable analysis of Hs-CRP. A log$_{10}$ transformation was applied to the Hs-CRP data and the log$_{10}$ transformed data was used in the over time analysis of Hs-CRP. A log$_{10}$ transformation is consistent with previous studies (Kilpatrick, Keevil *et al.* 2000).

Spearman’s rank correlations were used to test the linear association between two continuous variables. Comparisons of Hs-CRP at baseline across the treatment groups in the four hour study and the eight week study was made using Kruskal-Wallis tests. Comparison of Hs-CRP at baseline between sexes was made using a Mann-Whitney test.

The magnitude of the change in log$_{10}$ Hs-CRP over time for the four hour study and the eight week study separately within the each treatment groups were tested using linear mixed models. A linear mixed model can account for the within subject correlations (subject effect) from repeated Hs-CRP measurements on the same individual over the three periods. The linear mixed model used repeated contrasts to test the change in the expected mean of variables between successive time periods for both studies. That is,
mean change from baseline to two hours and mean change from two hours to four hours in the immediate effects study.

The change in Hs-CRP, FMD and GTN-induced dilatation from the start of the study to the end was calculated for each individual for the immediate effects study and eight week study. The linear association of the change in Hs-CRP over the study with the change in FMD and GTN-induced dilatation were assessed using Spearman’s rank correlations.

The subjects’ data was stratified into three groups, Hs-CRP < 0.25 mg/L, ≥ 0.25 to ≤ 0.7 mg/L and > 0.7 mg/L. This stratification was based on the paper by Järvisalo et al. (Jarvisalo, Harmoinen et al. 2002). Comparisons of the differences in continuous baseline variables across the three Hs-CRP groups were made using one-way analysis of variance (ANOVA). Comparisons of gender differences across the three Hs-CRP groups were made using Chi-squared analysis.

General linear models (GLM) were used to compare BMI and weight across the three Hs-CRP groups while controlling for insulin dose and sex.
Results

Four Hour Study

In the subjects (n= 35), attending for the four hour intervention with folate or vitamin B6, there was no significant difference in Hs-CRP between the groups at baseline (p= 0.29) (Table 3) and there was no significant change in Hs-CRP over four hours in any group (Table 32) (Figure 50).

Table 32. Change in High Sensitivity C-Reactive Protein

<table>
<thead>
<tr>
<th>Hs-CRP</th>
<th>Baseline</th>
<th>2 hours</th>
<th>4 hours</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>0.81 (0.25 - 2.8)</td>
<td>0.76 (0.25 - 2.6)</td>
<td>0.73 (0.25 - 2.9)</td>
<td>0.170</td>
</tr>
<tr>
<td>Folate</td>
<td>0.58 (0.25 - 20)</td>
<td>0.62 (0.25 - 23)</td>
<td>0.58 (0.25 - 21)</td>
<td>0.240</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>0.98 (0.25 -18)</td>
<td>0.90 (0.25 - 16)</td>
<td>0.91 (0.25-15)</td>
<td>0.147</td>
</tr>
</tbody>
</table>

Geometric mean (range) shown

Figure 50. Geometric mean of Hs-CRP over Four Hours
FMD improved significantly over four hours in the subjects receiving folate ($p< 0.001$) and vitamin B6 ($p< 0.001$) however there was no significant change in Hs-CRP over four hours in any group. The improvement in endothelial function over the four hours was independent of any change in Hs-CRP over the four hours ($r= 0.08$, $p= 0.67$).

GTN-induced dilatation did not change over time in any group and there was no relationship between change in GTN-induced dilatation and change in Hs-CRP over four hours ($r= 0.04$, $p= 0.83$).

There was no significant association in the change in Hs-CRP with both the change in FMD ($r= 0.08$, $p= 0.67$) and the change in GTN-induced dilatation ($r= 0.04$, $p= 0.83$). There was no significant change in Hs-CRP over four hours in the folate group ($p= 0.24$), vitamin B6 ($p= 0.15$) and placebo group ($p= 0.17$) (Figure 50).

**Eight Week Study**

Baseline Hs-CRP showed no significant differences between the groups (Table 18). The change in Hs-CRP in the folate ($p= 0.26$), vitamin B6 ($p= 0.23$) and placebo ($p= 0.56$) groups, over the study period, was not significant (Figure 51). The change in Hs-CRP in the folate/ B6 group was marginally significant ($p= 0.07$), there was a marginally significant decrease in Hs-CRP from baseline to four weeks ($p=0.07$) and no significant change between four and eight weeks ($p=0.73$).
In the subjects attending for the eight week assessment (n=121; data from two subjects were excluded from this analysis due to reported recent infection), there was no significant difference in Hs-CRP between the groups at baseline (p= 0.585). Baseline Hs-CRP did not significantly correlate with baseline FMD (r= 0.06, p= 0.51) or baseline GTN (r= 0.06, p= 0.48). Hs-CRP was significantly higher in females at baseline; the median Hs-CRP (range) in females was 0.91 (0.25 - 20.0) mg/l, and in males was 0.40 (0.25 - 6.4) mg/l, (p= 0.009). There was a significant positive association between Hs-CRP and BMI (kg/m²) (r= 0.48, p< 0.001), BMI z-score (r= 0.47, p< 0.001), and between Hs-CRP and weight (r= 0.33, p< 0.001) and weight z-score (r= 0.41, p< 0.001) at baseline.

When data was analysed in three Hs-CRP groupings: Hs-CRP < 0.25 mg/l, 0.25 - 0.7 mg/l and < 0.25 mg/l, mean weight z-score (p< 0.001) and mean BMI z-score (p< 0.001) were significantly different between the groups. Mean BMI z-score and mean weight z-score were significantly higher in the Hs-CRP > 0.7 mg/l group (Table 33).
### Table 33. Comparison of Baseline Variables between Hs-CRP Groups.

<table>
<thead>
<tr>
<th>Baseline Variables</th>
<th>Hs-CRP &lt; 0.25 (n=40)</th>
<th>Hs-CRP 0.25-0.7 (n=30)</th>
<th>Hs-CRP &gt; 0.7 (n=51)</th>
<th>Significance (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex M:F</td>
<td>28:12</td>
<td>18:12</td>
<td>25:28</td>
<td>0.08*</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>13.7 (2.7)</td>
<td>13.7 (2.7)</td>
<td>14.6 (3.1)</td>
<td>0.32</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>162 (16)</td>
<td>159 (14)</td>
<td>163 (14)</td>
<td>0.39</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>53 (15)</td>
<td>55 (14)</td>
<td>65 (20)</td>
<td>0.002</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>19.7 (2.3)</td>
<td>21.5 (3.2)</td>
<td>23.8 (4.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height z-score</td>
<td>0.54 (1.14)</td>
<td>0.08 (.96)</td>
<td>0.57 (.85)</td>
<td>0.08</td>
</tr>
<tr>
<td>Weight z-score</td>
<td>0.37 (.79)</td>
<td>0.56 (.76)</td>
<td>1.08 (.76)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI z-score</td>
<td>0.14 (.73)</td>
<td>0.64 (.67)</td>
<td>0.97 (.76)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>117 (14.9)</td>
<td>117 (16.2)</td>
<td>121 (16.1)</td>
<td>0.45</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>62 (8.4)</td>
<td>61 (6.9)</td>
<td>65 (8.3)</td>
<td>0.03</td>
</tr>
<tr>
<td>Duration of diabetes (yrs)</td>
<td>5.0 (3.6)</td>
<td>5.7 (3.6)</td>
<td>5.3 (3.6)</td>
<td>0.72</td>
</tr>
<tr>
<td>Insulin dose (Units/kg)</td>
<td>1.19 (.39)</td>
<td>1.27 (.31)</td>
<td>1.07 (.34)</td>
<td>0.06</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.4 (1.2)</td>
<td>8.9 (1.5)</td>
<td>8.8 (1.3)</td>
<td>0.30</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>12.5 (3.7)</td>
<td>13.6 (5.7)</td>
<td>14.1 (4.7)</td>
<td>0.24</td>
</tr>
<tr>
<td>Vitamin B6 (&lt;63%)</td>
<td>47.1 (7.6)</td>
<td>47.7 (7.2)</td>
<td>48.7 (9.0)</td>
<td>0.64</td>
</tr>
<tr>
<td>Serum Folate (µg/l) (3.4-15.0µg/l)</td>
<td>12.3 (4.0)</td>
<td>13.0 (2.7)</td>
<td>13.0 (3.1)</td>
<td>0.56</td>
</tr>
<tr>
<td>Red Cell Folate (µg/l) (168-388µg/l)</td>
<td>483 (142)</td>
<td>457 (122)</td>
<td>484 (150)</td>
<td>0.67</td>
</tr>
<tr>
<td>tHcy (µmol/l)</td>
<td>6.0 (1.7)</td>
<td>6.5 (1.7)</td>
<td>6.4 (1.4)</td>
<td>0.23</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>4.2 (.76)</td>
<td>4.7 (.82)</td>
<td>4.2 (.66)</td>
<td>0.02</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.7 (.42)</td>
<td>1.8 (.62)</td>
<td>1.5 (.32)</td>
<td>0.07</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>2.4 (.70)</td>
<td>2.6 (.73)</td>
<td>2.4 (.59)</td>
<td>0.24</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th></th>
<th>Folate</th>
<th>Vitamin B6</th>
<th>Placebo</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.44 (.29)</td>
<td>0.59 (.38)</td>
<td>0.54 (.45)</td>
<td>0.29</td>
</tr>
<tr>
<td>FMD (%)</td>
<td>3.6 (4.2)</td>
<td>3.6 (4.3)</td>
<td>4.1 (4.2)</td>
<td>0.82</td>
</tr>
<tr>
<td>GTN-induced dilatation (%)</td>
<td>21.6 (7.3)</td>
<td>21.6 (6.8)</td>
<td>21.2 (6.7)</td>
<td>0.95</td>
</tr>
<tr>
<td>FMD:GTN ratio</td>
<td>0.16 (0.20)</td>
<td>0.17 (0.18)</td>
<td>0.11 (.62)</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Conversion factor for SI units: folate and red cell folate, x 2.266 (nmol/litre).
Data are means (SD) unless otherwise stated.

* Chi-squared test was used for sex. One-way ANOVA was used for other comparisons.

FMD improved significantly over eight weeks in all intervention groups, i.e. subjects receiving folate and/or vitamin B6, however there was no significant change in Hs-CRP over eight weeks in any group. The improvement in FMD was independent of Hs-CRP ($r = -0.14$, $p = 0.15$).

GTN-induced dilatation did not change over time in any group and there was no relationship between Hs-CRP and GTN-induced dilatation over eight weeks ($r = -0.05$, $p = 0.62$).

