INTERVENTIONS AIMED TO IMPROVE AUTONOMIC AND ENDOTHELIAL FUNCTION IN CHRONIC HEART FAILURE AND CORONARY ARTERY DISEASE

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I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university and that to the best of my knowledge and belief, it does not contain any material previously published or written by another person except where due reference in made in the test.

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The current epidemic of cardiovascular disease has defined itself as the healthcare priority of this generation. It ranges from coronary artery disease (CAD) (presenting with acute symptoms) to chronic heart failure (CHF) (presenting with debilitating and gradual worsening symptoms). An improved understanding of the pathophysiology of the disease has lead to new medications and reduced disease specific mortality. However, increased prevalence and chronic symptoms have adversely affected quality of life. Persistent angina in CAD and dyspnoea and fatigue in CHF remain the cardinal symptoms restricting quality of life. These debilitating symptoms are a reflection of end stage disease and are resistant to current therapeutic options. This thesis explores new therapeutic options to manage these debilitating symptoms.

Recent studies have highlighted the potential benefit of non toxic agent's- folic acid, tetrahydrobiopterin and oxygen in patients with cardiovascular risk factors. Previous studies done by our group and others have shown oxygen to improve symptoms and exercise performance in patients with CHF while folic acid and tetrahydrobiopterin are shown to improve vascular endothelial function in these patients. The low cost and largely non toxic potential of these agents has driven their further assessment for this thesis.

Existing literature provides information on the influence of these agents in patients with cardiovascular risk factors. The scope of this thesis is to add to this knowledge and assess the effects of these agents on cardiopulmonary reflexes and endothelial function in patients with established CAD and CHF.

The first part of the thesis analysed the influence of low flow nasal oxygen on cardiopulmonary reflex during sub-maximal and maximal exercise testing in CHF and the second part analysed the influence of folic acid and tetrahydrobiopterin on endothelial function in CHF and CAD. The thesis also conducted a technical analysis of the reproducibility of pulse wave analysis, used to analyse vascular endothelial function.

Folic acid, tetrahydrobiopterin and oxygen were well tolerated and no significant side effects were reported. Patients with CHF were characterised by autonomic imbalance; predominantly inefficient ventilation during sub-maximal and maximal exercise. Low flow inhaled oxygen significantly improved this imbalance and reduced the ventilatory overdrive during sub-maximal, but not maximal exercise. However, it did not influence cardiovascular parameters. This ventilatory benefit might explain the improvement in exercise performance and quality of life seen with its use in CHF. Further studies are required to assess its impact on disease progression and mortality.

Folic acid significantly reduced serum ADMA level in patients with CHF. ADMA is a well defined surrogate marker of endothelial injury. However, it did not concurrently improve endothelial function. Furthermore, the combined administration of folic acid and tetrahydrobiopterin did not improve endothelial function in both the large (aorta) and small arteries (brachial) of patients with CAD. The lack of effect of folic acid and tetrahydrobiopterin might be explained by the advanced irreversible nature of endothelial function in patients with well established cardiovascular disease. The technical analysis of pulse wave analysis emphasised its

usefulness in assessing baseline endothelial function; however, it correlated poorly

when used to assess the impact of various therapeutic agents on endothelial function.

5-MTHF	5-methyl tetrahydrofolate
AA	ascending aorta
ACE	Angiotensin converting enzyme inhibitors
ADMA	serum asymmetrical dimethylarginine
AIx	augmentation index
ANS	autonomous nervous system
BH4	Tetrahydrobiopterin
BMI	body mass index
CAD	coronary artery disease
CHF	chronic heart failure
CaO2	arterial oxygen content
CvO2	venous oxygen content
cGMP	cyclic guanosine monophosphate
CMR	cardiac magnetic resonance imaging
CPET	Cardiopulmonary exercise testing
CV	coefficient of variation
DBP	blood pressure
DDA	distal descending aorta
DDAH	dimethylarginine dimethylaminohydrolase
EF	ejection fraction
eNOS	Endothelial NOS
FMD	Flow mediated dilatation
GTN	Glyceryl trinitrate
HR	heart rate
HIF-1	hypoxia inducible translation factor
ICC	intraclass correlation coefficient
IHD	ischaemic heart disease
L-NMMA	N monomethly-L-arginine
MI	myocardial infarction
MRI	Magnetic resonance imaging
NADPH	nicotinamide adenine dinucleotide phosphate
NOS	nitric oxide synthase
NO	nitric oxide
PDA	proximal descending aorta
PGC-1 γ	peroxisome-proliferator-activated receptor-γ co-activator 1
PP	pulse pressure
PWA	Pulse wave analysis
RER	Respiratory exchange ratio
ROS	reactive oxygen species
SDMA	symmetrical dimethylarginine
SV	stroke volume
sBP	systolic blood pressure
VO2	ventilatory oxygen
VCO2	ventilatory Carbon dioxide
Ve	ventilation
Vt	tidal volume

