Adaptations to 12 weeks of walking in women aged 65-74 years with type 2 diabetes

Adaptations to 12 Weeks of Walking in Women Aged 65 – 74 Years with Type 2 Diabetes

Michael James Simmonds

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School of Physiotherapy and Exercise Science

Griffith Health

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Correspondence

Michael J. Simmonds

School of Physiotherapy and Exercise Science

Gold Coast campus Griffith University Queensland 4222

AUSTRALIA

Telephone: + 61 424 113 284

Facsimile: + 61 7 5552 8674

Email: michaeljsimmonds@hotmail.com

Declaration

This work has not been previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

……

Michael J. Simmonds

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Preface

"The easier the exercise is to maintain, the more likely it is that people will do it. Therefore, mild to moderate exercise performed regularly will be of more benefit, than short bursts of high intensity exercise, which would be difficult to maintain over long periods."

Thomas D, Elliot EJ, and Naughton GA.

Exercise for type 2 diabetes mellitus

Cochrane Database of Systematic Reviews, 2006

I will open this thesis with a brief thought experiment that requires one simple rule.

Rule one: humans *are* rational creatures.

If this rule applied in a hypothetical world similar to ours, an important assumption could be derived without much more information. Given that these 'other worldly' humans were rational, upon the discovery of a new piece of information – let's call this 'research' – the population would (here's the assumption) systematically investigate the newly acquired information and subsequently determine whether to adopt the implications of the said research based on a logical cost-benefit analysis. *Does the benefit likely to be derived from the findings of the research outweigh the cost likely to be incurred from implementation?*

In this ‘other world’, researchers discovered a drug that simultaneously improved cardiac and vascular health, reduced adiposity, improved quality of life, helped manage glycaemic control, improved cholesterol and lipid profiles, increased bone-mineral density, had possible anti-ageing and anti-cancer effects, and practically improved nearly every aspect of health… beneficial? You bet! Consequently, the new drug was coined the ‘golden bullet’. Perhaps best of all, the golden bullet was a molecule that could be synthesised for next-to-nothing… clearly low cost! The conclusion of this thought experiment: was this hypothetical drug adopted by our *rational* experimental humans?

Would this drug be adopted in *our* world?

And so my research into exercise prescription in type 2 diabetes begins. It would border upon lunacy to suggest that it is unknown whether exercise is beneficial in the context of chronic disease – in many ways, exercise *is* the ‘golden bullet’. But why exercise participation and adherence rates remain so low, particularly for individuals with type 2 diabetes, is puzzling. The cost-benefit analysis clearly favours exercise training for nearly every one of the six billion individuals on this planet – doesn’t it?

Humans are not always rational.

In contrast, the Cochrane Review authors cited above suggested that “*The easier the exercise is to maintain, the more likely it is that people will do it”*. This statement indicated to me that a portion of the issues to be addressed in exercise-based research for type 2 diabetes had more to do with discovering the ‘easiest’ amount of exercise that

would be beneficial, not so much whether exercise *per se* is beneficial. Thus, when I began this work, my intention was simple: for individuals with type 2 diabetes, find the 'optimal' dose of exercise that improves the primary health outcomes of this metabolic disorder.

It is not the purpose of this preface to cover the 'who', 'how' and 'why' of this thesis; the answers to such questions are covered in subsequent chapters. I can safely inform the reader at this point, however, that determining the 'optimal' dose of exercise is not straightforward, particularly within the context of a metabolic disorder that has a large lifestyle component (aka influenced by environmental risk factors).

What is the 'cost' of exercise?

While important discoveries were made during the present studies, I also found that as many physical systems (such as electricity) flow along the paths of least resistance, many humans also choose the 'path of least lifestyle interference' when faced with the choice between pharmacological or lifestyle interventions.

The following work documents the journey that enabled me to appreciate, for the first time, the complexity of type 2 diabetes.

This thesis is structured as six chapters. Chapter One provides an overview of the health burden associated with type 2 diabetes and summarises several key areas that may be targeted by exercise interventions designed to manage type 2 diabetes. Given the recent understanding that the optimal management of type 2 diabetes may not necessarily be

realised if glycaemic control is the sole primary focus, the general aim of the present thesis was to implement exercise training to mediate changes in physiological markers that are known to reflect the complications/comorbidities of type 2 diabetes. Chapters Two, Three and Four are distinct bodies of work that subsequently address the general aim of this thesis through experimental research. The purpose of Chapter Five is to summarise the results presented in the various experimental studies and provide a synthesised interpretation of the findings. Finally, Chapter 6 provides an in-depth description and justification for the use of each method described in the experimental chapters. Chapter 6 was written with the intention that any reader should be able to replicate the research described within this thesis and gain an appreciation for the technical aspects of this work.

Abstract

The recent premature termination of the intensive glucose-lowering cohort of a major National Institute of Health study into diabetes control, due to increased mortality, has renewed interest in the primary goal(s) of type 2 diabetes management. There is accumulating evidence that improved prevention and management of the associated complications of type 2 diabetes (e.g., cardiovascular diseases; CVD) should be specifically considered rather than solely implementing glycaemic control, particularly for older individuals. The principle aim of this thesis was therefore to investigate the interactions between various known markers of CVD in older women with type 2 diabetes, and subsequently implement an individualised 12-wk walking intervention with the primary goal of improving those markers of CVD. The subjects of the present studies were all 65-74 yr old women with uncomplicated type 2 diabetes that were able to walk unaided and were not using medications known to interfere with any dependent variables. While a control group of non-diabetic women aged 65-74 yr was recruited for comparative purposes in the acute assessments in Study One, the subjects with type 2 diabetes acted as their own control in Studies Two and Three by visiting the laboratory for baseline assessments over a 6-wk lead-in period prior to exercise training. Subjects were randomly allocated into two groups where exercise frequency and session duration were manipulated (Group 1: 2 x 60 min·wk^{-1} or Group 2: 4 x 30 min·wk^{-1}), but intensity and accumulated weekly duration of exercise were consistent between groups (100% gas-exchange threshold; 120 min·wk^{-1}).

The purpose of Study One was to investigate whether haemorheological parameters were related to heart rate variability (HRV) in older women with type 2 diabetes. Study One demonstrated that significant relationships exist between impaired haemorheology and reduced short-term (5 min) HRV in 20 women aged 65-74 yr with type 2 diabetes, but not in 20 age-matched healthy women. Specifically, red blood cell (RBC) aggregation and RBC aggregability were significantly and positively related with low frequency (LF) power of HRV. Moreover, RBC aggregation and RBC aggregability

were significantly and positively associated with increased LF to high frequency (HF) power ratio (LF:HF ratio). The shear stress required for half-maximal elongation index ($SS_{1/2}$) – an important index of RBC deformability – was significantly and negatively related to the variability of cardiac intervals (SDNN). Given that these findings were only observed in older women with type 2 diabetes, but not healthy controls, suggested a shared pathophysiology of impaired haemorheology and HRV in type 2 diabetes.

The purpose of Study Two was to observe the changes in cardiovascular conditioning, haemorheology, and heart rate variability following 12 wk of varied-dose walking in older women with type 2 diabetes. Twelve weeks of exercise training significantly improved peak oxygen uptake, time to exhaustion, and the gas-exchange threshold ($p < 0.05$) compared with baseline values measured during the 6-wk lead-in (i.e., control) period, independent of exercise group. Exercise training significantly reduced RBC aggregation, RBC aggregability and RBC deformability ($p < 0.05$) compared with the control period for both groups. No change in HRV was observed for Group 1, whereas several key indicators of HRV were significantly improved in Group 2 ($p < 0.05$) compared with the control period. These findings were important given that no previous study had reported decreased RBC aggregation following an exercise-only study in type 2 diabetes. Moreover, Study Two demonstrated for the first time that exercise had the capacity to increase RBC deformability for individuals with type 2 diabetes. That HRV only improved for Group 2 suggested that cardiac autonomic modulation, particularly parasympathetic, may be sensitive to the frequency of exercise training.

Study Three investigated whether 12 wk of well-controlled and regular exercise training influenced the ratio of anti- to pro-inflammatory markers in older women with type 2 diabetes. It was unclear prior to this study whether an exercise-only intervention had the capacity to improve the anti- to pro-inflammatory ratio of individuals with type 2 diabetes. Twelve weeks of regular treadmill walking that significantly increased peak oxygen uptake ($p = 0.01$) and time to exhaustion ($p < 0.001$) did not elicit changes in body mass, waist to hip ratio, or glycaemic control. Regular walking did induce ~8%

decreased systolic and diastolic blood pressures ($p < 0.01$), a 9.6% reduction in cholesterol to high-density lipoprotein (HDL) ratio ($p = 0.01$), and significantly increased HDL concentration ($p = 0.01$). While 12 wk of regular walking did not alter plasma concentrations of interleukin-6 (IL-6), tumour necrosis factor-α, or C-reactive protein, plasma interleukin-10 (IL-10) concentration increased by 41% ($p < 0.04$). Consequently, the ratio of IL-10 to IL-6 increased by 54% ($p = 0.01$). Given that no change in glycaemic control or body mass/body mass index was observed, it is suggested that the improved inflammatory profile following exercise training was independent of reduced hyperglycaemia and/or adiposity.

The findings presented in this thesis confirm that older women with type 2 diabetes presented with significantly impaired haemorheology, reduced HRV, decreased cardiovascular conditioning, impaired glycaemic control, dyslipidaemia, and altered inflammatory cytokine profile, when compared with healthy age-matched controls or expected values. Important findings of the present thesis, however, suggest that many of the adverse changes that occur due to type 2 diabetes may be reversed with exercise training. Indeed, the capacity for a relatively short duration of weekly walking to improve multiple risk factors of CVD is important in the context of type 2 diabetes, where the uptake and compliance of regular exercise is notoriously low. It is concluded that regular walking, even when performed as infrequently as twice weekly, should form a key component for the management of type 2 diabetes in older women.

Publications arising from this thesis

Simmonds MJ, Sabapathy S, Marshall-Gradisnik SM, Christy RM, Haseler LJ, Gass GC, and Minahan CL. Heart rate variability is related to parameters of haemorheology in older women with type 2 diabetes. *Clinical Hemorheology and Microcirculation* (2011) 46 (1) 57-68.

Simmonds MJ, Minahan CL, Serre KR, Gass GC, Marshall-Gradisnik SM, Haseler LJ, Sabapathy S. Preliminary findings in the heart rate variability and hemorheology responses to varied frequency and duration of walking in women 65-74 yr with type 2 diabetes. *In press: Clinical Hemorheology and Microcirculation.*

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Simmonds MJ, Sabapathy S, Serre KR, Marshall-Gradisnik SM, Gass GC, Haseler LJ, Minahan CL. Increased interleukin-10 following regular walking in women 65-74 yr with type 2 diabetes. *Under review*.

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Simmonds MJ, Baskurt OK, Meiselman HJ, and Marshall-Gradisnik SM. A comparison of capillary and venous blood sampling methods for the use in haemorheology studies. *Clinical Hemorheology and Microcirculation* (2011) 47 (2) 111-119.

Abstracts presented to scientific conferences

Simmonds MJ, Minahan CL, and Sabapathy S. Haemorheology and heart rate variability in women with type 2 diabetes: is there a relationship? *Gold Coast Health and Medical Research Conference*. Gold Coast, Australia. December 2008.

Simmonds MJ, Sabapathy S, Haseler LJ, Marshall-Gradisnik SM, Gass GC, and Minahan CL. Parameters of Haemorheology and Heart Rate Variability are Related in Older Women with Type 2 Diabetes. *Human Movement Studies Postgraduate Conference*. Marine Research Station, North Stradbroke Island, Australia. April 2009.

Simmonds MJ, Sabapathy S, Haseler LJ, Marshall-Gradisnik SM, Gass GC, and Minahan CL. Impaired haemorheology is related to reduced heart rate variability in older women with type 2 diabetes. *National Heart Foundation Conference*. Brisbane, Australia. May 2009.

Simmonds MJ, Minahan CL, Gass GC, Haseler LJ, and Sabapathy S. Impaired Heart Rate Variability in Type 2 Diabetes: Roles of Major Cardiovascular Disease Risk Factors. *The American College of Sports Medicine 56th Annual Meeting*. Seattle, USA. May 2009.

Simmonds MJ, Sabapathy S, Haseler LJ, Marshall-Gradisnik SM, Serre K, Gass GC, Minahan CL. Cardiovascular disease risk factor responses to 12 wk of varied-dose walking in older women with type 2 diabetes. *Gold Coast Health and Medical Research Conference*. Gold Coast, Australia. December 2009.

Simmonds MJ, Minahan CL, Serre KR, Gass GC, Marshall-Gradisnik SM, Haseler LJ, Sabapathy S. Varied walking dose in women with type 2 diabetes: heart rate variability and hemorheology responses. *The American College of Sports Medicine 58th Annual Meeting* and *Second World Congress on Exercise is Medicine*. Denver, USA. May 2011.

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Chapter 1

Overview

A review of the literature and statement of purpose

1.1 BACKGROUND

Type 2 diabetes is a group of metabolic disorders characterised by a common manifestation: chronic elevations in blood glucose (hyperglycaemia). Chronic hyperglycaemia principally occurs in type 2 diabetes due to peripheral tissue insulin resistance and/or dysfunctional insulin secretion by pancreatic beta (β) cells. The level of hyperglycaemia observed in people with type 2 diabetes typically coincides with disease duration and severity. Chronic hyperglycaemia has many deleterious effects on the body that result in widespread organ damage and dysfunction, significantly increasing the morbidity and mortality observed in individuals with type 2 diabetes. The pathophysiology underlying the development of type 2 diabetes remains incompletely understood; however, it appears that both lifestyle and genetic factors influence an individual's susceptibility. Currently there is no cure for type 2 diabetes, thus treatment strategies aim to reduce disease progression and complications.

Significance and burden of type 2 diabetes

It is suggested that type 2 diabetes will become one of most significant threats to human health in the 21st century (Zimmet, 2000). Indeed, between 2010 and 2025, the number of individuals with diabetes is expected to double to 300 million worldwide (Zimmet et al., 2001). Given that the number of individuals with type 1 diabetes is remaining steady (Weets et al. 2002) or only marginally increasing (Bruno et al., 2001), new cases are principally attributed to type 2 diabetes. The accuracy of estimating the incidence of type 2 diabetes is questionable, however, as repeated blood measurements are necessary for diagnosis, but are inaccessible to many individuals. Consequently, most estimates of type 2 diabetes prevalence are based on self-reports that are notoriously inaccurate (Dunstan et al., 2002). Indeed, it is suggested that for every individual that self-reports type 2 diabetes, there is another that has the disease but remains undiagnosed (McCarty et al., 1996). Nevertheless, the escalating growth of type 2 diabetes observed worldwide has become a significant social, economic and health problem.

The DiabCost report characterised the financial costs that were directly (e.g., medications, hospitalisations etc) and indirectly (e.g., reduced productivity, loss of wages etc) attributable to type 2 diabetes in Australia (Colagiuri et al., 2003). The mean annual cost per individual with type 2 diabetes was $5360, of which $5325 was directly attributed to costs associated with managing the disease. Over 30% of the direct health costs were due to hospitalisation, ambulatory services and hypoglycaemic medications, whereas 26% of direct health costs were spent on managing comorbidities (e.g., hypertension, dyslipidaemia). The DiabCost report confirmed that complications associated with type 2 diabetes significantly increased the cost of disease management; individuals with type 2 diabetes without complications incurred $4025 of direct health costs per annum, whereas individuals with both microvascular and macrovascular complications incurred $9625 per annum. In addition to the costs attributed to managing type 2 diabetes, each individual with type 2 diabetes received an average of $5540 in benefits from the Australian Government. Figure 1.1 illustrates the costs

associated with disease management and the associated government benefits received per individual, with respect to the major complications of type 2 diabetes.

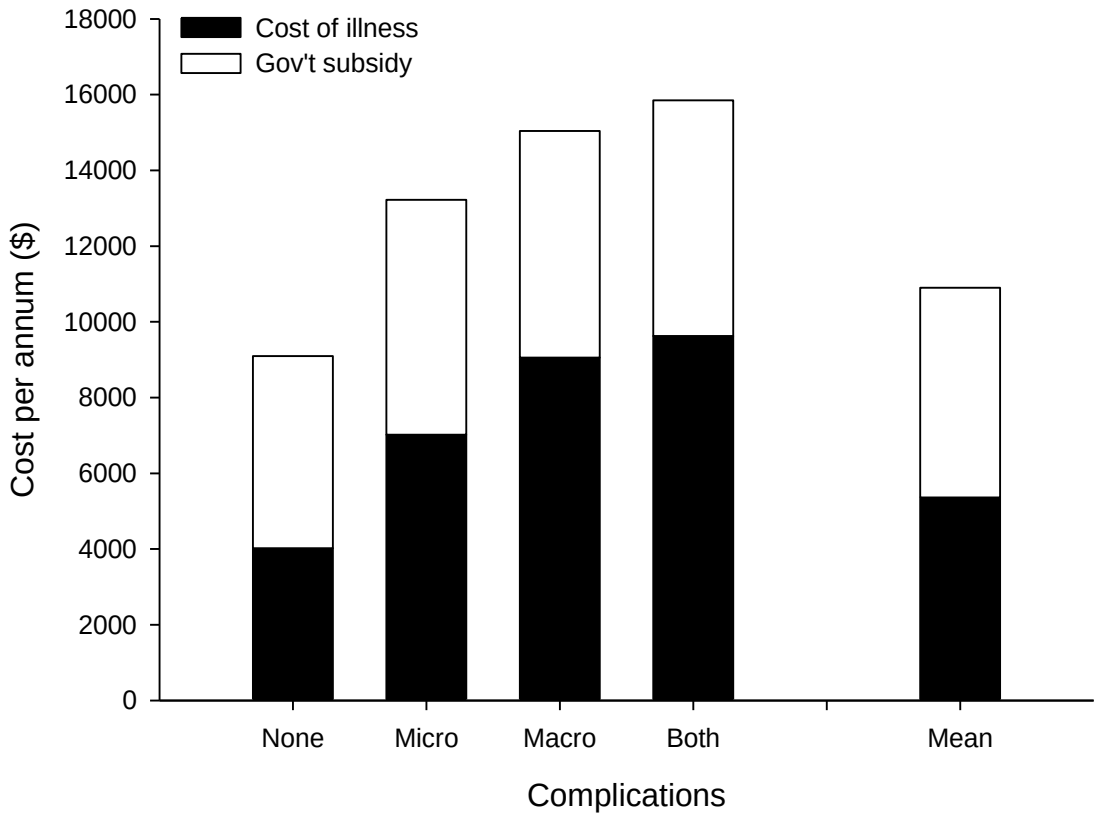

Figure 1.1. The costs associated with disease management (black) and Australian Government benefits received (white) per individual with type 2 diabetes. Note the increase in cost and Government benefits due to severity of complications. Gov't: Australian Government benefits. None: uncomplicated type 2 diabetes. Micro: microvascular. Macro: macrovascular. Both: individuals with microvascular and macrovascular complications. Figures produced from data presented in Colaguirui et al., (2003).

It is estimated that type 2 diabetes costs the Australian health budget $3 billion per annum (Colagiuri et al., 2003). Furthermore, significant reduction in quality of life, including impaired mobility and self-care, difficulty in performing daily activities, depression and pain,

is observed for individuals with type 2 diabetes – particularly among the elderly (Redekop et al., 2002, Holmes et al., 2000).

Older women with type 2 diabetes: a critical population

The age-specific prevalence of type 2 diabetes increased for both genders over the last three decades (Australian Institute of Health and Welfare, 2008b); however, women are overrepresented among new type 2 diabetes cases, particularly in the elderly (Dunstan et al., 2002). When the AusDiab Study (Dunstan et al., 2001) results were standardised to the national age profile for 1998, it was found that the prevalence of type 2 diabetes had increased more in women than for men, when compared with 1981 (Glatthaar et al., 1985).

Given that both inadequate weight control and physical inactivity are significant risk factors for the development of type 2 diabetes (Hansen, 1999), the increased incidence of type 2 diabetes in older women might be explained by lifestyle factors. It is reported that the prevalence of obesity peaks for Australian women between the ages of 55-74 yr, and participation rates in sports and physical recreation are lowest among women aged 65 yr and over (Australian Bureau of Statistics, 2006). Consequently, the highest incidence of type 2 diabetes is observed in women aged 65-74 yr (Australian Institute of Health and Welfare, 2008a). Given over half of all hospitalisations in persons over 65 yr are associated with type 2 diabetes (Australian Institute of Health and Welfare, 2008b), it might be that no other subset of the Australian population is more affected by type 2 diabetes than women aged 65-74 yr.

These findings highlight a significant challenge inherent in the ageing Australian population: the number of females aged 65-74 yr is predicted to rise from the present 682,000 to 1.28 million by 2021 – a relative increase greater than any other Australian population group (Australian Institute of Health and Welfare, 2004). Thus, understanding the mechanisms

responsible for type 2 diabetes might improve strategies for the reduction of burdens associated with type 2 diabetes.

Clinical presentation and diagnosis of type 2 diabetes

An individual with undiagnosed type 2 diabetes may present with random (or 'casual') blood glucose concentration greater than 11.1 $mmol \cdot L^{-1}$. Until recently, the American Diabetes Association diagnostic criteria required that elevated random blood glucose measurements be confirmed on a subsequent day using: A. *fasting* blood glucose measurement ($\geq$ 7.0 $mmol \cdot L^{-1}$); B. oral glucose tolerance test (2-h postload value $\geq$ 11.1 $mmol \cdot L^{-1}$); or C. symptoms* with *Random* blood glucose measurement ($\geq$ 11.1 $mmol \cdot L^{-1}$) (American Diabetes Association, 2009). However, the guidelines published in January 2010 acknowledge that glycated haemoglobin (HbA_{1c}) is at least as effective for defining hyperglycaemia and has superior technical attributes (i.e., less instability and biologic variability), when compared to fasting glucose (American Diabetes Association, 2010). Moreover, HbA_{1c} is a chronic marker of hyperglycaemia, reflecting the mean blood glucose concentration over the previous 2 to 3 mo, which is more clinically meaningful than acute markers such as fasting blood glucose concentration (International Expert Committee, 2009). Consequently, for the first time the American Diabetes Association included HbA_{1c} values $\geq$ 6.5% as a diagnostic threshold for diabetes in 2010 (American Diabetes Association, 2010).

Typically, individuals with type 2 diabetes present overweight or obese. Due to the slow progressive nature of type 2 diabetes, the associated hyperglycaemia may go undetected for years, generally because the slight elevations in blood glucose are asymptomatic. Despite the lack of symptoms, prediabetic individuals are at increased risk of developing the

* Classic symptoms of type 2 diabetes include polyuria (excessive production of urine), polydispia (excessive thirst), and unexplained weight loss.

complications associated with type 2 diabetes (Roman and Hancu, 2009). If aggressive preventative action (i.e., lifestyle intervention) is taken before the onset of overt disease, significant improvements in insulin resistance and glycaemic control are possible (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). Indeed, significant weight loss may even avert the onset of type 2 diabetes in the prediabetic individual (Khaodhiar et al., 2009). Nevertheless, a significant number of prediabetic individuals will continue to progress toward overt disease, with obesity, ageing and physical inactivity being significant risk factors. Women with a positive history for gestational diabetes also present with increased risk of developing type 2 diabetes.

Risk factors for the development of type 2 diabetes

Type 2 diabetes typically manifests later in life and is most likely the product of adverse interactions between environmental and genetic factors. The crucial role of genetic susceptibility is emphasised by the concordance for type 2 diabetes among monozygotic twins, which approaches 100% (Adeghate et al., 2006). Nevertheless, the contribution of environmental factors to the development of type 2 diabetes is probably better understood than the genetic components due to the heterogeneous nature of this metabolic disorder. Epidemiological research has indicated that the major environmental risk factors for type 2 diabetes include poor diet and physical inactivity, along with other 'mixed' factors such as obesity, dyslipidaemia and hypertension (Hansen, 1999). Obesity, dyslipidaemia and hypertension may be considered mixed factors given the genetic influences for each respective risk factor (Poulsen et al., 2001). The risk factors acknowledged to contribute to the development of type 2 diabetes are listed in Figure 1.2.

Much of the literature pertaining to the genetic influences of type 2 diabetes involve epidemiological studies of twins (Condon et al., 2008, Medici et al., 1999, Poulsen et al., 1999, Poulsen et al., 2001, Vaag et al., 1995). Despite the obvious merit of such studies, results should be interpreted with caution given that the shared environment each pair is

exposed to during crucial periods of development. For example, it is established that the intrauterine environment influences an individual's susceptibility for certain diseases that may develop during later adulthood, including type 2 diabetes (Pinney and Simmons, 2009, Simmons, 2009). The classic follow-up study of individuals conceived during the Dutch famine of 1944-45 highlights the significance of the intrauterine environment on health

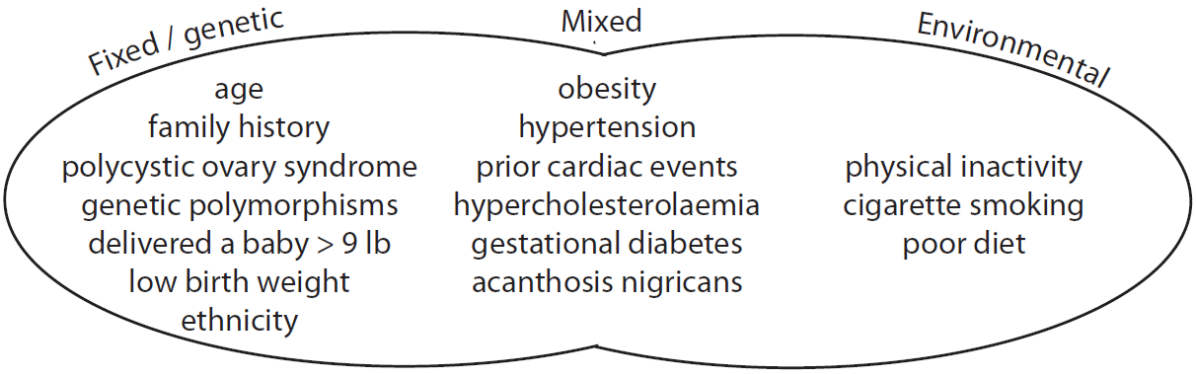

Figure 1.2. Risk factors contributing to the development of type 2 diabetes categorised according to modifiability. Original figure.

during adulthood; it was found that body mass index of women at age 50 yr was significantly higher than matched women born either one-year before or after the famine (Ravelli et al., 1999). Moreover, it was reported that infants born during the Dutch famine had significantly greater rates of impaired glucose tolerance than matched individuals by age 50 yr (Ravelli et al., 1998). While it is disputed whether Ravelli et al., (1998) defined their hypothesis *a priori* or retrospectively (Lumey, 2001), the data signify the potential importance of the intrauterine environment on subsequent disease susceptibility. Consequently, such findings demonstrate the limitations associated with the use of twin studies to ascertain the role of genetic factors contributing to the development of type 2 diabetes – perhaps the intrauterine environment is an *environmental* risk factor?

Physical inactivity and poor diet leading to obesity may be the principal environmental risk factors for the development of type 2 diabetes. Venables and Jeukendrup (2009) reported that physical inactivity constitutes one of the greatest influences for the development of type 2 diabetes. Indeed, it is reported that exercise reduces the risk of developing type 2 diabetes, slows the progression from prediabetes to overt disease, and reduces mortality due to type 2 diabetes (National Health and Medical Research Council, 2001). Additionally, it is suggested that weight loss should be a principal focus of interventions aiming to prevent type 2 diabetes, as every kilogram of weight loss achieved is associated with a decreased risk of type 2 diabetes by 16% (Hamman et al., 2006). It should be noted that while overweight/obesity causes increased risk for developing type 2 diabetes, individuals with type 2 diabetes who are not obese by traditional criteria may present with increased body fat (or fat cell size) in the abdominal region compared with non-diabetic controls (Kissebah et al., 1982).

Aetiology of type 2 diabetes

The sequence of events leading to overt type 2 diabetes may vary markedly between individuals, thus it is difficult to characterise the development of this metabolic disorder. Nevertheless, persistent and chronic hyperglycaemia – the hallmark sign of type 2 diabetes – often remains undetected until the progression of overt disease. It is established that an accumulation of impairments to the secretion of insulin and the subsequent metabolic responses precede the onset of type 2 diabetes. Hepatic glucose production also increases, compounding the hyperglycaemia observed in type 2 diabetes (Lin and Sun, 2010). Whether dysfunctional insulin secretion precedes insulin resistance remains controversial.

Insulin resistance was previously considered the principal contributor to the development of type 2 diabetes. Himsworth (1936) was the first to report that a significant number of individuals with diabetes were ‘insulin insensitive’. Yalow and Berson (1960) subsequently reported that patients with adult onset (i.e., type 2) diabetes demonstrated a hyperinsulinaemic response to an oral glucose tolerance test compared with nondiabetic

controls, suggestive of insulin resistance. Ginsberg et al., (1975), using eugylcaemic clamp methods, reported that the steady-state plasma glucose concentration of subjects with type 2 diabetes was more than three times greater than nondiabetic controls. Given that the corresponding steady-state insulin concentrations were similar between groups, it was concluded that subjects with type 2 diabetes were resistant to insulin. In light of previous findings demonstrating that individuals with type 2 diabetes had impaired insulin response to a glucose challenge (Karam et al., 1965, Reaven and Miller, 1968), Ginsberg et al., (1975) acknowledged that hyperglycaemia in type 2 diabetes may occur due to impaired insulin secretion, but was more likely caused by increased insulin resistance. Consequently, the contribution of impaired insulin secretion to the aetiology of type 2 diabetes was diminished and the focus on insulin resistance intensified.

Golay et al., (1986) investigated the degree of altered insulin-stimulated glucose uptake in subjects with impaired glucose tolerance, type 2 diabetes, and normal controls. The authors reported that impaired glucose tolerance and type 2 diabetes patients demonstrated impaired glucose uptake compared with control. Interestingly, all subjects with impaired glucose tolerance and type 2 diabetes presented with the same degree of reduced glucose uptake, irrespective of whether fasting glucose concentration was 5.5 or 13.75 $mmol \cdot L^{-1}$. The authors concluded that insulin resistance alone could not account for the severity of hyperglycaemia in impaired glucose tolerance or type 2 diabetes patients. It appeared that normal blood glucose concentrations were maintained by increased secretions of insulin. That is, even in subjects with severe insulin resistance, glucose tolerance may have been within normal ranges if the pancreas was able to secrete enough insulin. Thus, it is the ability of the β-cell to modify insulin secretions in response to changes in glucose concentration that may be most important in the development of type 2 diabetes.

Recent advances in human genetics have provided a considerably more complex and developed understanding of the aetiology of type 2 diabetes. Currently, it is accepted that many of the early-onset cases of type 2 diabetes (sometimes termed ‘maturity onset diabetes of the young’) may be caused by a defect in a single gene (monogenic). While some cases of

these rarer forms of diabetes have been associated with monogenic defects involved with insulin action (i.e., insulin resistance), most are attributed to defects in a gene associated with insulin secretion (Gill-Carey and Hattersley, 2007). The common form of type 2 diabetes, however, is clearly polygenic with an individual likely to have multiple polymorphisms that in isolation would not cause overt disease, but collectively predispose the individual to type 2 diabetes. At least 19 alleles have been identified that can be attributed to the development of type 2 diabetes, with the majority of alleles reported to have a significant influence on insulin secretion (McCarthy and Zeggini, 2009).

The current view on the development of type 2 diabetes involves polymorphisms in several genes, which may be 'triggered' by environmental factors. It is likely that a reduction in the ability of the pancreatic β-cells to secrete insulin leads to chronic accumulations in blood glucose concentration, concurrently reducing tissue sensitivity to insulin.

Pathways of glucose uptake

Blood glucose is tightly regulated in the healthy 'normoglycaemic' individual. Following a meal, blood glucose levels rise quite rapidly and reach peak values within 30 min (Brand-Miller et al., 2008). To maintain normal blood glucose concentration, glucose is actively transported out of the blood and into peripheral tissues, of which skeletal muscle represents the principal target (Perriott et al., 2001). While the transport of glucose into cells may occur slowly via 'passive' means, rapid transport is achieved via a member of the family of membrane proteins known as glucose transporters (GLUT). In the basal state, the GLUT1 isoform is responsible for glucose transport into cells. In response to pancreatic secretions of insulin, however, the GLUT4 isoform is responsible for the uptake of glucose in skeletal muscle and adipose tissue. The expression of GLUT4 at the cell surface is limited prior to insulin stimulation; rather, GLUT4 is predominately located in intracellular storage compartments (Roy and Marette, 1996). Thus, for glucose uptake to increase in response to elevations of blood glucose, translocation of GLUT4 from the intracellular compartments to

the cell surface is necessary. GLUT4 translocation may be triggered by two distinct methods – stimulation by either insulin or muscle contractions (Goodyear and Kahn, 1998).

The signalling mechanisms that occur between insulin/contraction stimulation and the eventual translocation of GLUT4 to the cell surface have not been fully elucidated. It is believed, however, that insulin and muscle contractions stimulate GLUT4 translocation by two interconnected, yet largely distinct pathways. The pancreatic β-cells constantly produce insulin despite blood glucose concentration and is stored in vacuoles (Lin and Sun, 2010). In response to post-prandial elevations in blood glucose concentration being detected by the pancreas, insulin is secreted into the blood stream (Freychet, 1990). Insulin is then circulated to the target tissue where insulin phosphorylates the membrane-bound insulin receptor, in turn activating the insulin receptor substrate (IRS) 1 and 2. The IRS proteins bind a number of downstream molecules, perhaps the most important being p85 – the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (Shepherd et al., 1998). PI3K signals several proteins that ultimately lead to an increase in GLUT4 translocation to the cell surface (Simpson et al., 2001), including Akt substrate of 160 kDa (AS160, or TBC1D4) (Howlett et al., 2007, Kane et al., 2002). The subsequent increase of GLUT4 expression at the muscle membrane results in a significant increase of blood glucose uptake into the muscle (Zierath et al., 1996).

Alternatively, exercise may stimulate GLUT4 translocation from intracellular compartments to the skeletal muscle membrane in the absence of insulin. The suggestion that insulin and exercise mediate glucose uptake by distinct pathways is based on evidence that muscle contractions have no effect on the insulin receptor, IRS or PI3K (Goodyear et al., 1995, Treadway et al., 1989), which are critical proteins involved in insulin-mediated glucose uptake. Moreover, muscle-specific knockout of the insulin receptor has no effect on contraction-mediated glucose uptake (Wojtaszewski et al., 1999). While the exact signalling pathway responsible for contraction-stimulated GLUT4 translocation remains poorly described, there is evidence that translocation may occur due to proteins sensitive to changes in intracellular calcium levels (e.g. Ca^{2+}/calmodulin-dependent protein kinase) (Witczak et al., 2007) or AMP:ATP ratio (e.g. AMP-activated protein kinase) (Kemp et al., 1999). It may

also be that protein kinase C or TBC1D4 may be the chief proteins involved in contraction mediated GLUT4 translocation; however, TBC1D4 is of particular interest as it is the most distal protein common to both insulin and contraction mediated glucose uptake (Cartee and Wojtaszewski, 2007).

While insulin-stimulated glucose uptake is reduced in type 2 diabetes due to impaired GLUT4 translocation, the precise intracellular mechanisms responsible remain incompletely understood. However, animal models of type 2 diabetes have been used to demonstrate that insulin receptor phosphorylation (Saengsirisuwan et al., 2004), IRS-1 protein level (Anai et al., 1998) and phosphorylation (Hevener et al., 2000), and PI3K activity (Saengsirisuwan et al., 2004, Anai et al., 1998) are impaired under basal conditions. Similar results have been observed in insulin-resistant skeletal muscle of humans, where insulin stimulation has been reported to exert reduced insulin receptor phosphorylation, reduced IRS-1 phosphorylation and reduced PI3K activity, compared with non-diabetic controls (Bjornholm et al., 1997). While these results provide some insight into skeletal muscle insulin resistance, it is known that contraction-mediated glucose uptake remains unaltered due to type 2 diabetes and reflects that of a healthy individual (Kennedy et al., 1999).

1.2 COMPLICATIONS ASSOCIATED WITH TYPE 2 DIABETES

Type 2 diabetes is associated with several comorbidities, most notably cardiovascular disease (CVD) and neuropathy, which contribute to increased rates of morbidity and mortality. The primary causes of diabetic comorbidities remain poorly understood; however, chronic hyperglycaemia was considered the principal candidate (Stratton et al., 2000). Recently, increased awareness of the importance of optimal control of blood pressure and cholesterol levels for the reduction in type 2 diabetes complications has emerged (United Kingdom Prospective Diabetes Study, 1998c, Colhoun et al., 2004). The complications associated type 2 diabetes may be broadly categorised as those principally affecting large (macrovascular) or small (microvascular) blood vessels.