There was no significant change in Hs-CRP over eight weeks in the folate group ($p = 0.26$), vitamin B6 group ($p = 0.23$) and placebo group ($p = 0.56$). The change in Hs-CRP in the folate/ B6 group was marginally significant ($p = 0.07$), and the repeated contrasts showed there was a marginally significant decrease in Hs-CRP from baseline to four weeks ($p = 0.07$) and no significant change between four and eight weeks ($p = 0.73$) (Figure 51).

There was no significant correlation between Hs-CRP and both baseline FMD ($r = 0.06$, $p = 0.51$) and baseline GTN-induced dilatation ($r = 0.06$, $p = 0.48$). There was no significant correlation between the change in Hs-CRP from baseline to eight weeks and both the change in FMD ($r = -0.14$, $p = 0.15$) and the change in GTN-induced dilatation ($r = -0.05$, $p = 0.62$) over the same period.
Further Analysis

Hs-CRP was assumed to be a continuous variable for the over time analysis therefore initial analysis was performed using Hs-CRP as a continuous variable. BMI, weight and female sex were associated with higher Hs-CRP. However, due to the large number of Hs-CRP < 0.25 mg/l and a small number of large Hs-CRP values, further analysis, with Hs-CRP not as a continuous variable but divided into groups based on Hs-CRP level, was performed.

The subjects’ data was initially stratified into three groups, Hs-CRP <0.25 mg/l, ≥0.25 to ≤ 0.7 mg/l and > 0.7 mg/l (Table 33). A small amount of additional information was gained by stratifying the data into these three groups, in particular data related to lipids. This initial stratification was based on the paper by Järvisalo et al. (Jarvisalo, Harmoinen et al. 2002). The rational for choosing this study as our standard was this study had used similar methods of measuring vascular function using brachial artery FMD and GTN-induced dilatation and studied the relationship between vascular function and Hs-CRP in a group of healthy children. A supporting paper for this stratification was the paper by Ridker (Ridker 2001) that described the use quintiles of Hs-CRP to determine cardiovascular risk in adults, with Hs-CRP < 0.7 mg/l being the lowest cardiovascular risk quintile in this report.

It was recommended by the Reviewers of the paper for ‘Pediatric Diabetes’ (Chapter 8), that the HS-CRP stratification be based on a large study of healthy children (Lambert, Delvin et al. 2004) as this provided better normative adolescent Hs-CRP data. This study examined Hs-CRP, insulin, lipids and blood pressure in a large cohort of school based adolescents aged 9, 13, and 16 years (n= 2224) in Quebec, Canada. Our analysis was therefore re-performed using the stratification from this study which used three groups: Hs-CRP <50th, 50-75th and >75th percentiles. The manuscript was changed accordingly and a table of this analysis is presented in the previous chapter. The strength of the associations did not change and there were no new correlations using this analysis.

One question raised by the Reviewers for the publication, was the possibility that the gender difference across the Hs-CRP groups could account for the difference in BMI z- and weight z-scores across the groups. The sex difference did not account for the difference for several reasons: age and sex are adjusted for in the calculation of BMI z- and weight z-scores. The mean (SD) weight for boys was 59.7 (17.6) kgs and 57.3 (17.9)
kgs in girls. Independent sample t-tests shown that there was no significant difference between sex in weight (p= 0.45) and BMI (p= 0.46). A comparison of weight and BMI across the three Hs-CPR groups, while controlling for the effect of sex were made using two-way ANOVAs. Two-way ANOVAs showed there was a significant difference in weight (p= 0.003) and BMI (p< 0.001) across the three Hs-CRP groups after controlling for sex.

Discussion

In this study, Hs-CRP did not correlate with endothelial function. FMD improved with folate and/or vitamin B6 treatment, however Hs-CRP did not correlate with the improvement in FMD which is consistent with other intervention studies (Vermeulen, Rauwerda et al. 2003).

Hs-CRP was evaluated in relation to endothelial function, specifically FMD and GTN-induced dilatation both cross-sectionally and longitudinally. No relationship was observed between Hs-CRP and measures of vascular function over the time periods examined.

Consistent with many epidemiological studies, the most significant association with Hs-CRP was BMI. BMI correlated with Hs-CRP and this finding was consistent over each test period. BMI is well recognised to be positively associated with Hs-CRP in children (Wu, Chu et al. 2003; Suheyl Ezgu, Hasanoglu et al. 2005) and in adults (Ford 1999; Festa, D’Agostino et al. 2001; Pieroni, Bastard et al. 2003) including adults with type 1 diabetes (Kilpatrick, Keevil et al. 2000; Targher, Bertolini et al. 2005). In the recent sub-analysis of the DCCT examining the effect of intensive therapy on levels of markers of inflammation linked to risk of CVD, intensive therapy increased Hs-CRP amongst those subjects that gained weight, suggesting the risk of atherosclerosis may be influenced by the degree of weight gain (Schaumberg, Glynn et al. 2005).

Female sex was associated with a higher Hs-CRP. The female/male difference may be an early, detectable indication of the increased risk of coronary heart disease in women with diabetes. These sex differences in Hs-CRP have been described in adults with type
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1 diabetes (Kilpatrick, Keevil et al. 2000) and in healthy adults (Pieri, Bastard et al. 2003).

Low vitamin B6 status has been shown to confer an independent risk of cardiovascular disease (Robinson, Arheart et al. 1998) and is associated with elevated CRP (Friso, Jacques et al. 2001). No association between vitamin B6 status and Hs-CRP was found in this study.

There were a small number of random large Hs-CRP values, eight measurements >10 mg/l, which were not easily explained. As acute infections elevate CRP levels in children this may be one possible explanation. Although no child was acutely unwell at the time of their assessment, some reported recent illness including colds and one reported recent gastroenteritis at the time of their studies. Undiagnosed coeliac disease may have accounted for an elevated Hs-CRP in one subject. Two subjects, who were randomised to the folate/B6 group, had elevated Hs-CRP levels at baseline only. Analysis was performed with this data removed however exclusion made no difference to the final results, therefore their data was not excluded from the final analysis.

Comparing other studies of Hs-CRP in T1DM, the Hs-CRP data was higher in the study from Kilpatrick et al. (Kilpatrick, Keevil et al. 2000) in which the median (IQR) Hs-CRP was 0.83 (0.45 to 1.80) mg/l, compared to this study 0.52 (0.25 to 1.30) mg/l. The Kilpatrick et al. study, had a lower limit of detection of Hs-CRP of < 0.1 mg/l and had no Hs-CRP measurements above 10 mg/l compared to this study which had eight measurements >10 mg/l. In the study by Targher et al. (Targher, Bertolini et al. 2005), Hs-CRP tended to be higher with a mean (SD) Hs-CRP in the control group of 1.52 (1.1) mg/l and higher in the T1DM groups, in the T1DM group with no complications Hs-CRP was 2.58 (1.3) mg/l. The lower Hs-CRP values in our study compared with these two studies may be a reflection of the younger age and shorter duration of T1DM of our subjects.

Further unexpected findings and limitations of the Hs-CRP analysis are discussed in Chapter 11.
Summary

Chapter 9 reviews the results of high sensitivity C-reactive protein as it relates to endothelial function and other baseline attributes in the children and adolescents with type 1 diabetes.

This chapter addresses and negates the hypothesis ‘High sensitivity C-reactive protein relates to endothelial function in children with type 1 diabetes’.

This study has confirmed that the factors already recognised to be associated with cardiovascular risk in type 1 diabetes. BMI and female sex are associated with elevated Hs-CRP levels. Despite the large body of evidence associating Hs-CRP with cardiovascular risk in adults, the association of endothelial dysfunction and Hs-CRP was not found in children with type 1 diabetes.
Chapter 10. GTN- Induced Vasodilatation

Introduction

The test of endothelial function used in this research includes a study of smooth muscle function which involves the administration of sublingual nitrates to examine the vasodilating effect of an exogenous source of nitric oxide (NO). Both FMD and GTN-induced dilatation allow the first changes in vascular function, critical to the development of atherosclerosis, to be detected early in life.

In this chapter, I have focused on the results of GTN-induced dilatation, as a marker of smooth muscle function, and its determinants in children and adolescents with type 1 diabetes. This chapter contains the extended analysis of the associations of GTN-induced dilatation not presented in earlier chapters.

Background

NO acts directly at the level of the arterial smooth muscle and produces an endothelium independent dilatation response. Therefore, smooth muscle function can be measured by glyceryl trinitrate (GTN)- induced dilatation. GTN is a nitric oxide donor that induces an increase in vessel diameter independent of the endothelium and assesses vascular smooth muscle response. GTN-induced dilatation is used as a control test for the FMD measurement to ensure that a decreased FMD response observed is truly a consequence of endothelial dysfunction and not a reflection of underlying smooth muscle dysfunction.

Recent evidence suggests that in addition to influencing endothelial function, atherosclerosis may also cause changes in the arterial response to exogenous NO (Adams, Robinson et al. 1998; Raitakari, Seale et al. 2001). Vascular smooth muscle dysfunction has been shown to be an independent risk factor for atherosclerosis in adults. Adult patients with coronary artery disease show impaired brachial GTN-induced dilatation compared with healthy controls (Raitakari, Seale et al. 2001).
The majority of vascular function studies in childhood do not include smooth muscle function. The limited data regarding vascular smooth muscle function has come from studies of children with cardiovascular risk factors such as diabetes (Singh, Groehn et al. 2003; Jarvisalo, Lehtimaki et al. 2004; Pena, Wiltshire et al. 2006), hypercholesterolaemia (Aggoun, Bonnet et al. 2000; Jarvisalo, Lehtimaki et al. 2004) and obesity (Tounian, Aggoun et al. 2001; Aggoun, Tounian et al. 2002; Pena, Wiltshire et al. 2006).

As part of this study we sought to examine vascular smooth muscle function and its associations in children with type 1 diabetes. The analysis is presented in this chapter.

**Statistical Analysis**

Spearman’s rank correlations were used to test the linear association between GTN-induced dilatation and other continuous variables of interest. Comparison of GTN-induced dilatation across three BMI centile groups was made using a one-way Analysis of Variance (ANOVA).
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Results

GTN-Induced Dilatation over the Immediate Effects Study.

As presented in Chapter 5, there was no significant difference in GTN-induced dilatation between the groups at baseline (p= 0.50). There was a decrease in GTN-induced vasodilatation in all groups over the four hours of the immediate effects study (Figure 23, Chapter 5). The change in GTN-induced vasodilatation in the placebo group from baseline to four hours was significant (p= 0.03). The change in GTN-induced dilatation over the four hours was not significant in the folate (p= 0.41) or the vitamin B6 (p= 0.11) groups.