CHAPTER 1

BACKGROUND AND OBJECTIVES

1.1   Introduction

The epidemic of cardiovascular disease has characterised the 20th century and defined itself as the health priority of the modern era. The significance of the heart and the cardiovascular system was first demonstrated in 1628 by Harvey who described the circular pattern of flow of blood in the heart. This caused a major shift from the previously held popular belief that the heart was primarily the source of heat for the body. The modern belief was further enriched by Mayow in 1674 when he described the obstruction of blood flow in the heart of patients with mitral stenosis causing ventricular dilatation and chronic haemodynamic overload (Katz 2003). The current epidemic of cardiovascular morbidity and mortality is predominantly due to disease processes affecting the heart and vascular compartments.

The introduction of antibiotics and improved sanitation in the industrialised nations saw a dramatic reduction in mortality by infectious disease. This process coincided with the concurrent and dramatic rise in atherosclerotic coronary artery disease (Luepker 2009). Early observations by De Langen, a Dutch physician, in 1916 from Indonesia, highlighted the differential prevalence of cardiovascular diseases in the colonial Dutch and native Javanese populations. Similar observations were also noted by Isadore Snapper between the Caucasians and Chinese populations in China. World War II saw a dramatic decline in the incidence of cardiovascular disease in the war neutral countries (Malmros 1950). A decline in the availability of food and modern amenities explained this reduction in disease pattern. These observations

lead to the understanding that differing lifestyles, diet and blood cholesterol levels resulted in the higher incidence of cardiovascular disease in the western population.

The first systematic study into epidemiology of cardiovascular disease began after World War II. The Minnesota Business and Professional Men Study in 1947, the Framingham Heart Study in 1948 and the Seven Countries Study in 1950 provided our first comprehensive insight into the causative factors of cardiovascular diseases. Both behavioural and physiological risk factors were identified in different population groups. Cigarette smoking, high blood pressure, diabetes, blood cholesterol level and family history were all identified as significant risk factors. The incidence and prevalence of differing cardiovascular outcomes including myocardial infarction, chronic heart failure and stroke were identified and documented. The identification of risk factors and predictability of disease outcomes lead to interventional studies for disease prevention.

The overwhelming spread of cardiovascular diseases in various parts of the world lead to a worldwide coordinated approach for its prevention. The world health organisation in the 1970's collaborated with various national healthcare organisations. A coordinated approach was developed to reduce the incidence and prevalence of cardiovascular disease. Secondary disease prevention focussed on smoking cessation, lifestyle modification, blood pressure control and medications. The success of these interventions in preventing disease progression lead to the introduction of large community based primary disease prevention strategies.

1.2 Epidemiology and pathogenesis of cardiovascular disease

The spectrum of cardiovascular disease involving the heart ranges from coronary artery disease (CAD) to chronic heart failure (CHF) while peripheral vascular pathology involvement leads to hypertension and stroke. Approximately 23% of all adult patients are affected by cardiovascular disease (Australian Bureau of statistics: Cardiovascular diseases in Australia: A snapshot 2004-05. 4821.0.55.001). The major mortality associated with cardiovascular disease is due to ischaemic heart disease (IHD) [also known as CAD] and CHF. They present the spectrum of disease affecting the heart during the various stages of life. Atherosclerotic plaques begin to build up in the early years of life. Various factors such as lipid metabolism, endothelial function, platelet adhesion and inflammation are believed to contribute to its progression. Atherosclerotic plaques develop over 10 to 15 years. Stable occlusive plaques lead to symptoms of stable angina. However, plaque rupture leads to acute ischaemic injury to the underlying myocardium. Ventricular remodelling following myocardial infarction occurs to accommodate for the chronic changes in vascular haemodynamics and leads to cardiac failure (Sutton, Sharpe 2000).