Macrovascular complications of type 2 diabetes

The principle complication that presents in type 2 diabetes is CVD. The prevalence of CVD is two-to-four times higher among individuals with type 2 diabetes, when compared with non-diabetic individuals (Buse et al., 2007, Hayes and Kriska, 2008). Haffner and colleagues (1998) monitored the development of myocardial infarction (MI), stroke and CVD-related mortality over 7 yr among individuals with and without type 2 diabetes. Over the 7-yr follow-up period, individuals with type 2 diabetes were significantly more likely to experience MI, stroke and death due to CVD when compared with non-diabetics. Haffner and colleagues (1998) subsequently categorised the individuals within each condition (type 2 diabetes or non-diabetic) into subgroups; those with a positive history of MI and those without a positive history of MI. As expected, a prior positive history of MI was associated with increased risk of CVD complications during the follow-up period for both groups. Perhaps unexpectedly, however, individuals with type 2 diabetes *without* a positive history of MI were just as likely to develop CVD complications as non-diabetics *with* a positive history of MI (Figure 1.3).

The mechanisms underlying the increased incidence of macrovascular complications observed in type 2 diabetes remain incompletely described. It is established, however, that CVD risk factors (e.g., hypertension, dyslipidaemia, obesity, smoking) are overrepresented in individuals with type 2 diabetes, resulting in increased morbidity and mortality, when compared with non-diabetic individuals. Hyperglycaemia, dyslipidaemia and insulin resistance are believed to culminate in alterations to the vascular endothelium, smooth muscle and platelet cells, thereby rendering arteries susceptible to atherosclerosis (Beckman et al., 2002). Atherosclerosis may be caused by age-related impairments to the endothelium that are exacerbated by type 2 diabetes. Type 2 diabetes is associated with an increased expression of adhesion molecules on damaged endothelial cells (Jager et al., 2000, Steiner et al., 1994), increasing the number of circulating monocytes that may subsequently transmigrate the endothelium. Consequently, increased passage of lipids and inflammatory cells occurs across

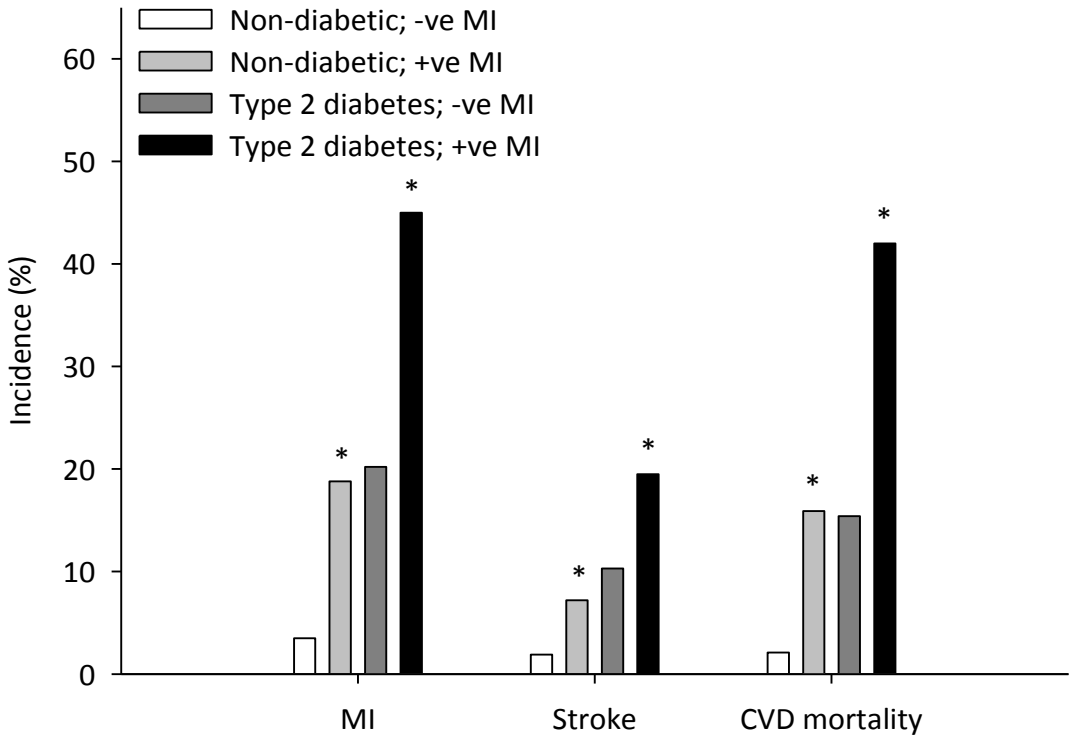

Figure 1.3. Incidence of cardiovascular complications in relation to history of myocardial infarction (MI) among individuals with and without type 2 diabetes. Note the similarity between the second and third columns in each cluster, demonstrating similar incidence of cardiovascular complications for type 2 diabetes without history of MI and non-diabetics with history of MI. * indicates positive history of MI significantly greater than negative history of MI ($p < 0.001$). CVD, cardiovascular disease; +ve/-ve; positive/negative history of MI. Figure produced from data presented in Haffner et al., (1998).

the artery wall. Once inside the arterial wall, the oxidation of low-density lipoproteins and activation of monocytes results in an inflammatory response, including the upregulation of interleukin (IL)-6, C-reactive protein (CRP), and tumour necrosis factor-alpha (TNF-α) (Pedersen, 2006, Petersen and Pedersen, 2005). It is clear that chronic low-grade inflammation has a primary role in the pathogenesis and progression of atherosclerosis and related vascular disorders in type 2 diabetes, given that increased concentrations of inflammatory markers may be observed in apparently healthy individuals that later develop type 2 diabetes (Pradhan et al., 2001, Thorand et al., 2003). Inflammatory markers such as

IL-6, CRP and TNF-α are not only upregulated in response to endothelial damage, but also potentiate the progression of atherosclerosis by activating monocytes/macrophages, enhancing the subsequent inflammatory response and thereby creating a cyclic process of monocyte/macrophage activation and further production of proinflammatory cytokines (Galis et al., 1995). This process leads to smooth muscle proliferation, calcification and ultimately, arterial enlargement or luminal narrowing.

Microvascular complications of type 2 diabetes

Most complications observed in type 2 diabetes may originate due to impairments in the microcirculation. Impaired blood flow, particularly within smaller blood vessels, reduces oxygenation and impairs nutrient delivery to target tissues. Reduced microcirculatory blood flow is implicated in the development of complications of the eye, kidney, and periphery (Mantskava et al., 2006), as well as the two principal complications of type 2 diabetes: nerve (Young et al., 1996) and cardiac-related (Kesmarky et al., 1998) complications.

Diabetic autonomic neuropathy manifests earliest in the longer autonomic nervous system (ANS) nerves. The longest of the ANS nerves is the vagus, responsible for ~75% of total parasympathetic activity (Ziegler, 1999). Given many organs of the body are dually innervated by both the sympathetic and parasympathetic branches of the ANS, it is believed that diminished parasympathetic activity resulting from damage to the vagus nerve might be intimately involved in the widespread organ damage associated with diabetic autonomic neuropathy (Vinik et al., 2003). In the context of type 2 diabetes, imbalances in the autonomic modulation of the myocardium may contribute to the increased prevalence of CVD. The ANS modulates both the electrical and contractile activity of the heart via a balancing act of the sympathetic and parasympathetic branches of the ANS (Sztajzel, 2004). Impairments in autonomic modulation of myocardial electrical activity may be detected by measuring beat-to-beat cardiac cycles. Reductions in beat-to-beat variability (i.e., heart rate variability; HRV) may be the earliest indicator of autonomic neuropathy (Maser and Lenhard,

2005). Beat-to-beat HRV has been used as an indicator of general cardiac health and also a predictor of CVD and sudden cardiac death (Malik et al., 1996). It is reported that individuals with type 2 diabetes demonstrate reduced HRV compared with healthy controls (Figueroa et al., 2007). Furthermore, among type 2 diabetes patients, those with chronic complications appear to have further reduced HRV compared with patients without chronic complications (Sztajzel, 2004).

While vascular function has previously been the primary focus of microvascular complications, there is accumulating evidence that the blood itself may be significantly altered within the context of type 2 diabetes. Blood is a suspension of formed elements (i.e., white/red blood cells, platelets) in a solution of organic molecules, proteins and salts (i.e., plasma) (Baskurt and Meiselman, 2003). The fluidity of blood has a substantial influence on vascular health and is largely determined by its cellular content, the viscosity of plasma, as well as the mechanical characteristics of the red blood cell (RBC).

In the healthy state, RBCs are highly deformable, which improves blood fluidity in both large vessel and microcirculatory conditions (Figure 1.4, Panel C). The high deformability of RBCs enables each cell to elongate into ellipses under increased arterial flow due to an increased shear stress, which causes the RBC to align with the direction of blood flow (Fischer et al., 1978). In the microcirculation, the high deformability of RBCs enables each cell to elongate and pass through capillaries as small as one-quarter of the original diameter of the RBC (Cooke and Lim, 2007). It is important to note that the deformability of RBCs is a reversible process, returning to its original biconcave shape when deforming forces are removed due to the RBC membrane's elasticity (Evans and La Celle, 1975).

Under conditions of low flow rate or stasis of blood, RBCs form clusters known as aggregates that are arranged in linear stacks, termed rouleaux. The process of RBC aggregation is influenced by two opposing forces; attraction promoted by plasma proteins (e.g., fibrinogen) and repulsion induced by the inherent negative charge of RBCs that is compounded by the disaggregating forces generated by blood flow. Two theoretical models

have been used to explain the tendency of RBCs to aggregate. The Bridging Model suggests that RBCs aggregate in response to plasma proteins (and macromolecules) being absorbed on cell membranes, reducing the flow across the cell surface and ultimately overcoming the extrinsic forces of disaggregation (Neu and Meiselman, 2002). Alternatively, the Depletion Model suggests that an *exclusion* of proteins or polymers at the cell membrane, relative to the suspending medium (plasma), causes fluid to move out of the intercellular space and ultimately draw RBCs together to form aggregates (Baumler et al., 1996). Recent evidence suggests that the Depletion Model may best reflect experimental data (Baskurt et al., 2002, Neu and Meiselman, 2002, Fenech et al., 2009). Red blood cell aggregation is a reversible process; once flow rate increases (i.e., increased shear force) sufficiently, RBCs disaggregate, thus allowing for increased blood fluidity.

In chronic disease populations, such as type 2 diabetes and CVD, RBCs become rigid and may also aggregate more readily. Cells may become more rigid due to hypercholesterolaemia, glycation, and glucose-induced oxidation of RBC proteins, which also appears to reduce the cells negative charge, therefore increasing aggregation (Babu and Singh, 2004, Babu, 2009, Schwartz et al., 1991, Shin et al., 2008). The concomitant increases in RBC aggregation and decreased RBC deformability have profound effects on microcirculatory blood flow and oxygen delivery (Figure 1.4, Panels B and D). Aggregation of RBCs draws the cells away from the vessel walls, known as axial migration. Axial migration of RBCs results in a cell-poor layer along the blood vessel wall, which can alter the haematocrit (thus, oxygen delivery) of side branches that are fed by the marginal blood stream (Figure 1.5, panel A). Moreover, blood flow through smaller blood vessels can be significantly reduced as a result of increased aggregation and decreased deformability of RBCs, as RBC aggregates must be dispersed before entering small vessels, and RBCs must substantially deform to pass through capillaries far smaller than the resting RBC diameter (Figure 1.5, panel B). The consequence of impaired haemorheology is significant: oxygen

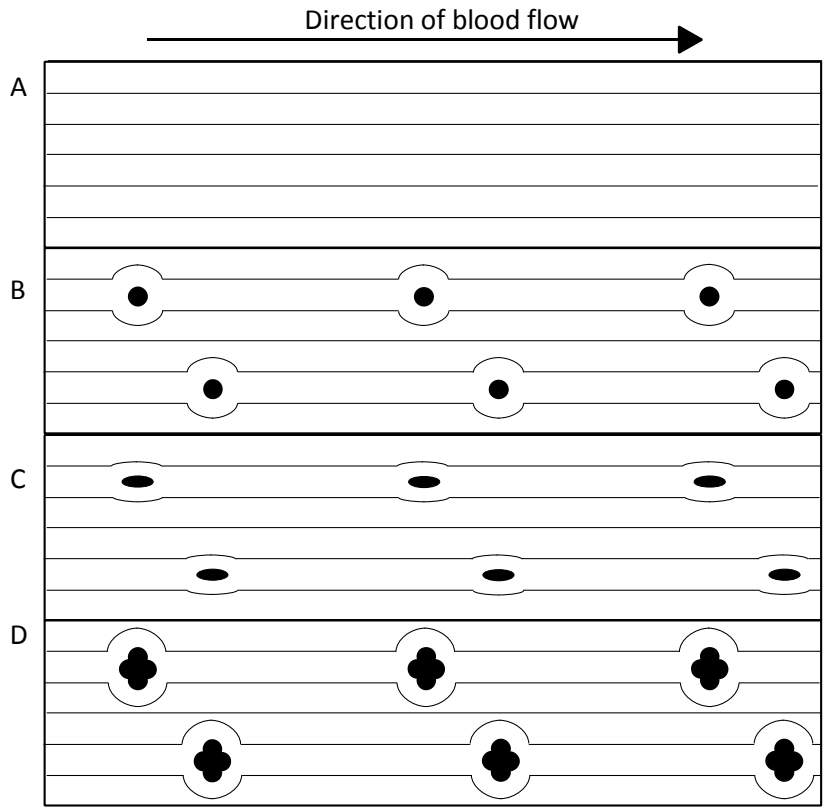

Figure 1.4. Changes in flow streamlines due to red blood cells (RBC) suspended in plasma. Panel A: flow streamlines of RBC-depleted plasma; Panel B: distorted flow streamlines due to RBCs with poor deformability; Panel C: reduced distortion of flow streamlines due to highly deformable RBCs; Panel D: flow streamlines distortion due to RBC aggregation. Adapted from Baskurt and Meiselman, (2003).

and nutrient delivery to various tissues may be altered, contributing to the complications observed in type 2 diabetes and other chronic diseases. Indeed, impairments in haemorheology have been reported for type 2 diabetes patients with various comorbidities, including diabetes-related CVD (Adak et al., 2008), and neuropathy (Husstedt et al., 1997, Young et al., 1996). While impaired haemorheology precedes overt neuropathy (Simpson, 1988), Husstedt and colleagues (1997) reported that increased RBC aggregation reflected the progression of neuropathy over a ~42 mo follow-up period. Impairments in haemorheology may contribute to the development of neuropathy, possibly due to haemorheological

disturbances that lead to decreased microcirculatory blood fluidity (Young et al., 1996, Mantskava et al., 2006).

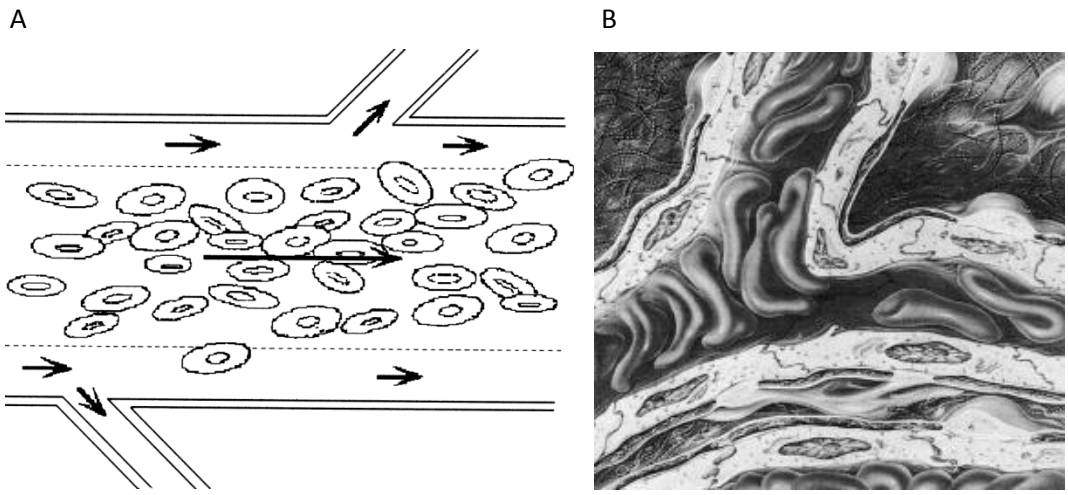

Figure 1.5. Panel A: red blood cell (RBC) aggregation draws cells away from the vascular wall, leading to a cell-poor layer that feeds many marginal blood streams. Panel B: RBCs must deform substantially to enter capillaries that are much smaller than the RBC resting diameter (from Baskurt & Meiselman, 2003).

1.3 STATEGIES FOR THE MANAGEMENT OF TYPE 2 DIABETES

The management of type 2 diabetes has been centred around the regulation of blood glucose concentrations to within normal 'healthy' ranges. Indeed, until very recently the American Diabetes Association recommended that optimal management of type 2 diabetes included reducing glycated haemoglobin (HbA_{1c}) to less than 7% for the prevention of diabetic complications (American Diabetes Association, 2008). The principal rationale for aggressively improving glycaemic control in type 2 diabetes patients was derived from observational studies reporting a dose-response relationship between the degree of glycaemic control and the incidence of macro- and microvascular complications (Stratton et al., 2000). Moreover, intense glycaemic control had previously been demonstrated to reduce the incidence of macro- and microvascular complications in individuals with type 1 diabetes; the

principal side-effect being an increased risk of severe hypoglycaemic events (The Diabetes Control and Complications Trial Research Group, 1993). Thus, the *seemingly* logical extrapolation of the observational findings by Stratton et al., (2000) in type 2 diabetics and the intervention findings in type 1 diabetics (The Diabetes Control and Complications Trial Research Group, 1993), suggested that intense glycaemic control would also reduce the incidence of complications in individuals with type 2 diabetes (Havas, 2009). An accumulation of evidence first being reported up to forty years ago (Meinert et al., 1970), however, suggested that aggressive glycaemic control was associated with increased rates of CVD-related (Meinert et al., 1970, Nissen and Wolski, 2007, Gerstein et al., 2008), diabetes-related (United Kingdom Prospective Diabetes Study, 1998a), or all-cause (Gerstein et al., 2008) mortality in individuals with type 2 diabetes. Indeed, a recent large-scale randomised controlled trial was prematurely terminated due to elevated mortality rates in subjects randomised to intensive glycaemic control (Gerstein et al., 2008).

Presently, debate continues regarding the principal goals of type 2 diabetes management on whether interventions should target hyperglycaemia or other risk factors for CVD (Versari et al., 2009, Stirban et al., 2009, Hanefeld et al., 2009, Del Prato et al., 2009, Davidson and Parkin, 2009, Conget and Gimenez, 2009). It is argued that the results of previous randomised controlled trials may be confounded due to: i. the class of hypoglycaemic drug investigated; ii. interactions between various hypoglycaemic drug-class types; iii. the level of blood pressure medication used; iv. changes in post-intervention body mass (Jenny-Avital et al., 2008). Indeed, while absolute measures of relevant risk factors (e.g., $HbA_{1c} < 7.0\%$) may be useful, it is becoming apparent that the way in which targets are achieved (i.e., type of drug; lifestyle intervention etc) is critically important (Krumholz and Lee, 2008).

Pharmacological interventions for managing type 2 diabetes

The goal of current pharmacological management strategies for type 2 diabetes involves improving blood glucose homeostasis via distinct mechanisms (for review, see Inzucchi,

2002). There are four primary classes of type 2 diabetes medications of varied efficacy and associated side effects. A brief overview of each major drug class is provided below.

Sulfonylureas improve glucose homeostasis by increasing insulin secretion from the pancreas. Sulfonylureas specifically bind a sulfonylurea receptor on the plasma membrane of pancreatic β-cells, which closes an associated ATP-dependent K^+ channel resulting in the stimulation of insulin release (Panten et al., 1996). Effectively, sulfonylurea's increase insulin secretion by lowering the glucose threshold required for insulin release by the pancreas (Zimmerman, 1997). The safety of sulfonylurea use has been questioned, however, due to the link between hyperinsulinaemia and CVD. For instance, earlier generations of sulfonylureas were demonstrated to significantly increase the risk of mortality (Goldner et al., 1971), whereas current generation sulfonylureas may reduce risk of all-cause mortality (United Kingdom Prospective Diabetes Study, 1998b). Side effects associated with sulfonylurea use include significant weight gain (Schade et al., 1998, United Kingdom Prospective Diabetes Study, 1998b) and hypoglycaemic episodes (United Kingdom Prospective Diabetes Study, 1998b, Zimmerman, 1997).

Alpha-glucosidase inhibitors aid in glucose homeostasis by delaying the absorption of carbohydrates across the intestine. The enzyme α-glucosidase resides in the epithelium of the small intestine and aids in the breakdown of complex carbohydrates. Consequently the inhibition of α-glucosidase delays the process of carbohydrate absorption and attenuates the subsequent elevations in postprandial blood glucose concentration (Goke and Herrmann-Rinke, 1998). Whereas α-glucosidase inhibitors sufficiently reduce postprandial blood glucose concentration, this class of drug is not effective for improving fasting blood glucose homeostasis (Hasche et al., 1999). Adverse effects associated with the use of alpha-glucosidase inhibitors include gastrointestinal irritability (i.e., flatulence and diarrhoea) and may increase the risk of hypoglycaemic events when used concurrently with other antidiabetic medications (Neumiller and Setter, 2009).

Thiazolidinediones are a unique antidiabetic drug that simultaneously enhances insulin sensitivity, increases peripheral glucose disposal and also diminishes hepatic glucose output (Triplitt, 2007). The mechanism of action by thiazolidinediones is not fully understood; however, it is known that thiazolidinediones alter transcription of genes associated with carbohydrate and lipid metabolism, as well as insulin action (Mudaliar and Henry, 2001). The principle effect of thiazolidinediones is a decrease in tissue insulin resistance that ultimately elevates skeletal muscle glucose uptake under insulin-stimulated conditions (Petersen et al., 2000). Preliminary evidence suggests that thiazolidinediones may also reduce blood pressure and lipid levels, while improving vascular function (Wang et al., 2004, Kelly and Bank, 2007). Several notable side effects are observed in individuals using thiazolidinediones, including weight gain, oedema, exacerbation of pre-existing heart failure (Lipscombe et al., 2007, Nesto et al., 2004), and increased rate and severity of fractures among older women (Kahn et al., 2008).

Biguanides act to decrease hepatic glucose output in the presence of insulin (Inzucchi et al., 1998) and increase peripheral glucose disposal (Bailey and Turner, 1996). Biguanides have similar glucose-lowering effects to other classes of antidiabetic medications, while exhibiting fewer severe side effects. Indeed, weight loss or absence of weight gain (Helvaci et al., 2008) (even in non-diabetics (Desilets et al., 2008), as well as lower incidence of hypoglycaemia have been associated with use of thiazolidinediones. Mild side effects are observed among users of biguanides, typically involving gastrointestinal distress (e.g. pain, nausea and diarrhoea) (Bailey and Turner, 1996).

The principle drawback of all pharmacological interventions for managing type 2 diabetes is that despite initially providing promising improvements in glycaemic control, it is clear that all pharmacological agents have a limited efficacy over longer time periods leading to blood glucose concentrations trespassing target thresholds (Del Prato et al., 2009). For instance, thiazolidinediones, biguanides and sulfonylureas have been demonstrated to fail (i.e., blood glucose did not reach target threshold at maximal-dictated or maximal-tolerated dose) to varying degrees within 5 yr of prescription as a monotherapy (Kahn et al., 2006a). Indeed,

biguanides ability to reduce HbA_{1c} to less than 7.0 % decreased significantly with time following prescription as a monotherapy: 3, 6, and 9 yr post prescription, failure rate was 56, 66 and 87%, respectively (Turner et al., 1999). Upon failure of anti-diabetic drugs, individuals with type 2 diabetes subsequently require i. multiple pharmacological therapies and ii. progression to exogenous insulin.

Despite the advances in pharmacological interventions for the purpose of type 2 diabetes management, it is reported that lifestyle interventions (e.g., diet, physical activity) are more effective than antidiabetic drugs. The Diabetes Prevention Program Research Group (Knowler et al., 2002) reported that a lifestyle intervention that comprised exercise and dietary modifications was significantly more effective at reducing the incidence of type 2 diabetes than biguanides over a ~2.8 yr period.

Dietary interventions for the management of type 2 diabetes

While diet interventions are one of the cornerstone therapies in the management of type 2 diabetes, the 'diabetic diet' is continually evolving due to recent research findings. The American Diabetes Association recommends that diet development for patients with type 2 diabetes should include individualised goals with special considerations for the patient (e.g. age, body mass index) (Posthauer, 2008). The traditional goal for most older individuals with type 2 diabetes is to reduce body fat (Aucott et al., 2004) and the symptoms of hyperglycaemia (American Diabetes Association, 2008), which is typically achieved by diets regulating total carbohydrate content.

As early as the 1920's it was understood that carbohydrates had a substantial effect on blood glucose (Robbins, 2008). More recently, evidence has accumulated suggesting that high carbohydrate diets may stimulate appetite and elevate energy intake, particularly among individuals with type 2 diabetes (Boden et al., 2005). Moreover, it has been reported that high carbohydrate diets that also emphasise low fat intake may exacerbate obesity and

hyperglycaemia (Arora and McFarlane, 2005). As a result, the carbohydrate content of recommended diets for individuals with type 2 diabetes has been heavily restricted.

The rationale for low carbohydrate diets was that a reduction in carbohydrate intake and the subsequent reduction in blood glucose levels would promote oxidative metabolism of body fat stores, leading to weight loss. In practice, there is evidence that low carbohydrate diets do indeed promote weight loss in non-diabetics in the short term. In addition, low carbohydrate diets have been reported to reduce plasma glucose levels (Gannon and Nuttall, 2004), increase insulin sensitivity (Boden et al., 2005) and reduce post-absorptive glycogenolysis (Allick et al., 2004). It is increasingly acknowledged, however, that the blood glucose response to food is not well predicted by the content of available carbohydrate in the food (Monro and Shaw, 2008) and that the positive health outcomes to low carbohydrate diets may be simply due to a reduction in energy intake (Arora and McFarlane, 2005). As a result, the need for meaningful values that describe a foods specific postprandial glycaemic response has been recognised.

Glycaemic index is a term that describes the blood glucose response and insulin demand resulting from an ingested meal. Jenkins and colleagues (1981) performed an elegant study that compared the blood glucose response to ingestion of common foods and sugars. Blood glucose levels were measured for 2 h and subsequently expressed relative to the glucose response curve measured when a comparable amount of pure glucose was consumed. This landmark study enabled food to be given a score that would approximate its effect on postprandial blood glucose measurements. A high glycaemic index value indicated that a given food was completely expressed as glucose within 2 h of consumption (e.g. potato), whereas low glycaemic index values indicated slower digestion and more gradual rise of blood glucose (e.g. lentils). It has been reported that glycaemic load (product of glycaemic index and volume of carbohydrate), but not protein, fat or low-carbohydrate diet score, predicts CVD (the principle complication of type 2 diabetes) development over a 20 yr follow up study (Halton et al., 2006).

Regular exercise is necessary for the optimal management of type 2 diabetes

It may be the consequences of physical *inactivity* that highlights the importance of regular exercise for the management of type 2 diabetes. When healthy individuals are subject to absolute bed rest for as little as 6-10 d (e.g., following trauma or severe illness), significant increases in fasting plasma insulin concentration (Stuart et al., 1988) and abnormally high insulin responses to glucose challenges (Mikines et al., 1989a, Sonne et al., 2010) are observed. Not surprisingly, physical inactivity increases an individual's risk of developing type 2 diabetes (Dunstan et al., 2002, Goodyear and Kahn, 1998, Venables and Jeukendrup, 2009). In contrast, regular exercise is associated with improvements in glycaemic control (Winnick et al., 2008, Eriksen et al., 2007), blood lipids (Halverstadt et al., 2007), blood pressure (De Feyter et al., 2007), cardiovascular conditioning (Larose et al., 2010), haemorheology (Chong-Martinez et al., 2003), and HRV (Zoppini et al., 2007).

Effects of exercise on glycaemic control

Despite the impaired insulin-stimulated glucose uptake that manifests in type 2 diabetes as insulin resistance, contraction-mediated glucose uptake is unaltered and reflects that of a healthy individual (Kennedy et al., 1999). Consequently, skeletal muscle contractions that occur constantly during exercise provide an effective method of reducing blood glucose concentration for individuals with type 2 diabetes. A single bout of exercise induces two separate effects on glucose transport within skeletal muscle. During exercise and for a short period afterwards, an increase in glucose uptake is observed independent of insulin stimulation (i.e., due to contraction-mediated glucose uptake) (Hansen et al., 1998). Moreover, glucose uptake remains elevated for several hours due to increased blood flow (Young et al., 1987) and GLUT4 expression at the muscle membrane (Goodyear et al., 1992). The second favourable effect that exercise exerts on glucose uptake is that peripheral tissues, such as skeletal muscle and adipose tissue, become hypersensitive to insulin stimulation for up to 16 h following the cessation of exercise (Ren et al., 1994). Consequently the prolonged

effects of exercise on insulin sensitivity might be particularly beneficial for individuals with impaired insulin sensitivity and may be one of the most important benefits of regular exercise in type 2 diabetes (Hansen et al., 1998). It must be noted, however, that the exercise-induced improvements in glycaemic control do not last longer than 3-4 d following an acute exercise bout.

Repeated exercise bouts (i.e., exercise training) are necessary to maintain the beneficial effects of exercise. As little as 7 d of 1-h daily exercise significantly improved peripheral insulin sensitivity and responsiveness, and significantly reduced hepatic glucose output (Kirwan et al., 2009). Improved insulin sensitivity following exercise training manifests as an increase in glucose uptake for a given insulin concentration, thus lowering the burden on pancreatic β-cells (Arciero et al., 1999). Christos and colleagues (2009) reported that 16 wk of exercise training reduced HbA_{1c} (15.0%) and plasma glucose (5.4%) compared with baseline, likely due to a decrease in insulin resistance. Alternatively, Brun and colleagues (2008) reported that 12 mo of exercise training, while reducing health-costs and increasing cardiovascular fitness, had no effect on HbA_{1c} or plasma glucose. Owing to contrasting findings suggesting that exercise training improves or has no effect on glycaemic control in type 2 diabetes, it now appears that the components of an exercise-training intervention (or how exercise is prescribed) must be carefully investigated to ensure optimal outcomes.

Gas-exchange adaptations to exercise training in individuals with type 2 diabetes

Individuals with overt type 2 diabetes perform less physical activity and demonstrate reduced cardiovascular conditioning when compared with non-diabetic individuals. Cardiovascular conditioning may be quantified by measuring the pulmonary gas-exchange responses to an incremental exercise test. The peak oxygen consumption ($\dot{V}O_2peak$) achieved during an incremental exercise test reflects the maximal rate at which ATP may be synthesised via oxidative metabolism and is an indicator of cardiovascular conditioning (Fletcher et al., 2001). Moreover, $\dot{V}O_2peak$ is inversely related to CVD-related and all-cause mortality

(Nocon et al., 2008). Another important measure of cardiovascular conditioning is the gas-exchange threshold (T_{ge}), which reflects the intensity during an incremental exercise test that elicits increased pulmonary ventilation and a disproportionate rise in carbon dioxide output ($\dot{V}CO_2$) with respect to oxygen consumption ($\dot{V}O_2$). Wasserman et al., (1977) suggested that the disproportionate increase in $\dot{V}CO_2$ at intensities above the T_{ge} occurred due to the production of non-metabolic carbon dioxide, which resulted in hyperventilation. In this model, non-metabolic carbon dioxide was supposedly generated as a result of plasma bicarbonate buffering of lactic acid (i.e., the hydrogen ion associated with the fermentation of pyruvate) inside the exercising muscles. Several lines of evidence against Wasserman's model have been presented, including: i. bicarbonate concentration does not match the increase in lactate (Stringer et al., 1992, Peronnet and Aguilaniu, 2006); ii. bicarbonate is not the principle intramuscular buffer (Hultman and Sahlin, 1980); iii. bicarbonate buffering does not result in non-metabolic carbon dioxide production (Peronnet and Aguilaniu, 2006), and; iv. the rate of carbon dioxide transport to the lungs is linear above and below the T_{ge} (Sun et al., 2001). While there is debate as to the exact cause(s) of the T_{ge}, recent evidence suggests H^+ accumulation in the blood causes a reduction in pH and thus blood bicarbonate is also decreased, resulting in a concurrent increase in ventilation and $\dot{V}CO_2$, compared with $\dot{V}O_2$ (for review, see Peronnet and Aguilaniu, 2006).

Cross-sectional studies demonstrate that well-trained individuals present with increased $\dot{V}O_2$peak and T_{ge} when compared to sedentary controls (Bircher and Knechtle, 2004). The increased $\dot{V}O_2$peak following exercise training is principally facilitated by improved oxygen delivery to (e.g., larger stroke volume; increased capillarisation etc) or utilisation by (e.g., increased mitochondrial density and oxidative enzymes etc) the active skeletal muscle. Improved T_{ge} may be observed following exercise training principally due to hypertrophy of type I muscle fibres and transformation of muscle fibres from type IIb to type IIa (Andersen and Henriksson, 1977), increased density of capillaries (Ingjer, 1979) and mitochondria (Suter et al., 1995), which ultimately reduce the muscles requirement for anaerobic energy provision during exercise (Jones and Carter, 2000).

Individuals with type 2 diabetes present with reduced $\dot{V}O_2peak$, lactate threshold (analogous with the T_{ge}) and time to exhaustion, when compared with non-diabetic controls matched for habitual physical activity and body mass (Regensteiner et al., 1995). Regensteiner and colleagues (1998) subsequently demonstrated that the rate response (kinetics) of $\dot{V}O_2$ at the onset of exercise was slower for individuals with type 2 diabetes, when compared with lean and obese non-diabetic controls. The findings of Regensteiner and colleagues (1995, 1998) suggest that key parameters of cardiovascular fitness are impaired in type 2 diabetes independent of body mass and habitual physical-activity levels (or training status). The mechanisms responsible for reduced $\dot{V}O_2peak$ and slower $\dot{V}O_2$ kinetics in type 2 diabetes are not well described, but likely involve a reduced ability to deliver (e.g., decreased stroke volume; Gusso et al., 2008) and/or utilise (e.g., decreased peripheral oxygen extraction; Baldi et al., 2003) oxygen within the active musculature. It is conceivable, however, that the complications of type 2 diabetes may directly impair pulmonary gas exchange and subsequently exercise performance. For instance, type 2 diabetes is associated with endothelial dysfunction (Rask-Madsen and King, 2007) and impaired haemorheology (Singh and Shin, 2009), which induce resistance to blood flow (thus, potentially oxygen delivery and extraction) particularly within the microcirculation (Parthasarathi and Lipowsky, 1999, Zhang et al., 2009). Moreover, microvascular complications reduced $\dot{V}O_2peak$ for individuals with type 2 diabetes, independent of disease duration, body mass index, age, sex and race (Estacio et al., 1998).

The benefits of exercise training for the improvement of cardiovascular fitness in type 2 diabetes are well established; however, individual responses to a given exercise training intervention may significantly vary (Bouchard et al., 1999). A meta-analysis of seven distinct studies reported that a mean duration of ~20 wk (range 8 – 52 wk) of aerobic exercise training was associated with a mean improvement in $\dot{V}O_2peak$ by ~11.8% for individuals with type 2 diabetes, when compared to pre-training (Boule et al., 2003). The mechanisms that elicit improvements in $\dot{V}O_2peak$ for individuals with type 2 diabetes remain poorly described; however, exercise training improved stroke volume (Mourot et al., 2009) and

capillary density (Kim et al., 2004), reversed endothelial dysfunction (Maiorana et al., 2003), and increased the activity of oxidative enzymes (i.e., citrate synthase and beta-hydroxy-acyl-CoA) (Bruce et al., 2004) for individuals with type 2 diabetes. Thus, it appears that exercise training may increase $\dot{V}O_2peak$ by improved delivery and utilisation of oxygen to skeletal muscles.

The effects of exercise training on heart rate variability in type 2 diabetes

While the conduction circuit of the heart contains various cells with intrinsic automaticity, the ANS continuously modulates the electrical activity of the myocardium (Sztajzel, 2004). Under increased sympathetic stimulation of the heart, an increase in the rate of pacemaker depolarisation occurs. Alternatively, parasympathetic modulation of the heart hyperpolarises the pacemaker cells, thus reducing the rate of depolarisation. Consequently, a 'balancing act' between the sympathetic and parasympathetic branches of the ANS modulates the rate and rhythm of the heart (Rajendra Acharya et al., 2006).

The variability of beat-to-beat heart rate provides insights into the ANS modulation of the heart. Specifically, high-frequency components of HRV reflect parasympathetic modulation of the cardiac cycle, whereas low-frequency power (expressed normalised to total power) reflects sympathetic modulation (Malik et al., 1996). Decreased HRV is associated with reduced cardiac health and increased risk of CVD-related and all-cause mortality. While HRV declines with ageing (De Meersman and Stein, 2007), type 2 diabetes accelerates this decline (Rajendra Acharya et al., 2006), particularly for patients with poor glycaemic control (Singh et al., 2000), autonomic neuropathy (Pagkalos et al., 2008), and microvascular complications (Ko et al., 2008, Jaffe et al., 1995). While the precise mechanism(s) underlying decreased HRV in type 2 diabetes is not understood, it is believed that vagal nerve dysfunction (thus, impaired parasympathetic modulation) resulting from a hypoxic nerve environment may be intimately involved (Vinik et al., 2003). Given that hypoxia may be

induced due to hyperglycaemia and/or impaired haemorheology, exercise training may mediate improvements in HRV due to improved glycaemic control and/or haemorheology.