GTN-Induced Dilatation over the Eight Week Study.

As presented in Chapter 6, there were no significant differences in baseline GTN-induced dilatation between the groups (p= 0.12). There was no significant change in GTN-induced dilatation over the eight weeks in any group (Figure 31, Chapter 6).

Correlates of GTN-Induced Dilatation

124 subjects with type 1 diabetes subjects are included in the following analysis. GTN-induced dilatation was associated with FMD, baseline vessel diameter, systolic blood pressure, age, insulin dose (U/kg), BMI Z-score and HDL-cholesterol (Table 20).

The most significant correlation with both baseline FMD and GTN-induced dilatation was baseline vessel diameter. There was also a significant association between FMD and GTN-induced dilatation (Table 21 and Table 22). The relationship between FMD and GTN-induced dilatation is demonstrated in the scatterplot diagram (Figure 52).
Figure 52. Scatterplot Diagram: The Relationship between Baseline GTN-Induced Dilatation and Baseline FMD.
GTN-Induced Dilatation and Systolic Blood Pressure

The relationship between systolic blood pressure and GTN-induced dilatation is demonstrated in the scatterplot diagram (Figure 53).

Figure 53. Scatterplot Diagram: The Relationship between Baseline GTN-Induced Dilatation and Systolic Blood Pressure.
GTN- Induced Dilatation and HDL- Cholesterol

The relationship between HDL-cholesterol and GTN- induced dilatation is demonstrated in the scatterplot diagram (Figure 54).

Figure 54. Scatterplot Diagram: The Relationship between GTN- Induced Dilatation and HDL-Cholesterol (ln).
GTN- Induced Dilatation and BMI

GTN- induced dilatation related to BMI z-score. This relationship is demonstrated in the scatterplot diagram (Figure 55).

Figure 55. Scatterplot Diagram: The relationship between GTN-Induced Dilatation and BMI Z-score.

The BMI centile was calculated with CDC normal data and stratified BMI into three centile groups (BMI <85th, 85-95th and >95th centile). 85 children (69%) were included in the <85th centile group, 25 children (20%) in the 85th-95th centile group and 13 children (10%) in the >95th centile group. BMI z-score was unable to be calculated for one child at baseline.

GTN- induced dilatation was significantly different across the BMI centile groups (<85th, 85-95th and >95th centile) (p=.030). GTN- induced dilatation was significantly lower in
obese subjects (>95\textsuperscript{th} centile group) with T1DM compared with non-obese subjects with T1DM (Figure 56).

**Figure 56. GTN-Induced Dilatation relates to BMI Z-Score**
Discussion

This work demonstrates that GTN- induced dilatation, the vasodilatory response to an exogenous source of NO, is closely associated with endothelial function in children and adolescents with T1DM. In our study, the main associations with impaired GTN- induced dilatation were FMD, vessel diameter, age, insulin dose (U/kg), systolic blood pressure, HDL-cholesterol and BMI z-score. These findings suggest that impaired GTN- induced dilatation occurs in children and adolescents with T1DM at further increased risk for atherosclerosis.

Similar associations with GTN- induced dilatation in children have been found in a study by Järvisalo et al., in which GTN- induced dilatation correlated directly with FMD, inversely with brachial artery baseline diameter, age and body mass index (Jarvisalo, Lehtimaki et al. 2004).

In our study, impaired GTN- induced dilatation was most closely associated with baseline vessel diameter. Vessel diameter was the most significant correlation with both baseline FMD and GTN- induced dilatation. The significant association between FMD and GTN-induced dilatation is likely to be because both FMD and GTN- induced dilatation were associated with vessel diameter, i.e. smaller vessels dilate more.

It has been discussed in earlier chapters, that endothelial dysfunction is an early event in atherosclerosis, however it is generally thought that in the early stages of atherosclerosis, the endothelium-independent nitrate mediated vasodilatory responses remain unaltered. In most previous studies in children and in adults, the GTN responses have actually been mildly reduced in high-risk individuals, although this result has not always reached statistical significance (Celermajer, Sorensen et al. 1992; Celermajer, Sorensen et al. 1993; Singh, Groehn et al. 2003). In adults with risk factors for atherosclerosis, and with established atherosclerotic disease, impaired arterial dilatation to GTN has been observed and occurred independently of endothelial dysfunction (Adams, Robinson et al. 1998; Raitakari, Seale et al. 2001).

GTN- induced dilatation was inversely associated with HDL- cholesterol in this study. Reduced GTN- induced dilatation is associated with serum cholesterol, independently of endothelial function in apparently healthy adults (Adams, Robinson et al. 1998).
Body mass index (BMI) above the 95th percentile for age and sex defines obesity in children and adolescents. Vascular dysfunction, including both endothelial and smooth muscle dysfunction, occurs in severely obese children (Tounian, Aggoun et al. 2001). In this study, BMI z-score was inversely associated with GTN-induced dilatation but it was not associated with FMD, suggesting obesity in children and adolescents with T1DM is associated with smooth muscle dysfunction. Obesity in children and adolescents with T1DM may therefore confer an additional negative effect on vascular function.

There was also an association between impaired GTN-induced dilatation and higher insulin dose in this study. While this association may relate to insulin resistance, well-known to be associated with obesity, hence tying up these two associations, there may also be a direct effect of insulin on the blood vessels. Insulin has been shown to directly affect the endothelium in healthy subjects. Insulin increases endothelium dependent (NO-mediated) vasodilatation and induces this vasodilatation by causing an endothelium dependent increase in blood flow. This occurs independently from glucose and is mediated via nitric oxide (Steinberg, Brechtel et al. 1994). Therefore, in type 1 diabetes, a higher insulin dose, resulting in a higher background insulin level, may in turn produce background vasodilatation which consequently results in a lesser observed response to GTN.

In another published study from our unit, the data from the children and adolescents with T1DM in this current study was incorporated into a larger study comparing obese, non-obese, and children with type 1 diabetes mellitus (T1DM). Children with obesity and T1DM had a similar degree of vascular dysfunction. BMI and weight adjusted for age and sex related to both endothelial and smooth muscle function in non-obese and obese children. However, in children with T1DM, BMI and weight adjusted for age and sex related to smooth muscle function only (Pena, Wiltshire et al. 2006).

This current study demonstrates that impaired GTN-induced dilatation occurs in children with type 1 diabetes with additional risk factors for atherosclerosis, including endothelial dysfunction, higher systolic blood pressure, poor lipid profile with low HDL-cholesterol, higher BMI (including obesity) and higher insulin dose which may be due to insulin resistance.

The unexpected findings and limitations of the analysis of GTN-induced dilatation are discussed in Chapter 11.
Summary and Implications

Impaired GTN-induced dilatation occurs in children with T1DM with additional risk factors for atherosclerosis including endothelial dysfunction, poor lipid profile with low HDL-cholesterol, higher systolic blood pressure, obesity and evidence of insulin resistance. Therefore, children and adolescents with T1DM with these additional risk factors for atherosclerosis are in a higher risk category for vascular disease than those with no additional risk factors.

Obesity, insulin resistance, poor lipid profile and higher systolic blood pressure in children with T1DM confer additional negative effects on vascular function. Therefore, regular weight management needs to be reinforced in the care of children and adolescents with T1DM. In addition, Clinicians need to maintain vigilance with regular monitoring of blood pressure and routinely check lipid profiles.
Chapter 11. Unexpected Findings and Limitations

The Placebo Group

The placebo group had significantly higher baseline FMD (p< 0.001) and GTN- induced dilatation (p= 0.05) than the three intervention groups. Despite this, all three treatment groups had a significantly higher FMD than the placebo group (p< 0.001) after intervention, indicating the effectiveness of folate and vitamin B6 in improving endothelial function.

Subjects were blindly randomised upon entry into the study. We did not randomise at the outset of the study on the basis of FMD, GTN- induced dilatation or on the characteristics of the group. Therefore, the variation seen in baseline between the groups is likely to be a result of this randomisation process.

The reproducibility of the FMD in the placebo group, CV 2.5%, indicates that there is no discrepancy with measurement variation, and that the differences in baseline FMD and GTN-induced dilatation are the result of randomisation. The FMD and GTN- induced dilatation remain constant throughout the study in the placebo group.

We found baseline variables, including sex, age, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, systolic blood pressure and resting vessel diameter (VD) were associated (with various strengths) with baseline FMD. The strongest associations were LDL-cholesterol and VD.

The placebo group tended to be younger, have lower triglycerides, lower LDL-cholesterol and lower vessel diameter; however none of these individually reached statistical significance. Although the combination of these factors may have contributed to a higher FMD, this doesn't totally explain the difference seen. A multivariable ANOVA, which controlled for any baseline characteristics that differed between groups, continued to suggest the placebo group had a higher FMD.
As discussed, the reason for this is likely to be chance. The randomisation was completed correctly with double blinding however a perfect result is never guaranteed. There was no measured variable that explained the higher FMD in the placebo group.

The repeated measure analysis used to investigate the change over time between the groups allowed for different starting scores and adjusted accordingly. These results are not likely to be misleading due to the higher baseline placebo FMD.

**Smokers**

None gave a history of smoking, however five subjects had an elevated serum cotinine (> 28 nmol/l) at baseline. Two, with levels consistent with passive smoking (< 115 nmol/l), were randomised to the placebo group. Three, with levels within the active smoking range (115- 5700 nmol/l), were randomised to the folate/vitamin B6 group (n= 2) or the folate group (n= 1).

The subjects with cotinine levels consistent with active smoking did have lower baseline FMD however with treatment with folate/ vitamin B6 and folate, FMD improved significantly in these subjects. These subjects were included in the analysis, as exclusion did not alter the final results.

**Resting Vessel Diameters**

As resting vessel diameter was inversely related to baseline FMD we performed subsequent analysis to determine that resting vessel diameter was constant throughout the study and that improvement in FMD with intervention with folate and/or vitamin B6 was not associated with changing resting vessel diameter.

The changes in FMD seen at two and four hours, as well as four and eight weeks were not related to the initial vessel diameter. There were no significant differences in mean resting vessel diameter within each group over the duration of the study in either the immediate effect study (Table 34) or the eight week study (Table 35).
There was no significant difference in mean resting vessel diameter in all groups over the duration of the study in either the immediate effects study (p= 0.80) or the eight week study (p= 0.80) (Table 34 and Table 35).