CHF thus represents the final stage of the continuum of cardiovascular disease (Senni et al. 1998). Over the last 25 years mortality associated with coronary artery disease has declined. However, rising life expectancy has meant that we have an exponential increase in the prevalence and disease burden (Black 2003). In 2004-05, 18% (approximately 3.5 million) of Australians reported having a long-term cardiovascular condition. Of those, 28% reported having angina, 12% a cerebrovascular disease, 35% oedema and heart failure and 27% reported having a disease of the arteries, arterioles and capillaries, stroke and vascular conditions

(Australian Bureau of statistics: Cardiovascular diseases in Australia: A snapshot 2004-05. 4821.0.55.001). People who had cardiovascular disease reported their overall health as being lower than those without a cardiovascular disease. Of those with cardiovascular disease 33% reported their own health as being poor/fair while of those without cardiovascular disease only 11% rated their own health as poor/fair (Australian Bureau of statistics: Cardiovascular diseases in Australia: A snapshot 2004-05. 4821.0.55.001).

1.3   Coronary artery disease

Coronary artery disease remains a major public health problem. It is the leading cause of death in Australia accounting for 16% of all registered deaths (3303.0 - Causes of Death, Australia, 2008). It develops due to limitation of coronary blood flow and the resultant myocardial ischemia. This limitation occurs primarily due to the build up of atherosclerotic plaques in the coronary arteries. Atherosclerosis is a chronic vascular disease with the build up of either focal or large segmental atherosclerotic plaques. Patients with a stable endoluminal plaque causing a fixed obstruction; presents with stable angina. Symptoms of angina are predictable resulting from a fixed increase in cardiac output. However, the unstable vulnerable plaque leads to sudden plaque rupture and intraluminal thrombosis. The vulnerable plaque is often smaller in size, more rich in lipids and infiltrated with macrophages. About one half of patients presenting to the hospital with a myocardial infarction (MI) have had preceding angina (Rouleau et al. 1996). Stable angina remains a significant problem and is 30 times more prevalent than patients with acute coronary syndromes such as MI (Gibbons et al. 2002). In patients with stable angina an atherosclerotic plaque limits myocardial perfusion during exertion, causing angina. The progressive ageing of the population, increased incidence of hypertension,

diabetes and epidemic of obesity will continue to increase the prevalence of atherosclerosis and stable angina. An improved understanding of the pathogenesis of atherosclerosis has focussed on its early prevention and treatment. Dietary modifications and the increased use of statins have lead to major improvements in the management of atherosclerosis. However, patients with advanced atherosclerosis have persistent symptoms of angina despite maximal medications. A large proportion of these patients are unsuitable for surgical bypass treatment and suffer from persistent symptoms despite maximal medications. There is an urgent need for newer therapeutic agents to treat symptoms in these end stage CAD patients.

The development of atherosclerosis in the coronary arteries does not occur in isolation. Endothelial dysfunction precedes and characterises the development of atherosclerosis (Celermajer et al. 1992).

Endothelial dysfunction and ischaemic heart disease

The endothelium forms a single layer of endothelial cells lining the coronary vessel wall in the direction of blood flow. It provides a smooth non thrombotic surface for blood flow. It is also involved in platelet activation, thrombus formation and inflammation. These properties render the endothelium of vital importance in the development of ischaemic heart disease. Endothelial dysfunction is seen in both early and late coronary artery disease (Suwaidi et al. 2000). It is also present in the pre-clinical stages of coronary atherosclerosis (Celermajer et al. 1992). Pro-atherogenic low-density lipoprotein inhibits nitric oxide synthase (NOS) activation and nitric oxide (NO) production leading to endothelial dysfunction and atherosclerosis (Creager et al. 1990b). Coronary endothelial vasodilator dysfunction independently predicts progression of coronary atherosclerosis and acute cardiovascular events in patients with and without detectable coronary artery disease. It is associated with all

the major risk factors for atherosclerosis such as hyperlipidemia, diabetes, hypertension and smoking (Creager et al. 1990b, Vanhoutte, Boulanger 1995).