Exercise training has been demonstrated to improve HRV in healthy older individuals (Stein et al., 1999), but it is unclear whether exercise training improves HRV for individuals with type 2 diabetes. Several studies have reported no change in HRV following moderate-intensity exercise training in type 2 diabetes, despite improvements in HbA_{1c}, $\dot{V}O_2peak$, and baroreceptor sensitivity (Kanaley et al., 2009a, Loimaala et al., 2003, Zoppini et al., 2007). Alternatively, Figueroa and colleagues (2007) investigated the effects of 16 wk of moderate-intensity exercise training in obese subjects, with and without type 2 diabetes. It was found that exercise training increased low-frequency and high-frequency power of HRV when all subjects were grouped together; however, subgroup analysis revealed that the obese subjects with type 2 diabetes did not significantly improve HRV, limiting the inferences of the findings (Figueroa et al., 2007). Pagkalos and colleagues (2008) reported that 6 mo of moderate-intensity exercise training improved time-domain measures of HRV for individuals with type 2 diabetes, with and without cardiac autonomic neuropathy. The improved time-domain HRV parameters were associated with improved $\dot{V}O_2peak$ and glycaemic control for all subjects; however, only those subjects *with* cardiac autonomic neuropathy increased high-frequency power of HRV, suggesting that exercise training improved parasympathetic modulation only for those with cardiac autonomic neuropathy. Consequently, the authors concluded that subjects with cardiac autonomic neuropathy would benefit most from exercise training, when compared with uncomplicated type 2 diabetes. The conclusion by Pagkalos and colleagues (2008) is somewhat confusing, given that the data reported in the paper suggested that only those type 2 diabetes subjects *without* cardiac autonomic neuropathy improved sympathovagal balance (determined by calculating the ratio of low- to high-frequency power). Thus, further investigations are required to provide insights into the effects of exercise training on HRV in type 2 diabetes.

Exercise training may improve haemorheology in type 2 diabetes

Type 2 diabetes significantly affects the way that blood travels throughout the body. Blood viscosity, the overall resistance for blood to flow within the circulation, is increased for individuals with type 2 diabetes, when compared with control (Le Dévéhat et al., 2004). Moreover, blood viscosity is markedly worse for individuals with type 2 diabetes that present with poor glycaemic control (Bauersachs et al., 1989a), suggesting hyperglycaemia may be involved in the processes underlying increased blood viscosity. Given RBC are the dominant cell type found in blood, the functional characteristics (e.g., aggregation) and structural properties (e.g., deformability) of RBCs also significantly influence blood viscosity (Zhang et al., 2009, Singh and Shin, 2009). Chronic hyperglycaemia decreases the compliance of RBCs and reduces the repulsion induced by the negative charge of the cell surface (Baba et al., 1978, Petropoulos et al., 2007). The functional consequences of decreased RBC compliance and reduced cell repulsion due to chronic hyperglycaemia are decreased RBC deformability and increased RBC aggregation, respectively.

There are very few studies that have previously investigated the effect of exercise training on haemorheology in type 2 diabetes; however, there is limited evidence from investigations in other populations suggesting that exercise may modify haemorheology. Twelve weeks of moderate-intensity treadmill exercise reduced blood viscosity at higher (450 s^{-1}) and lower (90 s^{-1}) shear rates in older sedentary, but otherwise healthy individuals (Coppola et al., 2004). Furthermore, 12 wk of 'pain-free' exercise training (walking) increased RBC deformability in patients with claudication (Mika et al., 2006); however, 8 wk of low-intensity cycling did not improve RBC aggregation or RBC rigidity (analogous with deformability) in obese, insulin resistant individuals (Dumortier et al., 2002, Aloulou et al., 2006). The only study pertinent to type 2 diabetes demonstrated that ~14 wk of intensified glycaemic management, incorporating insulin therapy, exercise training and dietary modification, reduced blood glucose concentrations, blood and plasma viscosity, and RBC aggregation (Chong-Martinez et al., 2003).

There is a lack of clear findings in the literature regarding the efficacy of exercise training for the improvement of haemorheology in type 2 diabetes. Indeed, the little that is known into the effect of exercise training on haemorheology in individuals with type 2 diabetes warrants further investigation. For instance, Chong-Martinez and colleagues (2003) reported promising results from a lifestyle intervention, demonstrating decreased RBC aggregation and lower high-shear blood viscosity (indicative of increased RBC deformability); however, the study design (i.e., multiple interventions) limits the ability to determine whether exercise training *alone* influenced haemorheology. Furthermore, the exercise intervention was not clearly defined; Chong-Martinez and colleagues (2003) simply stated that subjects were requested to perform 30 min of aerobic exercise (presumably home based) on 3 $d \cdot wk^{-1}$. Finally, no other studies have investigated the effects of exercise training on direct measures of RBC deformability in type 2 diabetes.

Exercise training may have anti-inflammatory effects in type 2 diabetes

As discussed earlier, type 2 diabetes is associated with an upregulation of proinflammatory markers that accelerate the pathogenesis and progression of atherosclerosis and therefore CVD. An acute bout of exercise causes a marked increase in plasma concentrations of inflammatory cytokines and acute-phase reactants relative to the intensity and duration of the exercise bout, as well as the muscle mass recruited (Moldoveanu et al., 2001, Petersen and Pedersen, 2005). Thus somewhat paradoxically, regular exercise has been demonstrated to reduce markers of inflammation. For instance, cross-sectional studies demonstrated that physically active individuals presented with decreased concentrations of proinflammatory markers (Abramson and Vaccarino, 2002, Geffken et al., 2001). Whether exercise training may reduce the concentration of proinflammatory cytokines in individuals with type 2 diabetes remains unclear.

Lifestyle modification, including low-fat diet and aerobic exercise, has been demonstrated to improve the anti- to pro-inflammatory balance in obese men (Roberts et al., 2006). Moreover,

Christiansen et al., (2010) demonstrated that in obese, but otherwise healthy individuals, 12 wk of lifestyle intervention (diet only; exercise only; diet and exercise combined) leading to significant weight loss improved markers of low-grade inflammation. Indeed, exercise training did not induce any further change in proinflammatory markers above that achieved by diet only (Christiansen et al., 2010). There is also limited evidence that lifestyle interventions may improve the inflammatory profile of individuals with type 2 diabetes. For instance, 6 mo of exercise training, including cycling, walking and calisthenics, lead to a significant improvement in anti- to pro-inflammatory markers (Kadoglou et al., 2007). It is unclear of the contribution that exercise influenced for this improvement, however, given that exercise intensity was not consistent (50-75% $\dot{V}O_2peak$), varied mode exercise was performed, and significant weight loss was also achieved (Kadoglou et al., 2007). Consequently, the capacity for exercise training to improve inflammatory markers in type 2 diabetes is not clear.

Prescription of exercise in type 2 diabetes

There is overwhelming consensus in the literature that exercise training is fundamental and necessary for the prevention and/or management of type 2 diabetes. Indeed, exercise-training interventions may be more beneficial for managing type 2 diabetes than the leading diabetes medications (Knowler et al., 2002). Nevertheless, before exercise training can be prescribed *en masse*, the goals and specific dose of exercise training for type 2 diabetes should be clarified and revised.

Clarifying the primary goal of exercise training in type 2 diabetes

The traditional focus of exercise training for managing type 2 diabetes was a reduction in blood glucose concentration (i.e., improved glycaemic control). The recent premature termination of the intensive glucose-lowering cohort of the ACCORD study, due to increased

mortality (Mayor, 2008), has renewed interest in what should be the primary goal of type 2 diabetes management. It is suggested that i. lowering blood glucose concentration should be performed with caution in older individuals, and ii. there is potentially a threshold below which lowering blood glucose concentration may be detrimental (Stirban et al., 2009). Thus, type 2 diabetes management may require a shift in priority toward improved prevention and management of the associated complications (e.g., CVD) rather than solely implementing glycaemic control, particularly for older individuals.

If exercise training could concurrently improve cardiovascular conditioning, haemorheology, HRV, hypertension, inflammation, and glycaemic control (among others), one would expect that the development and progression of complications associated with type 2 diabetes would be significantly reduced. Moreover, if the principal complications of type 2 diabetes (i.e., CVD, neuropathy, nephropathy) are subsequently diminished and/or delayed, it is plausible the CVD-related and all-cause mortality would also reduce. It is suggested that the ideal antidiabetic agent would normalise glycaemic control with minimal side effects, while reducing the development of complications and mortality (Del Prato et al., 2009). Presently, no anti-diabetic medication concurrently improves the multiple risk factors associated with type 2 diabetes; perhaps evidence-based exercise prescription may facilitate such improvements.

Clarifying the dose of exercise training for type 2 diabetes management

The American Diabetes Association currently recommends that individuals with diabetes perform at least 150 $min \cdot wk^{-1}$ of moderate-intensity aerobic exercise and where possible, perform resistance-exercise training 3 $d \cdot wk^{-1}$ (American Diabetes Association, 2010). The American Diabetes Association exercise guidelines for individuals with type 2 diabetes are based on technical reviews, however, that principally investigate the effectiveness of exercise training for improving glycaemic control and cardiovascular conditioning (Boule et al., 2001, Boule et al., 2003). For instance, the frequency of exercise-training sessions are suggested to

be performed no more than 72 h apart, citing that insulin sensitivity is elevated only for ≤ 72 h following an acute bout of exercise (Wallberg-Henriksson et al., 1998). The weekly volume of exercise (150 min·wk^{-1}) is suggested to decrease HbA_{1c} in individuals with type 2 diabetes, while the intensity of exercise is recommended given that increased exercise intensity appears to be associated with the greatest reductions in HbA_{1c} (Sigal et al., 2004). The current exercise guidelines for individuals with type 2 diabetes, therefore, appear to be heavily influenced by positive modifications in glycaemic control, with little additional attention to the primary complications of type 2 diabetes. Perhaps the optimal exercise intervention for individuals with type 2 diabetes would focus on the effects of exercise on markers of health that are associated with diabetic complications? Moreover, exercise adherence is a significant problem for individuals with type 2 diabetes, particularly among older women who are notoriously inactive. Yet, current exercise guidelines for individuals with type 2 diabetes suggest three aerobic exercise sessions and three resistance exercise sessions, requiring up to six sessions of exercise per wk. If exercise training could be performed using fewer weekly sessions (e.g., 2 vs. 4 d·wk^{-1}) and/or each session was of a shorter duration (e.g., 30 vs. 60 min·session^{-1}), and yet still have the capacity to deliver the benefits achieved during frequent and longer duration sessions, one would anticipate that adherence to exercise training would improve while reducing the impact of type 2 diabetes.

1.4 GENERAL AIMS

The principle aim of this thesis is to ascertain whether exercise training at an intensity which reflects the anaerobic threshold may improve various markers of CVD in older women with type 2 diabetes. The intensity of exercise training was prescribed at an intensity which reflected each individual's gas-exchange threshold (i.e., ≈ anaerobic threshold), principally because while being safe (Meyer et al., 2005), exercise training at such intensities is associated with decreased adipose tissue, blood glucose and lipid concentrations, and increased exercise capacity and peak oxygen uptake (Kim et al., 2009, Brandenburg et al., 1999, Gaskill et al., 2001). Moreover, the anaerobic threshold is profoundly modifiable following exercise training (Davis et al., 1979), and while training at high-intensities is most effective for increased anaerobic threshold, the elevated risk for cardiovascular events to occur during high-intensity exercise must be avoided. Consequently, training at an intensity that reflects the anaerobic threshold would elicit improved exercise capacity (including increased intensity at which the anaerobic threshold manifests), but minimise the probability of adverse events occurring during exercise. Given that exercise performed above the anaerobic threshold is associated with many uncomfortable side-effects (e.g., heavy breathing, rapid heart rate, skeletal muscle fatigue, etc) and cardiac drift, an increase of the intensity at which the anaerobic threshold occurs would likely improve safety and compliance of exercise. Decreased anaerobic threshold is associated with higher risk for premature death (Gitt et al., 2002, Robbins et al., 1999), therefore the capacity to increase the exercise intensity associated with the anaerobic threshold may also improve the prognosis for individuals at increased risk of CVD, such as those with type 2 diabetes.

Despite the recent realisation that glycaemic control, while important, should not be the sole focus of management for individuals with type 2 diabetes, it is surprising that few studies have investigated exercise-only interventions for the improvement of CVD risk factors in the context of this metabolic disorder. Study One addressed this principle aim by examining relationships between 'novel' markers of CVD in older women with type 2 diabetes and in non-diabetic controls. Study Two directly addressed the principle aim by investigating the

cardiovascular conditioning, haemorheology and HRV adaptations to 12-wk of varied-dose walking in older women with type 2 diabetes. Study Three investigated the anti-inflammatory effects of 12-wk of regular walking in older women with type 2 diabetes with the aim of clarifying whether exercise-only interventions might have an effect on anti- to pro-inflammatory markers. The specific aims of each study are provided below:

Study One: To determine whether changes in haemorheological parameters are related to heart rate variability in older women with type 2 diabetes.

Study Two: To observe the changes in cardiovascular conditioning, haemorheology, and heart rate variability following 12-wk of varied-dose walking in older women with type 2 diabetes.

Study Three: To investigate whether 12-wk of well-controlled and regular exercise training influences the ratio of anti- to pro-inflammatory markers in older women with type 2 diabetes.

Chapter 2

Heart rate variability is related to impaired haemorheology in older women with type 2 diabetes

Study One

Chapter preface During the study design phase of this thesis, it became clear that the focus of type 2 diabetes management was progressing from being almost exclusively centred on glycaemic control towards managing the risk factors for the complications associated with type 2 diabetes. Given that co-morbid CVD are almost impossible to separate from type 2 diabetes, it became obvious that investigating non-traditional risk factors for CVD and determining whether there is a shared aetiology and/or manifestation, might provide further insights for the design of subsequent health interventions.

2.1 INTRODUCTION

Chronic hyperglycaemia has been associated with microvascular and macrovascular complications that increase the morbidity and mortality attributed to type 2 diabetes. The most common comorbidity associated with type 2 diabetes is cardiovascular disease (CVD); a disease that occurs two-to-four times more in those with type 2 diabetes than in non-diabetics. CVD related complications that frequently present in type 2 diabetes include arteriosclerosis, cardiac autonomic neuropathy and myocardial infarction (Beckman et al., 2002). While large vessel damage in type 2 diabetes occurs principally via chronic low-level inflammation, there is accumulating evidence suggesting that microcirculatory impairments also have a critical role in the aetiology of diabetes-related vascular complications (Mantskava et al., 2006). Reductions in the deformability and increases in the aggregation of red blood cells (RBC) contribute to impaired microcirculatory flow (Baskurt and Meiselman, 2003) and are implicated in the development of CVD (MacRury et al., 1993) and neuropathy (Young et al., 1996).

Impairments to haemorheology are well documented in individuals with type 2 diabetes when compared with healthy controls. Increased blood viscosity and plasma fibrinogen concentration, increased RBC aggregation and reduced RBC deformability are observed in uncomplicated type 2 diabetes (for review, see Singh and Shin, 2009). Further impairments in haemorheology have been reported for type 2 diabetes patients with various comorbidities, including diabetes-related CVD (Adak et al., 2008), and neuropathy (Husstedt et al., 1997, Young et al., 1996), when compared with uncomplicated type 2 diabetes. While impaired haemorheology is known to precede overt neuropathy (Simpson, 1988), Husstedt and colleagues (1997) reported that increased RBC aggregation reflected the progression of neuropathy over a ~42 mo follow-up period. The authors did not report whether significant relationships existed between parameters of neuropathy and RBC aggregation. It has been suggested that impairments in haemorheology may contribute to the development of neuropathy, possibly due to haemorheological disturbances to microcirculatory blood fluidity (Young et al., 1996, Mantskava et al., 2006).

Neuropathy manifests earliest in the longer autonomic nervous system (ANS) nerves. Given the vagus is the longest ANS nerve and is the principal innervator of cardiac parasympathetic modulation, heart rate variability (HRV) may be used to noninvasively detect autonomic neuropathy. Indeed, reductions in HRV precede signs of neuropathy (Malik et al., 1996), thus it is suggested that reduced HRV may be the earliest indicator of neuropathy in type 2 diabetes (Maser and Lenhard, 2005). Moreover, beat-to-beat HRV is reported to be an indicator of overall cardiac health, as well as a strong predictor of CVD and sudden cardiac death (Rajendra Acharya et al., 2006).

While the relationships between autonomic neuropathy and haemorheological parameters have been previously described in type 2 diabetes (Young et al., 1996, Husstedt et al., 1997), it is unclear whether impaired HRV is related to changes in haemorheology. In an insightful study, Connes and colleagues (2008) reported that impaired haemorheology was significantly related to reduced HRV, particularly those HRV parameters that reflect parasympathetic ANS activity. However, that study examined young men expressing sickle cell trait – an atypical model of haemorheology. Given that impairments in HRV and reduced microcirculatory flow precede overt neuropathy, as well as significantly elevate risk of CVD, we investigated the relationships among parameters of HRV and the deformability and aggregation of RBCs in women aged 65-74 yr with uncomplicated type 2 diabetes. It was hypothesised that individuals with type 2 diabetes and impaired haemorheology would also present with decreased HRV.

2.2 METHODS

Subjects

Twenty women (age 69 ± 2 yr) with uncomplicated type 2 diabetes, and twenty healthy women (age 69 ± 3 yr) without diabetes (i.e., control), volunteered to participate as subjects in the present study. Subjects responded to invitations mailed from Diabetes Australia

(Queensland) or advertisements placed in the local media. Inclusion criteria for the present study were: i. clinical diagnosis of type 2 diabetes for at least 12 mo (diabetics only); ii. no documented history of significant comorbidities (e.g., cardiovascular, lung, renal disease; overt neuropathy); iii. currently not smoking, and; iv. currently not using exogenous insulin or any medication known to interfere with cardiac rhythm (e.g., beta blockers) or haemorheology (e.g., warfarin). Type 2 diabetes patients managing their: blood glucose concentration with sulfonylureas, biguanides and/or α-glucosidase inhibitors, and/or; blood pressure with calcium channel blockers, angiotensin-converting enzyme inhibitors and/or low-dose aspirin were eligible for participation. While aspirin may reduce the filterability of RBCs (Bilto, 1999), RBC aggregation of diabetics does not appear to be affected by aspirin (Vekasi et al., 2008). Moreover, HRV is not reported to be different following long-term therapy with calcium-channel blockers or angiotensin-converting enzyme inhibitors (Akselrod et al., 1981). Suitable individuals were subsequently invited to the laboratory for a formal screening session, comprising medical history, basic anthropometry, pulmonary function, a resting 12-lead electrocardiogram (ECG) and blood pressure measurements. Witnessed informed consent was also obtained during this visit. Individuals considered suitable based on screening results were then asked to visit their regular General Practitioner, who advised in writing that the individual had no significant comorbidities (e.g., renal disease, overt neuropathy) that would exclude her from continued participation in the study.

Experimental Design

Subjects were subsequently invited to visit the research laboratory on two occasions, each separated by no more than 48 h. During the first visit, blood was collected in the fasting state for the assessment of haemorheological parameters. On the subsequent visit, the measurement of beat-to-beat (RR) intervals of cardiac rhythm was performed. Measurements were collected in the morning and all subjects continued their physician's recommended medication regime. Measures of HRV were performed on a separate visit to blood sampling

to ensure any potential catecholamine and sympathetic responses associated with venipuncture would not influence the HRV parameters. The present study complies with the Declaration of Helsinki and the experimental protocol was reviewed and approved by the human research ethics committees at Griffith University and Bond University.

Acquisition and analysis of cardiac intervals

Measurements of RR intervals were performed in the morning. Subjects were instructed to refrain from vigorous activity and caffeine consumption for at least 12 h, and to consume a 'light' meal before visiting the laboratory; these instructions are in agreement with those recommended by Malik et al., (1996). Nevertheless, no subject reported performing exercise nor experienced a significant hypoglycaemic event prior to visiting the laboratory. Upon arrival at the laboratory, subjects were fitted with a chest strap transmitter that was coupled to a wristwatch receiver (Polar s810, Polar Electro OY, Kempele, Finland) to record RR intervals. Subjects were asked to lay supine on a clinical bed in the laboratory that was dim and quiet. Laboratory temperature was controlled between 22°C and 24°C throughout all tests. After 20 min of supine rest, RR intervals were recorded continuously for 10 min.

HRV was subsequently determined in both the time- and frequency-domains using software previously described (Niskanen et al., 2004) (HRV Analysis Software v1.1, Biosignal Analysis and Medical Imaging Group, University of Kuopio, Finland). Prior to analysis, each RR interval time series was visually inspected for outliers (e.g., ectopy); RR intervals differing by more than 20% from the previous were defined as outliers (Nunan et al., 2008). Outliers were excluded from subsequent analysis to minimise the influence on the spectral components. The following time-domain variables were calculated: i. the standard deviation of RR intervals (SDNN); ii. the root mean square of the differences between consecutive RR intervals (RMSSD); iii. the number of consecutive RR intervals differing by more than 50 ms (NN50); iv. NN50 expressed as a percentage of total RR intervals (pNN50). Frequency-domain analysis involved interpolating the raw RR intervals at 4 Hz to ensure an evenly

sampled signal was produced from the irregularly sampled time series. The power spectral density of the RR time series was calculated using autoregressive spectrum estimation (16^{th} order). Power and peak frequencies were determined for the low frequency (LF, 0.04 – 0.15 Hz), and high frequency (HF, 0.15 – 0.4 Hz) bands. Values for the very-low frequency band (< 0.04 Hz) were discarded as recommended for short-term recordings (Malik et al., 1996). The power measured within the LF and HF bands were expressed in absolute (ms^2) and in units normalised to total power (n.u.) exclusive of power in the very-low frequency range.

Blood collection

Subjects visited an accredited pathology laboratory for the measurement of fasting haematological parameters, including glucose, insulin, glycated haemoglobin (HbA_{1c}; type 2 diabetes only), cholesterol, low- and high-density lipoprotein, and homocysteine. Within 48 h of the initial blood sample and after an overnight (12 h) fast, subjects were asked to return to the laboratory for the measurement of RBC aggregation and deformability. Blood was collected in accordance with the guidelines published by Baskurt et al., (2009a), from a prominent antecubital vein into EDTA (1.8 $mg \cdot ml^{-1}$) collecting tubes, which were immediately placed on a tube roller until subsequent analysis. Haemorheology measures were completed within 3 h of venous blood collection.

Determination of RBC deformability

RBC deformability was determined using laser-diffractometry. Briefly, 7 µL of whole blood from an EDTA tube was transferred to a 2 mL microfuge tube and diluted in 700 µL of the manufacturers medium solution (5.5% Polyvinylpyrrolidone, $mol \cdot L^{-1}$ = 360,000, dissolved in 1 $mmol \cdot L^{-1}$ PBS, osmolality = 300 $mosmol \cdot kg^{-1}$). An aliquot of the diluted blood (600 µL) was transferred into the flow chamber (RSD-K01, Sewon Meditech Inc., Korea) and loaded into an ektacytometer (Rheoscan-D, Sewon Meditech Inc., Korea) for analysis at room

temperature (21 ± 1°C). The sample was subjected to a range of shear stresses (0 – 25 Pa) by aspiration across a microchannel (~200 µm), while a laser was emitted through the channel. A diffraction pattern for each sample was captured at 2 Hz by an integrated digital camera. The resultant images were transferred to a personal computer and analysed by fitting an ellipse to the diffraction pattern. An elongation index (EI) was calculated for shear stress levels ranging between 0 ~ 25 Pa, using the following equation: EI = (A – B)/(A + B), where A is the length of the major axis of the ellipse, and B is the length of the minor axis of the ellipse. The EI measurements were performed in duplicate and the average values are reported. The shear stress required for half of maximal deformation ($SS_{1/2}$) and the maximum elongation index at infinite shear stress (EI_{max}) were determined using a non-linear curve fitting algorithm based on the work by Baskurt and Meiselman (2004).

Determination of RBC aggregation

The haematocrit of whole blood collected into an EDTA containing tube was determined within 30 min of collection using an automated haematology analyser (Act.T diff, Beckman Coulter Inc., Fullerton, CA). Whole blood was then divided into two aliquots and centrifuged at 2000 *g* for 10 min. One aliquot of spun blood was adjusted to 40% haematocrit by adding or removing autologous plasma. The packed cells from the remaining aliquot of spun blood were separated from plasma and washed twice in isotonic PBS (285 ± 3 $mmol \cdot kg^{-1}$, pH 7.4). The packed cells were then washed once in an aggregating solution (3% DX70, dextran molecular mass 70 300; Sigma Chemical Co., St Louis, MO, USA), before being resuspended at 40% haematocrit in 3% DX70. DX70 is a water-soluble polysaccharide that at 3% mimics aggregation in whole blood, thus enabling aggregation to be measured without the influence of plasma factors (e.g., fibrinogen). RBC aggregation for both suspensions (i.e., adjusted plasma and DX70) were determined at room temperature (21 ± 1°C) from a 20 µL aliquot of each sample, using a computerised aggregometer system (Myrenne Aggregometer, Myrenne GmbH, Roetgen, Germany).

Two indices of RBC aggregation were determined for each suspension: i. M0, aggregation of RBCs at stasis within 10 s following an abrupt cessation of high shear (600 s^{-1}); ii. M1, aggregation of RBCs at a very-low shear (3 s^{-1}) within 10 s following the cessation of a high shear (600 s^{-1}). Indices of aggregation were determined in both haematocrit adjusted plasma ($M0_{PLA}$ and $M1_{PLA}$) and haematocrit adjusted DX70 ($M0_{DX}$ and $M1_{DX}$). Measurements were performed in duplicate and the average values are reported for M0 and M1.

Statistical analysis

Data reported as mean ± SD. Normality of the data was tested using the D'Agostino & Pearson omnibus normality test (GraphPad Software Inc, Release 5.0, USA). Heart rate variability data that did not pass normality testing (i.e., LF and HF power, normality $p < 0.05$) were transformed using the natural logarithm before analysis. Data for each group (type 2 diabetes and control) were then compared using an Independent Samples T Test to determine whether significant differences existed. Parametric correlation analyses were used to examine associations between dependent variables within each group (SPSS Inc, Release 17.0, USA). Significance was determined at an alpha level of 0.05.

2.3 RESULTS

The physical characteristics and haematology of the subjects are listed in Table 2.1. The subjects with type 2 diabetes were defined as 'well-controlled' based on HbA_{1c} levels, when referenced against the criterion published by the American Diabetes Association (2008). Mean concentrations for serum lipids were also within the recommended ranges for individuals with well-controlled type 2 diabetes (American Diabetes Association, 2008). No significant differences were observed between groups for age or body mass. Subjects with type 2 diabetes presented with significantly elevated fasting blood glucose and insulin, and

reduced HDL concentrations, compared with control. Cholesterol concentration was lower for individuals with type 2 diabetes, compared with control.

Table 2.1. Physical characteristics and haematology in older women with type 2 diabetes and matched women without diabetes.

	Control *n* = 20	Type 2 diabetes *n* = 20
Height (cm)	162 ± 6	158 ± 6
Body mass (kg)	69.3 ± 9.3	71.2 ± 10.5
Glucose ($mmol \cdot L^{-1}$)	5.1 ± 0.3	7.5 ± 1.8**
HbA_{1c} (%)	-	6.6 ± 0.8
Insulin ($mU \cdot L^{-1}$)	5.3 ± 1.8	11.9 ± 8.6**
Cholesterol ($mmol \cdot L^{-1}$)	5.7 ± 1.1	4.2 ± 0.8**
HDL ($mmol \cdot L^{-1}$)	1.8 ± 0.8	1.3 ± 0.3*
LDL ($mmol \cdot L^{-1}$)	3.3 ± 0.9	2.3 ± 0.7
Homocysteine ($\mu mol \cdot L^{-1}$)	10.4 ± 2.6	12.1 ± 5.0

Values are mean ± SD. *, $p < 0.05$. **, $p < 0.01$. HbA_{1c}: glycated haemoglobin. HDL: High-density lipoprotein. LDL: Low-density lipoprotein.

All subjects presented with normal haematocrit levels (Table 2.2). Differences between subject groups were observed for RBC aggregation; aggregation at stasis in haematocrit adjusted plasma ($M0_{PLA}$) and dextran 70 ($M0_{DX}$) were significantly higher (32.1 and 29.2%, respectively) for subjects with type 2 diabetes, compared with control ($p < 0.05$). There were no significant differences between groups for $M1_{PLA}$ and $M1_{DX}$ ($p > 0.05$).

Table 2.2. Red cell aggregation in older women with type 2 diabetes and matched women without diabetes.

	Control *n* = 20	Type 2 diabetes *n* = 20
Haematocrit (%)	40.8 ± 2.1	40.6 ± 3.7
$M0_{PLA}$	10.3 ± 2.9	13.5 ± 4.0*
$M1_{PLA}$	15.4 ± 5.7	16.2 ± 5.4
$M0_{DX}$	8.6 ± 2.4	11.1 ± 4.2*
$M1_{DX}$	15.7 ± 3.7	19.2 ± 5.3

Values are mean ± SD. *, $p < 0.05$. M0: aggregation at stasis following high shear rate; M1: aggregation at a low shear following high shear rate; $_{PLA}$: plasma suspension adjusted to 40% haematocrit; $_{DX}$: 3% dextran 70 suspension adjusted to 40% haematocrit.

RBC deformability was not significantly different between the groups across the range of shear stress levels measured (Figure 2.1, panel A). Accordingly, no difference was detected between groups for the shear stress required for half-maximal deformation ($SS_{1/2}$, Figure 2.1, panel B) or the maximum elongation index at infinite shear stress (EI_{max}, Figure 2.1, panel C).

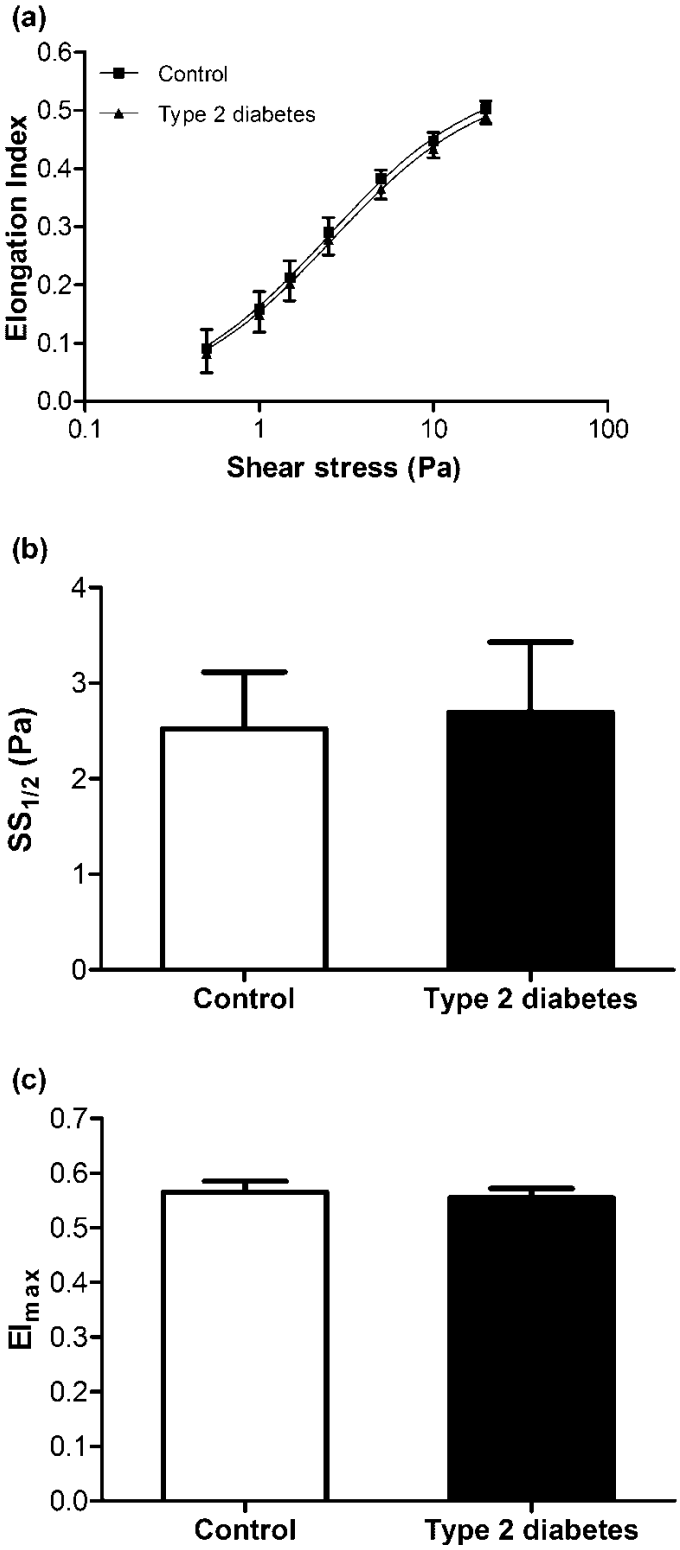

Figure 2.1. (a) Red blood cell deformability (elongation index) for healthy controls and subjects with type 2 diabetes measured at representative shear stresses between 0.5 and 20 Pa, presented on a semi-logarithmic scale for clarity. (b) Shear stress for half-maximal deformation ($SS_{1/2}$) and (c) maximum elongation index at infinite shear stress (EI_{max}) for both groups. Data are mean ± standard deviation.

Key time and frequency domain parameters of HRV analysis are presented in Table 2.3. No significant differences were detected between groups for any time-domain parameter of HRV. However, HF power expressed in normalised units was significantly reduced for those with type 2 diabetes, when compared with control ($p = 0.034$). It was also found that the ratio of low-frequency to high-frequency power (LF:HF ratio) was significantly higher for subjects with type 2 diabetes, compared with control ($p = 0.007$).

Table 2.3. Time and frequency domain parameters of heart rate variability in older women with type 2 diabetes and matched women without diabetes.

	Control *n* = 20	Type 2 diabetes *n* = 20
RR mean (ms)	904 ± 111	917 ± 117
SDNN (ms)	24.1 ± 13.4	20.8 ± 12.9
RMSSD (ms)	20.0 ± 12.7	19.5 ± 17.8
NN50 (count)	13.5 ± 28.0	14.0 ± 35.7
pNN50 (%)	4.5 ± 9.4	5.2 ± 14.3
lnLF power (ms^2)	4.2 ± 1.2	3.5 ± 1.8
LF power (n.u.)	37.3 ± 20.1	50.8 ± 26.6
lnHF power (ms^2)	3.7 ± 1.2	3.0 ± 1.4
HF power (n.u.)	45.3 ± 14.0	34.3 ± 21.8*
LF:HF ratio	1.1 ± 1.1	2.7 ± 2.6**

Values are mean ± SD. *, $p < 0.05$. **, $p < 0.01$. RR mean: mean duration of cardiac (RR) intervals; SDNN: standard deviations of RR intervals; RMSSD: root mean square of successive differences; NN50: count of RR intervals differing by > 50 ms; pNN50: NN50 expressed as percent of total RR intervals; ln: natural logarithm transformed; LF: low frequency; HF: high frequency.

No significant relationships were detected between parameters of haemorheology and HRV for the control group; however, significant relationships were observed between RBC aggregation and key frequency domain parameters of HRV for the subjects with type 2

diabetes (Table 2.4). For the subjects with type 2 diabetes, the M0 indices of RBC aggregation were positively related to LF power and the LF:HF ratio, and negatively associated with HF power, independent of suspending medium (i.e., plasma or DX70). The M1 indices demonstrated positive relationships with LF:HF ratio, independent of suspending medium for the subjects with type 2 diabetes. The shear stress required for half maximal deformation ($SS_{1/2}$) demonstrated a significant positive relationship with SDNN for the subjects with type 2 diabetes.

Table 2.4. Relationships between haemorheological parameters and heart rate variability in older women with type 2 diabetes.