Table 34. Resting Vessel Diameter (mm) for the Immediate Effect Study

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (mm)</th>
<th>2 hours (mm)</th>
<th>4 hours (mm)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>0.29 (.03)</td>
<td>0.30 (.04)</td>
<td>0.30 (.03)</td>
<td>0.6</td>
</tr>
<tr>
<td>Folate</td>
<td>0.31 (.07)</td>
<td>0.31 (.07)</td>
<td>0.32 (.07)</td>
<td>0.9</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>0.28 (.04)</td>
<td>0.29 (.04)</td>
<td>0.29 (.04)</td>
<td>0.9</td>
</tr>
<tr>
<td>Total Group</td>
<td>0.30 (.05)</td>
<td>0.30 (.05)</td>
<td>0.31 (.05)</td>
<td>0.8</td>
</tr>
<tr>
<td>p value</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

Data are provided as mean (standard deviation)

Table 35. Resting Vessel Diameter (mm) for the Eight Week Study

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (0 weeks)</th>
<th>4 weeks (mm)</th>
<th>8 weeks (mm)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>0.28 (.04)</td>
<td>0.29 (.05)</td>
<td>0.28 (.04)</td>
<td>0.6</td>
</tr>
<tr>
<td>Folate</td>
<td>0.30 (.06)</td>
<td>0.30 (.04)</td>
<td>0.30 (.05)</td>
<td>0.9</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>0.30 (.04)</td>
<td>0.29 (.04)</td>
<td>0.30 (.04)</td>
<td>0.9</td>
</tr>
<tr>
<td>Folate &amp; B6</td>
<td>0.28 (.04)</td>
<td>0.28 (.04)</td>
<td>0.28 (.04)</td>
<td>0.9</td>
</tr>
<tr>
<td>Total Group</td>
<td>0.29 (.05)</td>
<td>0.29 (.04)</td>
<td>0.29 (.04)</td>
<td>0.83</td>
</tr>
<tr>
<td>p value</td>
<td>0.04</td>
<td>0.3</td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>

There was no significant difference between the groups in the immediate effects study (Table 34), however there was a significant difference in baseline resting vessel diameter between the groups at baseline (p=0.04) and eight weeks (p=0.07) in the eight week study (Table 35). The resting vessel diameter was smaller in the placebo group and in the folate/vitamin B6 group. This difference in resting vessel diameter may partly explain the higher baseline FMD seen in the placebo group (Table 19, Figure 30) as a smaller
resting vessel diameter was associated with a greater improvement in FMD (r=-0.33, p<0.001) (Table 20 and Table 21).

Stimulus for FMD

In order to make comparisons between the groups regarding the effect of intervention on FMD, the stimulus for FMD (reactive hyperaemia), i.e. the percent increase in arterial blood flow velocity post cuff deflation, was subsequently analysed.

The stimulus for FMD was not significantly different between the groups and was the same at each point in the study in all groups.

There was no significant difference in stimulus for FMD, within each group over the duration of each study, between groups at each time point and no difference in stimulus for FMD in all groups over the duration of the study in either the immediate effects study (p= 0.50) or the eight week study (p= 0.40) (Table 36 and Table 37).

Table 36. Immediate Effect Study: Stimulus for FMD. % Increase in Blood Flow (m/s).

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (range)</th>
<th>2 hours (range)</th>
<th>4 hours (range)</th>
<th>P value §</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>493 (203-925.7)</td>
<td>330 (91-586.7)</td>
<td>352 (192.4-784.4)</td>
<td>0.5</td>
</tr>
<tr>
<td>Folate</td>
<td>490 (142-835.9)</td>
<td>548 (155.3-860)</td>
<td>391 (136.4-1258.1)</td>
<td>0.9</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>568 (235.3-933.8)</td>
<td>362 (185.8-815.8)</td>
<td>442 (179.5-885.5)</td>
<td>0.6</td>
</tr>
<tr>
<td>Total Group</td>
<td>536 (142-933.8)</td>
<td>380 (91-860)</td>
<td>389 (136.4-1258.1)</td>
<td>0.5</td>
</tr>
<tr>
<td>P value §</td>
<td>0.9</td>
<td>0.1</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

Data for Stimulus for FMD is median (range).

§ Kruskal-Wallis tests were used for Stimulus for FMD as these were non-normally distributed variables.
Table 37. Eight Week Study: Stimulus for FMD. % Increase in Blood Flow (m/s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (0 weeks)</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>P value&lt;sup&gt;§&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>507 (144.5-2413.3)</td>
<td>476 (185.9-1112.5)</td>
<td>485 (143.8-942.6)</td>
<td>0.9</td>
</tr>
<tr>
<td>Folate</td>
<td>462 (142.1-1015.4)</td>
<td>485 (148.5-889.7)</td>
<td>393 (190.7-976.7)</td>
<td>0.3</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>431 (121.1-1615.5)</td>
<td>432 (176.7-974.5)</td>
<td>465 (169.5-1455.0)</td>
<td>0.7</td>
</tr>
<tr>
<td>Folate &amp; B6</td>
<td>496 (147.5-878.0)</td>
<td>419 (37.1-1163.9)</td>
<td>448 (101.4-812.3)</td>
<td>0.8</td>
</tr>
<tr>
<td>Total Group</td>
<td>465 (121.1-2413.3)</td>
<td>439 (37.1-1163.9)</td>
<td>464 (101.4-1455.0)</td>
<td>0.4</td>
</tr>
<tr>
<td>P value&lt;sup&gt;§&lt;/sup&gt;</td>
<td>0.9</td>
<td>0.8</td>
<td>0.8</td>
<td></td>
</tr>
</tbody>
</table>

Data for Stimulus for FMD is median (range).

<sup>§</sup> Kruskal-Wallis tests were used for Stimulus for FMD as these were non-normally distributed variables.

**Improvement in FMD**

The pilot study to this randomised placebo controlled trial was a cross-over design trial which showed an improvement but not a normalisation of FMD with folate in T1DM with similar exclusion criteria.

This may be predominantly explained by the larger number of subjects in this study compared with the pilot study. Another contributing factor may have been the rigorous follow-up procedures undertaken in this study to ensure adherence to the study tablets and attendance at study assessments. Phone calls were made at two weeks to make the four week appointment and ensure no difficulties, at three weeks and two days prior to reconfirm the appointment. Further phone calls were made at six weeks again to ensure no difficulties and to make the eight week appointment and at seven weeks to reconfirm the appointment.
**GTN Dose**

One limitation of this study is the use of a single dose of GTN in all subjects. We administered a full pharmacologic dose of GTN (400 µg sublingually) that is known to elicit maximal vessel dilatation.

The dose of GTN used in this study is the standard dose used in our studies of endothelial function using flow mediated dilatation. We did not deviate from the original reporting of the technique (Celermajer, Sorensen et al. 1992) and as we were using previously studied controls (Wiltshire, Gent et al. 2002) it was important to keep the protocol identical. The dose of GTN is intentionally a supra-maximal dose to establish maximal vessel dilatation and is used as a positive control. Most other studies of vascular function use this dose of GTN.

It has been suggested the GTN dose of 25µgm be used to avoid possible side effects of the larger dose and to allow serial testing (Deanfield, Donald et al. 2005). We did not encounter side effects in our study from the higher dose and baseline vessel diameter did not change significantly during the immediate effects study.

The use of the full anti-anginal dose of GTN produces a plateau arterial dilatation which reflects the maximum arterial endothelium independent vasodilatory capacity. It can also be argued that the use of this large dose of GTN may lead to a misinterpretation that GTN-induced dilatation is not compromised in individuals at increased risk for atherosclerosis.

One study of arterial nitrate mediated dilatation in children, administered four consecutive 50 µg doses of GTN to acquire a dose response curve (Jarvisalo, Lehtimaki et al. 2004). In this study by Järvisalo et al., GTN responses were lower compared with previous studies in children. It was assumed that the maximum arterial endothelium independent vasodilatory capacity was not reached when the four consecutive 50 µg doses of GTN were given. This approach may be a more sensitive means to observe subtle impairments in endothelium independent vasodilatation.
Hs-CRP

Hs-CRP was a consistent finding in this study and in children with T1DM, Hs-CRP related to weight, BMI and female gender. These associations are consistent with other studies and not unexpected.

The finding that there was no relationship between Hs-CRP and vascular function however was contrary to expectation. A relationship between Hs-CRP and vascular function has been shown in a study of healthy children (Jarvisalo, Harmoinen et al. 2002) and in studies of adults with type 1 diabetes (Kilpatrick, Keevil et al. 2000; Targher, Bertolini et al. 2005). This may be due to the sample size although the lack of relationship between vascular function and Hs-CRP was consistent in both the cross sectional analysis in 123 subjects and in the longitudinal analysis in 33 placebo subjects in this study.

There are limited numbers of studies published comparing Hs-CRP between healthy children and children with type 1 diabetes, in the publication (Chapter 8) we enrolled a control group of healthy children that had been previously studied. The control group was enrolled to assess differences in relation to children with T1DM.

There was no relationship between BMI and Hs-CRP in our control subjects. The lack of this relationship between BMI and Hs-CRP was also unexpected and is likely due to the relatively small numbers of controls (n= 31) and the narrow range of BMI in our study compared with other studies of Hs-CRP. The relationship between BMI and Hs-CRP is a consistent finding in larger studies of healthy children (Jarvisalo, Harmoinen et al. 2002; Lambert, Delvin et al. 2004). Height and weight differences were not significant between control subjects and subjects with T1DM. Both height and weight tended to be higher in T1DM, however did not reach significance.

Insulin dose was significantly lower at highest centile Hs-CRP. The reason for this is unclear. Several cross-sectional epidemiologic studies have examined the relationship between CRP and measures of insulin resistance in adults of various ages and both sexes. Insulin resistance is associated with elevated CRP in adults, with chronic, low-grade inflammation an early event in the development of insulin resistance states (Festa, D'Agostino et al. 2000; Festa, D'Agostino et al. 2001; McLaughlin, Abbasi et al. 2002; Kopp, Kopp et al. 2003; Pradhan, Cook et al. 2003). These studies have theorised
several mechanisms for these findings. Firstly, that insulin resistance might induce inflammatory responses, secondly, that chronic inflammation may represent either the triggering factor or that inflammation plays an early role in the progression to insulin resistance, thirdly that elevated CRP may reflect pre existing and underlying atherosclerosis and finally, that this effect could occur via adipose tissue. With this in mind, our result that insulin dose was significantly lower at highest centile Hs-CRP, is contrary to expectation. Interestingly, the relationship between insulin dose and Hs-CRP is not documented in other studies examining Hs-CRP in T1DM (Kilpatrick, Keevil et al. 2000; Coulon, Willems et al. 2005; Targher, Bertolini et al. 2005; Picardi, Valorani et al. 2007). In the study by Lambert et al., a strong relationship between CRP and fasting insulin was found in healthy children, however this association was markedly weakened after adjustment for BMI to the extent that it was no longer statistically significant in girls (Lambert, Delvin et al. 2004). Other studies, that have specifically examined the relationship between Hs-CRP, insulin resistance and obesity, have established that BMI rather than insulin resistance is the major determinant of Hs-CRP (Escobar-Morreale, Villuendas et al. 2003; Kopp, Kopp et al. 2003). With these studies considered, it appears that if there is a biologically relevant independent association between CRP and insulin resistance, it is minor compared with the association between CRP and BMI. Our results are consistent with these studies with the relationship between Hs-CRP and BMI and the relationship between Hs-CRP and weight being independent of insulin dose.