The vascular endothelium is central to the pathogenesis of atherosclerosis and is strongly associated with disease outcome. It has important secretory, metabolic and immunological functions and impairment of these functions is an early step in the pathogenesis of atherosclerosis. Nitric oxide synthase (NOS) located in the endothelial cells, eNOS, synthesises nitric oxide. It regulates vascular tone and structure along with beneficial effects on both platelets and monocyte function (Vane 1994). The release of nitric oxide from the endothelium is regulated by shear induced vascular stress and polymorphism of the C allele at the T(-786)C endothelial nitric oxide synthase (Rossi et al. 2003, Cattaruzza et al. 2004). Endothelial dysfunction is the result of a reduction in nitric oxide availability. eNOS expression and nitric oxide release is significantly decreased in atherosclerosis and coronary artery disease (Cosentino, Katusic 1995, Oemar et al. 1998). Studies have suggested that enhanced eNOS activity and NO production have a beneficial effect on the cardiovascular system and therefore may develop into an important therapeutic target (Alp, Channon 2004).

L-arginine, a semi-essential amino acid is the only substrate for nitric oxide synthesis (Bennett-Richards et al. 2002). It is hydroxylated to N-hydroxy-L-Arginine and further oxidized to NO and L-citrulline (Heffernan, Vieira & Valentine 2008). NO from the endothelial cells diffuses into the underlying smooth muscles to activate cyclic GMP mediated smooth muscle relaxation. Dietary supplementation of L-arginine is shown to improve endothelial function in young patients with hypercholesterolemia (Clarkson et al. 1996).

Cofactors, namely nicotinamide adenine dinucleotide phosphate (NADPH), flavin, heme, calcium-calmodulin and tetrahydrobiopterin are required in addition to L-arginine for the synthesis of NO (Loscalzo 2004). Folic acid enhances the binding of L-arginine to NOS and enhances regeneration of tetrahydrobiopterin from its inactive form. Folic acid, an essential B-vitamin, occurs in the polyglutamate form in leafy vegetables and animal products. It is cleaved to the monoglutamate form in the jejunum, where it is absorbed. The recommended dietary allowance ranges from 200-400 micrograms/day and deficiency is mainly due to low dietary intake (Allen et al. 1993). Folic acid is known to improve endothelial function in patients with coronary artery disease, diabetes and hypercholesterolemia (Verhaar et al. 1999, Mangoni et al. 2005, Chambers et al. 2000). Though its beneficial endothelial effects were initially believed to be due to its homocysteine lowering effect, recent studies have demonstrated its effects to be independent of this effect (Doshi et al. 2002).

Tetrahydrobiopterin (BH4), a naturally occurring coenzyme for phenylalanine hydroxylase was first discovered in 1963 (Kaufman 1963). In its fully reduced form it is an essential cofactor for the biosynthesis of NO (Kwon, Nathan & Stuehr 1989). Oxidation of BH4 to BH3 and BH2 reduce its bioavailability. This leads to structural changes in NOS leading to the formation of superoxide/hydrogen peroxide (Rosen et al. 2002, Vasquez-Vivar et al. 2002). These cause oxidative stress and atherosclerosis (Moens, Kass 2006, Harrison et al. 2003).

Endothelial dysfunction and CHF

Impairment of endothelium-dependent vasodilatation is a characteristic of chronic heart failure (CHF) (Katz et al. 1993, Morgan et al. 2004, Nakamura et al. 2004, Sakane et al. 2000). It results from impaired endothelial release of nitric oxide (NO),

abnormal production of cyclooxygenase-dependent vasoconstricting factor and decreased vascular smooth muscle responsiveness to cyclic GMP-mediated vasodilatation. Endothelial dysfunction is characteristic of progression of atherosclerosis (Gonzalez, Selwyn 2003) and is evident in the peripheral as well as in the coronary and pulmonary circulation (Elkayam et al. 2002, Takahashi et al. 2005). Furthermore, it has major clinical implications as it is independently associated with increased cardiovascular mortality in CHF (Katz et al. 2005). An improvement in endothelial function in pro-atherosclerotic conditions is associated with improved survival (Gupta 2004, Kiliszek et al. 2007, Wilkinson, McEniery 2004). This highlights the significance of mediators that influence endothelium-dependent vasodilatation. The effects of reduced NO activity in CHF are evident on