	$M0_{PLA}$	$M1_{PLA}$	$M0_{DX}$	$M1_{DX}$	EI_{max}	$SS_{1/2}$ (Pa)
RR mean (ms)	-0.394	-0.397	-0.350	-0.228	-0.109	-0.206
SDNN (ms)	-0.188	-0.211	-0.136	-0.090	-0.332	-0.485*
RMSSD (ms)	-0.361	-0.286	-0.253	0.019	-0.420	-0.450
NN50 (count)	-0.446	-0.328	-0.250	0.091	-0.460	-0.414
pNN50 (%)	-0.454	-0.330	-0.255	0.096	-0.460	-0.406
lnLF power (ms^2)	0.203	0.094	0.179	0.168	-0.003	-0.344
LF power (n.u.)	0.572*	0.391	0.655**	0.325	0.158	-0.177
lnHF power (ms^2)	-0.312	-0.312	-0.350	-0.205	-0.201	-0.325
HF power (n.u.)	-0.468	-0.283	-0.532*	-0.214	-0.146	0.148
LF:HF ratio	0.561*	0.513*	0.665**	0.544*	-0.045	-0.256

Values represent Pearson's *r*. All data from subjects with type 2 diabetes; no significant relationships were observed for controls. * $p < 0.05$. ** $p < 0.01$. M0: aggregation at stasis following disaggregation; M1: aggregation at a low shear following disaggregation; $_{PLA}$: plasma suspension adjusted to 40% haematocrit; $_{DX}$: 3% dextran 70 suspension adjusted to 40% haematocrit; EI_{max}: maximum elongation index at infinite shear stress; $SS_{1/2}$: shear stress for half-maximal deformation; RR mean: mean duration of cardiac (RR) intervals; SDNN: standard deviations of RR intervals; RMSSD: root mean square of successive differences; NN50: count of RR intervals differing by > 50 ms; pNN50: NN50 expressed as percent of total RR intervals; ln: natural logarithm transformed; LF: low frequency; HF; high frequency; n.u.; normalised units.

2.4 DISCUSSION

The present study demonstrated that several key parameters of HRV were significantly related to the aggregation and deformability of RBCs in older women with type 2 diabetes, but not non-diabetic controls. Detrimental changes to haemorheology and HRV in patients with type 2 diabetes are independently associated with an increased risk of CVD and neuropathy. The present study is the first to show that RBC aggregation and RBC deformability are significantly associated with LF dominance and reduced SDNN in type 2 diabetes patients.

In the present study, RBC aggregation was increased for subjects with type 2 diabetes, when compared with control. It is reported that RBC aggregation is increased in individuals with type 2 diabetes due to altered serum and plasma proteins (e.g., globulin and fibrinogen) (Singh and Shin, 2009). In the present study, however, RBC aggregation was also increased for RBCs suspended in plasma-free dextran 70, suggesting that impairments intrinsic to the RBC may be responsible for the increased aggregation. Intrinsic alterations to the RBC in type 2 diabetes principally involve an impairment of the surface charge of RBCs, increasing the RBCs tendency to aggregate (Baba et al., 1978, Budak et al., 2004).

We also found that the HF power of HRV was lower for subjects with type 2 diabetes compared with control. Power in the HF spectrum of HRV reflects parasympathetic modulation of the cardiac cycle, suggesting that the individuals with type 2 diabetes presented with reduced parasympathetic control of heart rate. Time domain measures of HRV that correlate with parasympathetic modulation of cardiac cycles (e.g., RMSSD, pNN50) were not different between groups; however, correlations between frequency and time domain measures of HRV are strongest in long-term (i.e., 24 h) recordings and may not be detected in short-term HRV studies (Malik et al., 1996). The lower HF power for the subjects with type 2 diabetes was reflected by an increase in LF:HF ratio, when compared with control. Whereas absolute LF power reflects modulation by both branches of the ANS, when expressed in normalised units LF power principally represents sympathetic modulation of the

cardiac cycle (Malik et al., 1996). Thus, subjects with type 2 diabetes presented with a sympathetic dominance of ANS modulation of the cardiac cycle.

The M0 index of RBC aggregation for individuals with type 2 diabetes was significantly associated with increased LF and decreased HF power of HRV, independent of suspending medium. Our findings suggest that RBC aggregation is related to increased sympathetic and decreased parasympathetic modulation of the cardiac cycle, which is supported by the positive relationships between RBC aggregation and LF:HF ratio. Consistent with the findings of the present study, Connes and colleagues (2008) reported that a dominance of LF power was associated with increased high-shear blood viscosity and reduced oxygen availability to tissues in sickle cell trait carriers. The authors suggested that high sympathetic activity (i.e., increased LF power) and low parasympathetic activity (i.e., decreased HF power) may be the result of the heart adapting its firing rate to compensate for reduced oxygen availability to tissues, secondary to elevated blood viscosity. Given that RBC aggregates strongly influence low-shear blood viscosity (Zhang et al., 2009), our results support the findings of Connes and colleagues (2008) in that (at least low-shear) blood viscosity may be related to HRV as a consequence of increased RBC aggregation.

No difference in RBC deformability was observed between individuals with type 2 diabetes and control in the present study. Babu (1996) reported that RBC filterability (analogous with deformability) is impaired for type 2 diabetics with hypercholesterolaemia, when compared to type 2 diabetics with normal cholesterol concentrations. The individuals with type 2 diabetes of the present study had significantly lower cholesterol (likely due to management) compared with control, possibly contributing to the similar RBC deformability between the groups. While RBC deformability was not significantly different between individuals with type 2 diabetes and control, a significant negative relationship was observed between $SS_{1/2}$ and the overall variability of the cardiac cycle (SDNN) only for subjects with type 2 diabetes. An increase in $SS_{1/2}$ reflects reduced RBC deformability, as a greater shear stress is required to achieve one half of EI_{max} (Baskurt et al., 2010). While SDNN reflects global autonomic modulation of cardiac cycles, decreased SDNN is associated with several risk factors for

cardiac mortality following myocardial infarction, including reduced left-ventricular ejection fraction and baroreflex sensitivity (La Rovere et al., 1998, Casolo et al., 1992). The relationship between decreased SDNN and increased $SS_{1/2}$ for the subjects with type 2 diabetes suggests that individuals with impaired RBC deformability also present with decreased HRV, which might explain, in part, the elevated risk of CVD in type 2 diabetes patients, as both indices are independently associated with the development of CVD.

To understand how impairments in the functional properties of RBCs could be related to HRV only among subjects with type 2 diabetes, it is necessary to view these relationships within the context of type 2 diabetes. Chronic hyperglycaemia increases RBC fragility, decreases the compliance of RBCs and reduces the repulsion induced by the negative charge of the cell surface (Baba et al., 1978, Petropoulos et al., 2007, Kung et al., 2009). A reduction in cell repulsion would result in increased aggregation of RBCs (Rogers et al., 1992). The findings of the present study confirm that individuals with type 2 diabetes present with increased RBC aggregation, when compared with controls. Given that the circulation of blood is dependent upon interactions between the properties of the blood (haemorheology) and the vasculature (for review, see Fenech et al., 2009), it is suggested that the concomitant alterations to the RBC (for example, glycation and glucose-induced oxidation of RBC proteins) that result in increased aggregation and reduced deformability in patients with type 2 diabetes, culminate in 'sluggish' flow in the microcirculation (Singh and Shin, 2009, Neu and Meiselman, 2002). For instance, reduced RBC deformability impedes blood flow into and throughout capillaries (Parthasarathi and Lipowsky, 1999); however, RBC deformability for type 2 diabetes was not different to control in the present study. Nevertheless, elevated RBC aggregation may also impair microcirculatory blood flow by increasing effective blood viscosity, thereby increasing the resistance to flow in small vessels (Zhang et al., 2009). Moreover, RBC aggregates in the arterial circuit require separation before entry into capillaries of similar size to the RBC, compounding the resistance to flow (Baskurt and Meiselman, 2008).

Impaired haemorheology and reduced blood fluidity are suggested to disrupt the delivery of oxygen to various tissues, including nerves (Cameron et al., 1991, Neu and Meiselman, 2002). Reduced blood flow is believed to expose susceptible nerves to chronic hypoxia, causing impaired conduction and nerve dysfunction that precede overt neuropathy (Veves and King, 2001). Given that nerve oxygenation is dependent on microvascular blood flow, impaired haemorheology may contribute to the development of neuropathy. Neuropathy manifests earliest in the long ANS nerves such as the vagus, which is the principal modulator of cardiac parasympathetic activity (Vinik et al., 2003). Not surprisingly, HRV measures that reflect parasympathetic activity are sensitive to changes in vagal nerve function prior to overt signs of neuropathy presenting (Maser and Lenhard, 2005). Thus, in the context of type 2 diabetes, it is plausible that there may be a mechanistic association – either direct or indirect – between the impaired haemorheology and reduced HRV observed in older women with type 2 diabetes. In the present study, the increased RBC aggregation for older women with type 2 diabetes, when compared with controls, was associated with a sympathetic dominance of HRV (i.e., increased LF:HF ratio). Furthermore, while RBC deformability was not significantly different between subjects with type 2 diabetes and control, significant negative relationships between RBC deformability and HRV were only observed for older women with type 2 diabetes. However, a clear cause-effect relationship was not assessed in the present study; future studies may provide mechanistic insights into the nature of the observed relationships.

In the present study the subjects with type 2 diabetes were not directly assessed for neuropathy by the investigators. Rather, each subjects' General Practitioner advised in writing that the individual had not previously presented with overt neuropathy. Future studies might investigate whether criterion measures of neuropathy (e.g., nerve conduction velocity) are influenced by altered haemorheology. Nevertheless, the relationships between HRV and haemorheology in the present study suggest a common pathophysiology in patients with type 2 diabetes, given that decreased HRV is perhaps the earliest indicator of cardiac autonomic neuropathy (Maser and Lenhard, 2005).

The present study found that key parameters of HRV were related to RBC aggregation and deformability in women aged 65-74 yr with type 2 diabetes, but not in controls. It might be that impaired microcirculatory blood flow, resulting from modifications to the structural and functional properties of RBCs in type 2 diabetes, leads to changes in the sympathovagal balance of cardiac autonomic control. While reduced HRV and impairments in haemorheology independently elevate the risk of CVD, a concomitant rise in both indices may be interpreted as compounding the risk of CVD in type 2 diabetes. Alternatively, we propose that reduced HRV and impaired haemorheology may be linked mechanistically, or at least, share a common underlying cause (e.g., hyperglycaemia) in type 2 diabetes and therefore may not increase CVD risk additively.

Chapter 3

Heart rate variability and haemorheology responses to varied frequency and duration of walking in women 65-74 yr with type 2 diabetes

Study Two

Chapter preface The results observed in Study One demonstrated that haemorheology and heart rate variability were impaired, and related to each other, in older women with type 2 diabetes. Consequently, it was intended that the subsequent exercise intervention should aim to reverse or at least minimise these impairments. However, given the previous focus on glycaemic control of nearly all exercise interventions in type 2 diabetes, little was known regarding the basic training principals (i.e., frequency, intensity, volume etc) required to improve heart rate variability or haemorheology in type 2 diabetes. Indeed, conflicting results for heart rate variability and concurrent pharmacological-exercise interventions for haemorheology made it unclear whether exercise training *alone* had any effect at all on these measures in type 2 diabetes.

3.1 INTRODUCTION

Type 2 diabetes accelerates and exacerbates the age-related decline in heart rate variability (HRV) and impairment of haemorheology (Singh and Shin, 2009, Rajendra Acharya et al., 2006). Reduced HRV is strongly correlated with premature mortality in those with type 2 diabetes (Maser et al., 2003). Furthermore, the elevated blood glucose concentration associated with type 2 diabetes may induce defects in the red blood cell (RBC) membrane, which lead to increased RBC aggregation and decreased RBC deformability (Singh and Shin, 2009). Such negative alterations to the RBC may contribute to the increased burden of cardiovascular disease that is associated with type 2 diabetes (Adak et al., 2008) and it was recently reported that impaired haemorheology and reduced HRV are correlated in uncomplicated type 2 diabetes patients (Simmonds et al., 2010). Regular exercise is one of the cornerstone therapies for the prevention and management of type 2 diabetes; however, little is known regarding the optimum dose of exercise training for improving HRV and haemorheology, particularly in older women with type 2 diabetes.

Exercise training is reported to improve HRV in healthy individuals (Stein et al., 1999), and patients with hypertension (Collier et al., 2009) and coronary artery disease (Iellamo et al., 2000). Whether exercise training improves HRV in individuals with type 2 diabetes remains unclear, as significant and non-significant changes in HRV have been reported following exercise training (Bhagyalakshmi et al., 2007, Kanaley et al., 2009b). The inconsistency in the adaptation of HRV to exercise training in individuals with type 2 diabetes may be due to different exercise dose (e.g., mode, frequency, intensity), or due to the heterogeneity of subject populations, given that both age (Iyengar et al., 1996) and gender (Huikuri et al., 1996) significantly influence HRV. Further research into the effects of exercise training to improve HRV in individuals with type 2 diabetes, when well-controlled for age and gender, is warranted.

While exercise training is reported to decrease RBC aggregation (Ernst and Matrai, 1987) and increase RBC deformability (Mika et al., 2006) in patients with peripheral vascular disease, it

is unknown whether similar exercise-induced adaptations occur in individuals with type 2 diabetes. Chong-Martinez et al. (2003) reported that intensified type 2 diabetes management for 14 wk incorporating exercise, dietary modification and insulin therapy, resulted in a significant reduction in RBC aggregation, plasma viscosity, and whole blood viscosity. Given that insulin, dietary interventions and exercise training were concurrently implemented (Chong-Martinez et al., 2003), the contribution of exercise training in reducing RBC aggregation was not discernable. To our knowledge, there are no studies that have investigated the effects of exercise training on RBC deformability in type 2 diabetes patients. Furthermore, whether the frequency and duration of exercise sessions influences RBC aggregation and deformability is also unknown.

The purpose of the present study was to investigate the effect of varying the frequency and duration of exercise sessions, while maintaining constant accumulated weekly work, on HRV and haemorheology in women 65-74 yr with type 2 diabetes. Given hyperglycaemia is reported to contribute to the impaired HRV and haemorheology in persons with type 2 diabetes (Singh and Shin, 2009, Singh et al., 2000), and blood glucose disposal is influenced by the frequency of exercise sessions (for review, see Praet and van Loon, 2007), it was hypothesised that more frequent exercise-training sessions per week would be more effective for improving HRV and haemorheology.

3.2 METHODS

Subjects

Sixteen women aged 65-74 yr with a stable history of type 2 diabetes satisfied the following inclusion criteria and were accepted into this study. Volunteers responded to invitations mailed-out by Diabetes Australia to their member database, and/or advertisements placed in the local media and diabetes clinics. Volunteers were initially screened by a telephone interview to ascertain their suitability for participating in the present study. Inclusion criteria

for the present study were: i. clinical diagnosis of type 2 diabetes for at least 12 months; ii. no documented history of significant comorbidities (e.g. cardiovascular or lung disease, nephropathy, neuropathy or retinopathy); iii. the ability to walk unaided; iv. currently not smoking; v. currently not using medications known to interfere with the exercise response (e.g., thiazolidines), cardiac rhythm (e.g., beta blockers) or haemorheology (e.g., warfarin); vi. not currently using exogenous insulin. Suitable volunteers were invited to the laboratory for the measurement of basic anthropometry, pulmonary function, and supine and standing 12-lead ECG and blood pressure, at rest. Witnessed, written informed consent was obtained. The experimental protocol was approved by the institutional Human Research Ethics Committees.

Volunteers visited an accredited pathology laboratory to provide fasting blood samples for the assessment of basic haematology. Each volunteer was then asked to visit her personal General Practitioner with the screening results, for a medical opinion regarding her suitability for continuation in the present study. The General Practitioners were aware of the inclusion criteria for the present study and reported in writing that each volunteer had no significant comorbidities that would exclude them from the study. Of the 72 respondents to the recruitment drive, 24 individuals did not meet the inclusion criteria, and 48 individuals were not interested in further participation. Time commitment and disinterest in an exercise intervention were the most commonly cited reasons. Sixteen women, aged 65-74 yr, agreed to participate, with 15 subjects (one drop-out) completing the study. The subjects were randomly allocated into Group 1 (n = 8) or Group 2 (n = 7). No subject modified their medication regime during the study. Medications that were used by the subjects during the study period are listed in Table 3.1.

Experimental design

The present study investigated the pulmonary gas exchange, basic haematology, HRV, and haemorheology adaptations to 12 wk of moderate-intensity treadmill walking. Subjects were

randomly allocated into two groups that varied in exercise frequency and duration (Group 1: 2 x 60 min sessions per wk or Group 2: 4 x 30 min sessions per wk). The intensity of exercise training, equivalent to the gas-exchange threshold (T_{ge}), and the accumulated weekly exercise duration (120 min·wk^{-1}) were the same for both groups. Dependent variables were measured 6-wk before (-6 wk), immediately before (0 wk), and 6 and 12-wk after exercise training commenced. Subjects were instructed to continue their normal daily activity for the 6 wk before commencing exercise training. On a separate visit and before the first testing session, subjects practiced walking on a motor-driven treadmill at various speeds (2.0 – 6.0 km·h^{-1}) and treadmill grades (0 – 10%) to simulate a typical exercise session. Each subject's preferred walking speed was determined (at 1% grade; as described in Chapter 6, *'Familiarisation of experimental procedures and equipment'*) and was used during all subsequent exercise tests.

Table 3.1. Medications used by the subjects with type 2 diabetes during the study.

	Group 1 (2x60 min·wk^{-1}, *n* = 8)	Group 2 (4x30 min·wk^{-1}, *n* = 7)
Biguanide	4	3
Sulfonylurea	1	2
Statin	6	4
ACE inhibitor	4	2
Calcium channel blocker	1	2
Angiotensin II inhibitor	1	0
Diuretic	1	1
Aspirin	3	2

Values are numbers of subjects using the specific medication.

Determination of peak oxygen uptake and gas-exchange threshold

The subjects performed a physician-supervised incremental exercise test to volitional fatigue for the determination of peak oxygen uptake ($\dot{V}O_2peak$), time to exhaustion and $T_{ge,}$ on a

motor driven treadmill ('Valiant'; Lode B.V., Groningen, Netherlands) with an integrated safety harness to prevent falls. Subjects commenced walking at 3.0 $km \cdot h^{-1}$ at 0% grade for 4 min, before the speed was increased every 60 s until the previously determined preferred speed was attained. Treadmill grade was then increased by 2% every 60 s until volitional fatigue or signs and symptoms precluded further exercise. During the incremental exercise test, cardiac rhythm was monitored using a 12-lead ECG (Cardio Perfect, Welch Allyn Inc., Skaneateles Falls, USA) and brachial artery blood pressure was measured and recorded every 3 min. Oxygen uptake, carbon dioxide output ($\dot{V}CO_2$), and minute expired ventilation were measured breath-by-breath using a calibrated open-circuit metabolic measurement system (Ultima CPX, Medical Graphics Corporation, St Paul, USA). Peak exercise values were determined as the average of the two highest consecutive 30-s values measured before volitional fatigue. The T_{ge} was determined using the simplified V-slope method (Schneider et al., 1993). Peak power was calculated using the following equation: Power (W) = F (N) x V ($m \cdot s^{-1}$) x grade (%), where F was the force of each individual's body mass against gravity, V was the peak treadmill velocity, and grade was the percent gradient of the treadmill at peak exercise (Powers and Howley, 2004).

Acquisition and analysis of HRV

Measurements of cardiac (RR) intervals were performed using a validated (Gamelin et al., 2006) chest-strap coupled to a wristwatch receiver (Polar s810, Polar Electro OY, Kempele, Finland). Subjects were instructed to refrain from vigorous activity and caffeine consumption for at least 12 h before visiting the laboratory. No subject reported to the laboratory within 12 h of a significant hypoglycaemic event. After 20 min of supine rest in a dim and quiet laboratory (23±1°C), RR intervals were recorded continuously for 10 min. The parameters of HRV were subsequently determined in the time and frequency-domains for a 5-min segment, using validated software (Kubios HRV 2.0, Biosignal Analysis and Medical Imaging Group, University of Kuopio, Finland) (Tarvainen et al., 2009). Data points greater than 3.5 times the

interquartile range from the median value were considered extreme outliers and excluded from subsequent analysis to minimize the influence of outliers on the spectral components (Lippman et al., 1994).

Time-domain variables included: i. the mean of RR intervals (RR); ii. the standard deviation of RR intervals (SDNN); iii. the root mean square of the differences between consecutive RR intervals (RMSSD). Prior to frequency-domain analysis, the raw RR intervals were interpolated at 4 Hz to produce an evenly sampled dataset from the irregularly sampled time series. The power spectral density of the interpolated RR time series was calculated using autoregressive modelling (16^{th} order), which is the superior method for short-term spectral analysis (Fagard et al., 1998, Pichon et al., 2006). Power and peak frequencies were determined for the low frequency (LF, 0.04 – 0.15 Hz), and high frequency (HF, 0.15 – 0.4 Hz) bands. The power measured within the LF and HF bands were expressed in the natural logarithm of absolute units (ms^2).

Blood collection

Subjects visited an accredited pathology laboratory for the measurement of fasting glucose, insulin, glycated haemoglobin (HbA_{1c}), and homocysteine. Within 48 h of the initial blood sample and after an overnight fast, subjects returned to the experimental laboratory for the measurement of RBC aggregation and deformability. Blood was collected from a prominent antecubital vein into EDTA collecting tubes. Plasma was separated from whole blood within 30 min, and haemorheology measures were completed within 3 h of blood collection.

Determination of RBC deformability

RBC deformability was determined at 21±1°C using laser-diffractometry as described and validated by Shin et al., (2007). Briefly, 7 µL of whole blood from an EDTA tube was diluted

in 700 µL of medium solution (5.5% Polyvinylpyrrolidone, $mol \cdot L^{-1}$ = 360,000, dissolved in 1 $mmol \cdot L^{-1}$ PBS, osmolality = 300 $mosmol \cdot kg^{-1}$). Diluted blood (600 µL) was transferred to a test kit loaded into an ektacytometer (Rheoscan-D, Sewon Meditech Inc., Korea) for analysis. The sample was subjected to a range of shear stresses (0 – 25 Pa) by aspiration across a microchannel (~200 µm), while a laser was emitted through the channel. The laser-diffraction pattern for each sample was captured at 2 Hz by an integrated digital camera. The resultant images were analysed by fitting an ellipse to the diffraction pattern, and an elongation index (EI) calculated for shear stress levels ranging between 0~25 Pa using the following equation: EI = (A – B)/(A + B), where A is the length of the major axis of the ellipse, and B is the length of the minor axis of the ellipse. Measurements were performed in duplicate and the average EI values were subsequently fit with a non-linear curve (Prism, GraphPad Software Inc, Release 5.0, USA) to identify two parameters that describe RBC deformability as previously described (Baskurt et al., 2009b): $SS_{1/2,}$ the shear stress required for half of maximal deformation, and; EI_{max}, the maximum elongation index at infinite shear stress.

Determination of RBC aggregation

The haematocrit of whole blood was determined within 30 min of collection using an automated haematology analyser (Act.T diff, Beckman Coulter Inc., Fullerton, CA). Whole blood was then divided into two aliquots and centrifuged at 2000 *g* for 10 min. One aliquot of spun blood was adjusted to 40% haematocrit by adding or removing autologous plasma. The packed cells from the remaining aliquot of spun blood were separated from plasma and washed twice in isotonic PBS (285±3 $mmol \cdot kg^{-1}$, pH 7.4). The packed cells were then washed in an aggregating solution (3% DX70, dextran molecular mass 70 300; Sigma Chemical Co., St Louis, MO, USA), before being suspended in 3% DX70 at 40% haematocrit. The DX70 suspension enabled aggregation to be measured free from the influence of plasma factors (e.g., fibrinogen). RBC aggregation for both suspensions were determined at room temperature (21±1°C) from a 20 µL aliquot of each sample using a computerized

aggregometer system (Myrenne Aggregometer, Myrenne GmbH, Roetgen, Germany) as previously described (Bauersachs et al., 1989b). Two indices of RBC aggregation were determined for each suspension: i. M0, aggregation of RBCs at stasis, 10 s after an abrupt cessation of high shear (600 s^{-1}); ii. M1, aggregation of RBCs at a very-low shear (3 s^{-1}), 10 s after the cessation of a high shear (600 s^{-1}). Indices of aggregation were determined in both haematocrit adjusted plasma ($M0_{PLA}$ and $M1_{PLA}$) and haematocrit adjusted DX70 ($M0_{DX70}$ and $M1_{DX70}$). Measurements were performed in duplicate and the average values are reported.

Exercise training protocol

During exercise training, subjects were monitored with a remote telemetry system (X12+, Mortara Instrument Inc., Milwaukee, USA) that provided continuous ECG tracings. Blood pressure and heart rate were measured at rest in the upright exercise position. Subjects then performed 3 min of warm-up walking at 3.0 $km \cdot h^{-1}$ and 0% grade. The treadmill speed and grade were then increased to the predetermined speed/grade that elicited a $\dot{V}O_2$ value corresponding to 100% T_{ge} for the remainder of the exercise session (i.e., 30 or 60 min). Following each exercise session, 3 min of active recovery was performed at 3.0 $km \cdot h^{-1}$ and 0% grade. Blood pressure and heart rate were recorded every 5 min throughout the exercise training session, while cardiac rhythm was monitored continuously. After each exercise session, 3 min of active recovery was performed at 3.0 $km \cdot h^{-1}$ and 0% grade. All exercise sessions were scheduled and completed between 06:30 h and 11:30 h.

The speed and grade that corresponded with the T_{ge} for each subject was determined during the first exercise training session. Briefly, treadmill speed and grade were adjusted incrementally until steady steady-state $\dot{V}O_2$ matched the T_{ge} measured during the incremental exercise test. Breath-by-breath $\dot{V}O_2$ was measured as described for the incremental exercise test. This training intensity was maintained for 6 wk. The training intensity was then adjusted following the incremental exercise test performed during wk 6 testing, ensuring the training intensity reflected the T_{ge} for the entire 12 wk duration.

Data Analyses

Results are reported as mean±SD unless otherwise stated. Data were examined using two-way ANOVA with repeated measures (time: wk -6, 0, 6, and 12) within each group. Pair-wise comparisons were performed when appropriate with Bonferroni adjustments. Time to exhaustion was compared only within each group, rather than between groups, as the incremental exercise test was performed at preferred walking speed – thus the temporal data was only comparable within individuals, but not between groups. Bivariate correlations were performed to detect significant relationships between dependent variables. An alpha level of 0.05 was chosen to indicate statistical significance. Statistical analyses were performed with SPSS PC (SPSS Inc, Release 17.0, USA).

3.3 RESULTS

No significant differences were detected between Group 1 and 2 for age (Group 1: 68.6±2.8 yr; Group 2: 69.3±2.5 yr), body mass, or systolic and diastolic BP measures (see Table 3.2). Exercise training for 12-wk significantly reduced systolic ($F = 8.507$, $p = 0.012$) and diastolic BP ($F = 11.945$, $p = 0.004$) in both groups, when compared with wk -6 and 0.

Peak $\dot{V}O_2$ ($L \cdot min^{-1}$) was significantly higher for Group 1 (2 x 60 $min \cdot wk^{-1}$) at wk -6 and 0 ($F = 4.858$, $p = 0.046$) when compared with Group 2 (4 x 30 $min \cdot wk^{-1}$), but no significant differences between groups were observed at wk 6 or 12. When $\dot{V}O_2peak$ was expressed relative to body mass ($mL \cdot kg^{-1} \cdot min^{-1}$), however, no differences were detected between groups at any time point ($F = 1.492$, $p = 0.244$). Both exercise groups significantly increased $\dot{V}O_2peak$ following 12 wk of training ($F = 13.486$, $p = 0.003$). Accordingly, exercise tolerance improved following exercise training for both groups, which was reflected by significant improvements in time to exhaustion ($F = 28.558$, $p < 0.001$) and peak power ($F = 25.123$, $p < 0.001$).

Table 3.2. Physical characteristics and peak exercise values during incremental exercise testing before (wk -6, 0) and after 12-wk exercise training in women aged 65-74 yr with type 2 diabetes.

	Group 1 (2x60 min·wk^{-1}, *n* = 8)			Group 2 (4x30 min·wk^{-1}, *n* = 7)		
Variable	Week -6	Week 0	Week 12	Week -6	Week 0	Week 12
Body mass, kg	77.9±13.0	77.0±13.3	77.7±13.1	76.2±15.3	76.0±15.5	75.9±14.9
Body mass index, kg·m^2	30.5±5.1	30.1±5.2	30.4±5.2	30.1±5.6	30.0±5.5	29.6±5.1
TE, s	703±125	705±94	816±105*	584±103	608±96	786±90*
$\dot{V}O_2$peak , L·min^{-1}	1.60±0.11	1.58±0.18	1.67±0.16*	1.44±0.15†	1.35±0.14†	1.52±0.21*
$\dot{V}O_2$peak , mL·kg^{-1}·min^{-1}	21.0±3.4	21.1±4.6	22.2±4.4*	19.2±2.5	18.0±2.8	20.3±2.3*
Peak power, W·kg^{-1}	1.8±0.5	1.8±0.4	2.4±0.4*	1.4±0.3	1.4±0.3	2.2±0.4*
Peak HR, beat·min^{-1}	141±11	141±14	142±12	146±14	141±15	144±11
$\dot{V}O_{2\ Tge}$, L·min^{-1}	1.09±0.06	1.09±0.05†	1.17±0.10*,†	0.91±0.09	0.89±0.16†	1.00±0.18*,†

Values are mean±SD. *, $p < 0.05$, post-training significantly different to wk 0. †, $p < 0.05$, Group 1 significantly different to Group 2. BP, blood pressure; $\dot{V}O_2$peak, peak oxygen uptake; HR, heart rate; $\dot{V}O_{2\ Tge}$, oxygen uptake measured at the gas-exchanged threshold; T_{ge}, gas-exchanged threshold.

Differences were revealed between groups for $\dot{V}O_2$ measured at the T_{ge}; Group 1 had significantly higher T_{ge} than Group 2 ($F = 9.722$, $p = 0.008$) at all data collection points. The $\dot{V}O_2$ measured at the T_{ge} significantly increased following 12 wk of training ($F = 6.426$, $p = 0.001$). When T_{ge} was expressed as a percentage of $\dot{V}O_2$, however, no differences were observed between groups, or due to exercise training.

No significant differences were observed between groups for the parameters of haematology and haemorheology across any time point (see Table 3.3). In addition, no significant differences were detected between pre- and post-training concentrations of blood glucose, insulin, HbA_{1c} or homocysteine.

Table 3.3. Haematology and red blood cell deformability before (wk -6, 0) and after 12-wk exercise training in women aged 65-74 yr with type 2 diabetes.

	Group 1 (2x60 min·wk^{-1}, *n* = 8)			Group 2 (4x30 min·wk^{-1}, *n* = 7)		
Variable	Week -6	Week 0	Week 12	Week -6	Week 0	Week 12
Glucose, mmol·L^{-1}	7.1±0.6	7.2±1.0	7.2±0.9	7.6±2.4	7.5±2.6	7.1±2.0
Insulin, mU·L^{-1}	13.6±10.1	13.4±9.7	15.1±10.6	7.7±3.4	7.7±3.8	7.8±3.1
HbA_{1c} (%)	6.2±0.4	6.3±0.4	6.5±0.3	7.0±1.0	6.9±1.1	6.9±1.0
Cholesterol, mmol·L	4.4±0.8	4.5±0.6	4.1±0.7	4.4±1.0	4.2±0.9	4.3±0.9
Triglyceride, mmol·L	1.5±0.8	1.5±0.5	1.4±0.5	1.2±0.5	1.1±0.4	1.2±0.4
HDL, mmol·L^{-1}	1.3±0.3	1.3±0.3	1.4±0.3*	1.4±0.3	1.4±0.3	1.5±0.3*
LDL, mmol·L^{-1}	2.4±0.7	2.5±0.8	2.1±0.7	2.4±0.9	2.3±0.8	2.3±0.7
Homocysteine, mmol·L	14.2±6.4	13.8±6.5	12.9±3.4	10.1±2.0	10.2±1.8	9.4±1.7
EI_{max}	0.567±0.012	0.563±0.013	0.574±0.011*	0.555±0.020	0.551±0.023	0.562±0.022*
$SS_{1/2}$, Pa	2.58±0.40	2.52±0.41	2.87±0.42	2.53±0.59	2.74±0.74	2.64±0.71

Values are mean±SD. *, $p < 0.05$, post-training significantly different to wk 0. HbA_{1c}, glycated haemoglobin; EI_{max}, maximum elongation index (i.e., deformability) at infinite shear stress; $SS_{1/2}$, shear stress for half-maximal deformation.

While no change in $SS_{1/2}$ was observed due to exercise training, EI_{max} was significantly increased for both groups at 12 wk when compared with pre-training ($F = 3.586$, $p = 0.022$). The time-course changes in RBC aggregation during the exercise-training intervention are illustrated in Figure 3.1. The magnitude of change in RBC aggregation, due to exercise training, was not significantly different between Group 1 and 2. Whereas M0 was significantly reduced by 12-wk training in DX70 ($F = 6.257$, $p = 0.016$) compared with wk 0 for both groups, M1 was significantly reduced in both PLA ($F = 6.137$, $p = 0.019$) and DX70 ($F = 5.141$, $p = 0.029$) suspensions at wk 12, when compared with wk 0 for both groups. The decrease in $M0_{PLA}$ and the decrease in $M0_{DX70}$ with exercise training were negatively correlated with the training-induced increase in $\dot{V}O_2peak$ ($r = -0.64$, $p = 0.010$ and $r = -0.66$, $p = 0.008$, respectively).

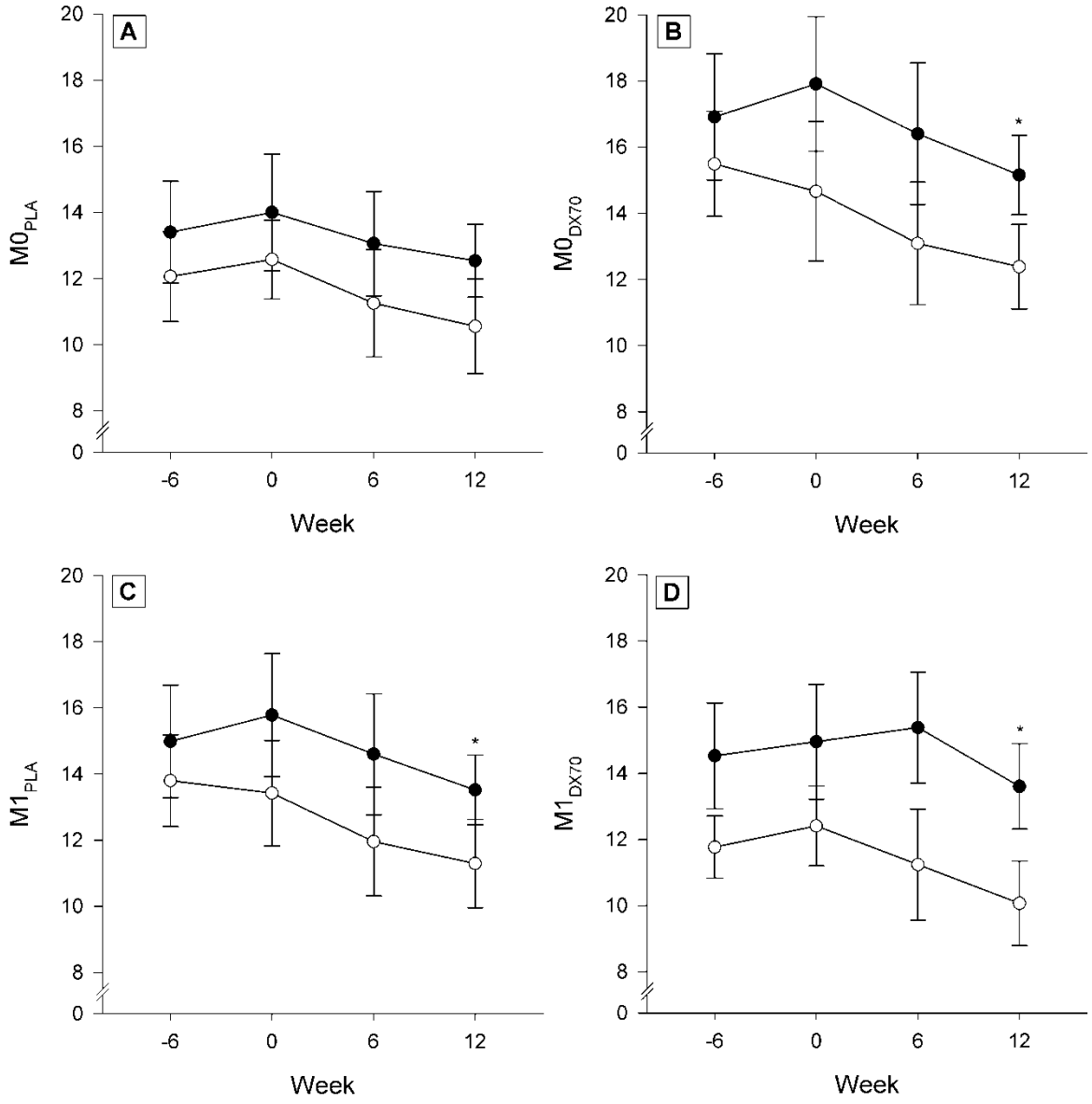

Figure 3.1. Changes in RBC aggregation due to exercise training for Group 1 (●) and Group 2 (○). Values are mean±SEM. *, $p < 0.05$, significantly different to pre-training. M0, aggregation at stasis following high shear disaggregation. M1, aggregation at low shear following high shear disaggregation. Aggregation was determined in two suspending mediums: PLA, plasma adjusted to 40% haematocrit, and; DX70, dextran 70 adjusted to 40% haematocrit.