Recent evidence has emerged that insulin therapy can exert some unexpected biologic effects in relation to vasodilatation (Steinberg, Brechtel et al. 1994), as discussed in the previous chapter, and anti-inflammatory activities. These anti-inflammatory effects of insulin may explain the observation seen in this study that insulin dose was significantly lower at highest centile Hs-CRP. In vitro studies have shown that insulin can inhibit the NF-κB in human aortic endothelial cells suggesting an anti-inflammatory effect (Aljada, Ghanim et al. 2001). Insulin has also been shown to attenuate the systemic inflammatory response in various animal models (Jeschke, Klein et al. 2004; Dandona, Chaudhuri et al. 2007; Emanuele, Emanuele et al. 2007). Treatment with an insulin infusion to maintain normoglycaemia has been shown to reduce systemic inflammation, including Hs-CRP, in acute cardiac syndromes (Dandona, Chaudhuri et al. 2007; Dandona, Chaudhuri et al. 2009) and in patients including children who are critically ill (Jeschke, Klein et al. 2004; Dandona, Chaudhuri et al. 2007). Insulin treatment has also been shown to significantly decrease of low-grade inflammation markers, again including Hs-CRP, in newly diagnosed type 2 diabetes (Mao, Liu et al. 2009). Despite the expectation that a higher
insulin dose would be associated with insulin resistance and therefore a raised Hs-CRP, this evidence documenting anti-inflammatory properties of insulin may in fact explain the observation seen in this study that insulin dose was lower at highest centile Hs-CRP.

One of the limitations in the analysis of Hs-CRP was that the lowest detectable level of Hs-CRP in our laboratory was <0.25 mg/l, other major studies have been performed with reporting Hs-CRP to the lowest value of <0.1 mg/l. There were a large number of subjects with Hs-CRP <0.25 mg/l (32% at baseline). The statistical analysis of our results using Hs-CRP <0.25 mg/l as the lowest detectable level of Hs-CRP may therefore lead to some differences when comparing with similar studies.
Chapter 12. Discussion

Introduction

Microvascular and macrovascular complications are the major cause of long term morbidity and mortality in type 1 diabetes. Poor metabolic control increases the risk of developing these complications (1993) however does not account for all the increased risk.

The origins of the vascular complications of diabetes have their beginnings in childhood before the signs of subclinical disease can be detected. Endothelial dysfunction is the earliest measurable event in the development of vascular disease occurring early in type 1 diabetes (Clarkson, Celermajer et al. 1996). Endothelial dysfunction can be measured non-invasively in children using flow mediated dilatation.

Folate is gaining increased recognition as a potential new therapy in vascular disease, exerting a positive effect on endothelial function in a wide variety of study populations. With the devastating vascular complications of diabetes in mind and knowledge of work demonstrating a benefit of folate on endothelial function it is pertinent to pursue this work in children with type 1 diabetes.

Work performed in our unit has shown that children with type 1 diabetes have significant endothelial dysfunction (Wiltshire, Gent et al. 2002) and a pilot, cross-over design trial has provided preliminary data that supplemental folate is beneficial to endothelial function in children with type 1 diabetes (Pena, Wiltshire et al. 2004) and this work needed to be substantiated by a randomised control trial.

The hypothesis that vitamin B6 is beneficial to endothelial function in children with type 1 diabetes had not been tested. However vitamin B6 has shown initial promise in improving endothelial function studies in adults.

I set out at the beginning of this research to confirm that folate was beneficial as well as to test the hypothesis that vitamin B6 was also beneficial to endothelial function.
With recent evidence suggesting that folate had acute effects on the endothelium (Doshi, McDowell et al. 2002), I also set out to test these findings in our population of children with type 1 diabetes to gain more insight as to the mechanism of folate action acutely. This study by Doshi et al., suggested that the improvement in endothelial function with folate seen over four hours, occurred by a mechanism independent of homocysteine.

The previous work had shown that children with diabetes have lower tHcy levels compared with age matched controls (Wiltshire, Thomas et al. 2001) and in the cross-over trial improvement in endothelial function was independent of homocyst(e)ine (Pena, Wiltshire et al. 2004). I hypothesised that any effect of folate and vitamin B6 on endothelial function would also be independent of tHcy.

In this chapter, I discuss the findings of this research, its implications for people with type 1 diabetes and strategies for ongoing work in this area. I also discuss the possibility of recommending the instigation of folate as an adjunct to therapy in light of this research.

Before discussing these issues, I will review the strengths and weaknesses of the methodology I have used, its validity and wider applicability.

**Strengths and Weaknesses**

I have used a randomised controlled trial (RCT) to test the hypotheses that are set out in the beginning chapters. This was the best method available to test such hypotheses. As the name suggests, RCTs involve the random allocation of different interventions to subjects.

Randomised control trials are ones in which:

- Patients are assigned to one of two or more groups to be offered different therapeutic measures
- Chance alone dictates whether a patient will be assigned to a particular group
- Patients in each group are monitored for the possible outcome or efficacy of the interventions.
The primary role of an RCT is to assess the efficacy of therapy, although they also have an important role to play in identifying adverse effects that are relatively common, and occur relatively soon after therapy has been initiated. RCTs provide results that can be interpreted relatively easily. RCTs are relatively labour intensive, and generally entail a high cost per patient. The cost for this study was considerable. A budget was calculated at the onset of the study and included in the appendices (Appendix 3). The other problem with RCTs is that there may be difficulties encouraging patients to participate actively in the study preventing enrolment of large numbers of participants (Rothman and Greenland 1988). Although a large number of subjects were required, most subjects approached were willing to participate.

RCTs have the least source or error of scientific studies. They are generally considered to be the most reliable form of scientific evidence because they eliminate spurious causality and bias. Double-blind RCTs are preferred, as they tend to give the most accurate results. Double-blinding was used in this study.

Despite RCTs being considered the most robust of studies, potential error includes both study precision and study validity.

**Precision**

Precision refers to the degree of random error in a study. The precision of a study can be improved primarily by reducing sampling size error, i.e. by enlarging the size of the study by studying more subjects (Rothman and Greenland 1988).

One determinant of precision is the power of a study to detect a significant difference between subjects and controls if a difference exists. This depends on not only the sample size but also the distribution of values within the sample, the size of the difference between the groups and the strength of association between variables. Sample size calculations at the start of a study therefore depend on a number of assumptions, in particular related to the distribution of values and the size of difference which may actually prove to be inaccurate once the study has commenced.
For the studies of endothelial function, I based the sample size calculations on the previous cross-over design trial performed in our unit. Based on this, the study with 30 subjects in each treatment group had 74% power at 0.05 to detect a difference in FMD of 2.0 +/- 3%. This is a smaller difference than that noted in studies of folate supplementation in adults and the standard deviation noted in our previous FMD studies in 36 adolescents.

Because the power calculations for this study were based the pilot study undertaken in our unit, on subjects studied using the same methods, under the same conditions, it is unlikely there has been an error related to the precision in this study.

Validity

Validity refers to the degree of systematic error in a study. Validity is usually separated into two components; internal and external validity.

Internal Validity

Internal validity is dependent on the amount of error in measurements. It refers to the design of the study, the way it has been conducted and its' analysis. Internal validity therefore refers to the degree to which a study supports the conclusion drawn from the results. Inferences are said to possess internal validity if a causal relation between variables is properly demonstrated. Good internal validity implies a lack of error in measurement and suggests that inferences may be drawn at least as they pertain to the subjects under study.

When considering internal validity, the methodology used in the study must be considered, it must be appropriate for the study and able to generate data to test the hypotheses raised. The laboratory methods used in this study were well established, reproducible and with levels or variation within acceptable limits for laboratory data. The measure of vascular function, flow mediated dilatation, including GTN-induced dilatation,
is well established and accepted. The technique has been used in our unit over several years and the coefficient of variation has previously been 3.9%.

With any study it is important to consider potential sources of bias that may influence the validity of a study. There are three potential sources of bias: selection bias, information bias and confounding.

**Selection Bias**

Selection bias refers to differences between groups that may interact with the independent variable and thus be 'responsible' for an observed outcome. An example of selection bias is volunteer bias. Subjects/volunteers bring to the research characteristics that may result in selection bias. For example, sex, weight and other physical attributes, but also attitudes like motivation or willingness to participate. Volunteers may not be representative of the true population. Selection bias may be reduced when selection processes are controlled for and group assignment is randomised. However, in most cases, it may never be ruled out completely as relevant between-group differences may exist.

Subjects in this study were approached and selected if they were children and adolescents of an appropriate age attending the Diabetes Clinic held at WCH and willing to participate. As subjects had to attend for early morning investigations in a fasting state this was a limiting factor, but not exclusion criteria, for subjects who lived outside of Adelaide. This factor did not deter some subjects from outside of Adelaide, even as far as three hours drive away participating in the study. Studies were performed weekdays and weekends so as to not preclude subjects with working parents. There were no indigenous Australian subjects in the study, T1DM is rare in this population and no subjects approached to enter the study were of indigenous Australian decent. Children from a multitude of different ethnic backgrounds participated in the study and where language was a barrier, official interpreters were used.
Information Bias

Information bias occurs when the nature and completeness of the data collected are in some way influenced. Information bias is unlikely to have affected this study to any great extent as the study was double blinded and data collection was for the most part complete. Laboratory data was not able to be collected on only one subject at baseline and one subject withdrew from the study after the four week assessment. The laboratory measurements were robust and were carried out in a completely blinded fashion. The measures of vascular function were also carried out blinded. The studies of endothelial function and smooth muscle function, flow mediated dilatation and GTN-induced dilatation, were recorded onto VHS tapes and interpreted at the end of the study by two independent observers. The initial interpretation of the scans was performed at the WCH, Adelaide, the tapes were then sent to Wellington, New Zealand, where they were read by Dr Wiltshire.

Confounding

Confounding refers to the complex interplay that occurs between variables associated with a disease or variable being investigated.

A confounding variable, or confounder, is a variable that correlates, positively or negatively, with both the dependent variable and the independent variable and thus resulting in erroneous conclusions that the dependent variables are in a causal relationship with the independent variable. Therefore, confounding is a major threat to the validity of inferences made about cause and effect as the observed effects should be attributed to the confounder rather than the independent variable.