- Vascular tone

- Platelets

- Left ventricular remodelling

Endothelial dysfunction and vascular tone

The discovery of endothelial derived relaxing factor by Furchgott and Zawadski, later known as endothelial nitric oxide, derived from L-arginine and oxygen, catapulted the understanding of the role of endothelium in maintaining vascular tone (Furchgott 1999). The endothelium forms a single layer of cells lining the vascular compartment and has important secretory, metabolic and immunological functions and plays an important role in local vascular homeostasis. This is accomplished by maintaining an active balance between vasodilatation and vasoconstriction. Nitric oxide secreted by the eNOS is the most important endothelium-derived vasodilator (Palmer, Ferrige & Moncada 1987). Nitric oxide mediated vasodilation depends on

stimulation of guanylate cyclase and its product cyclic guanosine monophosphate (cGMP) (Cohen, Vanhoutte 1995). Endothelium dependent vasodilators such as acetylcholine produce a time and concentration dependent increase in cellular cGMP levels which precedes the onset of vasodilation (Gruetter et al. 1981, Ignarro et al. 1981). These endothelial cells are hyperpolarized by endothelium dependent vasodilators. The hyperpolarization is transmitted to the smooth muscle cells of the arteries through myoendothelial gap junctions. Vasodilatation is achieved by the closing of voltage dependent calcium channels in the smooth muscle cells (Beny, Koenigsberger & Sauser 2006). An imbalance in this activity leads to endothelial dysfunction.

NO-mediated vasodilatation in response to hormonal stimulation and/or shear stress is significantly impaired in patients with CHF (Katz et al. 1992). Katz et al was the first to demonstrate the differential effect of endothelium dependent vasodilators on the femoral artery of patients with CHF. The mean blood flow velocity increased significantly in 9 healthy individuals to an intra arterial infusion of acetylcholine. However, femoral artery vasodilatation in response to acetylcholine was seen in only 20% of patients with CHF. This phenomenon alters peripheral autoregulation and reduces the supply of oxygen and nutrients to peripheral organs and tissues, thus reducing exercise capacity and quality of life (Katz et al. 1996, Nakamura et al. 1994). Moreover, the consequent increase in arterial stiffness induces a progressive increase in systolic blood pressure (SBP) and diastolic dysfunction. (Moore et al. 2003, Bonapace et al. 2003, Kawaguchi et al. 2003). Chen-Huan Chen et al studied the correlation between arterial stiffness and ventricular systolic function in 57 patients undergoing cardiac catheterisation for chest pain (Chen et al. 1998). Continuous left ventricular pressure volume data was recorded using a conductance

catheter and arterial stiffness was calculated by the end ventricular systolic pressure by the stroke volume. The study provided the first direct evidence that left ventricular systolic pressure was accompanied by an increased arterial load. Animal studies by Leite-Moreira studied demonstrated the interaction between increased afterload and diastolic dysfunction (Leite-Moreira, Correia-Pinto & Gillebert 1999). Nine anaesthetised open chest rabbits underwent multiple graded aortic occlusions in the ascending aorta during the diastole separating two heartbeats. The preceding beat was used as control for the following beat. Afterloads exceeding 80% of peak isovolumetric pressure increased diastolic pressures and resulted in an increase in the left ventricular pressure to end diastolic volume indicating diastolic dysfunction. These haemodynamic changes impose a double burden in patients:

1) An increase in SBP leads to an increased cardiac afterload with subsequent development of left ventricular hypertrophy; and

2) Diastolic dysfunction may critically reduce coronary perfusion pressure during diastole, thus triggering ischaemia (Moore et al. 2003, Bonapace et al. 2003, Kawaguchi et al. 2003).

Vascular tone is regulated by the availability of endothelial NO. Physiological stress and gene expression of eNOS control the release of NO and thus vascular tone. CHF leads to reduced left ventricular output with resultant reduction in shear stress on the major blood vessels. This is evident from reduced NO activity in the larger blood vessels (Comini et al. 1996). There is a resetting of endothelial NO activity in the aorta leading to reduced vasodilation, however, the endothelial NO activity in skeletal muscle vasculature is intact (Comini et al. 1996). This highlights the

selective lack of vasodilatation in the larger blood vessels due to lack of shear stress in these vessels.