Significant differences were detected between groups for the HRV parameters (Table 3.4). An interaction effect was observed for SDNN ($F = 4.072$, $p = 0.030$), RMSSD ($F = 4.953$, $p = 0.016$), and the natural log of HF (nlHF) power ($F = 8.855$, $p = 0.001$). While exercise did not

influence HRV for subjects training 2 x 60 min·wk^{-1} (Group 1), SDNN, RMSSD, and nlHF significantly increased for subjects training 4 x 30 min·wk^{-1} (Group 2) ($p < 0.05$).

Table 3.4. Heart rate variability before (wk -6, 0) and after 12-wk exercise training in women aged 65-74 yr with type 2 diabetes.

	Group 1 (2x60 min·wk^{-1}, *n* = 8)			Group 2 (4x30 min·wk^{-1}, *n* = 7)		
Variable	Week -6	Week 0	Week 12	Week -6	Week 0	Week 12
RR, ms	950±87	961±79	948±94	899±96	891±98	928±108
SDNN, ms	19.6±7.4	17.3±7.5	14.5±4.6	14.8±8.4	13.8±9.0	17.8±10.1*
RMSSD, ms	20.6±8.8	18.5±8.4	15.0±4.5	13.4±6.6	14.2±10.5	18.4±11.2*
lnLF, ms^2	4.7±0.7	4.9±1.0	4.4±0.7	4.2±1.4	4.2±1.4	4.5±1.4
lnHF, ms^2	4.6±1.1	4.9±0.9	4.2±0.5	4.1±1.5	4.0±1.1	4.5±1.1*
lnLF:lnHF, ratio	1.06±0.19	1.02±0.19	1.04±0.15	1.06±0.23	1.06±0.25	0.99±0.13

Values are mean±SD. *, post-training significantly different to wk 0. RR, mean duration of cardiac (RR) intervals; SDNN, standard deviation of normal-to-normal RR intervals; RMSSD, root mean square of the differences between consecutive RR intervals; lnLF, natural log of low-frequency power; lnHF, natural log of high-frequency power.

3.4 DISCUSSION

The principal finding of the present study was that 12 wk of treadmill walking for 120 min·wk^{-1} at an intensity equivalent to the T_{ge}, significantly decreased RBC aggregation and increased RBC deformability in women 65-74 yr with type 2 diabetes, irrespective of the frequency and duration of exercise sessions. In contrast, significant improvements in HRV were only observed in those subjects performing more frequent exercise sessions (4 x 30 min·wk^{-1}; Group 2). These findings suggest that walking for 120 min·wk^{-1} is effective in improving haemorheology; however, the improvement of cardiac autonomic modulation may be dependent on the frequency of exercise sessions.

Our hypothesis, that frequent exercise training would be more effective for the improvement of haemorheology was not supported by the results. Red blood cell aggregation was

significantly decreased and RBC deformability was significantly increased following exercise training, irrespective of training frequency. Impaired haemorheology has been suggested to precede and contribute to the development of CVD (Zilberman-Kravits et al., 2006). Intense management of type 2 diabetes (comprising insulin therapy, dietary modification and exercise training) lead to improved glycaemic control reduced RBC aggregation (Chong-Martinez et al., 2003). The present study demonstrated that an exercise-only intervention significantly improved RBC aggregation and deformability in individuals with type 2 diabetes without a concomitant improvement in glycaemic control. Our findings are important, as it has been suggested that the chronic hyperglycaemic environment characteristic of type 2 diabetes increases RBC aggregation by reducing the negative surface charge of RBCs (Rogers et al., 1992) and increased plasma factors (e.g., fibrinogen) (Singh and Shin, 2009). We observed a decrease in RBC aggregation for cells that were suspended in a plasma-free medium (DX70), suggesting that plasma factors do not explain the magnitude of decrease in RBC aggregation. Consequently, it appears that RBC aggregation decreased via mechanisms intrinsic to the RBC, independent of glycaemic status and the frequency of exercise training. Given that RBC deformability also improved due to exercise training, it is plausible that intrinsic changes to the RBC accounted for reduced aggregation and improved membrane compliance.

In the present study, significant improvements in HRV were only observed in Group 2 (4 x 30 min·wk^{-1}), whereas HRV did not significantly change in Group 1 (2 x 60 min·wk^{-1}). Our hypothesis that more-frequent exercise training would be more effective for improving HRV is supported by the present results. Measures of cardiac variability (SDNN), as well as parameters reflecting parasympathetic cardiac modulation (RMSSD, HF power) increased significantly following 12 wk of exercise training for Group 2, when compared with pre-training values measured at wk -6 and 0. This finding suggests that cardiac autonomic modulation, particularly parasympathetic, may be sensitive to the frequency of exercise training and might explain why previous studies, using different exercise training stimuli, have reported disparate results regarding the effects of exercise training on HRV (Bhagyalakshmi et al., 2007, Kanaley et al., 2009a). Increases in parasympathetic cardiac

modulation post-training are suggested to be responsible for associated decreases in resting heart rate (Ekblom et al., 1973). In the present study, however, mean RR intervals (heart rate) at rest did not significantly change with exercise training for either group, despite improved HRV for Group 2. Katona et al., (1982) pharmacologically blocked each branch of the autonomic nervous system and demonstrated that the lower resting heart rate of trained individuals was due to decreased intrinsic cardiac rate, rather than increased parasympathetic cardiac modulation. Katona et al., (1982) suggested a dissociation of parasympathetic cardiac modulation and resting heart rate, which might explain why parasympathetic parameters of HRV were increased following exercise training in the present study despite no significant change in mean RR intervals. It is possible that increased HRV may have been due to altered vasomotor tone and baroreflex sensitivity (Aubert et al., 2003).

The exercise dose of the present study did not improve glycaemic control in either exercise group. Brun et al., (2008) reported that 12 mo of walking twice weekly (2 x 30-45 min·wk^{-1} at ~T_{ge}) was also insufficient in improving blood glucose concentration in type 2 diabetics. It has been suggested that individuals with type 2 diabetes should expend ~5 MJ·wk^{-1} during exercise training, which equates to brisk walking for ~19 km·wk^{-1} (Praet and van Loon, 2007). In the present study, the energy expenditure during exercise training was calculated to be ~3 MJ·wk^{-1} and subjects walked ~10 km·wk^{-1}. Thus, the exercise dose of the present study may not have been sufficient to improve glycaemic control. However, to achieve the previous recommendation (Praet and van Loon, 2007) an older individual would be required to walk for ~4-5 h per wk. This may be overly optimistic for older women with type 2 diabetes, given that i. physical inactivity increases with ageing, and; ii. the compliance to exercise for individuals with type 2 diabetes is notoriously low (Zhao et al., 2008). It is plausible that the exercise training could not improve glycaemic control over-and-above that exerted by hypoglycaemic medications for the subjects in the present study.

Systolic and diastolic BP at rest were significantly reduced in both exercise groups. Acute reductions in BP occur after a single bout of exercise (i.e., post-exercise hypotension) that may be sustained for at least 13 h after the cessation of exercise (Pescatello et al., 1991).

Ishikawa-Takata et al., (2003) reported that >30 min·wk^{-1} of moderate-intensity walking by patients with essential hypertension significantly decreased systolic and diastolic BP, irrespective of whether exercise was performed 1-2 or >5 sessions·wk^{-1}. Moreover, increasing the duration of exercise beyond 30 min·wk^{-1} did not induce any further significant improvements in systolic and diastolic BP (Ishikawa-Takata et al., 2003). The effectiveness of even low volumes of exercise and at a moderate intensity for improving BP at rest may be especially important for the prescription of exercise for individuals with poor exercise compliance, such as those with type 2 diabetes (Praet and van Loon, 2007), who are also at an increased risk for CVD.

The mechanism for reduced $\dot{V}O_2$peak in type 2 diabetes, when compared with healthy controls, is unresolved (Brandenburg et al., 1999, Segerström et al., 2008); however, it is suggested that impaired cardiac and/or endothelial function may reduce oxygen delivery to skeletal muscle, ultimately reducing $\dot{V}O_2$peak and exercise tolerance (Brandenburg et al., 2003). Our findings demonstrate that exercise training for 120 min·wk^{-1} over 12 wk, at an intensity corresponding with the T_{ge}, increases $\dot{V}O_2$peak in older women with type 2 diabetes, regardless of whether training was performed 2 or 4 times per wk. Improvements in the $\dot{V}O_2$peak of women with type 2 diabetes may result from an increased rate of oxygen uptake due to increased delivery and/or utilization of oxygen (Brandenburg et al., 1999). Given that increased RBC aggregation may contribute to reduced blood flow (Durussel et al., 1998), at least in capillaries of a horizontal alignment (Baskurt and Meiselman, 2008), the decreased RBC aggregation following exercise training in the present study may have improved microcirculatory blood flow and consequently, peripheral oxygen extraction. The significant negative relationships detected between RBC aggregation and $\dot{V}O_2$peak in the present study suggest that decreased RBC aggregation may indeed contribute to the increase in $\dot{V}O_2$peak following exercise training.

The reduced T_{ge} in individuals with type 2 diabetes, when compared with age-matched controls (Regensteiner et al., 1998), may represent one of the limitations to prolonged exercise in this population. In the present study, both exercise groups, independent of

exercise frequency and duration, demonstrated significant increases in the T_{ge} following exercise training. Improvements in T_{ge} following exercise training have been attributed to intramuscular adaptations broadly relating to decreased lactate production or increased lactate clearance during exercise (for review, see Faude et al., 2009). Functionally, an improved T_{ge} is a positive outcome of endurance-exercise training that permits an individual to perform physical activity at a higher absolute intensity and/or for a longer duration, before the onset of fatigue. Given that exercise participation and adherence is reduced in individuals with type 2 diabetes, increasing the T_{ge} may enhance exercise compliance by attenuating the unfavourable effects of fatigue associated with physical activity.

While the findings of the present study contribute to the mounting evidence for the benefits of regular physical activity in type 2 diabetes, challenges remain with respect to translating this into increased exercise participation in this population. The present study involved highly targeted recruitment strategies in a large urban area. Despite many individuals that were eligible for inclusion (48 of 72 volunteers) in the study reporting an awareness of the importance for regular exercise, a substantial majority (32 volunteers) were not willing to commit to regular and supervised exercise training. Future studies may benefit from the incorporation of behaviour modification practices to improve subject compliance and increased exercise participation by individuals with type 2 diabetes .

In summary, moderate-intensity walking for 120 $min \cdot wk^{-1}$ significantly improved RBC aggregation and deformability, systolic and diastolic BP, $\dot{V}O_2peak$ and T_{ge}, independent of the frequency and duration of exercise sessions. It appears that the accumulated weekly exercise duration, therefore, may be the most important training component for the prescription of exercise in older women with type 2 diabetes. Exercise frequency should be considered when prescribing exercise for the improvement of HRV.

Chapter 4

Increased interleukin-10 following regular walking in women 65-74 yr with type 2 diabetes.

Study Three

Chapter preface The results of Study Two suggested that regular exercise had the capacity to alter markers of CVD in the context of type 2 diabetes. Given the central role of inflammation in CVD and indeed type 2 diabetes, it was surprising that the influence of exercise training – in the absence of concurrent lifestyle interventions – on inflammatory markers in type 2 diabetes remained largely unknown. Thus, rather than investigating whether the specific dosage of exercise was important for improving inflammation, the purpose of Study Three was simple: to investigate whether exercise training *alone* could improve the ratio of pro- to anti-inflammatory markers in type 2 diabetes.

4.1 INTRODUCTION

Prospective studies demonstrate that hyperglycaemia is positively related to the incidence of macro- and microvascular complications in type 2 diabetes (Fowler, 2008). Type 2 diabetes impairs the function of endothelial and smooth muscle cells, leading to vascular dysfunction (for review, see Beckman et al., 2002). Low-grade inflammatory processes are reported to be involved in the initiation and progression of atherosclerosis in individuals with and without type 2 diabetes (Dandona et al., 2003). The role of inflammation in the development of type 2 diabetes is also becoming recognised, given that increased plasma concentrations of inflammatory markers may be observed in apparently healthy individuals that later develop type 2 diabetes (Pradhan et al., 2001, Thorand et al., 2003). Elevated concentrations of inflammatory markers, such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), may also contribute to insulin resistance (Kahn et al., 2006b), supporting that inflammation may be common to cardiovascular diseases and type 2 diabetes. Therefore, strategies to minimise the adverse effects of pro-inflammatory proteins may improve the therapeutic management of type 2 diabetes.

An effective therapeutic strategy for preventing and managing type 2 diabetes is regular aerobic and/or resistance exercise. Dynamic aerobic and resistance exercise are reported to increase insulin sensitivity and facilitate the translocation of glucose transporters to the outer membrane of peripheral tissues, resulting in an improved uptake of glucose and therefore modulating hyperglycaemia (Mikines et al., 1989b, Goodyear et al., 1992). Given the central role of inflammatory processes in atherosclerotic development, myocardial ischaemia and other cardiac-related diseases, there is increased awareness of the importance for managing inflammation in type 2 diabetes. Paradoxically, given that acute exercise is associated with increased concentrations of inflammatory cytokines and acute-phase reactants (Moldoveanu et al., 2001), lifestyle modification including chronic exercise has been demonstrated to reduce markers of inflammation in some (Esposito et al., 2003, Kadoglou et al., 2007, Dekker et al., 2007, Stewart et al., 2007), but not all studies (Christiansen et al., 2010). Exercise training may also increase interleukin-10 (IL-10) concentration, which inhibits the gene

expression and production of proinflammatory cytokines (e.g., IL-6, TNF-α) (Tinsley et al., 2010). The specific anti-inflammatory effect of IL-10 is complex, but may be mediated by the inhibition of proinflammatory cytokine release from T-cells, in addition to reduced antigen presenting capabilities of macrophages (for review, see Asadullah et al., 2003). Consequently, the capacity for exercise training to concurrently decrease circulating proinflammatory cytokines and increase anti-inflammatory cytokine concentration is of clinical interest. Whether aerobic exercise training alone, however, improves the balance between anti- to proinflammatory cytokines remains less clear, given previous studies included mixed dietary and exercise interventions (Esposito et al., 2003, Dekker et al., 2007) and/or the mixed-mode of exercise prescribed (e.g., combination of cycling, treadmill, resistance) (Stewart et al., 2007, Kadoglou et al., 2007).

It is important to determine the effect of aerobic exercise training *alone* on inflammatory markers in individuals with type 2 diabetes, given that i. weight loss following dietary intervention significantly influences inflammatory markers, and ii. regular walking is the preferred method of exercise among the elderly, yet previous studies incorporated multiple modes of exercise. It was hypothesised that 12 wk of walking would improve the balance of pro- to anti-inflammatory markers.

4.2 METHODS

Subjects

Eighteen women (69 ± 3 yr) with type 2 diabetes volunteered to participate in the present study. Individuals responded to invitations mailed-out by Diabetes Australia (Queensland) and/or advertisements placed in the local media and diabetes clinics. Individuals were initially screened by a telephone interview to ascertain their suitability for participating in the present study. Inclusion criteria for the present study were: i. clinical diagnosis of type 2 diabetes for at least 12 months; ii. no documented history of significant comorbidities (e.g.

cardiovascular or lung disease, nephropathy, neuropathy or retinopathy); iii. the ability to walk unaided; iv. currently not smoking; v. currently not using medications known to interfere with the exercise response (e.g., thiazolidines), cardiac rhythm (e.g., beta blockers); and vi. not currently using exogenous insulin. Suitable individuals were invited to the laboratory for a detailed clinical examination, the measurement of basic anthropometry, pulmonary function, supine and standing 12-lead ECG and blood pressure at rest. Witnessed, written informed consent was obtained from volunteers willing to continue their participation. Volunteers were subsequently asked to visit an accredited pathology laboratory to provide a fasting blood sample for the assessment of basic haematology. Each volunteer was then asked to visit their personal General Medical Practitioner with the screening results, for a medical opinion regarding their suitability to participate in the present study. The General Medical Practitioners were aware of the inclusion criteria for the present study and reported in writing that each volunteer had no significant comorbidities that would exclude them from the study. The experimental protocol was conducted in accordance with the Declaration of Helsinki and was approved by the Human Research Ethics Committees at Griffith University and Bond University.

Experimental design

The present study comprised a 12-wk exercise training intervention that was preceded by a 6-wk 'control' period. Thus, each subject served as their own control, prior to performing an individualised walking intervention. Subjects performed 120 min of treadmill walking each week for 12 wk, which was delivered as either 60 min of walking twice per wk, or 30 minutes of walking four times per wk. The intensity of exercise training was equivalent to each individual's gas-exchange threshold (T_{ge}) that was determined during an incremental exercise test. On a separate visit and before the first testing session, subjects practiced walking on a motor-driven treadmill at various speeds (2.0 – 6.0 $km \cdot h^{-1}$) and treadmill grades (0 – 10%) to simulate a typical exercise session. Each subject's preferred walking speed was determined

(at 1% grade) and was used during all subsequent exercise tests. Dependent variables were measured 6-wk before (-6 wk), immediately before (0 wk), and 6 and 12-wk after exercise training commenced. Subjects were instructed to continue their normal daily activity for the duration of the study (i.e., -6 wk to 12 wk). Dependent variables included gas-exchange data obtained during an incremental exercise test, anthropometry, resting supine blood pressure, basic haematology, and plasma concentrations of fibrinogen, C-reactive protein (CRP), interleukins 6 and 10 (IL-6, IL-10), and tumour necrosis factor-alpha (TNF-α).

Exercise training protocol

The exercise training intensity was determined for each individual during the first exercise training session. Breath-by-breath $\dot{V}O_2$ was measured while the treadmill speed was increased by 0.5 $km \cdot h^{-1}$ increments every 3 min until the subjects previously determined preferred walking speed had been attained. Treadmill grade was subsequently increased by 1% increments every 3 min until steady-state $\dot{V}O_2$ approximated the T_{ge} measured during the previous incremental exercise test; speed and grade were recorded and used during all exercise training sessions for the first 6 wk. Exercise training intensity was re-adjusted, as described above, following the incremental exercise test performed during week 6.

Each morning before commencing exercise training, subjects were fitted with a remote telemetry system (X12+, Mortara Instrument Inc., Milwaukee, USA) that provided continuous ECG recordings (leads II, V2 and V5). Blood pressure and heart rate were then recorded at rest in the upright exercise position. Warm-up exercise involved the subject walking at 3.0 $km \cdot h^{-1}$ and 0% grade for 3 min. The treadmill speed and grade were subsequently increased to the pre-determined training speed/grade for the remainder of the exercise session. After each exercise-training session, 3 min of active recovery was performed at 3.0 $km \cdot h^{-1}$ and 0% grade. Blood pressure and heart rate were recorded every 5 min throughout the training session, while cardiac rhythm was monitored continuously.

Determination of peak oxygen uptake and gas-exchange threshold

A physician-supervised incremental exercise test to volitional fatigue was performed for the determination of peak oxygen uptake ($\dot{V}O_2peak$), time to exhaustion and $T_{ge,}$ on a motor driven treadmill ('Valiant'; Lode B.V., Groningen, Netherlands) with an integrated safety harness to minimise the risk of falls. Subjects commenced walking at 3.0 $km \cdot h^{-1}$ and 0% grade for 4 min, before the speed was increased every 60 s until each subject's previously determined preferred walking speed was attained. Treadmill grade was then increased by 2% every 60 s until volitional fatigue or signs and symptoms precluded further exercise. During the incremental exercise test, cardiac rhythm was monitored using a 12-lead ECG (Cardio Perfect, Welch Allyn Inc., Skaneateles Falls, USA) and blood pressure was measured at the left brachial artery and recorded every 3 min. Oxygen uptake, carbon dioxide output, and minute expired ventilation were measured breath-by-breath using an open-circuit metabolic measurement system (Ultima CPX, Medical Graphics Corporation, St Paul, USA). The gas analysers and pneumotachograph were calibrated before each incremental exercise test using precision reference gases and a 3-L calibration syringe (Hans Rudolph Inc., Kansas City, USA). Peak exercise values were determined as the average of the two highest consecutive 30-s values measured before volitional fatigue. The T_{ge} was determined using the simplified V-slope method (Schneider et al., 1993).

Biochemical assays

Subjects visited the research laboratory between 0700–0830 h after an overnight (~12 h) fast to provide a blood sample for the assessment of plasma fibrinogen, CRP, IL-6, IL-10 and TNF-α. Blood was collected into ethylenediaminetetraacetic acid (EDTA, 1.8 $mg \cdot ml^{-1}$) or sodium citrate (3.2%) collection tubes. Plasma was separated from whole blood within 30 min of collection by centrifugation (1500 *g* for 10 min) and then immediately aliquoted into 500 uL samples that were stored at -80°C until analysis. Within 48 h of the initial blood sample and after an overnight fast, subjects visited an accredited pathology laboratory

between 0700–0830 h for the measurement of fasting glucose, insulin, cholesterol, low- and high-density lipoprotein, and homocysteine concentrations. The validated 'nonlinear homeostasis model assessment' (HOMA2) (Wallace et al., 2004) was used to determine insulin sensitivity (%S), beta-cell function (%β), and insulin resistance (IR) (HOMA Calculator, Release 2.2.2, Diabetes Trial Unit, University of Oxford, UK).

Plasma fibrinogen, cytokine and CRP concentrations were determined *en masse* upon the completion of the study. Fibrinogen concentration was measured in sodium citrate plasma using a random access coagulation analyser (CA-6000, Sysmex Corporation, Hyaga, Japan) by the method first described by Clauss (2001). Quantitative detection of plasma CRP was performed using a commercially available enzyme-linked immunosorbent assay (ELISA, Bender MedSystems GmbH, Vienna, Austria), while IL-6, IL-10, and TNF-α in EDTA plasma were measured using high-sensitivity ELISA from the same manufacturer. The limits of detection were: CRP, 0.00015 $mg \cdot L^{-1}$; IL-6, 0.03 $pg \cdot mL^{-1}$; IL-10, 0.05 $pg \cdot mL^{-1}$; TNF-α, 0.13 $pg \cdot mL^{-1}$. The intra-assay coefficient of variances calculated for each ELISA using duplicate samples at the four time points were: CRP, 7.5%; IL-6, 6.3%; IL-10, 6.0%; TNF-α, 5.1%.

Anthropometry and resting blood pressure

Subjects were instructed to refrain from vigorous activity as well as caffeine and alcohol consumption for at least 12 h before visiting the laboratory. Upon arrival at the laboratory, the inflatable cuff of a mercury sphygmomanometer (Baumanometer Standby model, W.A. Baum Co. Inc., Copiague, NY) was fitted to the volunteers left arm. The subject was asked to lay supine on a clinical bed for 15 min of rest in a quiet laboratory with minimal lighting. Blood pressure was subsequently measured using a stethoscope and brachial artery occlusion, and the first and fourth Korotkoff sounds were used to determine systolic and diastolic blood pressures, respectively. During the same visit, body mass was measured to the nearest 0.01 kg using precision scales (2391 Eye-Level Physician Scale, Cardinal Scale Mfg. Co., Webb

City, USA) after the volunteer had removed their shoes and excess clothing (e.g., coats). Waist and hip circumferences were measured using a flexible inelastic tape (Lufkin W606PM, Cooper Tools, Raleigh, USA) against the skin without compressing the underlying tissue. Measurements were always performed by the same investigator.

Data Analyses

Results are reported as mean±SD unless otherwise stated. Normality of the data was tested using the D'Agostino & Pearson omnibus normality test (GraphPad Software Inc, Release 5.0, USA). No differences were observed between those subjects that performed exercise training for 30 min on 4 d or 60 min on 2 d per wk; consequently all data were grouped for analyses. Data were analysed using a full factorial ANOVA with repeated measures for time, with Bonferroni adjustments used when appropriate to determine differences between time points. Two subjects did not complete the entire 12 wk of the walking program due to personal circumstances – their data was excluded from repeated measures analyses. Bivariate correlations were performed to detect significant relationships between dependent variables. An alpha level of 0.05 was chosen to indicate statistical significance. Statistical analyses were performed with SPSS PC (SPSS Inc, Release 17.0, USA).

4.3 RESULTS

The baseline characteristics of the subjects are provided in Table 4.1. Two subjects only completed 6 wk of the walking program due to personal circumstances – their data was excluded from repeated measures analyses – while all other subjects completed >90% of exercise training sessions. All subjects demonstrated the ability to complete the prescribed duration of the exercise sessions, suggesting that the intensity and duration of walking were well tolerated. Fasting glucose concentration was elevated with respect to the expected range for healthy individuals. Insulin resistance as determined by HOMA2 computer modelling

(IR) was elevated for this cohort when compared to normal values (Geloneze et al., 2009); the subjects had substantially decreased %β and reduced %S. The percent change in the dependent variables measured after the control period (i.e., at week 0), and after 6 and 12 wk of walking are presented in Table 4.2. No significant difference was observed for any dependent variables measured at wk -6 and wk 0, indicating a reliable control period for comparisons with exercise-training data.

Table 4.1. Baseline characteristics.

	Week -6
Body mass, kg	77.9±13.2
BMI, $kg \cdot m^{-2}$	30.5±5.0
Waist:hip ratio	0.87±0.07
Systolic BP, mmHg	133.3±12.5
Diastolic BP, mmHg	76.4±7.4
Time to exhaustion, s	620.9±143.6
$\dot{V}O_2peak$, $L \cdot min^{-1}$	1.50±0.16
$\dot{V}O_2peak$, $mL \cdot kg^{-1} \cdot min^{-1}$	19.6±3.27
Glucose, $mmol \cdot L^{-1}$	7.43±1.61
Insulin, $mU \cdot L^{-1}$	11.0±7.7
Cholesterol, $mmol \cdot L^{-1}$	4.59±1.11
Triglyceride, $mmol \cdot L^{-1}$	1.41±0.68
HDL, $mmol \cdot L^{-1}$	1.34±0.28
LDL, $mmol \cdot L^{-1}$	2.61±0.97
Chol:HDL	3.53±0.99
Homocysteine, $mmol \cdot L^{-1}$	11.9±5.0
%β	66.0±37.4
%S	79.1±46.5
IR	1.53±1.04

Values are mean±SD. BMI: body mass index. BP: blood pressure. $\dot{V}O_2peak$: peak oxygen uptake. HR: heart rate. HDL: high-density lipoprotein. LDL: low-density lipoprotein. %β: beta-cell function. %S: insulin sensitivity. IR: insulin resistance.

Anthropometry, cardiovascular conditioning and blood pressure.

The walking program did not elicit any change in body mass, BMI or waist:hip ratio. The time to exhaustion measured during maximal exercise testing increased significantly ($F = 24.436$, $p < 0.001$) after 6 and 12 wk of the walking program. While $\dot{V}O_2peak$ tended to increase after 6 wk of the walking program, a significant increase was not observed until 12 wk of regular walking had been completed ($F = 6.451$, $p = 0.001$). Systolic blood pressure decreased after 6 wk and 12 wk of regular walking when compared with baseline ($F = 5.249$, $p = 0.003$). A trend for reduced diastolic blood pressure was observed after 6 wk of regular walking ($p = 0.050$); however, significance was only observed after 12 wk ($F = 4.943$, $p = 0.005$). At baseline, six individuals presented poorly controlled hypertension (i.e., systolic 140-159 mmHg or diastolic > 90-99 mmHg) despite concurrent pharmacological intervention (Chobanian et al., 2003); after 6 wk of regular walking, two of the six individuals were still poorly controlled, but after 12 wk of regular walking, only one individual still presented with systolic blood pressure > 140 mmHg.

Glycaemic control and blood lipids

Fasting glucose and insulin concentrations did not change during the control period nor due to regular walking when compared with baseline. Consequently, no change in %S, IR or %β were observed following 12 wk of regular walking. Regular walking did not alter fasting total cholesterol, triglycerides, or LDL concentration; however, 6 and 12 wk of regular walking lead to significantly increased HDL concentration compared to baseline ($F = 4.259$, $p = 0.010$). Consequently, Chol:HDL was increased following 12 wk of regular walking ($F = 3.247$, $p = 0.030$).

Table 4.2. Change in physical characteristics, peak exercise values during incremental exercise testing, and blood chemistry in women aged 65-74 yr with type 2 diabetes.

	Week 0			Week 6			Week 12		
	Change (%)	95%CI	*p* value	Change (%)	95%CI	*p* value	Change (%)	95%CI	*p* value
Body mass, kg	-0.8	-1.4,0.0	-	-0.65	-1.7,0.4	-	-0.5	-1.2,0.3	-
BMI, $kg \cdot m^{-2}$	-0.8	-1.4,-0.1	-	-0.65	-1.7,0.4	-	-0.5	-1.2,0.3	-
Waist:hip ratio	0.1	-1.4,1.5	-	1.1	-1.7,3.8	-	0.9	-2.3,4.1	-
Systolic BP, mmHg	-3.6	-7.5,0.4	0.08	-6.6	-12.0,-1.1	0.02	-8.5	-14.0,-3.0	<0.01
Diastolic BP, mmHg	-3.1	-8.2,2.1	0.21	-3.4	-7.1,0.3	0.05	-7.2	-12.0,-2.4	<0.01
Time to exhaustion, s	3.8	-3.4,11.0	0.51	21.0	8.4,33.0	<0.01	28.0	17.0,40.0	<0.001
$\dot{V}O_2peak$, $L \cdot min^{-1}$	-2.8	-6.9,1.2	0.16	3.4	-1.5,8.3	0.21	6.2	1.9,11.0	0.01
$\dot{V}O_2peak$ $mL \cdot kg^{-1} \cdot min^{-1}$	-2.2	-6.5,2.1	0.32	3.8	-0.95,8.6	0.15	6.6	2.4,11.0	<0.01
Glucose, $mmol \cdot L^{-1}$	1.8	-6.7,10.0	-	-2.4	-10.0,5.3	-	0.43	-7.5,8.4	-
Insulin, $mU \cdot L^{-1}$	-4.9	-16.0,6.2	-	3.6	-9.0,16.0	-	5.6	-9.0,20.0	-
Cholesterol, $mmol \cdot L^{-1}$	-0.7	-6.2,4.8	-	-0.9	-7.2,5.4	-	-3.5	-11.0,3.9	-
Triglyceride, $mmol \cdot L^{-1}$	2.5	-11.0,16.0	-	-3.0	-18.0,12.0	-	0.1	-13.0,14.0	-
HDL, $mmol \cdot L^{-1}$	3.1	-1.4,7.6	0.21	7.0	1.0,13.0	0.04	7.8	1.6,14.0	0.01
LDL, $mmol \cdot L^{-1}$	-1.8	-9.9,6.2	-	-1.0	-13.0,11.0	-	-6.5	-19.0,6.3	-
Chol:HDL	-2.8	-9.8,4.2	0.21	-5.6	-14.0,2.6	0.07	-9.6	-17.0,-1.7	0.01
Homocysteine, $mmol \cdot L^{-1}$	-0.8	-6.7,5.1	-	-7.4	-15.0,0.6	-	-4.3	-13.0,4.3	-
%β	-0.21	-19.0,19.0	-	10.0	-7.3,28.0	-	6.8	-7.4,21.0	-
%S	10.0	-4.0,25.0	-	1.5	-9.5,12.0	-	-1.3	-16.0,14.0	-
IR	-4.8	-16.0,6.4	-	2.9	-9.5,15.0	-	9.1	-7.5,26.0	-

Values are percent change relative to baseline data (Week -6) with respective lower and upper 95% confidence intervals. Significance (*p*) values only presented for data with significant main effect. BMI: body mass index. BP: blood pressure. $\dot{V}O_2peak$: peak oxygen uptake. HR: heart rate. HDL: high-density lipoprotein. LDL: low-density lipoprotein. %β: beta-cell function. %S: insulin sensitivity. IR: insulin resistance.

Plasma fibrinogen, cytokines and C-reactive protein

Regular walking significantly improved plasma fibrinogen concentration by -6.9% after 12 wk, when compared with baseline. Plasma IL-6, TNF-α and CRP did not change due to exercise training. Plasma IL-10 steadily increased following the onset of regular walking, resulting in a significant increase of 41% from baseline, when measured after 12 wk of walking ($F = 3.104$, $p = 0.038$). The ratio between IL-10 and IL-6 was 1.15±1.14 when measured at wk -6 (pretraining) and increased by 54% following 12 wk of regular walking to 1.78±1.09 ($F = 4.128$, $p = 0.012$). The ratio between IL-10 and TNF-α did not change due to regular walking ($F = 1.718$, $p = 0.179$).

Correlations

The change between baseline and after 6 and 12 wk of regular walking was determined for each dependent variable. Significant relationships were observed between the exercise-induced increase in IL-10 and the increase in time to exhaustion (at wk 6, $r = 0.58$; at wk 12, $r = 0.54$), $\dot{V}O_2peak$ (at wk 6, $r = 0.66$), and TNF-α (at wk 6, $r = 0.62$; at wk 12, $r = 0.73$). Positive relationships were observed between the change in TNF-α and systolic blood pressure (at wk 6, $r = 0.68$; at wk 12, $r = 0.64$), diastolic blood pressure (at wk 12, $r = 0.68$), cholesterol (at wk 12, $r = 0.69$), and LDL (at wk 12, $r = 0.77$).

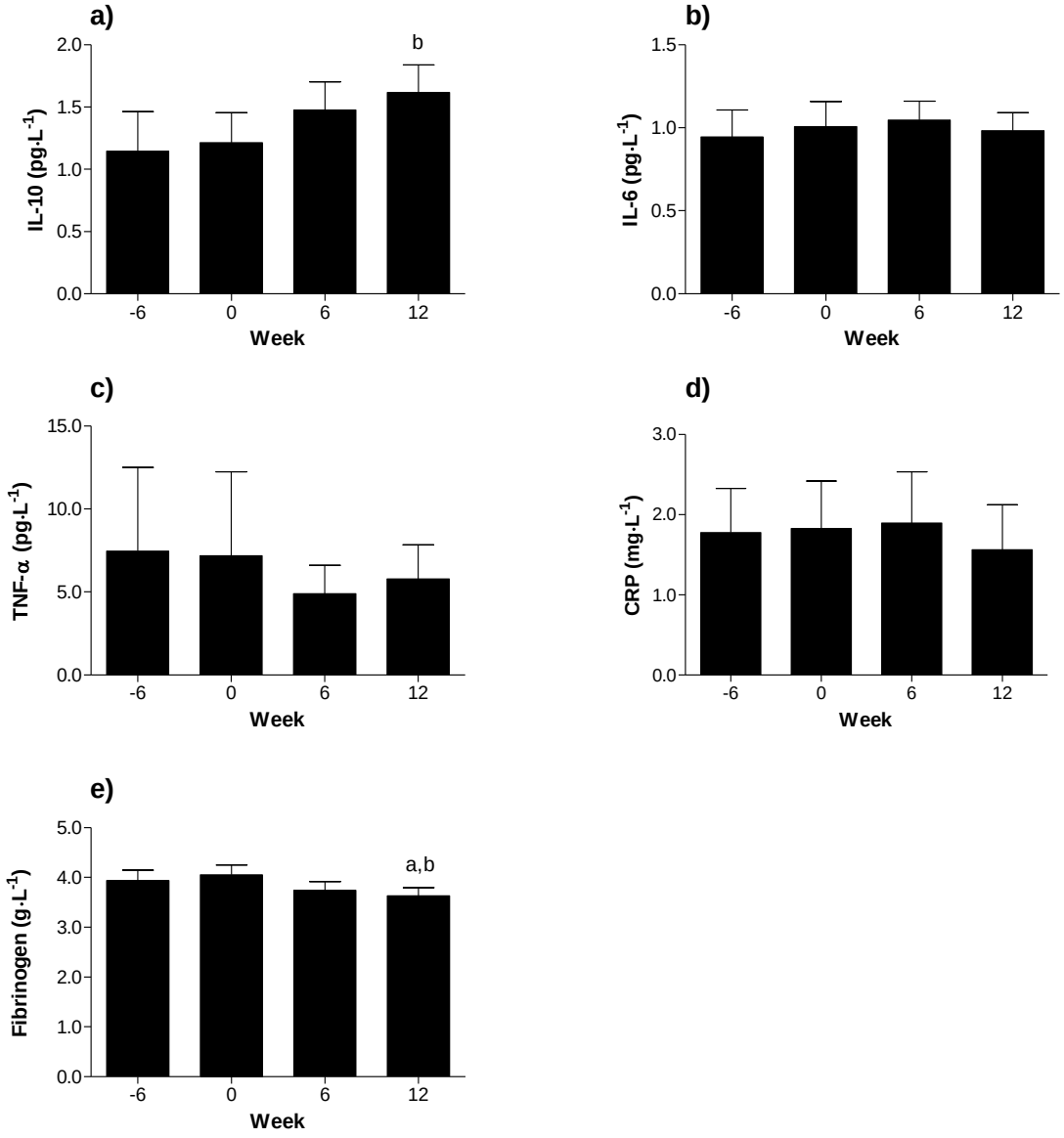

Figure 4.1. Interleukins 10 (panel a) and 6 (panel b), tumour necrosis factor-alpha (panel c), c-reactive protein (panel d) and fibrinogen (panel e) concentration measured before, during and after 12-wk of exercise training in women 65-74 yr with type 2 diabetes. Data are mean±SEM. Note y-axes unequal between panels. [a], significantly different to wk -6. [b], significantly different to wk 0. Note: p values in text.