Examples of possible measurable confounders in this study would have been age, sex, duration of diabetes, smoking. One way of controlling for confounding is by measuring the known confounders and adjusting for them during the analysis of the data using multi-variate statistical analysis. I attempted to identify and control for all potential confounders as part of the statistical analysis of this study.

One major problem is that confounding variables are not always known or measurable. This leads to 'residual confounding' or incompletely controlled confounding. Confounding
may be minimised by randomisation. If the numbers in the groups in an RCT are sufficient, this ensures that all confounding variables, both known and unknown, will be evenly distributed across all study groups.

This study was double-blinded and randomised. The numbers studied were adequate yet despite this, there was a difference seen in endothelial function at baseline between the groups that was not able to be accounted for. This is discussed in depth in the previous chapter, and while mostly likely due to chance, may be an example of either selection bias or confounding.

**External Validity**

The question raised from any research is how applicable the results are to individuals and populations outside of the study population. External validity, also known as generalisibility, pertains to this, i.e., the extent to which the study group is representative of the population from which the sample was drawn and the extent to which there can be generalisation of the results beyond the studied group to the target population or even beyond that population to a more universal statement.

Internal validity is a prerequisite for external validity. Therefore minimising bias makes a study more generalisable. I believe the results of this study are generalisable as the subjects studied were representative sample of children with type 1 diabetes. Sources of error were minimised and analysis attempted to account for potential sources of bias.
Summary

The major strength of this study was that it was a randomised controlled trial. The RCT is recognised to be the most scientifically sound methodology for an intervention study such as this, as potential sources of bias are minimised but not excluded. In this research I have attempted to recognise sources of bias and account for these in the analysis. The major negative aspect of an RCT is that it is labour intensive and requires large numbers of subjects.

The other major weakness of this study is the end points measured. We measured vascular function using a non-invasive technique, flow mediated dilatation, in children in whom microvascular or macrovascular disease was not apparent. Although FMD is well recognised as a marker of vascular disease and correlates with invasive tests of vascular function, we assumed, from the extensive supporting evidence, that endothelial function is a surrogate marker for vascular disease. Only long term follow-up will definitively confirm this assumption. The intervention with folate and vitamin B6 improved endothelial function measured by FMD, however whether this will correlate with improved long term outcome from diabetic vascular disease is speculative. This study has raised many questions worthy of future research and these are later discussed.
Study Results and Conclusions

I will now summarise the findings of my research and its implications and discuss possible strategies for ongoing research.

The Immediate Effects of Folate and Vitamin B6 on Endothelial Function in Children and Adolescents with Type 1 Diabetes.

In this study I have shown that endothelial function is impaired in children with type 1 diabetes. This is consistent with the previous work from our unit as well as studies of older individuals with type 1 diabetes (Clarkson, Celermajer et al. 1996; Wiltshire, Gent et al. 2002; Pena, Wiltshire et al. 2004).

Major findings of the study were that both folate and vitamin B6 improved endothelial function acutely. Endothelial function was normalised with both folate and vitamin B6 within the four hours of the immediate effects study. This confirms my original hypotheses that folate improves endothelial function acutely and that vitamin B6 improves endothelial function acutely.

The finding that folate improves endothelial function acutely is consistent with studies in adults that have shown improvement in endothelial function within hours of additional oral folate (Doshi, McDowell et al. 2002) and within minutes of intravenous 5-MTHF, the active form of folate (Verhaar, Wever et al. 1998; van Etten, de Koning et al. 2002).

This is the first study to examine the immediate effects of vitamin B6.

Interestingly in the immediate effects study only, insulin dose (U/kg) was associated with improved FMD. In the immediate effects study, unlike the eight week effects study, FMD was studied prior to and at two and four hours after administration of subcutaneous insulin. Acute administration of insulin causes vasodilatation by increasing endothelium derived nitric oxide (Steinberg, Brechtel et al. 1994). Therefore this improvement in FMD may be a direct effect of the insulin administered with breakfast.
The Effect of Folate and Vitamin B6 on Endothelial Function in Children with Type 1 Diabetes

Dr Karen E MacKenzie

In the immediate effects study, we demonstrated the improvement in endothelial function, with either folate or vitamin B6, was independent of change in homocysteine. This supports the results from the previous cross-over trial from our unit as well as the studies in adults which also show improvement in endothelial function with folate independent of homocysteine (Doshi, McDowell et al. 2002; Pena, Wiltshire et al. 2004). This finding confirms one of the initial hypotheses that supplemental folate improves endothelial function in children with type 1 diabetes mellitus independent of lowering homocysteine.

The Eight Week Effect of Folate and/or Vitamin B6 on Endothelial Function in Children and Adolescents with Type 1 Diabetes.

In this study, in a large cohort of children with type 1 diabetes, endothelial function has again been shown to be impaired. This study has confirmed that endothelial dysfunction occurs early in T1DM as FMD was not associated with disease duration and abnormal FMD occurred in patients with shorter duration of diabetes.

High levels of LDL cholesterol and serum triglycerides correlated with impaired FMD. Baseline FMD results in this study, showed an inverse relation with serum triglycerides and LDL cholesterol. This result is supported by work from Clarkson, Celermajer et al., that also demonstrated that LDL cholesterol is associated with impaired FMD response in type 1 diabetes (Clarkson, Celermajer et al. 1996). In our previous work we have also demonstrated an inverse relationship with triglycerides and FMD (Wiltshire, Gent et al. 2002).

Major findings of this study were that both folate and vitamin B6 improved endothelial function and maintained this improvement over eight weeks. There was no detectable significant difference between the combination of folate/ vitamin B6 and either folate or vitamin B6 alone.

Improvement in FMD related to increase in serum folate and red cell folate. There is substantial evidence to support the benefits of folate on endothelial function. There is also increasing research that provides evidence for an understanding of the mechanism of action of folate. This study contributes to the extensive literature showing positive
effect of folate in vascular disease. It also contributes to the small but growing database of information on folate supplementation in diabetes. It provides a substantial contribution to the literature in children with type 1 diabetes as this is the first randomised control trial of this kind. In this work there is a large study population and we have shown that folate normalises endothelial function in children with type 1 diabetes.

This is the first study to examine the effects of vitamin B6 in children. Improvement in FMD was related to improvement in vitamin B6 status. Poor vitamin B6 status also correlated with impaired baseline FMD. Several studies have shown the low vitamin B6 is an independent predictor of adverse vascular outcome (Robinson, Mayer et al. 1995; Robinson, Arheart et al. 1998; Kelly, Shih et al. 2003; Wilmink, Welch et al. 2004). Experimental work has shown that there is increased utilisation of vitamin B6 in diabetes. This association with low vitamin B6 status and vascular disease as well as increased utilisation of vitamin B6 may in part explain the aetiology of vascular disease in diabetes.

**High Sensitivity C-Reactive Protein**

In addition to flow mediated dilatation as a marker of vascular disease, I examined high sensitivity C-reactive protein. Hs-CRP is also a strong marker of risk of coronary heart disease and has been used to predict the risk of cardiovascular events (Ridker, Cushman et al. 1997; Ridker, Rifai et al. 2002; Danesh, Wheeler et al. 2004). In adults with diabetes, Hs-CRP has been shown to be elevated and elevated Hs-CRP is associated with female sex, diabetes duration, glycaemic control, advanced glycation end products, BMI, HDL cholesterol, triglycerides and systolic blood pressure (Schalkwijk, Poland et al. 1999; Kilpatrick, Keevil et al. 2000; Colhoun, Schalkwijk et al. 2002; Schram, Chaturvedi et al. 2003; Targher, Bertolini et al. 2005).

The relationship between Hs-CRP and endothelial function had not been examined in children with diabetes.

In our study Hs-CRP was associated with BMI, weight and female gender in T1DM, both at baseline and over eight weeks. There was no relationship with vascular function. Hs-CRP did not correlate with either baseline endothelial function or the improvement in endothelial function that occurred with either folate or vitamin B6 treatment.
There was no difference in Hs-CRP between children with T1DM and healthy control subjects in this study. Despite this, the association between Hs-CRP and BMI, weight, female gender was not seen in the control subjects. There was also no relationship between Hs-CRP vascular function in control subjects.

**Study Implications**

I have confirmed the beneficial effects of folate on endothelial function in children with type 1 diabetes both acutely and over eight weeks. I have for the first time shown that vitamin B6 also has a beneficial effect on endothelial function in children with type 1 diabetes both acutely and over eight weeks.

The benefits of folate supplementation over one year have been reported (Woo, Chook et al. 2002), raising the possibility of long term benefits of folate and possibly vitamin B6 therapy on vascular disease progression. This therefore remains an exciting area for future research.

The prospect of being able to intervene effectively at an early stage in childhood, in addition to optimising metabolic control, should have a major impact on long term risk of vascular complications. A safe, cheap intervention such as folate is extremely promising and warrants significant consideration.

As elevated Hs-CRP was seen in children with T1DM and a higher BMI, these children may be at additional risk of future cardiovascular events. Maintenance of a healthy BMI in T1DM may be important in the prevention of vascular complications of diabetes. Promotion of a healthy weight may improve long-term cardiovascular outcomes for children with T1DM.
Directions for Future Research

Endothelial function is impaired in children with type 1 diabetes. Flow mediated dilatation and GTN- induced dilatation are widely accepted markers of vascular disease yet they remain proxy markers. Studies over time of vascular function in children with T1DM will determine the significance of these markers of endothelial and smooth muscle function.

In future research, other markers of vascular disease, such as carotid intima media thickness (cIMT) and aortic intima media thickness (aIMT) could be employed in conjunction with and/ or in direct comparison to FMD to study the effects of folate and vitamin B6 on vascular function.

cIMT is a well established index of early atherosclerosis that correlates with prevalent and incident coronary heart disease and stroke (Bots, Hoes et al. 1997; O'Leary, Polak et al. 1999). cIMT assesses the combined thickness of the intima and media walls and like FMD, uses high resolution B-mode ultrasound. cIMT has been shown to correlate with impaired FMD in children with type 1 diabetes (Jarvisalo, Raitakari et al. 2004), who inconsistently show increased cIMT compared with age matched controls (Jarvisalo, Raitakari et al. 2004; Krantz, Mack et al. 2004). The Epidemiology of Diabetes Intervention and Complications (EDIC) study showed decreased progression of cIMT in subjects randomised to intensive therapy in the Diabetes Control and Complications Trial (DCCT) (Nathan, Lachin et al. 2003). cIMT has been used as a primary outcome measure these landmark intervention trials, for this reason it would be useful to develop and utilise cIMT in future research in our unit.