Regulation of genetic expression controlling NO release in the endothelium is another significant contributor in CHF. Heart failure induced by external pacing in animal models, lead to significant changes in their vascular NO availability. In the initial stages of heart failure there is an increased production of NO leading to vasodilation. However, after the development of heart failure, this increase in vascular NO independently regulates the genetic expression of NO production. There is a subsequent down regulation of mRNA required to produce endothelial NO (Smith et al. 1996, Wang et al. 1994). The exact cause of this shift in genetic expression is unknown. Animal studies indicate that this shift in genetic expression takes place after 4 weeks of onset of heart failure (Zhao et al. 1995). The endothelial genetic expression of nitric oxide producing mRNA is initially upregulated, however, after the development of heart failure there is a down regulation of this genetic expression. This indicates the crucial role of genetic expression of endothelial nitric oxide production in decompensated heart failure.

Another reason for reduced NO bioactivity in CHF is its increased breakdown in the endothelium. Several enzymes including NADH oxidase, xanthine oxidase and angiotensin II are capable of releasing electrons that reduce molecular oxygen and act as an antioxidant. This effect is mediated through the formation of reactive oxygen species (ROS) and superoxide anions (O-). They react with bioactive NO leading to peroxynitrite. This process is exaggerated in CHF and contributes to endothelial dysfunction (Bauersachs et al. 1999). Another important cofactor required for the NO production is folic acid and tetrahydrobiopterin (BH4). In its

fully reduced form it combines with NOS to produce NO resulting in vasodilatation (Moens, Kass 2006). A close relationship exists between the availability of BH4 and NO synthesis. BH4 deficiency influences vascular damage in patients with diabetes, hypercholesterolemia, hypertension and smokers. BH4 deficiency leads to structural changes in NOS termed uncoupling of NOS. Uncoupling of NOS leads to superoxide production causing vascular damage. Recently tetrahydrobiopterin deficiency has also been shown to play a major role in cardiac ischaemic/reperfusion injury, hypertrophy and remodeling (Moens, Kass 2006).

Endothelial dysfunction and platelets

In addition to the vascular endothelium, nitric oxide is secreted by platelets. There is a complex relationship between nitric oxide secretion by platelets and endothelial dysfunction in CHF (Oemar et al. 1998). Platelet derived nitric oxide inhibits platelet activation and aggregation. Its secretion is reduced in CHF, leading to rapid platelet aggregation. Endothelial dysfunction and reduced vascular nitric oxide availability further aggravates platelet activation and aggregation (Bauersachs, Widder 2008). The resulting prothrombotic state leads to increased venous and arterial thrombosis in patients with CHF.

In CHF, the diminished nitric oxide availability in platelets is due to nitric oxide breakdown by superoxide radicals (Schafer, Eigenthaler & Bauersachs 2004). As in the vascular endothelium, there is an uncoupling of endothelial nitric oxide synthase and its cofactor tetrahydrobiopterin (BH4). This leads to increased superoxide production and nitric oxide breakdown. Inflammatory cytokines such as TNF-α are elevated in CHF. These independently have a negative influence on nitric oxide production in platelets (Schafer, Eigenthaler & Bauersachs 2004, Chung, Lip 2006).

The level of platelet activation correlates significantly with symptoms in CHF. Activated platelets secrete C-C chemokine, a potent chemo attractant. These are elevated independent of the aetiology of CHF and levels are highest in patients with NYHA class 4 symptoms (Aukrust et al. 1998). Platelet activation in CHF also leads to a prothrombotic state in CHF. This leads to increased incidence of cerebral strokes and arterial thrombosis in CHF (Schafer, Eigenthaler & Bauersachs 2004).

Endothelial dysfunction and left ventricular remodelling

Left ventricular remodelling after ischaemic injury is the major contributor to the development of CHF. It involves cardiomyocyte hypertrophy, chamber dilation, and interstitial fibrosis in response to increased mechanical and neurohumoral stress. This leads to contractile dysfunction and heart failure (Sutton, Sharpe 2000). Nitric oxide (NO) generated by the endocardial NOS plays an important role in postinfarction ventricular remodelling and heart failure (Prabhu 2004). It also protects against myocyte apoptosis and hypertrophy (Brutsaert 2003).