4.4 DISCUSSION

The primary finding of the present study was that 12 wk of moderate-intensity regular walking for 120 min·wk^{-1} significantly improved the basal concentrations of various anti-inflammatory mediators, despite no corresponding change in measures of glycaemic control or body mass. It is increasingly recognised that the optimal management of type 2 diabetes, particularly among older individuals, should emphasise the prevention of cardiovascular-related complications in addition to the traditional goal of glycaemic control. Given the important anti-inflammatory functions of IL-10 and HDL, 120 min of regular walking per wk appears to provide a stimulus sufficient to improve the cardiovascular-risk profile of older women with type 2 diabetes.

IL-10 is produced by Th2 lymphocytes, monocytes and B cells, and subsequently plays an integral role in immune function. In the context of CVD, the anti-inflammatory effects of IL-10 are important. IL-10 inhibits cytokine and chemokine production by Th1, monocytes and macrophages, which are critical for the progression of macrophage/monocyte activation and low-level inflammation (Petersen and Pedersen, 2005). In a hypertensive and pregnant rat model, recombinant IL-10 injections normalised hypertension and aortic endothelial dysfunction (Tinsley et al., 2010). It is clear that an acute exercise bout leads to a significant but transient increase in plasma IL-10 (Petersen and Pedersen, 2005) and there is accumulating evidence that exercise training may improve basal plasma IL-10 concentration. Kadoglou et al., (2007) reported that a 6-mo intervention that included cycling, treadmill exercise and calisthenics that lead to significant improvements in glycaemic control increased plasma IL-10 concentration in individuals with type 2 diabetes. Balducci et al., (2010) demonstrated that mixed exercise sessions including cycling and walking, with or without resistance training, that lead to improved glycaemic control, was associated with improved inflammatory profile in individuals with type 2 diabetes. In the present study, a 12 wk walking-only program did not induce any change in glycaemic control, but lead to a significant increase in plasma IL-10 concentration. Moreover, while IL-10 to TNF-α ratio did not change, the IL-10 to IL-6 ratio significantly increased following 12 wk of regular

walking. The balance of pro- to anti-inflammatory cytokines have been suggested to be related to hyperglycaemia (Esposito et al., 2002, Shanmugam et al., 2003) and adiposity (Balducci et al., 2010); given that no change in glycaemic control or anthropometry was observed in the present study, it appears that there are multiple and independent mechanisms contributing to the anti-inflammatory effects of regular exercise. Indeed, positive correlations were observed between the exercise-induced increase in IL-10 and the changes in $\dot{V}O_2peak$ and time to exhaustion, supporting that regular walking that improves cardiovascular conditioning has an anti-inflammatory effect.

No significant difference was observed in basal concentrations of CRP, IL-6 or TNF-a following regular walking. It is unclear whether exercise training may improve these markers of low-grade inflammation, given that some (Wolever et al., 2008, Stewart et al., 2007, Martins et al., 2010), but not all (Donges et al., Hammett et al., 2004, Campbell et al., 2008), report improvements post-intervention. A common trait observed in those studies reporting improved proinflammatory markers is the capacity for the exercise intervention to induce weight loss; indeed, Martins et al., (2010) suggested that the improvement in CRP following exercise training was related to reduced adiposity. Given that no change in any anthropometrical parameter was observed in the present study, it might be that a reduction in adipose tissue is necessary for the improvement of some proinflammatory markers.

It is clear that HDL concentration provides protection against atherosclerosis and CVD (Murphy et al., 2009b, Toth, 2004). The capacity for HDL to mobilise cholesterol from non-hepatic tissue and the subsequent transportation to the liver for disposal (i.e., reverse cholesterol transport) is perhaps the most well-described mechanism for HDL-induced CVD risk reduction (Rothblat et al., 1986). The results of the present study demonstrated improved cholesterol to HDL ratio following 12 wk of regular walking, suggesting an increase in the capacity to clear cholesterol occurred following exercise training. Moreover, HDL may be anti-atherogenic due an inhibition of the oxidation of LDL and also the HDL-induced reduction in platelet fibrinogen binding and aggregation (Murphy et al., 2009a, Nofer et al., 1998). The anti-inflammatory role of HDL is gaining recognition; HDL may inhibit

monocyte migration across the endothelium (Navab et al., 1991) likely due to reduced expression of adhesion molecules (Calabresi et al., 1997, Kimura et al., 2006). Moreover, HDL may suppress transcription factors that regulate proinflammatory gene expression (Robbesyn et al., 2003). The present study suggests that 120 min of regular walking is sufficient for increased HDL concentration in older women with type 2 diabetes. These results build on the findings of the STRRIDE group (Kraus et al., 2002), which reported that the distance walked per wk was more important than the intensity of exercise for improving plasma HDL concentration.

Plasma fibrinogen, a key glycoprotein involved in blood coagulation, was significantly improved by regular walking in the present study. Fibrinogen acts as a bridge between activated platelets, stimulates platelet aggregation, and significantly influences low-shear blood flow due to enhanced aggregation of red blood cells (Kozek-Langenecker et al., 1999, Baskurt and Meiselman, 2003). Consequently, high fibrinogen concentrations may be intimately involved in the development of CVD. Indeed, a 13.5 yr follow-up study demonstrated that baseline fibrinogen concentration was strongly related to subsequent myocardial infarction and stroke (Wilhelmsen et al., 1984). While ageing is related to increased fibrinogen concentration and reduced fibrinolysis (McBane et al., 2010), type 2 diabetes exacerbates the age-related increase in fibrinogen concentration, possibly due to an increased fibrinogen concentration in response to acute changes in insulin concentration (Barazzoni et al., 2003). In the present study, no change in insulin concentration nor any other index of glycaemic control was observed, though it has also been suggested that HDL – which did increase significantly following regular walking – reduces fibrinogen-induced platelet binding and aggregation (Nofer et al., 1998), which may contribute to the cardioprotective effects of regular walking. The mechanism for improved fibrinogen concentration in response to exercise training remains unclear, but fibrinolytic activity is higher in physically active when compared with inactive individuals (DeSouza et al., 1998), and three weeks of aerobic exercise training in post-menopausal women significantly reduced fibrinogen concentration and increased fibrinolytic activity (Jahangard et al., 2009). The

findings of the present study demonstrate that the type 2 diabetes induced elevation in fibrinogen concentration is reversible following regular walking.

The difference in TNF-α values measured pre- and post-training was positively associated with several traditional markers of CVD. A positive relationship was observed between the change in TNF-α and both systolic and diastolic blood pressure. Proinflammatory cytokines are implicated in the development and progression of atherosclerotic and metabolic diseases and there is limited evidence suggesting that hypertension may also be influenced by proinflammatory cytokines (Orshal and Khalil, 2004). A positive correlation between TNF-α and systolic blood pressure has been previously reported (Zinman et al., 1999), possibly due to improved ratio of TNF-α receptors 1 and 2 (Fernandez-Real et al., 2002). In a hypertensive rat model, systemic and vascular inflammation was demonstrated to be mediated by an upregulation of proinflammatory cytokines (including TNF-α and IL-6) and a downregulation of IL-10 (Schiffrin, 2010). The present study supports the previous findings that proinflammatory cytokines may contribute to hypertension in type 2 diabetes, as the exercise-induced decrease observed in systolic and diastolic blood pressure was associated with the decrease in TNF-α concentration. Similar relationships were observed for the change in TNF-α and the change in cholesterol and LDL concentrations; collectively these changes suggest an improvement in the global risk profile for CVD.

The results of the present study demonstrated that only 120 min of walking per wk was sufficient to improve indices of cardiovascular conditioning, blood coagulation, the balance of pro- to anti-inflammatory cytokines, blood pressure, and blood lipids in older women with type 2 diabetes. Collectively, these findings are important for exercise prescription in the context of older individuals, particularly with type 2 diabetes who are at increased risk for CVD: the anti-atherogenic and anti-inflammatory effects of exercise may be achieved through regular walking of an achievable intensity.

Supplemental data to accompany Table 4.2

Table 4.3. Physical characteristics, peak exercise values during incremental exercise testing, and blood chemistry in women aged 65-74 yr with type 2 diabetes.

	Week -6	Week 0	Week 6	Week 12
Body mass, kg	77.9±13.2	77.3±13.4	77.4±13.5	77.5±13.3
BMI, $kg \cdot m^{-2}$	30.5±5.0	30.3±5.0	30.3±5.0	30.4±5.0
Waist:hip ratio	0.87±0.07	0.86±0.07	0.87±0.05	0.87±0.06
Systolic BP, mmHg	133.3±12.5	128.0±10.6	123.8±12.4	121.4±11.6
Diastolic BP, mmHg	76.4±7.4	73.6±7.3	73.6±7.7	70.0±6.5
Time to exhaustion, s	620.9±143.6	633.9±121.6	724.2±103.5	786.4±112.0
$\dot{V}O_2peak$, $L \cdot min^{-1}$	1.50±0.16	1.45±0.20	1.54±0.18	1.59±0.19
$\dot{V}O_2peak$, $mL \cdot kg^{-1} \cdot min^{-1}$	19.6±3.3	19.3±4.1	20.3±3.3	21.1±3.5
Glucose, $mmol \cdot L^{-1}$	7.43±1.61	7.54±1.89	7.27±1.94	7.47±1.98
Insulin, $mU \cdot L^{-1}$	11.0±7.7	10.4±7.7	12.1±9.1	11.6±8.4
Cholesterol, $mmol \cdot L^{-1}$	4.59±1.11	4.49±0.81	4.47±0.79	4.34±0.91
Triglyceride, $mmol \cdot L^{-1}$	1.41±0.68	1.39±0.63	1.31±0.54	1.39±0.66
HDL, $mmol \cdot L^{-1}$	1.34±0.28	1.38±0.28	1.42±0.29	1.44±0.30
LDL, $mmol \cdot L^{-1}$	2.61±0.97	2.47±0.76	2.45±0.75	2.26±0.71
Chol:HDL	3.53±0.99	3.35±0.75	3.23±0.63	3.11±0.83
Homocysteine, $mmol \cdot L^{-1}$	11.9±5.0	11.7±4.9	10.5±3.0	11.1±3.1
%β	61.6±32.4	60.9±38.6	73.2±54.9	70.7±48.7
%S	92.2±53.6	100.4±61.0	94.3±58.4	90.6±57.8
IR	1.53±1.04	1.44±1.02	1.60±1.22	1.78±1.68

Values are mean±SD. BMI: body mass index. BP: blood pressure. $\dot{V}O_2peak$: peak oxygen uptake. HR: heart rate. HDL: high-density lipoprotein. LDL: low-density lipoprotein. %β: beta-cell function. %S: insulin sensitivity. IR: insulin resistance.

Chapter 5
Conclusions

SUMMARY OF THE FINDINGS

Red blood cell aggregation was increased, and RBC deformability was decreased, in older women with type 2 diabetes, when compared with healthy controls, which plausibly leads to impaired nutrient delivery and flow of blood throughout the microcirculation. Parameters of HRV that relate to parasympathetic modulation of the cardiac cycle were reduced in the same study cohort, indicating a 'sympathetic dominance' of the cardiac cycle. Impaired haemorheology and reduced HRV have been independently associated with disease progression and CVD in type 2 diabetes; however, the present thesis demonstrated for the first time that significant and consistent relationships were observed between impaired haemorheology and reduced HRV. Several lines of evidence support the hypothesis that

impaired microcirculatory blood flow may lead to hypoxic nerve conditions and thus, result in the reduced HRV observed in type 2 diabetes.

Prior to the subsequent exercise interventions, the 6-wk control period (i.e., without intervention) that was implemented before the 12-wk walking intervention in the present studies demonstrated stable and repeatable measurements for all dependent variables. When compared with age-predicted values, subjects presented with increased body mass and BMI, elevated coagulation indices (e.g., fibrinogen), and also decreased aerobic conditioning (e.g., $\dot{V}O_2peak$, time to exhaustion), impaired glycaemic control (e.g., increased Hb_{A1c}, impaired HOMA2), impaired haemorheology (e.g., increased RBC aggregation, decreased RBC deformability) and finally, reduced HRV (e.g., HF power). Collectively, these findings support that type 2 diabetes induces many deleterious changes to the body that may explain the high incidence of CVD observed in this metabolic disorder.

The 12-wk walking intervention demonstrated that many of the impairments described above may be reversible following lifestyle change. Moreover, irrespective of whether individuals walked 2 or 4 sessions per wk, when total work was matched at 120 min per wk, significant improvements in aerobic conditioning, lipid profile, haemorheology, and blood pressure were observed. Heart rate variability was the exception in the present thesis, where only those individuals that trained 4 sessions per wk demonstrated a significant increase in SDNN, RMSSD and HF power. Given that impaired HRV is a powerful predictor of future CVD-related and all-cause mortality, the capacity for regular walking to improve HRV in older women with type 2 diabetes is an important finding.

It was also observed in the present research that an exercise-only intervention had the capacity to improve the ratio of anti- to pro-inflammatory cytokines, and plasma fibrinogen concentration, in older women with type 2 diabetes. These findings were important given that previous studies that had demonstrated improved inflammatory profile, had also implemented multiple and concurrent pharmacological interventions and/or modes of exercise. Moreover, glycaemic control and body mass did not change due to exercise training, suggesting

improved inflammatory profile following exercise training was independent of reduced adiposity and/or hyperglycaemia.

SPECIFIC CHALLENGES

It was my experience throughout the subject recruitment phase of this research to learn that an alarming number of individuals with type 2 diabetes were not willing to commit to participating in formal exercise. It seems that while individuals may take the time and effort to contact the investigators running the exercise intervention (which in itself requires *some* motivation), so many of these individuals declined to participate in formal exercise. Thus, it is clear that increasing the uptake of exercise in the entire population with type 2 diabetes represents a significant challenge.

Subject recruitment for the exercise-based studies commenced in October 2007 with a local newspaper advertisement that has a 205,000 readership. Concurrently, an almost identical advertisement was placed in the same newspaper for individuals *without* type 2 diabetes for a separate study conducted by an associated research group. While the advertisement for the non-diabetic project produced much interest (i.e., > 50 phone calls), only three phone calls were received for the type 2 diabetes project. As a consequence, in December 2007, Diabetes Australia (Queensland) agreed to "mail-out" 200 invitations to members that directly matched the inclusion criteria and within select postcodes that were specifically chosen to ensure easy access to the laboratory. Over the subsequent 24 mo regular recruitment efforts included: i. a further 400 mail-out invitations posted to unique Diabetes Australia (Queensland) addresses; ii. two interviews with Gold Coast radio stations; iii. community announcements that were run regularly on three Gold Coast radio stations; iv. an interview with Gold Coast television news; v. 4 d of providing free diabetes-related information at two local shopping centres; vi. flyers were posted at nearby pharmacies, medical centres, diabetes clinics, ophthalmologist practices, pathology laboratories, and gymnasiums; vii. two information sessions were presented at a city-wide seniors expo; viii. an invitation letter was

sent to all seniors in the local electorate from the then Shadow Minister for Ageing, Mrs Margaret May MP. The result of the recruitment efforts over >24 mo were some 82 phone calls from 'interested' individuals, 27 of which did not meet inclusion criteria (typically due to concurrent CVD or an inability to walk unaided). The remaining 55 eligible subjects were invited to an information session at the laboratory, of which two-thirds subsequently suggested that they were too busy to participate in an ongoing exercise study. Given that the 2006 census reported the number of women aged 65-74 yr living on the Gold Coast was 17,548 (Australian Bureau of Statistics, 2007), an estimate of the number of these women with type 2 diabetes would range between 1,316 (7.5%) and 2,194 (12.5%). The number of individuals recruited during the present thesis, relative to the target population, highlights the need for behavioural-change strategies if any meaningful gains in type 2 diabetes management are to be realised.

FUTURE DIRECTIONS

Future studies would likely benefit from the implementation of behavioural-change strategies to improve exercise uptake in this special group. It was clear that all individuals that had contacted the investigators were aware of the importance for regular exercise, thanks largely to regular visits to their General Practitioner. Despite the difficulty in subject recruitment, it would be incorrect to suggest that 'the message' is not being heard; rather, it might be that education in isolation is simply inadequate for individuals with a lifestyle disease. In the case of behavioural modification, a direct benefit for future researchers would be an increased sample size from which to conduct exercise studies.

In the event that large cohorts of individuals with type 2 diabetes could be recruited, a true dose-response for exercise prescription could be ascertained. While the present thesis demonstrated that there were very few differences between training 2 or 4 d per wk as long as total work was matched, it is possible that one, 3 or 5 d of training could be more or less beneficial. Using the logic gleaned by the literature focusing on glycaemic control, one would

predict that 1 d of exercise training per wk would be insufficient to mediate meaningful change – after all, GLUT4 protein expression returns to baseline within 90 h of ceasing exercise (Kawanaka et al., 1997). The results of the present thesis, however, suggest that when meaningful markers of CVD and related complications are investigated following exercise training in type 2 diabetes, significant improvements may be attained with as little as 2 d of exercise training per wk. Thus, if adequate numbers of subjects could be recruited, the study design of the present study could be extended to investigate 1, 3 or 5 d of weekly training. Once the 'optimum' weekly training frequency was ascertained, it could be investigated whether an optimum weekly training volume (i.e., how many min per wk) of exercise might confer the greatest benefits in the context of type 2 diabetes.

A truly fascinating area of research has recently emerged due to the rapid scientific and technological advancements in the sequencing of deoxyribonucleic acid. The ability to detect single nucleotide polymorphisms may lead to an entirely new wave of exercise prescription. The issue of 'responders' and 'non-responders' to interventions – pharmacological, lifestyle or otherwise – may reflect small changes in an individual's genome that either promote or impede on adaptations following a given intervention (Marian, 2010, Chen et al., 2006). While the potential for single nucleotide polymorphisms has been enthusiastically embraced by pharmacologists and related disciplines in the context of drug discovery, it is likely that variations in the genome may also dictate whether an individual responds to a particular exercise intervention. The power of such technology would enable an individual to have select sequences of the genome analysed prior to lifestyle interventions, and exercise might then be personally prescribed to ensure that the individual might 'respond' to a given exercise dose and mode. While this information would not directly address exercise uptake or compliance, compliance would potentially improve if beneficial results could be guarenteed. Nevertheless, if 'personalised medicine' is to be realised as predicted (Marian, 2010), surely 'personalised exercise prescription' should form a core of this new wave of medicine?

CONCLUSION

Type 2 diabetes is a heterogeneous metabolic disorder that clearly has an environmental trigger. Despite the accumulation of research over many decades that demonstrates that lifestyle interventions are the most effective method of preventing and managing many chronic diseases, including type 2 diabetes, the number of individuals performing regular exercise remains low. Given that participation rates in sports and physical recreation are lowest among Australian women aged 65 yr and over, it is not surprising that women are overrepresented among new type 2 diabetes cases.

Perhaps the prescription of a convenient volume and manageable intensity of walking – a preferred mode of exercise that is available to most able bodied individuals – would improve the adherence to regular exercise? In such a case, it is almost certain that the incidence of chronic metabolic disorders would decline, thus reducing the costs associated with treating the complications that arise so frequently in these diseases. A major limitation to prescribing exercise within the context of type 2 diabetes is that the current American Diabetes Association guidelines are based on the findings of technical reports that have often been performed in younger study populations and with the aim of improving glycaemic control. Clearly the exercise prescription for a middle-aged male with type 2 diabetes should be vastly different to an older woman with the same disease, given that sound prescription of exercise requires the consideration of age, gender and disease-progression specific limitations (American College of Sports Medicine, 2009). As discussed in Chapter One, despite decades of research suggesting that large reductions in fasting blood glucose concentration and/or glycated haemoglobin were associated with increased mortality, it is only recently that the focus of type 2 diabetes management has started to shift toward a more holistic and perhaps even cardiovascular-specific approach (Stirban et al., 2009). Thus, the primary aim of this thesis was to improve the understanding of the responses to regular walking by older women with type 2 diabetes, with a specific focus on the changes in physiological and biochemical markers that are associated with CVD.

This thesis presented for the first time that there may be links between the type 2 diabetes induced changes in haemorheology and cardiac autonomic modulation. It has been suspected that peripheral neuropathy that is commonly observed in type 2 diabetes might occur due to disrupted microcirculatory blood flow (Young et al., 1996). The results of Study One built upon earlier findings (Young et al., 1996) by demonstrating that HRV – suggested to be the earliest sign of cardiac autonomic neuropathy (Maser and Lenhard, 2005) – was significantly related to impaired haemorheology (i.e., increased RBC aggregation and decreased RBC deformability). The increased RBC aggregation and the sympathetic dominance of cardiac cycles observed in the older women with type 2 diabetes independently represent an increased risk for CVD (MacRury et al., 1993, Rajendra Acharya et al., 2006); the subsequent exercise interventions presented in Study Two and Three investigated changes in cardiovascular risk profile in response to varied-dose and regular walking.

Regular walking at an intensity associated with the T_{ge} significantly decreased RBC aggregation and increased RBC deformability of the older women with type 2 diabetes in Study Two to within similar ranges observed for the healthy controls in Study One. This finding suggests that the impaired haemorheology observed in type 2 diabetes is reversible to within 'healthy levels' following only 12 wk of regular walking. One of the principle aims of Study Two was to observe whether the efficacy of regular walking was related to the interaction between the frequency and duration of exercise sessions. In this respect, 120 min of walking per wk was sufficient to improve haemorheology, irrespective of whether exercise was performed 2 or 4 d per wk. This finding was important given that no previous study had demonstrated decreased RBC aggregation following an exercise-only study in type 2 diabetes. Moreover, Study Two demonstrated for the first time that exercise had the capacity to increase RBC deformability for individuals with type 2 diabetes.

In contrast to the homogenous haemorheology findings observed for all subjects irrespective of whether exercise was performed 2 or 4 d per wk, significant improvements in HRV were observed only in those individuals that walked 4 d per wk; this is an important finding given that regular walking improved all other dependent variables in the same direction (that is, if

'Variable A' decreased for Group 1, the same was observed in Group 2) and were of a similar magnitude. Thus, that HRV only improved for Group 2 suggests that improvements in HRV may be fundamentally linked to the number of rest-to-exercise transitions (i.e., exercise frequency) or that more frequent 'assaults' on homeostasis are required to induced improvements in cardiac autonomic modulation, synonymous with how GLUT4 expression requires frequent stimuli given the short half-life of this protein.

It was unclear prior to this thesis whether an aerobic exercise-only intervention had the capacity to improve the anti- to pro-inflammatory ratio of individuals with type 2 diabetes. Thus, the primary focus of Study Three was not whether varied-dose walking would improve the inflammatory profile of individuals with type 2 diabetes, but rather whether aerobic exercise in the absence of insulin therapy, dietary intervention or concurrent resistance exercise (all previously described in the literature) could achieve such benefits. Nevertheless, it was observed that no dependent variable was significantly different between the subjects that performed 2 or 4 d of regular walking per wk, and improvements were consistent for both Groups. Twelve wk of regular walking for 2 h per wk at an intensity associated with the T_{ge} significantly increased plasma HDL, IL-10 and also improved the ratio of IL-10 to IL-6, suggestive of an improved anti-inflammatory profile. These changes were important given the likely causal role of atherosclerosis/arteriosclerosis in many of the complications observed in type 2 diabetes.

It was also observed that plasma fibrinogen significantly decreased following regular walking at an intensity associated with the T_{ge}. This finding has implications for reducing cardiovascular risk, given plasma fibrinogen was strongly related to subsequent myocardial infarction and stroke (Wilhelmsen et al., 1984). Moreover, fibrinogen is a key glycoprotein involved in blood coagulation and RBC aggregation. The results of Study Two and Three collectively suggest that coagulation and RBC aggregation were reduced following regular walking. While fibrinogen clearly enhances RBC aggregation, the exercise-induced decrease in RBC aggregation observed cannot be solely attributed to the decrease in plasma fibrinogen, given that RBC aggregability (i.e., RBC aggregation measured in a standard

aggregating medium – dextran 70) in the absence of plasma (and therefore fibrinogen) also decreased following regular walking. These findings suggest that haemorheology may improve following regular exercise due to concurrent intrinsic (e.g., enhanced surface charge) and extrinsic (e.g., reduced plasma fibrinogen concentration) changes to the RBC for older women with type 2 diabetes.

Regular walking was prescribed at an intensity associated with the T_{ge} due to the evidence for health benefits, as reported in Chapter One. While the T_{ge} is a physiological/biochemical phenomenon most accurately detected using technical methods, subjective methods have also been proposed to prescribe exercise at similar intensities. For instance, given that ventilation increases disproportionately to exercise intensity above the anaerobic threshold, maintenance of speech may become difficult, thus the 'talk test' has been proposed as a valid (Goode et al., 1998, Foster et al., 2008) and indirect method to identify the T_{ge}. Moreover, some (Parfitt et al., 2006) but not all (Malatesta et al., 2004) suggest that when individual's are asked to exercise at their individual 'preferred' walking speed, most choose an intensity that generally reflects the T_{ge}. Consequently, the findings of the present thesis should readily translate into practise for exercise prescription purposes: if patients with type 2 diabetes are recommended to regularly exercise at their preferred walking speed that may also elicit difficulty in maintenance of prolonged speech, it is plausible that the health benefits described within this thesis may be expected.

Underlining the findings of each individual chapter was that improvements in many of the dependent variables were independent of exercise frequency, changes in body mass and/or glycaemic control. That 2 and 4 d of regular walking were equally effective for inducing positive change in markers for CVD is important for individuals who are 'time poor' – such as older women who often facilitate the sole care of partners – that may only perform less frequent exercise sessions. Moreover, many complications that present in type 2 diabetes have been attributed to elevated blood glucose concentration, hyperinsulinaemia, or obesity. The findings of the present thesis demonstrated that body mass, waist to hip ratio, glycated haemoglobin, fasting blood glucose, and the homeostasis model assessment of insulin

resistance, were not altered following 12-wk of regular walking, suggesting that the exercise-induced improvements of the dependent variables were mediated by means other than the traditional markers of type 2 diabetes. Consequently, the findings of this thesis are timely given the recent realisation that glycaemic control should not be the sole focus of type 2 diabetes management.

Based on the findings presented in this thesis, I support the notion that moderate-intensity exercise, irrespective of the frequency of exercise sessions, is a powerful intervention for the management of type 2 diabetes. I also support that the benefits of regular exercise extend far beyond the traditional goals of glycaemic control and reduced body mass for individuals with type 2 diabetes. For widespread exercise uptake and therefore substantial improvements in health management, however, I suggest significant behavioural change must be realised to ensure those with type 2 diabetes do not follow 'the path of least lifestyle interference'.

Chapter 6

Methodology

A detailed exploration of the methods

6.1 SUBJECT RECRUITMENT & PRE-PARTICIPATION HEALTH SCREENING

Women between 65 and 74 yr with type 2 diabetes were invited to participate in the experimental studies. Interested individuals volunteered to participate in response to:

i. *Personal invitations.* Invitations were mailed to individuals on a Diabetes Australia (Queensland) database. Three separate mail-outs were commissioned between October 2007 and July 2008, which were sent to Diabetes Australia (Queensland) members that matched the following criteria:

- female
- aged between 65 and 74 yr
- type 2 diabetes with no history of type 1 diabetes
- not using exogenous insulin
- registered residential postcode within 50 km of the laboratory.

ii. *Paper advertisement.* Posters/flyers were placed in Gold Coast: General Practitioners practices; ophthalmology practices; diabetes clinics; pharmacies; pathology laboratories; fitness centres

iii. *Seminars.* Educational presentations that focused on the health benefits of regular exercise during ageing were provided for older women. Concluding each presentation, individuals were invited to participate in the study. Educational presentations were made to various Gold Coast community groups, including: University of the Third Age; seniors expositions; women's church meetings; aged care facilities

iv. *Media releases.* Interviews were conducted by journalists with the investigators by: two radio stations; one television network; two newspapers. In addition, advertisements were placed in Gold Coast newspapers over 12 mo, and 'community noticeboards' run by Gold Coast region television and radio networks over the same duration.

Interested volunteers contacted the investigator (typically via telephone), who provided an overview of the respective study, explaining the expected duration, requirements and outcomes of participation. Due to the elevated risk of exercise testing in older individuals with type 2 diabetes, an extensive pre-participation health screening and evaluation process was initiated upon the volunteers continued interest.

Pre-participation health screening

Interested volunteers were asked a series of eleven questions over the telephone by the investigator to establish general suitability for participation in the present study. Only those

individuals free of diagnosed and overt comorbidities were asked to attend the laboratory for further investigation. Individuals not suitable were referred to their General Practitioner for guidelines on clinically-guided exercise. Suitable individuals were asked to provide their contact details so that an information package could be mailed to the individual, which included the Explanatory Statement (detailed explanation of the study design), Physical Activity Readiness Questionnaire, and a Medical History Questionnaire. Volunteers were then mailed the information package and subsequently completed the questionnaires. Upon receipt of the completed questionnaires contained in the information package, the investigators reviewed the individual's responses and assessed whether the individual was within the inclusion criteria for the particular study. Suitable individuals were then invited to attend the laboratory for further pre-participation health screening procedures.

During the initial visit to the laboratory, volunteers were asked to closely follow the investigator as he read the Explanatory Statement in detail. The volunteers were encouraged to ask questions during the reading of the Explanatory Statement to ensure clarity of the written and spoken content. The recital of the Explanatory Statement typically required 30 min, after which an independent investigator assisted the volunteer to perform The Mini-Mental State Examination. The Mini-Mental State Examination was conducted to ensure that the volunteer could adequately receive and understand written and spoken communications, and was performed by an independent party to minimise the potential bias of recruiting unsuitable volunteers. If the Mini-Mental State Examination was successfully completed, volunteers were then asked to provide written and witnessed Informed Consent. If the volunteer provided written consent, the investigator invited the volunteer to undertake in pre-participation physical assessment. The physical assessment consisted of anthropometric, 12-lead electrocardiogram (ECG), blood pressure, and pulmonary function measurements.

Anthropometric measurements

The anthropometric measurements were performed by an investigator who had certification from the International Society for the Advancement of Kinanthropometry. Measurement included stature, body mass, and girth measurements of the waist and hip. Stature was measured to the nearest 0.01 m while the volunteer was barefooted using a stadiometer. Body mass was measured to the nearest 0.01 kg after the volunteer had removed excess clothing using precision scales (2391 Eye-Level Physician Scale, Cardinal Scale Mfg. Co., Webb City, USA). Waist and hip circumferences were measured using a flexible inelastic tape (Lufkin W606PM, Cooper Tools, Raleigh, USA) against the skin without compressing the underlying tissue and after the volunteer had exhaled normally. Volunteers were asked to stand with their arms relaxed at their side and with their weight distributed evenly on both feet, which were separated approximately in line with the shoulders. The waist measurement was made through a horizontal plane halfway between the inferior-border of the lowest rib and the crest of the ilium. The hip measurement was made through a horizontal plane from the most posterior protuberance of the buttocks. Circumference measurements were made at the end of each exhalation to the nearest 0.1 cm and were repeated twice, the mean of which is reported in the present study.

12-lead electrocardiogram and blood pressure

Each volunteer was asked to lay supine on a clinical examination bed after removing any necklaces or undergarments containing metal. The inflatable cuff of a sphygmomanometer was fitted to the volunteers left arm, ensuring that the artery marker aligned with the volunteer's brachial artery that was found via palpation. A piece of tape that was approximately one-third of the length of the cuff was used to fix the cuff to the volunteers arm. The investigator then applied a 12-lead ECG to the chest of the volunteer. Ten electrodes were placed on skin that was cleaned using 70% ethanol and gauze. The configuration for electrode placement was standard for precordial leads, while limb leads

were moved to the torso to minimise muscle and movement artefact likely to be observed in subsequent exercise bouts. After 5 min of supine rest with the volunteer's arms beside their torso and legs straightened (i.e., not crossed), a 12-lead ECG and rhythm strip of leads II, V2 and V5 were recorded. Once the ECG recordings were visually inspected by the investigator and marked "Limb leads on torso", a blood pressure measurement was performed while the volunteer remained supine.

Volunteers were subsequently asked to slowly sit up and assume a standing position for the subsequent measurement of a second 12-lead ECG recording and blood pressure measurement. Volunteers were asked to stand unaided (e.g., without leaning on the clinical bed) and to ensure that they did not cross their limbs. At least 2 min were allowed for the volunteer's haemodynamic response to reach equilibrium after standing to their feet before a 12-lead ECG and rhythm strip of leads II, V2 and V5 were recorded. Once the ECG recordings were visually inspected by the investigator and marked "Limb leads on torso", a blood pressure measurement was performed while the volunteer remained standing. In the event that arrhythmias or abnormalities were observed on the ECG, notes were made on the recording and were filed in the report that was to be referred to the volunteer's General Practitioner.

All blood pressure measurements were performed while the sphygmomanometer cuff was at the level of the heart; while supine, the volunteer's arm was slightly elevated by placing the investigators fingers under the arm during the measurement. Moreover, the volunteer was always reminded to relax their arm and measurements were not performed until the arm was 'limp'. The blood pressure of the volunteer was obtained by inflating the sphygmomanometer cuff to ~180 mmHg, before deflating at 2 $mmHg \cdot s^{-1}$. If auscultations were observed within 20 mmHg (e.g., >160 mmHg) of commencing deflation of the sphygmomanometer cuff, the bladder was reinflated to ~200 mmHg to ensure that the first Korotkoff was not underestimated due to an auscultatory gap. Nevertheless, upon observing the first clear 'tapping' sound, deflation of the cuff was slowed to confirm the pressure of the first Korotkoff; two consecutive beats at the first Korotkoff was recorded as systolic blood

pressure. Once systolic blood pressure was noted, the sphygmomanometer cuff was deflated at an increased rate of 4-6 $mmHg \cdot s^{-1}$ for approximately 20 mmHg, to minimise the duration required for the measurement of blood pressure. The rate of release of the sphygmomanometer cuff was subsequently decreased back to 2 $mmHg \cdot s^{-1}$ to ensure an accurate observation of diastolic blood pressure. The point at which auscultations became 'muffled' or 'thumping' after a period of 'clear and tapping' (i.e., third Korotkoff) reflected the fourth Korotkoff, which was noted and subsequently recorded as diastolic blood pressure. Deflation of the sphygmomanometer cuff was continued for a further 20 mmHg below the fourth Korotkoff to ensure that a silence was observed (i.e., fifth Korotkoff) and that diastolic blood pressure was not overestimated due to an auscultatory gap. Upon completion of blood pressure measurement, systolic and diastolic blood pressures were recorded immediately. In the event that blood pressure was considered normotensive, blood pressure was reassessed during the volunteer's next visit for confirmation; however, if the volunteer presented with hypertension, another measurement was made 10-15 min after the first and while the subject remained resting to minimise the potential for artificially increased systolic blood pressure due to the 'white-coat effect'.

The technique for measuring blood pressure while standing was the same described for supine measurements, ensuring that the sphygmomanometer cuff was at the level of the heart during the measurement. The sphygmomanometer cuff and ECG electrodes were subsequently removed, before the pulmonary function of the volunteer was examined.

Pulmonary function test

Pulmonary function of the volunteers was determined while seated on a chair by performing repeated forced vital capacity (FVC) manoeuvres using an open-circuit metabolic measurement system (Ultima CPX, Medical Graphics Corporation, St Paul, USA), in accordance with the guidelines published by the American Thoracic Society. The FVC manoeuvre consisted of measuring the rate and maximal volume of gas that was able to be

forcefully exhaled over ~6 s, following a maximal (but not forceful) inhalation. Before the FVC manoeuvres were performed, the flow meter of the metabolic measurement system was calibrated using a calibration syringe of known volume (Hans Rudolph Inc., Kansas City, USA) and the volunteer's current height and body mass was updated. The volunteer was asked to remain seated during the FVC manoeuvre with an upright posture and feet flat on the ground. Volunteers were instructed specifically not to hunch or lean forward during exhalation to minimise the increase in expiratory flow induced by physical compression of the abdomen and thorax. The FVC manoeuvre was performed by the investigator using a spare mouthpiece and noseclip as a demonstration before the experimental measurement was performed by the volunteer. Once the volunteer expressed comfort and an understanding of the FVC manoeuvre, they were fitted with a sterile mouthpiece and noseclip.

The volunteer was then instructed to quietly breathe as normally as possible to establish a baseline tidal breathing pattern. Following six normal breaths, the volunteer was instructed to maximally inhale and "fill your lungs as much as possible". Once the volunteer was unable to inhale any more air, the volunteer was instructed to exhale maximally as "fast and forcefully as possible"; the volunteer was then encouraged to continue exhaling as forcefully as possible for a further 5-6 s. Once the metabolic measurement system demonstrated that the test was successful, the volunteer was encouraged to take a deep breath to maximally fill their lungs once again, before returning to normal respiration. Following each FVC manoeuvre, the mouthpiece and noseclip were removed and a cup of water was provided to moisten the throat. The results were displayed immediately on a computer screen and feedback was provided by the investigator to assist the volunteer in improving their technique for further attempts. The FVC manoeuvre was typically repeated approximately three times, and a composite of the highest or best values were derived for reporting of pulmonary function parameters.