Atherosclerosis develops first in the abdominal aorta where changes precede those seen in the carotid arteries (McGill, McMahan et al. 2000). In children with hypercholesterolaemia or type 1 diabetes, aIMT is has been shown to be an earlier marker than cIMT of early atherosclerosis (Jarvisalo, Jarti et al. 2001) and therefore would be a useful measure of vascular disease in our study population.

Clearly, the short term improvements in vascular function seen in this study will need to be assessed in longer term studies that have clear clinical end points. The relationship between vascular function and microvascular and macrovascular end-points still needs to be determined with long term follow up studies. Nevertheless, the encouraging results
from this study of folate and/or vitamin B6 supplementation, support the need for further studies of this simple strategy to improve endothelial function and restore vascular health in our children with type 1 diabetes.

Folate, in many different study populations has been shown to have a positive effect on endothelial function. This study has shown in children with type 1 diabetes, that folate also has a positive effect. This may have a huge impact to these children as interventions at this early stage in the disease process may curb the devastating long term vascular complications. The precise mechanism of folate on endothelial function is unclear although in vitro research is providing clues to potential mechanisms. Whatever the molecular mechanism, the observation that this simple intervention improves endothelial function has great clinical implications. The results of this study along with the combined work of many other clinical studies of folate showing that folate ameliorates the endothelial dysfunction associated with many conditions, advocates for the broad use of folate for patients at risk of vascular disease.

Folate fortification of food lowers homocysteine levels in the normal population but these small doses appear inadequate to improve endothelial function in disease states. Vitamin supplementation including folate, vitamin B6 and vitamin B12 combinations have improved endothelial function in adults but have generally been disappointing in reducing clinical vascular outcomes in adults with established disease (Lonn, Yusuf et al. 2006). However, a recent meta-analysis of the impact of folate supplementation specifically on stroke showed a significant reduction in the incidence of stroke, with most evidence of benefit in primary prevention of stroke and also some evidence of a dose-response relationship (Wang, Qin et al. 2007). Collectively, this data suggests early intervention with folate supplementation is more likely to be effective than intervention in established atherosclerosis. As children do not have established vascular disease, they are likely to be more amenable to treatment and the prospect of reducing vascular disease safely and simply, by folate and other B vitamin supplementation, requires further investigation.

In the short term, this study raises the question of a dose response relationship of folate on the endothelium. As folate improves endothelial function acutely, a study to determine the dose response of endothelial function to different doses of folate may be readily answered.
The ultimate and probably most importantly question, raised from this work is what will be the long term impact of supplemental folate on diabetic vascular disease. Any work attempting to address these questions will be massive undertaking as work will need to be conducted over many years in a large population to be able to determine the effect of such an intervention. The possible next step with an intervention with folate is for the international diabetes community to review the possible benefits and consider a collaboration to instigate an intervention trial and monitor the long term effects.

Parents and children are keen to learn of promising adjuncts to insulin therapy. Folate is not known to have long term side effects. In saying that, clinicians need to be aware of the masking of vitamin B12 deficiency associated anaemia and neuropathy when prescribing folate and screen for vitamin B12 accordingly. If any long term study of folate therapy is commenced, long side effects would need to be examined.

As demonstrated in this study, vitamin B6 also improves flow mediated dilatation. There is now a recognised association with low vitamin B6 status and vascular disease. Experimental work has shown increased utilisation of vitamin B6 in diabetes and case control studies have shown low vitamin B6 levels in both children and adults with diabetes. These associations may in part explain the aetiology of vascular disease in diabetes however they also pose unanswered questions as to its contribution to the aetiology of vascular disease in diabetes. As yet there is little understanding as to how vitamin B6 exerts its effects on endothelial function. More work will need to be done to confirm vitamin B6 effect on the endothelium. The mechanism of action of B6 on the endothelium is poorly understood and the results of this study may open this as a question for further experimental work. Finally in terms of the effect of vitamin B6, there is little understanding of the altered vitamin B6 metabolism in diabetes. An understanding of the pathophysiology of vitamin B6 in diabetes may provide new insights into the disease process. This also opens doors for further research. However, due to the theoretical concerns of neurotoxicity with vitamin B6 therapy, the possibilities of further clinical intervention studies and long term intervention with vitamin B6 are limited.

There is limited data examining Hs-CRP in children with T1DM. Increased BMI, a known risk factor for vascular disease, and female sex are associated with elevated Hs-CRP in this study. Interestingly, despite Hs-CRP being a recognised marker of risk of vascular disease, Hs-CRP was not associated with endothelial function in our study. This also raises the possibility of further work in this area. One question regarding BMI and Hs-
CRP that was raised, resulted in a study of the effect of obesity on endothelial function, this has subsequently been published with results showing that obese children and children with T1DM have a similar degree of endothelial dysfunction (Pena, Wiltshire et al. 2006). Further questions are raised about the role Hs-CRP in the pathophysiology of the vascular disease of T1DM. Long term analysis of Hs-CRP in subjects with T1DM to determine whether Hs-CRP and the progression of Hs-CRP correlates microvascular and macrovascular end-points may be warranted.

This work has examined the effects of two vitamins on vascular function in children with T1DM in an attempt to gain insight into possible interventions to limit the devastating vascular complications of diabetes. To me, the prospect of an adjunct to optimal diabetes management with a safe, cheap intervention such as folate and possibly vitamin B6 is extremely exciting. To be able to intervene effectively at an early stage in childhood is paramount.
The Effect of Folate and Vitamin B6 on Endothelial Function in Children with Type 1 Diabetes

Dr Karen E MacKenzie
The Effect of Folate and Vitamin B6 on Endothelial Function in Children with Type 1 Diabetes

Dr Karen E MacKenzie

Appendices

Appendix 1. Consent Form

WOMEN'S & CHILDREN'S HOSPITAL RESEARCH ETHICS COMMITTEE
CONSENT FORM

I ____________________________________________________________

hereby consent to my/**my child's involvement in the research project entitled:

Effect of folate and vitamin B6 on endothelial function in children and adolescents with type1 diabetes mellitus.

1. The nature and purpose of the research project described on the attached information sheet has been explained to me. I understand it, and agree to (**my child) taking part.

2. I understand that I (**my child) may not directly benefit by taking part in this study.

3. I acknowledge that the possible risks and/or side effects, discomforts and inconveniences, as outlined in the Information Sheet, have been explained to me.

4. I understand that while information gained in the study may be published, I (**my child) will not be identified and information will be confidential.

5. I understand that I can withdraw (**my child) from the study at any stage and that this will not affect medical care or any other aspects of my (**my child's) relationship with this hospital.

6. I understand that there will be no payment to me (**my child) for taking part in this study.
7. I have had the opportunity to discuss taking part in this research project with a family member or friend and/or have had the opportunity to have a family member or friend present whilst the research project was being explained by the researcher.

8. I am aware that I should retain a copy of the consent form, when completed, and the information sheet.

9. a) I consent to specimen of blood being taken from me (**my child) and being used in the above project.

b) I consent to the blood samples being used in any other research project, provided the project has the approval of the Women's & Children's Hospital Research Ethics Committee.

10. I understand that I am free to stop donating blood samples or partaking in flow mediated dilatation studies at any stage, without giving any reason, and that my action of donating/not donating a sample will not affect (i) my prospects in any position; (ii) any other conceivable situation.

Signed: ..........................................................

Relationship to Patient: ......................................................

Full name of patient: ..............................................................

Dated:............................

I certify that I have explained the study to the parent (**patient)(**and/or child) and consider that he/she understands what is involved.

Signed: ..........................................................

Title: ....................................................... 

Dated: ..............................................

** Please delete either the phrase, or the brackets, as appropriate.
Appendix 2. Information Sheets

Information Sheet. Immediate Effects

Effect of Folate and Vitamin B6 on Endothelial Function in Children and Adolescents with Type 1 Diabetes Mellitus

1) What is the study and why is it being done?

The aim of this study is to determine if folate and vitamin B6 are beneficial to improving the function of blood vessel walls in children and adolescents with Type 1 diabetes.

Our diabetes unit has been studying the way blood vessels work in children with type 1 diabetes for several years. We have found that if children and adolescents with type 1 diabetes have good folate levels their blood vessels work well. This is important as changes in how the blood vessel works are involved in the long term complications of diabetes. In this study we aim to find out how quickly folate and another B vitamin, vitamin B6, work on the blood vessel function and also if there is any added benefit from these B vitamins over several weeks.

2) What would I/my child be asked to do if I/my child took part?

• Children and adolescents involved in this study will be asked to take 2 tablets every day for 2 months. Neither the researchers nor you will know whether the 2 tablets are folate, vitamin B6, or a placebo (blank tablet).

• You/ your child will be required to attend the hospital on three occasions.

1) First Day. We will perform a test called flow mediated dilatation on a blood vessel in the elbow and do the first blood test. During the first blood test an intravenous cannula will be left in place for further blood tests this day. After this, you/ your child will be given the first tablet. Next, we will look at the effect this has on the blood vessel by performing flow mediated dilatation again and take another blood sample. These first tests will be performed over 4 hours. There will be 3 measurements of flow mediated dilatation during this time and 3 further blood samples.

2) After 4 weeks of taking the 2 tablets we will again perform a blood test and take one measurement of flow mediated dilatation to look at the blood vessel.

3) After 8 weeks of taking the 2 tablets we will perform the final study. This will be a final blood test and another Flow mediated dilatation test.
Emla cream, a local anaesthetic cream applied to the skin, will be provided for all blood tests. This cream is very effective at numbing the skin. Occasionally there is a mild local irritation from using this cream.

- On each of the days that we perform flow mediated dilatation, it is important not to have breakfast or have insulin prior to the FMD. Please bring your insulin with you and we will provide breakfast for you/your child on these days at the hospital after the first part of the test has been performed. On these mornings, if your/your child’s blood sugar level is low, please give glucose (e.g. jelly beans) and plain bread. Please do not have protein or fat. If you have any concerns please telephone us.
- It is important during the 2 months of the study that you/your child does not take other vitamin supplements.
- It is also vital that you/your child does not smoke cigarettes. Both taking vitamin supplements and cigarette smoking interferes with the results.

3) What is Flow Mediated Dilatation?

Flow mediated dilatation is a technique which has been well established to look at how the blood vessel is working. It uses an ultrasound on an artery in the arm. During the technique, a blood pressure cuff is inflated and after the blood pressure cuff is released the ultrasound measure the size of the blood vessel. After 15 minutes of resting, a medicine called glyceryl trinitrate (GTN) is given under your/your child’s tongue which cause the blood vessels to dilate. The ultrasound is again used to measure the size of the blood vessel. GTN is a safe medicine and is routinely used in people with angina. The technique takes about 30 minutes and each ultrasound recording takes only a few minutes.