Three isoforms of Nitric oxide synthase, NOS 1 (neuronal NOS), NOS 2 (inducible NOS) and NOS 3 (endothelial NOS) are present in the endocardium. Inducible NOS (iNOS) is located in the sarcoplasmic reticulum and is activated by inflammatory cytokines or ischaemic injury. Its activation leads to increased nitric oxide production in the cytosol. Endothelial NOS (eNOS) is located in the sarcolemma (SL) in close proximity to ß-adrenergic receptors and L-type calcium channels (Barouch et al. 2002). eNOS derived NO enhances cardiac contractility by inhibition of ß-adrenergic receptors. Thus the two isoforms of NOS exert divergent but beneficial effect on the myocardium under different situations. eNOS is shown to play a significant role in ventricular remodelling after myocardial infarction. Studies

by Jansen et al in transgenic mice with cardiac limited over expression of eNOS demonstrated the beneficial effects of eNOS (Janssens et al. 2004). The mice were protected against maladaptive remodelling after myocardial infarction. Myocardial contractility, left ventricular systolic and diastolic function was better preserved. Histological analysis did not reveal any difference in the degree of fibrosis but myocyte width in the non infracted myocardium was less in the transgenic mice. These findings suggest that cardiac-specific over expression of NOS3 attenuates the development of LV remodelling.

In conclusion, cardiovascular disease is characterised by endothelial dysfunction and the development of atherosclerotic plaques in the coronary arteries. Nitric oxide synthase expression and nitric oxide production are vital for endothelial function and its deficiency plays a key role in CHF. Both folic acid and tetrahydrobiopterin are vital in the synthesis and regeneration of nitric oxide. Early treatment strategies focussed on the endothelium are essential to prevent the development and progression of cardiovascular disease. Folic acid and tetrahydrobiopterin may present as an early and non-toxic strategy in the prevention and treatment.

1.4   Chronic heart failure

CHF defines the opposite end of the spectrum of patients presenting with cardiovascular disease. Pathologically it encompasses a complex blend of structural, functional and biological alterations evoking profound changes in the neurohormonal, skeletal muscle and respiratory function. Over the last 25 years mortality associated with cardiovascular disease has declined (3303.0 - Causes of Death, Australia, 2008). However, with the aging population, prevalence of CHF has increased (Senni et al. 1998). The increased prevalence of CHF in an aging

population has focussed attention on the impact of the disease on the patient's functional status. This disabling and lifestyle limiting disease has significantly reduced quality of life.

The functional status of a patient with CHF is assessed using the New York Heart Association classification. It is a powerful prognostic marker for mortality. Dyspnoea and fatigue, at rest and/or during exercise, are the cardinal symptoms of CHF. They significantly impact quality of life in these patients (Ekman et al. 2005a). Independent of the functional assessment and ejection fraction they serve as powerful prognostic predictors in these patients (Ekman et al. 2005b).

Recent improvements in the understanding of the pathophysiology of CHF have lead to the addition and increased usage of beta-blockers, ACE-inhibitors and diuretics in the management tof CHF. However, despite these improvements patients with CHF have a profound limitation of their daily activities due to dyspnoe and fatigue. Symptomatic CHF patients express a strong preference for better symptom management (Lewis et al. 2001, Stanek et al. 2000). Despite improved diagnostic and therapeutic management, there is a lack of emphasis on the control of symptoms and quality of life in CHF (Nordgren, Sorensen 2003). There is an urgent need to address and treat lifestyle restricting symptoms in CHF to improve quality of life in these patients (Barnes et al. 2006). There in an unmet need for new therapeutic agents to treat symptoms of dyspnoea and fatigue in patients with CHF to improve quality of life.

Dyspnoea is a major contributor to exercise intolerance in CHF. Two major factors influence respiration in CHF. An abnormal increase in minute ventilation with respect to carbon dioxide production (Chua et al. 1997b) and activation of several

neuroendocrine systems(Francis et al. 1990). Elevated plasma levels norepinephrine (Cohn et al. 1984), atrial natriuretic peptide (Gottlieb et al. 1989) and renin-angiotensin levels (Anonymous 1987a) in CHF indicate the activation of the neuroendocrine systems. The afferent arm of neuroendocrine activation originates from the skeletal muscle and is known as the ergo reflex. It contributes to increased minute ventilation and inefficient ventilation (Piepoli et al. 1996). Thus the resultant dyspnoea occurs due to a heightened ventilatory and sympathetic response in CHF.