The metabolic measurement system was then used to create a report that included: FVC in absolute volume (L) and percent predicted value (%); FEV_1, the forced expiratory volume between 0.0 and 1.0 s of the FVC manoeuvre, reported as absolute volume (L) and percent

predicted value (%); FEV_1/FVC, the ratio of the expiratory volume forcefully exhaled between 0.0 and 1.0 s compared with the total volume exhaled during the manoeuvre; PEFR, the peak expiratory flow rate, expressed as a rate ($L \cdot s^{-1}$) and percent predicted value (%).

The results obtained during the preliminary screening visit were compiled into a report that was provided to the volunteer. A pathology request form was also provided to the volunteer for a detailed haematological assessment, including fasting measures of glucose, cholesterol, lipids, glycated haemoglobin and full blood count. The volunteer was asked to visit the pathology laboratory for the haematological assessment and subsequently visit her general practitioner with the report from the preliminary screening session and the haematology results.

General Practitioners endorsement for further participation

During the Explanatory Statement reading, special emphasis was placed on the volunteers existing management strategies (e.g., diabetic care plan) determined by her General Practitioner and/or diabetes clinic. The investigator made every effort to ensure that the volunteer understood that participating in the exercise study should in no way annul the treatment strategies that had been developed previously for the individual. Moreover, the investigator explained that participation in the exercise study required acknowledgement and endorsement in writing from the volunteer's general practitioner, given that this medical professional would have a far greater understanding of the individuals medical history and the potential benefit and/or risks of participating in an exercise study that would involve multiple maximal exercise tests.

The general practitioner was provided with the results of the preliminary screening session, along with the haematological profile, and was asked to provide written endorsement for the volunteer to participate in an exercise study that would include medical and health tests, including several maximal exercise tests. In making an endorsement, the general practitioner

also advised that the volunteer had no known history of any comorbidities that were listed on the exclusion criteria provided. Volunteers that received endorsement from their general practitioner were subsequently considered as a subject for the experimental studies.

6.2 PRE-PARTICIPATION EXERCISE TESTING

Upon receiving endorsement to participate, subjects were invited to the laboratory for familiarisation with the experimental procedures and equipment. Prior to the collection of any dependent variables, subjects were randomly allocated to one of the two walking groups. De-identified subject codes were provided for each subject in random order and subsequently the subject codes were input into a freeware program specifically written to randomise lists (Random Allocation Software 1.0, M. Saghaei, Isfahan University of Medical Sciences, Iran). Walking group allocation was determined by the randomisation of subject codes into the two groups using a single block design. The 'sample size' was set to the number of subjects for the given cohort and groups were set to '2'.

Before commencement of the walking program, it was important to teach subjects to walk on a moving platform (i.e., treadmill), particularly because the subjects would be performing maximal exercise while fitted with a mouthpiece for pulmonary gas-exchange measurements and a safety harness. Subjects were also familiarised with breathing through a mouthpiece while wearing a noseclip.

Familiarisation of experimental procedures and equipment

Subjects were invited to the laboratory for familiarisation with the experimental procedures and protocols prior to performing any experimental measures. Upon arrival at the laboratory, the purpose of the visit was reiterated with special attention to the safety aspects of treadmill walking. Subjects were then fitted with an ECG that provided leads II, V2 and V5 via

telemetry. A sphygmomanometer cuff was also fitted to the subjects left arm as described earlier for the *Prescreening Blood Pressure Measurements*.

Prior to mounting the treadmill, subjects were fitted with a safety harness that would suspend the subject off the floor in the event of tripping. The safety harness was attached to an overhead support that was on a free-running railing, enabling the harness to move in the posterior-anterior direction while the subject was walking on the treadmill. This was an important design feature of the safety harness, so that the subject could walk freely up and down the treadmill and find their preferred position without concern of the harness anchor point. Once the safety harness was attached to the support, the investigator demonstrated how to: i. safely mount the stationary treadmill; ii. position feet once the treadmill belt had started but the test had not yet commenced; iii. move feet from the stationary sides of the treadmill onto the moving belt; iv. use the treadmill supports for balance without supporting the subjects body mass; v. use hand gestures to signal 'yes', 'no', 'stop'; vi. safely dismount the treadmill once the treadmill belt had been stopped. Once the subject was satisfied with the demonstration, the subject was invited to stand on the stationary treadmill. Measurements were then made of blood pressure and heart rate and rhythm while the subject was in the exercise position (i.e., standing on the treadmill).

The subject was then asked to place their feet on the stationary sides of the treadmill before the belt started moving. Once the subject was comfortably standing on the stationary sides of the treadmill, the treadmill was set to 3.0 $km \cdot h^{-1}$ at 0% grade. The subject was then asked to use one leg to 'get a feel for the speed of the treadmill' by supporting their weight with their hands on the treadmill supports and one of their legs on the stationary side of the treadmill, and then placing their preferred foot on the moving treadmill belt. The subject was instructed to allow their foot to move with the belt in a posterior direction until they felt like taking a step, at which point then they picked their foot up and placed their foot in front of them and allowed their foot to move backwards in a posterior direction again. This one-legged 'skating' action was repeated several times until the subject was comfortable with the speed,

before the subject timed the skating action to allow their non-preferred leg to also step onto the treadmill and commence walking.

Subjects were encouraged to initially use their hands to support their body weight and to try various walking methods. We noticed that many subjects would initially drag their feet along the treadmill belt, thus by telling subjects to 'pick up your foot and place it in front of you when you want to step' (rather than sliding their feet along the treadmill belt), the subjects improved their treadmill walking pattern (gait). The gait of the subject was monitored while walking at 3.0 $km \cdot h^{-1}$ at 1% grade, with the most common problem being subjects leaning forward and recruiting their hamstring and gluteal muscle groups more than necessary, thus many subjects were also instructed to walk with a more upright posture until they felt comfortable. Once subjects felt comfortable, the speed of the treadmill was increased by 0.5 $km \cdot h^{-1}$ increments every few minutes until the subject was walking at a brisk pace (typically 5.0 $km \cdot h^{-1}$). Interestingly, gait appeared more normal for most subjects once the speed was 4.0 to 4.5 $km \cdot h^{-1}$, probably because the initial speed of 3.0 $km \cdot h^{-1}$ was slower than their typical overground walking speed. Upon subjects suggesting that they had reached a comfortable walking pace, the speed of the treadmill was increased by 0.5 $km \cdot h^{-1}$ each minute for 1-2 min, with the subjects approval, to ensure that the subject had not underestimated their preferred walking speed. Once the subject indicated that the treadmill speed was slightly too fast, treadmill speed was decreased by 0.5 $km \cdot h^{-1}$ until the subject was comfortable with the walking pace; the corresponding treadmill speed was recorded immediately and subsequently used as the individual's 'preferred walking speed'.

Once subjects were walking at a brisk and comfortable pace, the treadmill grade was increased to 3-4% so that subjects could feel the difference of walking on a slight incline compared with flat ground. While the subject was walking on the treadmill, heart rate and rhythm were continuously monitored; if heart rate exceeded 80% of age-predicted maximal heart rate (typically 120 $beat \cdot min^{-1}$), treadmill speed and / or grade were reduced. Once the subject had walked at a brisk pace and against a slight incline, the treadmill speed and grade was returned to 3.0 $km \cdot h^{-1}$ at 0% and the subject practiced the dismount procedures, which

involved walking at an ever slower pace until the belt became stationary. The subject then placed their feet on the stationary sides of the treadmill and dismounted from the rear.

During the familiarisation of treadmill walking, subjects were also familiarised with breathing via the MedGraphics mouthpiece and nose clip to be used in subsequent maximal exercise tests. While walking at a brisk pace, the subject was fitted with the nose clip, and then the subject would insert the mouthpiece in her mouth. The subject subsequently breathed through the mouthpiece while wearing a nose clip for several minutes, before removing and reinserting the mouthpiece several times.

Maximal exercise testing – peak oxygen uptake

In the days before the maximal exercise test, subjects were contacted by telephone to reiterate the goal and procedures of the test. During this conversation, subjects were also reminded to:

- have a 'light' meal approximately 2 h and consume water prior *ad libitum* to visiting the laboratory
- abstain from caffeinated / alcoholic beverages for 12-h before visiting the laboratory
- wear comfortable and loose-fitting clothes
- wear footwear suitable for walking
- report to the investigator their present health status, with emphasis on anything that would preclude performing maximal exercise (e.g., common cold, headaches etc).

Prior to each maximal exercise testing session, the supervising physician reviewed each subjects medical history and the results from previous and current experimental tests (e.g., full blood count, maximal exercise tests etc). Once the subject arrived at the laboratory, the physician examined the subject's respiratory and circulatory systems using auscultation and obtained a recent medical history, providing opportunities for the subject to ask questions.

The physician specifically enquired about any conditions that may cause adverse reactions to strenuous exercise (e.g., symptoms of common cold/respiratory infections etc). When the physician was satisfied that the individual had no contraindications for performing maximal exercise, the investigators prepared the subject for the maximal exercise test.

The subject was asked to lie on a clinical bed while they were fitted with a 12-lead ECG and blood pressure cuff (as described in *Electrocardiogram* and *Blood pressure*, respectively). When both supine and standing ECG and blood pressure were obtained, the physician inspected the traces of the ECG. Subjects who were normotensive that presented with normal sinus rhythm were then fitted with a safety harness that was attached to the treadmill supports. The safety harness and the method used to mount/dismount the treadmill are described in *Familiarisation*.

Immediately before the maximal exercise test and while the treadmill was still stationary, the investigators reminded the subject of the purpose of the test. During this explanation, subjects were also reminded of the 'normal' and 'abnormal' responses to maximal exercise. Subjects were reminded that it is normal to: feel warm; sweat; feel tired, particularly in the legs; breathe frequently and heavily, and even feel short-of-breath towards completing the test; experienced increased heart rate. Subjects were then reminded that it is abnormal to: feel light headed or faint; feel tingling in their fingers or toes; experience pain or tightness across their chest; feel excessive and particularly 'sharp' pain in muscles/joints; experience intense shortness of breath that is out of proportion to the effort (e.g., during the early stages of the maximal exercise test); lose vision. In the event that any abnormal responses were experienced, subjects were told to immediately inform the investigators by giving a predefined hand signal ('thumbs down'). Subjects were also informed that they were in control of the test, meaning that they could stop the test at any time and without penalty or consequence. Similarly, subjects were also informed that the physician and/or investigators may stop the test at any time if in their opinion it was unsafe to continue the test. Reasons that the investigators would terminate the exercise test included: a decrease in systolic blood pressure of ≥10 mmHg compared with resting levels with another sign of ischaemia; onset of

moderate-to-severe angina; signs of dizziness or ataxia; signs of poor perfusion such as cyanosis or pallor; ECG abnormalities, either technical or physiological. Just prior to commencing the maximal exercise test, the investigators reminded the subject of how to rate their perceived exertion by reading a standardised paragraph that explained the 6-20 Borg Scale (Borg, 1970). The subject was then offered an opportunity to ask any questions pertaining to the maximal exercise test and offered a sip of water, before the mouthpiece and noseclip were fitted.

Subjects were then asked to move their feet from the stationary treadmill belt and place them onto the sides of the treadmill. One minute of resting gas exchange was collected while the subject was standing stationary, before the treadmill belt was turned on. Each subject performed a similar protocol for the determination of peak exercise values based on their preferred walking speed. Specifically, all subjects performed 3 min of 'warm-up' walking at 3.0 $km \cdot h^{-1}$ and 0 % grade. At the completion of the warm-up period, the treadmill speed was increased by 0.5 $km \cdot h^{-1}$ increments each minute until the subjects 'preferred walking speed' was attained (e.g., 4.5 $km \cdot h^{-1}$), but the treadmill was still at 0 % grade. Once the subject reached their preferred walking speed, the subject was informed that the treadmill speed would not get any faster, but only treadmill grade would increase. The grade of the treadmill was increased by 2 % increments each minute until the termination of the test. Upon termination of the test, the mouthpiece and noseclip were removed as soon as possible and a rating of perceived exertion was recorded for the legs and chest (i.e., tiredness of the lower limbs and breathing difficulty). Concurrently, the treadmill was returned to 3.0 $km \cdot h^{-1}$ and 0 % grade for 10 min of active recovery. Time to exhaustion was recorded to the nearest millisecond.

Throughout the maximal exercise test, pulmonary gas-exchange was monitored and recorded continuously by the investigators and the physician. The ECG was monitored and recorded continuously throughout the test and also during the 10 min of active recovery. Blood pressure was measured and recorded every 3 min of the test, immediately post-exercise, and then every 5 min post-exercise. Following the 10 min of active recovery, the subject was

invited to sit down on a chair next to the treadmill so that the ECG and blood pressure could be monitored. The subject was monitored until heart rate and blood pressure reflected pre-exercise baseline values.

At the end of each testing session and once the subjects were no longer in the laboratory, the data from each maximal exercise test were exported from the proprietary software of the metabolic measurement system into Microsoft Excel spreadsheets to enable offline analysis. The pulmonary gas-exchange parameters and the heart rate derived from the 12-lead ECG were exported into the spreadsheets as i. breath-by-breath measurements; ii. values averaged over 30 s; ii. values averaged over 60 s.

Determination of gas-exchange thresholds

The data obtained during each maximal exercise test was analysed for the determination of the gas-exchange threshold. The gas-exchange threshold is a useful non-invasive estimate of an individual's anaerobic threshold (also known as the blood lactate threshold) and was determined using the simplified V-slope method (Schneider et al., 1993). The simplified V-slope analysis involved plotting the carbon dioxide production ($\dot{V}CO_2$) against oxygen uptake ($\dot{V}O_2$) that was measured during the maximal exercise test on equal axes (Figure 6.1).

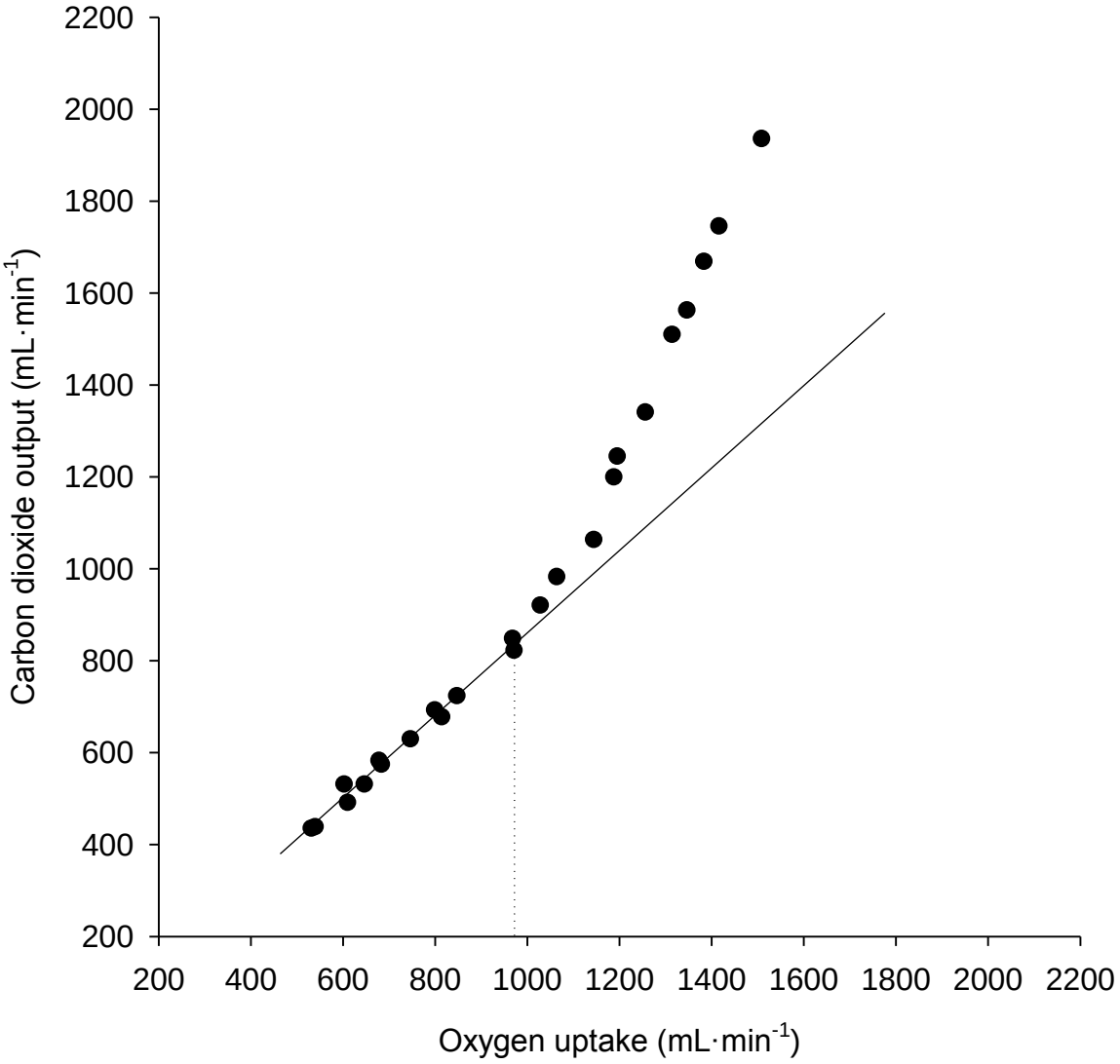

Figure 6.1. The simplified V-slope method of estimating the anaerobic threshold. The solid line represents the line of identity for the carbon dioxide ouput-oxygen uptake plot during the early stages of the maximal exercise test that were performed below the anaerobic threshold. The last data point before the departure from the line of identity is considered the gas-exchange threshold (dotted drop-line).

Given that breath-by-breath pulmonary gas-exchange fluctuates depending on instantaneous changes in heart rate, stroke volume, and tidal volume, data were averaged over 30-s to improve the clarity of the data set. When exercise is performed at intensities below an

individual's anaerobic threshold, $\dot{V}CO_2$ and $\dot{V}O_2$ maintain a linear relationship because $\dot{V}CO_2$ is believed to reflect mainly metabolic processes. Alternatively, when exercise is performed at an intensity greater than the anaerobic threshold, $\dot{V}CO_2$ increases disproportionately to $\dot{V}O_2$, departing from the linear relationship observed below the anaerobic threshold. While the mechanism(s) that cause the gas-exchange threshold are debatable, it is relatively well established that non-metabolic production of carbon dioxide adds to the metabolic production of carbon dioxide when exercise is performed above the anaerobic threshold, causing an increase in $\dot{V}CO_2$. Consequently, when $\dot{V}CO_2$ is plotted against $\dot{V}O_2$ on equal axes, the first departure from a linear relationship of the data may be used to non-invasively detect the anaerobic threshold. Given that gas-exchange indices are used to determine the anaerobic threshold, it is commonly referred to as the gas-exchange threshold. In some circumstances, the gas-exchange threshold may be difficult to detect due to 'noisy' pulmonary gas exchange, often caused by large fluctuations in ventilation. Thus, the gas-exchange threshold for each individual was confirmed by comparison with another non-invasive method that uses the 'ventilatory equivalents' of pulmonary gas-exchange to determine the anaerobic threshold.

The ventilatory equivalents technique of estimating the anaerobic threshold involves plotting the quotient of minute ventilation ($\dot{V}_E$) and $\dot{V}CO_2$ (i.e., $\dot{V}_E / \dot{V}CO_2$), and the quotient of $\dot{V}_E$ and $\dot{V}O_2$ (i.e., $\dot{V}_E / \dot{V}O_2$), against exercise duration or intensity (Figure 6.2). The ventilatory equivalents non-invasively estimates the anaerobic threshold using the relationships between ventilation, $\dot{V}CO_2$ and $\dot{V}O_2$ throughout incremental exercise. As explained for the simplified V-slope method, below the anaerobic threshold, $\dot{V}CO_2$ and $\dot{V}O_2$ maintain a linear

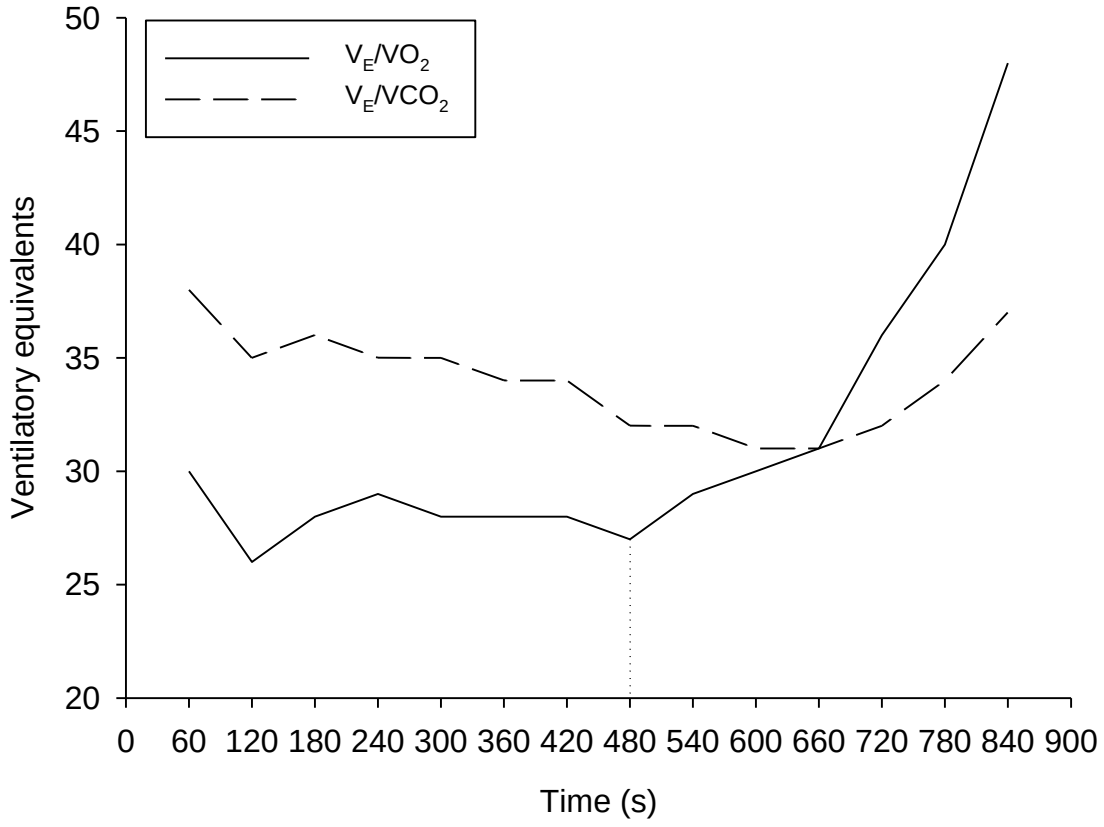

Figure 6.2. The ventilatory equivalents method of estimating the anaerobic threshold. The first systematic increase in the ventilatory equivalent for oxygen ($\dot{V}_E / \dot{V}O_2$; solid line) without an associated increase in the ventilatory equivalent for carbon dioxide ($\dot{V}_E / \dot{V}CO_2$; dashed line) is termed the ventilatory threshold (analogous with the gas-exchange threshold). The dotted-drop line represents the exercise time associated with the ventilatory threshold.

relationship, but above the anaerobic threshold, non-metabolic production of carbon dioxide increases $\dot{V}CO_2$ disproportionately to $\dot{V}O_2$. Given that carbon dioxide is a volatile acid, the increased non-metabolic production of carbon dioxide causes a decrease in pH. The body may remove the carbon dioxide and thus return pH to physiological levels by increasing respiration. This 'compensatory hyperventilation' is reflected by a non-linear increase in $\dot{V}_E$ at exercise intensities above the anaerobic threshold. Given that both $\dot{V}CO_2$ and $\dot{V}_E$ increase disproportionately to exercise intensity above the anaerobic threshold, $\dot{V}_E / \dot{V}CO_2$ remains

constant. In contrast, given that $\dot{V}O_2$ maintains a linear increase with exercise intensity, the disproportionate increase in $\dot{V}_E$ at the anaerobic threshold increases $\dot{V}_E / \dot{V}O_2$. Consequently, the intensity during a maximal exercise test that results in an increase in $\dot{V}_E / \dot{V}O_2$ without an associated increase in $\dot{V}_E / \dot{V}CO_2$ may be used to non-invasively detect the anaerobic threshold. Two investigators performed this process independently, before discussing their findings. If the investigators could not agree on a particular gas-exchange threshold, a third investigator was consulted.

Peak exercise values

The 30-s averages of pulmonary gas-exchange values and the associated heart rate data derived from the ECG were used to determine peak exercise values. The data were initially inspected to determine the onset of warm-up exercise, given that 'baseline' and resting data were collected for approximately 60 s prior to commencing the maximal exercise test. The duration of baseline data collection was noted to the nearest millisecond, so that this duration could be subtracted from the final test time to determine time to exhaustion. The time to exhaustion for each individual was determined by monitoring the breath-by-breath data recorded during the incremental exercise test for the last data point before the treadmill speed and/or grade were reduced for active recovery. The time associated with the final exercise data point was recorded to the nearest millisecond, the duration of baseline data collection was subtracted, and the result was recorded as the time to exhaustion.

The 30-s averaged data sets were then inspected to ascertain peak exercise values. Data recorded during the baseline period at rest, and data recorded beyond the time to exhaustion, were excluded from analyses as these data did not reflect values recorded during exercise. Peak exercise values were calculated as the average of the two final 30-s bins for each dependent variable. Usually the final 30-s value did not represent a full 30-s bin; for instance, if an individual's time to exhaustion was 13 min 46 s, the final 30-s value would only include 16 s (the data between 13:30 and 13:46). Only 30-s bin values were included if more than 20

s were included in analysis (i.e., a minimum of 50 s of the final min) – if the final bin included <20 s of data, that data was excluded from analyses to minimise the potentially lower signal-to-noise ratio that may be caused by aberrant breaths. Dependent variables included peak values for $\dot{V}O_2$, $\dot{V}CO_2$, $\dot{V}_E$, respiratory exchange ratio and heart rate.

6.3 HEART RATE VARIABILITY

Data acquisition

Subjects were scheduled to attend to laboratory on a separate visit for the measurement of HRV. Subjects were instructed to refrain from consuming caffeine and performing vigorous exercise during the 12 h prior to visiting the laboratory. Upon arrival at the laboratory, subjects were ask to complete a recall questionnaire to monitor whether they had refrained from using caffeine and vigorous exercise. The investigators then explained the purpose of the test and the subject was given opportunity to ask any questions. Once complete, subjects were then asked to stand while the skin that would be underlying the chest strap was cleaned using 70% ethanol and a gauze pad. The alcohol was given time to evaporate, before the subject was fitted with a chest strap (Polar Electro OY, Kempele, Finland) that sampled beat-to-beat heart rate at a frequency of 1000 Hz. To ensure optimal conductivity, the electrode contacts on the chest strap were moistened using warm water. Once the chest strap was fitted, a sphygmanomometer cuff was fitted to the subjects' left arm as described in *Blood pressure*. The subject was then asked to lie on a clinical bed, with a pillow for neck support. The subject was also offered a small pillow to place under the knees and a blanket for warmth. Once supine, the subject was instructed to relax and keep a clear mind, to minimise movement and to keep their upper and lower limbs relaxed and by their side. The investigator then synchronised the chest strap with a receiver (Polar s810, Polar Electro OY, Kempele, Finland) that enabled real-time observation of cardiac (RR) intervals via an infrared connection to a personal computer. Real-time observation of RR intervals enabled the signal quality to be assessed remotely. Laboratory temperature was controlled between 22°C and

24°C throughout all tests. Immediately before the acquisition of RR intervals, the laboratory lights were dimmed. After 20 min of supine rest, RR intervals were recorded continuously for 10 min. The RR intervals were recorded at 1000 Hz onto a personal computer in real-time and were saved as ANSI (single-byte ISO-8859) text files.

Heart rate variability analysis

Prior to analysis of HRV, the raw RR intervals were imported into a Microsoft Excel spreadsheet. The raw RR intervals were then plotted against time to visually inspect for outliers. Given that outliers can significantly influence the spectral components of HRV, outliers were removed using the interquartile range analysis of the data. The RR intervals that were greater than 3.5 times the interquartile range (IQR) from the median value were considered extreme outliers and excluded from subsequent analysis.

To determine the IQR, data was required to be arranged from shortest to longest RR intervals, which was performed by the 'sort ascending' function in Excel. Once data were sorted from shortest to longest RR intervals, the number of RR intervals was determined using the 'count' function of Excel. Subsequently, the numerical place of the 25^{th} quartile (Q1) was determined by the formula 'count*0.25', which did not return Q1, but rather the numerical place or rank of Q1. Thus, the RR interval that corresponded with Q1 was then visually detected and recorded as the Q1. This process was repeated to determine the 50^{th} and 75^{th} quartiles (i.e., median and Q3, respectively). Subsequently, the IQR was determined by the formula: IQR = Q3 – Q1.

Once the IQR was determined, the raw RR intervals were once again imported from the text file into an adjacent column of the spreadsheet because the initial data set had been sorted. Using the unsorted raw RR intervals, outliers were removed by using logic code (i.e., IF, OR etc) to assess whether a data point was ± 3.5 times the IQR from the median (see Table 6.1).

Table 6.1. Outlier removal of cardiac (RR) intervals.

	A	B	C	D
1	639	905	=IF(OR(B2<B44, B2>B43),"OUTLIER",B2)	905
2	817	817	=IF(OR(B3<B44, B3>B43),"OUTLIER",B3)	817
3	833	833	=IF(OR(B4<B44, B4>B43),"OUTLIER",B4)	833
4	890	890	=IF(OR(B5<B44, B5>B43),"OUTLIER",B5)	890
5	905	2115	=IF(OR(B6<B44, B6>B43),"OUTLIER",B6)	OUTLIER
6	929	1041	=IF(OR(B7<B44, B7>B43),"OUTLIER",B7)	1041
7	958	1153	=IF(OR(B8<B44, B8>B43),"OUTLIER",B8)	1153
8	**993**	1121	=IF(OR(B9<B44, B9>B43),"OUTLIER",B9)	1121
9	999	1099	=IF(OR(B10<B44, B10>B43),"OUTLIER",B10)	1099
10	1026	1071	=IF(OR(B11<B44, B11>B43),"OUTLIER",B11)	1071
11	1029	1123	=IF(OR(B12<B44, B12>B43),"OUTLIER",B12)	1123
12	1041	1118	=IF(OR(B13<B44, B13>B43),"OUTLIER",B13)	1118
13	1041	1063	=IF(OR(B14<B44, B14>B43),"OUTLIER",B14)	1063
14	1063	1065	=IF(OR(B15<B44, B15>B43),"OUTLIER",B15)	1065
15	1065	1041	=IF(OR(B16<B44, B16>B43),"OUTLIER",B16)	1041
16	**1065**	1102	=IF(OR(B17<B44, B17>B43),"OUTLIER",B17)	1102
17	1067	1090	=IF(OR(B18<B44, B18>B43),"OUTLIER",B18)	1090
18	1071	1067	=IF(OR(B19<B44, B19>B43),"OUTLIER",B19)	1067
19	1090	1137	=IF(OR(B20<B44, B20>B43),"OUTLIER",B20)	1137
20	1092	1099	=IF(OR(B21<B44, B21>B43),"OUTLIER",B21)	1099
21	1099	1092	=IF(OR(B22<B44, B22>B43),"OUTLIER",B22)	1092
22	1099	1100	=IF(OR(B23<B44, B23>B43),"OUTLIER",B23)	1100
23	1100	1065	=IF(OR(B24<B44, B24>B43),"OUTLIER",B24)	1065
24	**1102**	639	=IF(OR(B25<B44, B25>B43),"OUTLIER",B25)	OUTLIER
25	1110	1473	=IF(OR(B26<B44, B26>B43),"OUTLIER",B26)	OUTLIER
26	1118	999	=IF(OR(B27<B44, B27>B43),"OUTLIER",B27)	999
27	1121	958	=IF(OR(B28<B44, B28>B43),"OUTLIER",B28)	958
28	1123	929	=IF(OR(B29<B44, B29>B43),"OUTLIER",B29)	929
29	1137	993	=IF(OR(B30<B44, B30>B43),"OUTLIER",B30)	993
30	1153	1026	=IF(OR(B31<B44, B31>B43),"OUTLIER",B31)	1026
31	1473	1029	=IF(OR(B32<B44, B32>B43),"OUTLIER",B32)	1029
32	2115	1110	=IF(OR(B33<B44, B33>B43),"OUTLIER",B33)	1110
33				
34	=COUNT(A2:A34)	32	RRmean	1039.2
35	=0.25*A34	993	SDNN	89.9
36	=0.75*A34	1102	NN50	11
37	=0.5*A34	1065	pNN50	37.9

Cells A1:A32 represent raw RR intervals that have been 'sorted' from shortest to longest duration. Cells B1:B32 represent raw RR intervals as recorded. Cells C1:C32 contain the code used to test whether the raw RR intervals in column C are within 3.5 times the interquartile range from the median. Cells D1:D32 demonstrate the output observed from the code in column C. Cell B34 used to count the number of RR intervals. Cells B35, B36 and B37 used to calculated 25^{th}, 50^{th} and 75^{th} quartiles, respectively.

Given that statistically, values of 1.5 times the IQR of the median are considered 'mild' outliers, it may be argued that 3.5 times the IQR is very conservative. The investigator chose 3.5 times the IQR because the aim was not to 'condition' the data set; after all, the variability of RR intervals is the basis of HRV. Rather, 3.5 times the IQR was chosen because this would likely only remove technically induced artefact (e.g., signal interference etc) without reducing the inherent variability of the RR interval data set. When outliers were detected, they were deleted and the data set were 'moved up' to fill the vacant line.

Time domain analysis of heart rate variability

Perhaps the simplest calculation of HRV involves analysis of the so-called time domain. Time domain analyses typically measure the variation in the time/interval duration between consecutive cardiac cycles. Once the RR intervals have been recorded and outliers removed, simple statistical calculations can provide reliable measures of HRV representative of the recording period, including:

- RRmean: the average duration of RR intervals
- SDNN: the standard deviation of RR intervals
- NN50: the number of consecutive RR intervals varying by $\geq$ 50 ms
- pNN50: the percentage of NN50 relative to total RR intervals
- RMSSD: the square root of the mean squared differences of consecutive RR intervals

The time domain analyses can be performed simply and cost-effectively using Microsoft Excel formulae. For example, RRmean and SDNN could be determined by applying the 'AVERAGE' and 'STDEV' formulae to cells D1:D32 previously listed in Table 6.1. The NN50 and pNN50 require another column of formulae to determine whether each RR interval is $\geq$ 50 ms of the previous RR interval (using logic statements). For clarity, analysis is provided below in Table 6.2.

Table 6.2. Select time domain analysis of heart rate variability using spreadsheet software.

	A	...	D	E	F
1	639	...	905	=IF(OR(D3-D2>50,D2-D3>50),"NN50","")	
2	817	...	817	=IF(OR(D4-D3>50,D3-D4>50),"NN50","")	NN50
31	1473	...	1029	=IF(OR(D4-D3>50,D3-D4>50),"NN50","")	
32	2115	...	1110	=IF(OR(D4-D3>50,D3-D4>50),"NN50","")	NN50
33		**HRV parameter**		**Formula**	**Value**
34		RRmean		=AVERAGE(D1:D32)	1039.2
35		SDNN		=STDEV(D1:D32)	89.9
36		NN50		=COUNTIF(F2:F32,"NN50")	11
37		pNN50		=(F36/COUNT(D2:D32))*100	37.9

Cells E1:E32 represent the code used to determine whether the RR interval (Column D) was ± 50 ms of the previous RR interval (NN50). The total occurrences of NN50 was reported in Cell F36, and NN50 expressed as a percentage of total RR intervals was reported in Cell F37.

.

Other spreadsheet formulae may be used to determine the RMSSD; in an adjacent column to the RR intervals, each RR interval was subtracted from the successive RR interval resulting in a column of 'differences'. In an adjacent column to the differences, the differences may be squared (using '^2') to determine the 'squared differences'. The mean of the squared differences was determined using the 'AVERAGE' formula. Finally, the square root (using 'SQRT') of the squared differences may be calculated, as demonstrated in Table 6.3.

Table 6.3. Calculation of the square root of the mean squared differences of consecutive RR intervals using spreadsheet software.

	A	...	D	E	F	G	H
1	639	...	905	=D2-D1	*-88*	=E1^2	*7744*
2	817	...	817	=D3-D2	*16*	=E2^2	*256*
	↓	↓	↓	↓	↓	↓	↓
31	1473	...	1029	=D32-D31	*81*	=E31^2	*6561*
32	2115	...	1110	=D33-D32	*32*	=E32^2	*1024*
33		**Parameter**		**Formula**			**Value**
34		Mean of squared differences		=AVERAGE(H1:H32)			2828.89
35		RMSSD		=SQRT(H34)			53.19
36							
37							

Cells E1:E32 represent the code used to find the difference between successive RR intervals, the output of which is displayed in Cells F1:F32 for illustrative purposes. Cells H1:H32 represents the output of the code in G1:G32 used to square each difference of successive RR intervals. The average of the squared differences was then calculated (H34), before the square root of the mean of successive differences was calculated (H35).