4) Are there any risks to Flow mediated dilatation?

There are no risks to flow mediated dilatation. The procedure is very safe and has been used around the world in a large number of children and adults including children and adults with diabetes.

During the first part of the test, when the blood pressure cuff is left up can be uncomfortable although most children have not been bothered by this. The medication GTN is very safe and only remains active for a very short period of time. The side effects of this medication include, brief headaches in some people, light headedness, facial flushing, however this is usually not the case in children. These side effects are rare in children.
5) Are there any risks with Folate or Vitamin B6?

Folate and vitamin B6, all occur naturally in our diets. Folate is a vitamin which is found in leafy green vegetables and many breads and cereals have folate added. Vitamin B6 is found in meat, nuts, grains, fish and green vegetables.

The doses of the vitamins that we have chosen are safe and there have been no reported side effects at these doses. The dose of folate has been used in adults and has been shown to improve blood vessel function.

6) What will be done with the information from the study?

With the results of this study we will compare the vitamin levels from the blood test and compare it with the results of the flow mediated dilatation to see if there is any improvement in blood vessel function. These results will be published in medical journals to help other children with type 1 diabetes. All of the information will remain confidential and no child will be mentioned by name.

7) Do I have to take part in the study?

No, The study is optional.

8) Can I change my mind later if I decide not to participate?

Yes, You can choose to leave the study at any time.

9) Will the study benefit me/my child in any way?

It is not certain whether you/your child will directly benefit from this study in the short term. Information obtained from the study however will be useful in the long term and may benefit all children with Type 1 diabetes.

10) Has permission been given to perform this study?

Permission for this study has been granted by the Research Ethics Committee at the Women’s and Children’s Hospital. If you would like to discuss the approval process by which approval is granted for this research or any concern or complaint, please contact the Secretary of the Committee, Ms Brenda Penny. Ph 81616521.

Funding for the study has come from the Juvenile Diabetes Research Foundation & Channel 7.

11) What if I have any other questions?

Any further questions will be answered by Dr Karen MacKenzie, Endocrinology and Diabetes Fellow at the Women’s and Children’s Hospital. Ph 81616403 or 81616402 (Secretary-Julie). Associate Professor Jenny Couper may also be contacted. Ph 81616242 or 81617000 + pager 4127.
Information Sheet. 8 Week Study.

Effect of Folate and Vitamin B6 on Endothelial Function in Children and Adolescents with Type 1 Diabetes Mellitus

1) What is the study and why is it being done?
   The aim of this study is to determine if folate and vitamin B6 are beneficial to improving the function of blood vessel walls in children and adolescents with Type 1 diabetes.

   Our diabetes unit has been studying the way blood vessels work in children with type 1 diabetes for several years. We have found that if children and adolescents with type 1 diabetes have good folate levels their blood vessels work well. This is important as changes in how the blood vessel works are involved in the long term complications of diabetes. In this study we aim to find out how quickly folate and another B vitamin, vitamin B6, work on the blood vessel function and also if there is any added benefit from these B vitamins over several weeks.

2) What would I/my child be asked to do if my child took part?
   • Children and adolescents involved in this study will be asked to take 2 tablets every day for 2 months. Neither the researchers nor you will know whether the 2 tablets are folate, vitamin B6, or a placebo (blank tablet).

   • You/ your child will be required to attend the hospital on three occasions.
     1) First Day. We will perform a test called flow mediated dilatation on a blood vessel in the elbow and do the first blood test. After this, you/ your child will be given the first tablets to take. Breakfast will be provided and insulin can be given at this stage.
     2) After 4 weeks of taking the 2 tablets we will again perform a blood test and take one measurement of flow mediated dilatation to look at the blood vessel.
     3) After 8 weeks of taking the 2 tablets we will perform the final study. This will be a final blood test and another Flow mediated dilatation test.

   Emla cream, a local anaesthetic cream applied to the skin, will be provided for all blood tests. This cream is very effective at numbing the skin. Occasionally there is a mild local irritation from using this cream.

   • On each of the days that we perform flow mediated dilatation, it is important not to have breakfast or have insulin prior to the FMD. Please bring your insulin with you and we will
provide breakfast for you/your child on these days at the hospital after the first part of the test has been performed.
On these mornings, if your/your child’s blood sugar level is low, please give glucose (e.g. jelly beans) and plain bread. Please do not have protein or fat. If you have any concerns please telephone us.
- It is important during the 2 months of the study that you/your child does not take other vitamin supplements.
- It is also vital that you/your child does not smoke cigarettes.
Both taking vitamin supplements and cigarette smoking interferes with the results.

3) What is Flow Mediated Dilatation?
Flow mediated dilatation is a technique which has been well established to look at how the blood vessel is working. It uses an ultrasound on an artery in the arm. During the technique, a blood pressure cuff is inflated and after the blood pressure cuff is released the ultrasound measure the size of the blood vessel. After 15 minutes of resting, a medicine called glyceryl trinitrate (GTN) is given under your/your child’s tongue which cause the blood vessels to dilate. The ultrasound is again used to measure the size of the blood vessel. GTN is a safe medicine and is routinely used in people with angina.
The technique takes about 30 minutes and each ultrasound recording takes only a few minutes.

4) Are there any risks to Flow mediated dilatation?
There are no risks to flow mediated dilatation. The procedure is very safe and has been used around the world in a large number of children and adults including children and adults with diabetes.

During the first part of the test, when the blood pressure cuff is left up can be uncomfortable although most children have not been bothered by this. The medication GTN is very safe and only remains active for a very short period of time. The side effects of this medication include, brief headaches in some people, light headedness, facial flushing, however this is usually not the case in children. These side effects are rare in children.

5) Are there any risks with Folate or Vitamin B6?
Folate and vitamin B6, all occur naturally in our diets. Folate is a vitamin which is found in leafy green vegetables and many breads and cereals have folate added. Vitamin B6 is found in meat, nuts, grains, fish and green vegetables.
The doses of the vitamins that we have chosen are safe and there have been no reported side effects at these doses. The dose of folate has been used in adults and has been shown to improve blood vessel function.
6) What will be done with the information from the study?
With the results of this study we will compare the vitamin levels from the blood test and compare it with the results of the flow mediated dilatation to see if there is any improvement in blood vessel function.
These results will be published in medical journals to help other children with type 1 diabetes.
All of the information will remain confidential and no child will be mentioned by name.

7) Do I have to take part in the study?
No, The study is optional.

8) Can I change my mind later if I decide not to participate?
Yes, You can choose to leave the study at any time.

9) Will the study benefit me/my child in any way?
It is not certain whether you/your child will directly benefit from this study in the short term.
Information obtained from the study however will be useful in the long term and may benefit all children with Type 1 diabetes.

10) Has permission been given to perform this study?
Permission for this study has been granted by the Research Ethics Committee at the Women's and Children's Hospital. If you would like to discuss the approval process by which approval is granted for this research or any concern or complaint, please contact the Secretary of the Committee, Ms Brenda Penny. Ph 81616521.

Funding for the study has come from the Juvenile Diabetes Research Foundation and Channel 7.

11) What if I have any other questions?
Any further questions will be answered by Dr Karen MacKenzie, Endocrinology and Diabetes Fellow at the Women's and Children's Hospital. Ph 81616403 or 81616402 (Secretary- Julie). Associate Professor Jenny Couper may also be contacted. Ph 81616242 or 81617000 + pager 4127.
Appendix 3. Budget

1) **Salaries**

**Ultrasonographers:**

Paid independently from hospital commitments. The work is supplementary to routine service and paid at 1.5 time. Salary on costs are not relevant.

**Short term study:**

- 45 patients:
  - 2 patients per day = 7 hours
  - Subsequent visits, 2 x 1 hour
  - $45/2 \times 7 = 157 \text{hours}$
  - $45 \times 2 = 90 \text{hours}$

- Total short term study: $157 + 90 = 247 \text{hours}$

**Long term study:**

- 75 patients:
  - 3 x 1 hour visits
  - $75 \times 3 = 225 \text{hours}$

- Total long term study: $247 + 225 = 472 \text{hours}$

- 472 hours @ $32\text{per hour}$: $15,104.00$

My salary over two years is being met by The Juvenile Diabetes Research Foundation Fellowship.

$50,000.00$
2) **Laboratory Assays**

<table>
<thead>
<tr>
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<th>Direct</th>
<th>Total Cost</th>
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<td>$ 41.00</td>
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<tr>
<td>Folate</td>
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<td>$ 23.35</td>
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<tr>
<td>B12</td>
<td>$ 14.20</td>
<td>$ 40.60</td>
<td>$ 30.20</td>
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<tr>
<td>B6</td>
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<tr>
<td>Homocysteine</td>
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<td>$ 24.35</td>
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<tr>
<td>Hs-CRP</td>
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<td><strong>subtotal</strong></td>
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<td><strong>$ 202.75</strong></td>
<td><strong>$ 151.70</strong></td>
<td><strong>$ 113.90</strong></td>
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<td>VWF</td>
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<td><strong>subtotal</strong></td>
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<td><strong>$ 221.70</strong></td>
<td><strong>$ 166.40</strong></td>
<td><strong>$ 203.40</strong></td>
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It was agreed that we would pay 80% of the Medicare fee.

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<tr>
<th>Test</th>
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<td>80% of MBS fee</td>
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<tr>
<td>MTHFR Genotype</td>
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<td>To be done as part of other research – no cost incurred directly</td>
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<td>Serum MTHF</td>
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<td>Serum VitB12</td>
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<td>Homocysteine</td>
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<td>VWF</td>
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From the above quotation and previous experience with vWF, it was decided to not perform this assay.
The Effect of Folate and Vitamin B6 on Endothelial Function in Children with Type 1 Diabetes

Dr Karen E MacKenzie

<table>
<thead>
<tr>
<th>Test</th>
<th>Price per individual test</th>
<th>Total price per test per patient. Acute Study</th>
<th>Total price per test per patient. 8 Week Study</th>
<th>Total price (120 patients)</th>
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<td>$32.80</td>
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3) Funding Sources

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<td>Juvenile Diabetes Research Foundation Fellowship</td>
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</table>
The Effect of Folate and Vitamin B6 on Endothelial Function in Children with Type 1 Diabetes

Dr Karen E MacKenzie
References


Craig, M., N. Howard, et al. (2003). Female gender bias and high incidence of type 1 diabetes in NSW. Australasian Paediatric Endocrine Group Annual Scientific Meeting, Melbourne.


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Wiltshire, E., University of Otago.