While Microsoft Excel provides an adequate working environment to perform HRV analysis, the time required to perform all of the statistical analyses is significant. The *Biosignal Analysis and Medical Imaging Group* of the University of Kuopio provide free-to-use custom written software (Kubios HRV 2.0, Biosignal Analysis and Medical Imaging Group, University of Kuopio, Finland) that performs HRV analysis almost instantaneously, significantly improving efficiency. Despite the Kubios software having been previously reported to be valid and accurate (Niskanen et al., 2004, Tarvainen et al., 2009), it was important to ensure that the HRV parameters derived using this software reflected the values that could be calculated manually using Excel. Using the above spreadsheet technique on RR intervals recorded from four different individuals, there was no difference (0.0%) between the HRV indices calculated using Kubios and manual calculations. Thus, all HRV analyses performed during this dissertation were conducted using the custom written software.

Frequency domain analysis of heart rate variability

Another method of analysing signals that has become popular for HRV studies involves estimation of the power spectral density of the RR interval time series. These analyses are collectively referred to as belonging to the 'frequency domain' and enable identification of the rhythmical fluctuations that characterise the RR interval time series. The principal goal of frequency domain analyses is to separate the various components that make up a signal. Credit is given to Joseph Fourier (1768-1830) for fundamental work that suggested that periodic signals could be reduced to a set of sine/cosine waves (see Figure 6.3).

Similar methods have been applied to the RR interval time series to describe how the power ($ms^2 \cdot Hz^{-1}$) of the RR intervals (ms) is distributed with frequency (Hz). Analysis of the RR interval time series using frequency domain analysis has enabled the various oscillatory components that contribute to HRV to be distinguished based on power and peak frequencies, thus discrete frequency bands have been described. The most well-established frequency bands are the ultra low frequency (0.0-0.003 Hz), very low frequency (0.003-0.04 Hz), low frequency (LF, 0.04-0.15 Hz), and high frequency (HF, 0.15-0.40 Hz) bands. During short-term recordings (i.e., 5-10 min), the ultra low frequency and very low frequency bands have little physiological meaning, thus are not included in this dissertation. Alternatively, power within the LF and HF bands are well-described and have provided physiologically meaningful analyses. It should be noted that while the frequency bands have well defined boundaries, the distribution and peak frequencies observed during short-term recordings tend to vary based on the dynamic modulation of the cardiac cycle by the autonomic nervous system (ANS).

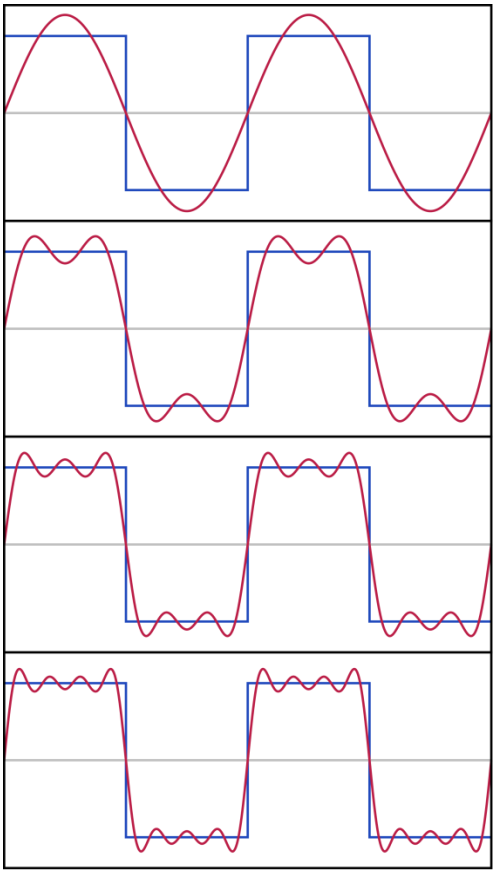

Figure 6.3. Construction of a 'square wave' by Fourier series. The square wave may be estimated with increasing accuracy using sets of sine waves. Note: this image illustrates the first four approximations only. Public domain image.

Studies altering vagal modulation of the heart (e.g., electrical stimulation; muscarinic receptor blockade) demonstrate that power within the HF band directly reflects parasympathetic ANS modulation of the RR interval time series (Pomeranz et al., 1985, Akselrod et al., 1981). Alternatively, the LF band of HRV has been suggested to reflect modulation of the cardiac cycle by both branches of the ANS, largely because the blockade of the parasympathetic ANS and interventions to increase sympathetic modulation result in reductions in LF power (Malik et al., 1996). It is important to note, however, that total power is also reduced in each of these cases. Thus, when LF power is expressed relative to total power, there is a clear increase in LF power (Malik et al., 1996) under sympathetic excitation.

Consequently, LF power expressed in units normalised to total power reflects the sympathetic modulation of the cardiac cycle.

The method used for frequency domain analysis in this thesis belong to the parametric family of spectral analyses as recommended for short-term recordings (Malik et al., 1996). Parametric analyses are superior during short-term recordings because they provide an accurate power spectral density even for small data sets, unlike non-parametric analyses such as Fast Fourier Transformations that require relatively larger data sets. Frequency domain analysis was performed using the same RR time series that was used during time domain analysis. That is, the RR time series for each subject were inspected for outliers, outliers were removed, and then both time and frequency domain analyses were performed on the same data.

Fluctuations in the rate of normal sinus rhythm results in a RR time series that is inherently unevenly sampled. Spectral analyses require an evenly sampled data set because small deviations in data density can cause significant error in the power spectral density. Thus, the raw RR intervals were interpolated at 4 Hz to produce an evenly sampled dataset from the irregularly sampled time series. The power spectral density of the interpolated RR time series was subsequently calculated using autoregressive modelling (16^{th} order) as illustrated in Figure 6.4.

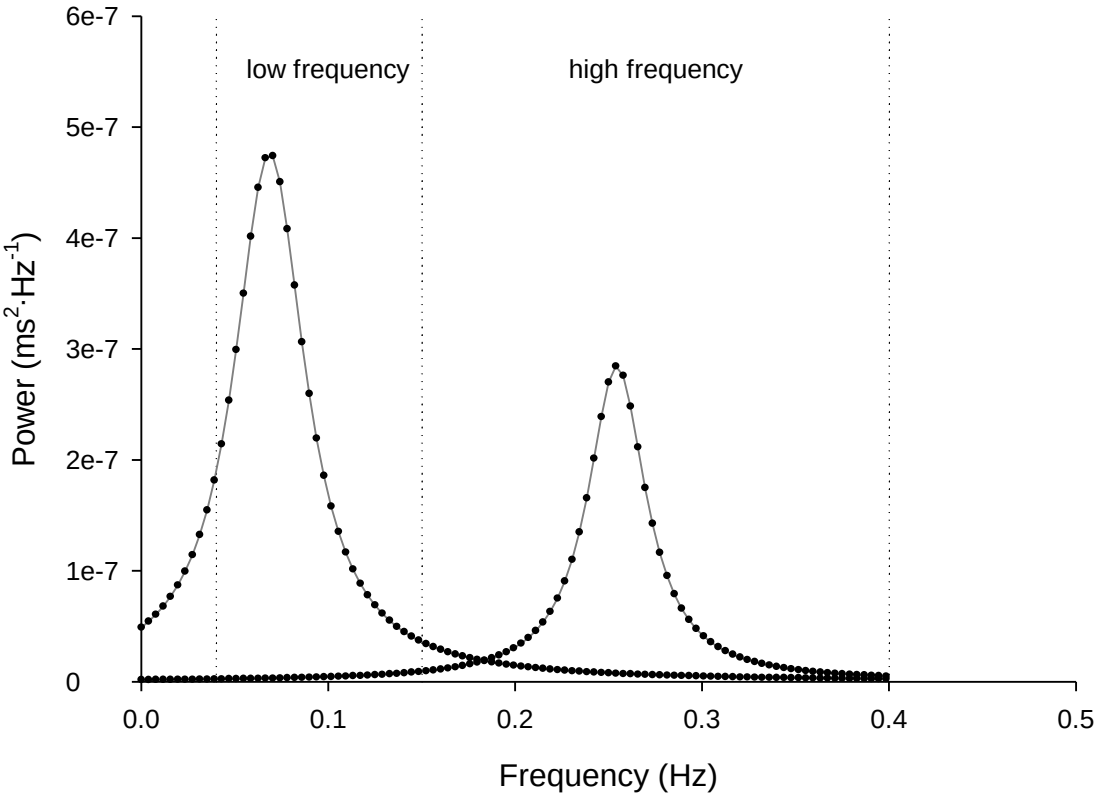

Figure 6.4. Power spectral density of a cardiac (RR) interval time series determined using parametric autoregressive modelling (16th Order). This power spectral density is typical of resting supine values, where absolute power in the low frequency band represents a mixture of sympathetic and parasympathetic autonomic modulation, whereas the high frequency band represents principally parasympathetic autonomic modulation. The integrated area under the plot is calculated as the power in each frequency band. Dotted vertical lines represent the boundaries of the low and high frequency bands. The two peaks have been factorised into the separate frequency components to aid visual clarity.

The autoregressive model and model order were chosen to comply with the recommendations of the Task Force of The European Society of Cardiology and The North American Society of Pacing and Electrophysiology and because the order should be at least twice the number of spectral peaks that are observed in the data set (Malik et al., 1996, Tarvainen et al., 2009). Given that two-to-three peaks are typically observed in the power spectral density of an RR time series, the 16th order was appropriate.

6.4 BLOOD RHEOLOGY

Blood sampling

Subjects visited the laboratory after an overnight (~12 h) fast to provide blood samples. Blood samples were collected between 0700 and 0900 h by an accredited phlebotomist according to the guidelines published by Baskurt et al., (2009a). Briefly, the guidelines for haemorheological assessments suggest that diurnal variation, hydration, food and fluid intake, physical activity, drugs and environmental conditions influence haemorheological parameters. Consequently, it was important to standardise the blood sampling methods to ensure that the results of the present study were not confounded by non-experimental influences. Subjects were instructed to refrain from moderate-to-vigorous physical activity, and the consumption of alcohol and caffeine for at least 24 h prior to visiting the laboratory. Moreover, all blood samples were collected following an overnight (~12 h) fast to standardise food and fluid intake. Subjects were specifically requested to only consume water in the morning prior to providing blood samples. The laboratory was maintained at 22 ± 1°C during blood collection.

The site and method of blood collection was also designed to align with the guidelines for haemorheological studies (Baskurt et al., 2009a). Tourniquet application significantly alters local blood composition; however, if blood is sampled within 90 s while the tourniquet is still applied, the influence is diminished (Connes et al., 2009). Narrow bore needles increase the shear stress applied to blood during collection (Lippi et al., 2006). Moreover, ethylenediaminetetraacetic acid (EDTA) has been demonstrated to induce less perturbation to haemorheology compared with lithium heparin. Consequently, blood samples for the haemorheological studies in this thesis were collected:

- within 90 s of tourniquet application and while the tourniquet was still applied
- using 21 gauge needles
- using EDTA (1.8 $mg \cdot mL^{-1}$) as an anticoagulant*.

*Note: blood for immunoassays were collected into tubes containing other anticoagulents; however, all blood for haemorheological assays was collected into EDTA tubes.

Blood was collected from a prominent antecubital vein into EDTA (1.8 $mg \cdot mL^{-1}$), sodium citrate (3.2% buffered tri-sodium citrate) or serum-separating collection tubes, which were immediately placed on a tube roller until subsequent analysis and/or centrifugation. Plasma was separated from whole blood within 30 min and stored at -80°C until subsequent analyses, and all haemorheology measures were completed within 3 h of blood collection.

Determination of red blood cell deformability

Whole blood (7 µL) was aspirated from an EDTA tube and dispensed into a 2 mL tube containing 700 µL of a viscous, isotonic polymer solution (5.5% polyvinylpyrrolidone 360 kDa dissolved in PBS, osmolality = 300 $mosmol \cdot kg^{-1}$), to produce a suspension of ~1% haematocrit. Given that the viscous, isotonic polymer solution is stored at 4°C, the solution was dispensed into the tubes early in the morning prior to analysis to ensure that it was always at room temperature before the whole blood was added. This step was incorporated to ensure that the cells would not be lysed due to the low temperature. The tube was subsequently inverted several times to mix the suspension, before the tube was placed on a roller for 10 min. The deformability of RBC was subsequently determined at 21 ± 1°C using a 600 µL sample of the diluted RBC suspension by ektacytometry.

Ektacytometry is a technique that calculates RBC deformability by measuring the laser diffraction of a RBC suspension (i.e., RBC in a high-viscosity medium) under varying shear stress (0~25 Pa). The laser diffraction was produced by emitting a low-powered laser diode (operating at 1.5 mW and a wavelength of 635 nm) through a concentric cylinder through which the RBC suspension flowed. The laser beam was diffracted by the RBC in the suspension; by aspirating the RBC suspension at differential pressures over 30 s, the diffraction of the laser beam was dependent upon the shape of the RBC (Figure 6.5).

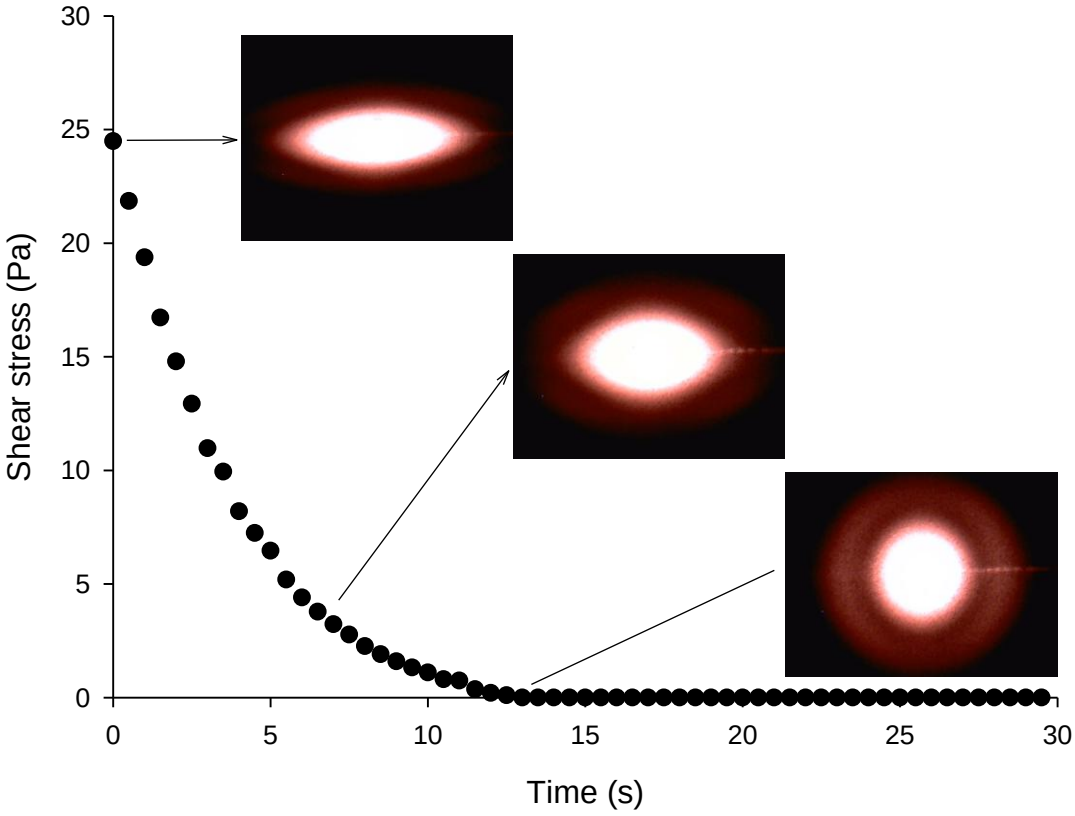

Figure 6.5. The pressure gradient used to 'deform' the red blood cells (RBC) with images representative of the laser diffraction at each region of the curve. Data points represent the shear stress corresponding with each laser diffraction image that was captured by the camera (2 Hz).

The resultant laser diffraction was captured by a 'charge-coupled device' video camera at 2 Hz over the 30-s duration and enabled real-time observation of RBC deformability. The video camera was connected to a personal computer with custom-written software (Deform-Ace ver. 2.2, Sewon Meditech Inc., Korea) that fitted an ellipse to the 60 (30-s at 2 Hz) diffraction patterns. The ellipse was then measured to provide the lengths of the major and minor axes of the diffraction pattern and an 'elongation index' (EI; analogous with RBC deformability) was calculated using the equation: $EI = (A - B) \div (A + B)$, where A represents the major axis and B represents the minor axis of the ellipse. Thus, an EI was calculated for 60 data points over

the 30-s duration of each test. Figure 6.6 demonstrates an example of how the elongation index was measured for one diffraction pattern.

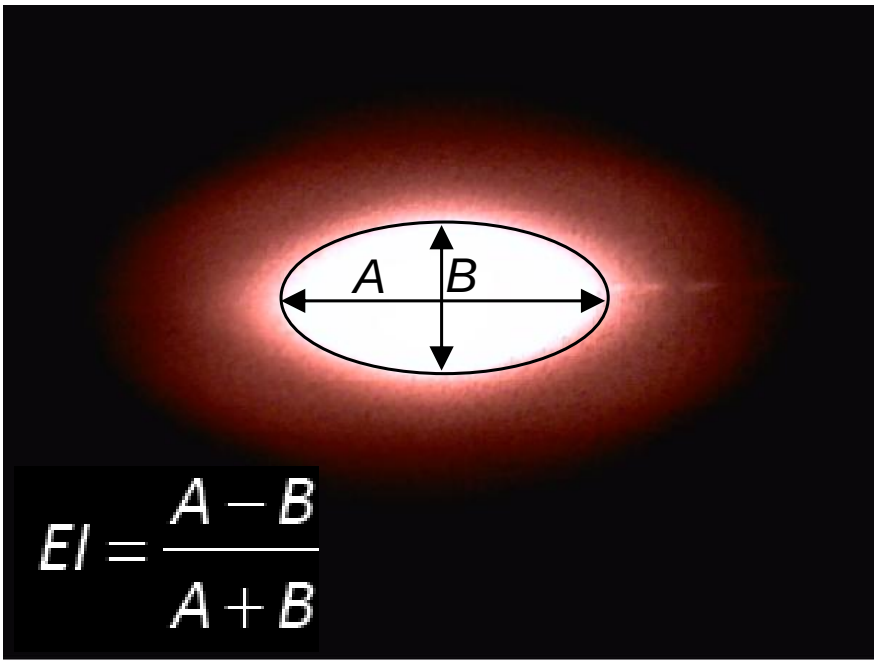

Figure 6.6. The measurement of red blood cell (RBC) deformability using ektacytometry. The white region in the centre represents the area of the RBC, while the pink 'halo' represents the scattered laser beam. The black outline of the ellipse is not the actual curve-fit used for data analysis, but is representative of the curve fitting process. *A*, the major axis of the ellipse. *B*, the minor axis of the ellipse. EI, elongation index.

Given that the EI of an RBC is dependent upon the applied shear stress, comparisons between individuals or longitudinally within an individual are complicated given that there are at least 60 data points per test (30-s at 2 Hz). Baskurt and Meiselman suggested that while the multiple data points are of particular interest when investigating the mechanical properties of RBC, it may be more appropriate in a clinical/experimental setting to use 'global' parameters that describe the shear stress-elongation index curve (Baskurt and Meiselman, 2004). Non-linear curve fitting techniques have provided two meaningful parameters that enable

simplified comparisons within/between subjects in experimental studies. The calculation of the maximal elongation index (EI_{max}) and the shear stress required for half maximal deformation of the RBC ($SS_{1/2}$) described the entire shear stress-elongation index curve. For clarity, EI_{max} and $SS_{1/2}$ have been plotted on a shear stress elongation index curve (Figure 6.7).

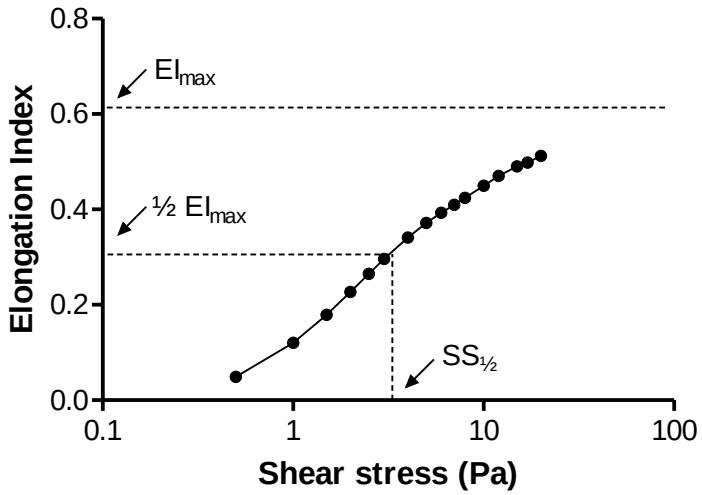

Figure 6.7. Shear stress-elongation index curve with parameterised values added for illustrative purposes. EI_{max}, maximal elongation index. $SS_{1/2}$, the shear stress required for half maximal deformation of the RBC. The use of these two parameters aids comparisons made within an individual at multiple time points or between individuals at a discrete time.

Determination of red blood cell aggregation

Red blood cell aggregation for all suspensions was determined at room temperature (21 ± 1°C). A sample (20 µL) of each suspension was aspirated from an EDTA tube and dispensed onto a transparent cone-plate shearing system (Myrenne Aggregometer, Myrenne GmbH, Roetgen, Germany) for the determination of RBC aggregation.

The principal of the aggregometer is based on the transmission of infrared light through a RBC suspension. The sample is placed onto a glass cone (angled at 2°), before a glass plate is lowered onto the cone to form a sealed 'measuring chamber'. Given that RBC aggregation occurs within the collection tube and even on the glass cone because the suspension is at stasis, the sample must be 'disaggregated' prior to measuring RBC aggregation. Disaggregation of the sample is achieved by rotating the cone at a high-shear rate (600 s^{-1}) for 10 s, thus separating all pre-existing RBC aggregates. Infrared light is then transmitted through the RBC suspension and recorded for 10-s by an inbuilt photometer set to the infrared range. Two measurements may be calculated from the resultant light transmission data (Figure 6.8), depending on the shear rate applied during the 10-s recording period: i. M0, aggregation of RBC at stasis during 10 s following an abrupt cessation of a high shear (600 s^{-1}); ii. M1, aggregation of RBC at a very-low shear (3 s^{-1}) within 10 s following the cessation of a high shear (600 s^{-1}).

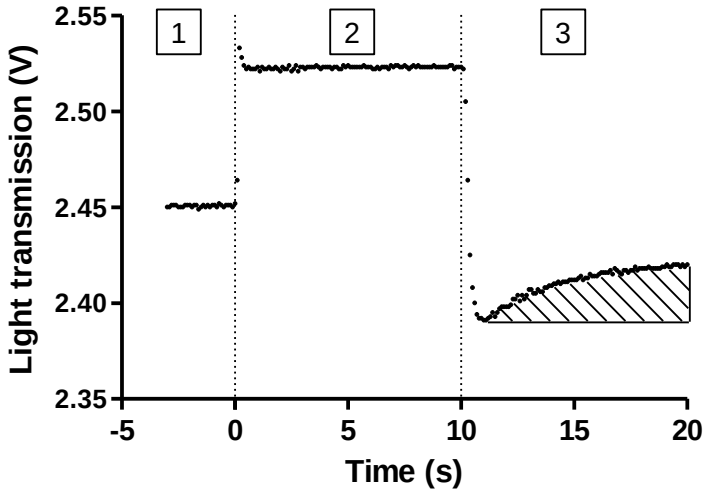

Figure 6.8. The infrared-light transmission curve used to determine red blood cell (RBC) aggregation. The RBC are already aggregated upon being dispensed on the cone (Region 1), thus the suspension is subjected to a high shear for 10 s to disaggregate the RBC (Region 2). The cone is then either stopped (M0 index) or rotated at a low shear (M1 index) for 10 s and the resultant light transmission curve is integrated (shaded area) to calculate RBC aggregation (Region 3).

The calculation of aggregation indices (M0, M1) was achieved by integrating the light transmission curve that was recorded over the 10-s recording period, immediately after the 10-s disaggregation period. The intensity of the light transmission detected by the photometer reflected the amount of RBC aggregation: i. for highly aggregating suspensions, light transmission was high, because large 'gaps' emerged between aggregates of RBC; ii. for lower aggregating suspensions the light path was blocked by the dispersed RBC, resulting in lower light intensity detection. Each index of RBC aggregation (M0, M1) was determined in duplicate, and the average value reported in this thesis.

Red blood cell aggregation is influenced by both intrinsic (e.g., membrane compliance, negative surface charge) and extrinsic (e.g., plasma fibrinogen concentration) factors, as well as haematocrit levels. Thus, to determine whether RBC aggregation was principally influenced by intrinsic or extrinsic factors in the present studies, aggregation was measured for RBC suspended in: i. plasma at native haematocrit; ii. plasma adjusted to 40% haematocrit; 3) a standard aggregating medium (3% dextran 70 kDa in isotonic PBS) adjusted to 40% haematocrit.

Aggregation of RBC in plasma at native haematocrit was determined by aspirating 20 µL of whole blood from an EDTA tube and dispensing the sample onto the cone for immediate measurement. The remaining two suspensions were adjusted to 40% haematocrit, thus the haematocrit of whole blood collected into an EDTA containing tube was determined within 30 min of collection using an automated haematology analyser (Act.T diff, Beckman Coulter Inc., Fullerton, CA). Whole blood was then divided into two aliquots and centrifuged at 2000 g for 10 min. One aliquot of spun blood was adjusted to 40% haematocrit by adding or removing autologous plasma. The packed cells from the remaining aliquot of spun blood were separated from plasma and washed twice in isotonic PBS (285 ± 3 $mmol \cdot kg^{-1}$, pH 7.4). The packed cells were then washed once in the standard aggregating medium, before being resuspended at 40% haematocrit in 3% dextran 70.

6.5 PLASMA ANALYSES

Determination of plasma fibrinogen concentration

The supernatant plasma from a sodium citrate (3.2% buffered tri-sodium citrate) tube was used to determine plasma fibrinogen concentration. Frozen plasma was thawed in a 37°C water bath before ~50 μL plasma was aspirated into a random access coagulation analyser (CA-6000, Sysmex Corporation, Hyaga, Japan). The assay used to determine plasma fibrinogen was based on the method first described by Clauss (1957). According to the Clauss assay, a high concentration of thrombin (35 – 200 $U \cdot mL^{-1}$) was added to dilute the plasma. Thrombin is an enzyme that converts soluble fibrinogen into insoluble fibrin (a clotting protein). Thus, the clotting time associated with the conversion of fibrinogen to fibrin may be measured and compared against a calibration curve. The calibration curve was prepared automatically by the coagulation analyser, which measured the clotting rate of a series of reference samples with known fibrinogen concentration. The clotting time of each plasma sample was compared with the calibration curve, resulting in the fibrinogen concentration ($g \cdot L^{-1}$) being recorded on the digital display of the coagulation analyser. The assay was performed in duplicate for all samples and the mean of the two results was recorded as the fibrinogen concentration.

Common procedures for enzyme-linked immunosorbent assay

Given that basal concentrations of inflammatory markers may be very low even in individuals with overt type 2 diabetes, it was necessary to use high-sensitivity assays. Various methods may be used to determine cytokine concentration; however, enzyme-linked immunosorbent assay's (ELISA) are particularly suitable because following the standard 'sandwich' assay, additional steps may be incorporated to amplify the signal. Consequently, lower concentrations of the target protein are able to be detected. All assays used to

determine the concentration of plasma inflammatory markers in this thesis were performed in the same manner.

Blood was collected into EDTA (1.8 $mg \cdot mL^{-1}$) containing tubes as described earlier in this thesis under the section *Blood sampling*. Plasma was separated within 30 min of collection by centrifugation at 1000 x *g* for 10 min, before being dispensed into 8 x 450 µL aliquots that were immediately stored at -80°C until analysis. To minimise freeze-thaw cycles, a single aliquot of plasma was slowly thawed to room temperature (22 ± 1°C) only immediately before each ELISA.

Immediately before each ELISA, reagents and the microtiter plate were removed from storage (4°C) and the microtiter plate was washed twice with 400 µL of wash buffer. All wash steps were performed using an automated washer (Fluido, Anthos Labtec Instruments, Eugendorf, Austria) with purpose-written protocols for each ELISA; typically, wash steps involved 6 cycles, with 10-15 s soak in between the wash buffer being dispensed and aspirated. After completing each wash step, the microtiter plate was inverted onto absorbent paper to remove excess wash buffer. The standards for each ELISA underwent 6 serial dilutions, ensuring that each dilution was thoroughly mixed by repeated aspiration and dispensing before performing successive dilutions. The absorbance of each ELISA was read using an automated 96-well spectrophotometer (Anthos 2020, Anthos Labtec Instruments, Eugendorf, Austria) using a primary wavelength of 450 nm and a reference wavelength of 620 nm. A standard curve was produced by plotting the mean absorbance of the duplicate standards 1-7 against the known concentration of each standard. A 5-parameter curve fit the absorbance of the standards to produce a standard curve. The absorbance of each sample was subsequently compared to the standard curve to determine concentrations. Where necessary, the concentration read from the standard curve was multiplied by the dilution factor to quantitate sample concentrations.

C-reactive protein

The standard curve for C-reactive protein (CRP) was produced using serial dilutions as described above except that two extra serial dilutions (i.e., 9 total) were added to increase the lowest limit of detection. Thus, the top standard corresponded to 10.0 $ng \cdot mL^{-1}$ and the lowest standard corresponded to 0.039 $ng \cdot mL^{-1}$. The samples were diluted 1:4000 in wash buffer (Tris Wash Buffer in water) to ensure that the CRP concentration in the samples was able to be detected within the range of the standard curve. To the microtiter plate, 100 µL of diluted standard and sample were dispensed into the corresponding wells of the plate. The plate was then incubated for 2 h on a temperature-controlled microplate shaker (Thermomixer comfort, Eppendorf, Hamburg, Germany) set to 200 $rev \cdot min^{-1}$ and 22°C and a further ~10 min with substrate until adequate colour production was observed. The absorbance was measured and quantified as described above in the '*Common procedures for enzyme-linked immunosorbent assay*'.

Interleukin-6, Interleukin-10 and Tumour necrosis factor-alpha

Interleukin (IL)-6, IL-10 and Tumour necrosis factor- alpha (TNF-α) were determined using high-sensitivity ELISA (BMS213HS/BMS215HS/BMS223HS; Bender MedSystems GmbH, Vienna, Austria). The standard curve was produced with 6 serial dilutions; for IL-6, the top standard corresponded to 5.0 $pg \cdot mL^{-1}$ and the lowest standard corresponded to 0.08 $pg \cdot mL^{-1}$; for IL-10, the top standard corresponded to 25.0 $pg \cdot mL^{-1}$ and the lowest standard corresponded to 0.39 $pg \cdot mL^{-1}$; for TNF-α, the top standard corresponded to 20.0 $pg \cdot mL^{-1}$ and the lowest standard corresponded to 0.31 $pg \cdot mL^{-1}$. The samples were diluted 1:2 in sample diluent. To the microtiter plate, 100 µL of diluted standard and sample were dispensed into the corresponding wells of the plate. The plate was then incubated for 2 h on a temperature-controlled microplate shaker (Thermomixer comfort, Eppendorf, Hamburg, Germany) set to 200 $rev \cdot min^{-1}$ and 22°C with the biotin conjugate; 1 h with streptavidin-HRP; 15 min for the first amplification (Biotinyl-Tyramide); 30 min for the second amplification (Streptavidin

HRP); and ~10 min with the substrate reactive with Streptavidin HRP until adequate colour production was observed. The absorbance was measured and quantified as described above in the '*Common procedures for enzyme-linked immunosorbent assay*'.

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Abbreviations and Symbols

Dot [·] above any variable indicates a temporal derivative

SYMBOLS

β	beta
µ	micro
F	F statistic
p	p-value test statistic
x	mathematical operator for multiplication

UNITS OF MEAUREMENT

95% CI	confidence intervals of the 95^{th} percentile
$beat \cdot min^{-1}$	beats per minute
cm	centimetre
d	day(s)
$d \cdot wk^{-1}$	days per week
g	gravitational acceleration
$g \cdot L^{-1}$	grams per litre
h	hour(s)
Hz	hertz
IQR	interquartile range
kg	kilogram
$km \cdot h^{-1}$	kilometres per hour
$km \cdot wk^{-1}$	kilometres per week
L	litre(s)

$L \cdot min^{-1}$	litres per minute
$L \cdot sec^{-1}$	litres per second
ln	natural logarithm transformed
mg	milligrams
$mg \cdot L^{-1}$	milligrams per litre
$mg \cdot mL^{-1}$	milligrams per millilitre
$MJ \cdot wk^{-1}$	megajoules per week
mL	millilitre
$mL \cdot kg^{-1} \cdot min^{-1}$	millitres per kilogram per minute
mmHg	millimetres of mercury
$mmHg \cdot s^{-1}$	millimetres of mercury per second
min	minute(s)
$min \cdot session^{-1}$	minutes per session
$min \cdot wk^{-1}$	minutes per week
$mmol \cdot kg^{-1}$	millimoles per kilogram
$mmol \cdot L^{-1}$	millimoles per litre
mo	month(s)
$mol \cdot L^{-1}$	moles per litre
$mosmol \cdot kg^{-1}$	milliosmoles of solute per kilogram of solvent
ms^2	power; milliseconds squared
$ms^2 \cdot Hz^{-1}$	power; milliseconds squared per hertz
$mU.L^{-1}$	international milliunits per litre
mW	milliwatt
n	sample size
$ng \cdot mL^{-1}$	nanogram(s) per millilitre
nm	nanometre(s)
n.u.	normalised units
Pa	Pascal
$pg \cdot mL^{-1}$	picograms per millilitre

Q1	25th quartile
Q3	75th quartile
r	Pearson's correlation coefficient
rev·min^{-1}	revolutions per minute
s	second(s)
s^{-1}	shear rate
SD	standard deviation
SEM	standard error of the mean
sessions·wk^{-1}	sessions per week
U.L^{-1}	international units per litre
W	watt(s)
W·kg^{-1}	watts per kilogram
wk	week(s)
yr	year(s)
µL	microlitre(s)
µm	micrometre(s)
µmol·L^{-1}	micromoles per litre

VARIABLES AND ABBREVIATED TERMS

%β	beta-cell function calculated via nonlinear homeostasis model
%S	insulin sensitivity calculated via nonlinear homeostasis model
Akt	a serine–threonine kinase
AMP	adenosine monophosphate
ANS	autonomic nervous system
AS160	see TBC1D4
ATP	adenosine triphosphate
BMI	body mass index
BP	blood pressure
Ca^{2+}	calcium

Chol:HDL	cholesterol to high-density lipoprotein ratio
CRP	C-reactive protein
CVD	cardiovascular disease(s)
DX70	dextran; molecular weight 70 000
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
EI	elongation index
EI_{max}	maximal elongation index
ELISA	enzyme-linked immunosorbent assay
FEV_1	forced expiratory volume in the first second of expiration
FVC	forced vital capacity
GLUT	glucose transporter
GLUT1	glucose transport type 1
GLUT4	glucose transport type 4
HbA_{1c}	glycated haemoglobin
HDL	high-density lipoprotein
HF	high frequency
HOMA2	nonlinear homeostasis model assessment
HRP	horseradish peroxidase
HRV	heart rate variability
IL	interleukin
IL-6	interleukin-6
IL-10	interleukin-10
IR	insulin resistance calculated via nonlinear homeostasis model
IRS	insulin receptor substrate
K^+	potassium
LF	low frequency
LF:HF ratio	low to high frequency ratio

M0	red blood cell aggregation at stasis in plasma ($M0_{PLA}$) or dextran 70 ($M0_{DX}$)
M1	red blood cell aggregation at 3 s^{-1} in plasma ($M1_{PLA}$) or dextran 70 ($M1_{DX}$)
MI	myocardial infarction
NN50	the number of consecutive cardiac intervals differing by more than 50 milliseconds
p85	subunit of phosphatidylinositol 3-kinase
PBS	phosphate buffered saline
pNN50	the number of consecutive cardiac intervals differing by more than 50 milliseconds expressed as a percentage of total cardiac intervals
PEFR	peak expired flow rate
PI3K	phosphatidylinositol 3-kinase
RBC	red blood cell
RMSSD	root mean square of the differences between consecutive cardiac intervals
RR	beat-to-beat cardiac interval measured between R waves
SDNN	standard deviation of normal-to-normal cardiac cycles
$SS_{1/2}$	shear stress required for half-maximal elongation index
TBC1D4	Akt substrate of 160 kDa
T_{ge}	gas-exchange threshold
Th1	T helper cell type 1
Th2	T helper cell type 2
TNF-α	tumour necrosis factor-alpha
$\dot{V}CO_2$	carbon dioxide output
$\dot{V}_E$	minute expired ventilation
$\dot{V}_E / \dot{V}CO_2$	quotient of minute ventilation and carbon dioxide output
$\dot{V}_E / \dot{V}CO_2$	quotient of minute ventilation and oxygen uptake
$\dot{V}O_2$	oxygen uptake
$\dot{V}O_2$peak	peak oxygen uptake