Fatigue in CHF is a result of the complex neuroendocrine abnormality, which appears to have a greater peripheral rather than central focus of origin. Inability to increase cardiac output during exercise does not appear to be the limiting factor causing fatigue (Coats 2001). Abnormalities in the skeletal muscle, peripheral blood flow, endothelial function and their neuroendocrine influence on respiration lead to fatigue in CHF (Sullivan et al. 1989a, Drexler et al. 1992, Wilson et al. 1984). Minotti et al showed that neither the central motor drive nor impaired neuromuscular transmission influence the symptom of fatigue in CHF (Minotti et al. 1992).

CHF and dyspnoea

Dyspnoea is a cardinal feature of CHF. Symptom progression from exertional dyspnoea to dyspnoea at rest and paroxysmal nocturnal dyspnoea indicates progression and severity of disease. It is a major cause of exercise intolerance (Davie et al. 1997, Wang et al. 2005). Traditionally the pathogenesis of dyspnoea was attributed solely to haemodynamic changes in CHF. Increased left ventricular pressures required to maintain cardiac output lead to pulmonary oedema and dyspnoea. However, the degree of left ventricular dysfunction does not correlate with progressive symptoms of dyspnoea (Coats 2001, Harrington, Anker & Coats 2001).

Furthermore, at similar workloads, slowly incremental exercises such as the bicycle test lead to fatigue while rapidly incremental treadmill exercises tend to cause dyspnoea (Witte, Clark 2008, Fink, Wilson & Ferraro 1986). Patients with exercise intolerance due to dyspnoea do not have a worse prognosis over patients with exercise limiting fatigue (Witte, Clark 2008).

The pathophysiology of dyspnoea leading to exercise intolerance in CHF is complex and contributed to

- Abnormalities in the respiratory muscle.

- An excessive ventilatory response

- Heightened ventilatory response to peripheral receptor reflexes in the skeletal muscle and artery.

Abnormalities in the respiratory muscle

Patients with CHF develop significant changes in the skeletal muscle (Mancini et al. 1992b). These changes extend to also involve the muscles of respiration. Studies by Mancini et al demonstrated deoxygenation of the accessory muscles of respiration during exercise (Mancini et al. 1991). This leads to reduced strength and endurance of the respiratory muscles. During respiration both expiratory and more prominently inspiratory pressures are significantly reduced (Hammond et al. 1990, McParland et al. 1992). The diaphragm performs most of the work of respiration. The diaphragmatic workload is increased in CHF leading to maximal perfusion and oxygen extraction. However, muscle fatigue in the diaphragm was not demonstrated (Mancini et al. 1992a). Selective respiratory muscle training focused on isocapnic

hyperpnea, resistive breathing and strength training have shown to improve sub maximal and maximal exercise capacity in patients with CHF (Mancini et al. 1995).

CHF is characterised by an increased ventilatory response during exercise (Higginbotham et al. 1983, Reed, Ablett & Cotes 1978). Arterial CO_2 level during exercise is the primary determinant of ventilation. It is maintained within a narrow range during all stages of exercise indicating normal ventilatory control mechanisms (Sullivan, Higginbotham & Cobb 1988). Arterial CO_2 level is balanced by increased ventilatory CO_2 output (VCO_2). VCO_2 is the primary ventilatory drive in humans (Whipp 1983). VCO_2 levels are determined by cellular activity and increases linearly with exercise. They depend on the CO_2 carrying capacity of blood, tissue exchange and cardiac output. Unlike O_2; CO_2 is more soluble in blood and measured ventilatory CO_2 is also dependent on ventilation. The ventilatory variables that balance the VCO_2 include ventilation (Ve), tidal volume (Vt) and ventilatory dead space (Vd). The ventilatory dead space includes both the anatomic dead space (gas in the conducting areas of the respiratory tract that are not in contact with the alveolar surface) and physiological dead space (underperfused alveoli). Vd/Vt is an index of the gas exchange efficiency. It is the fraction of each breath (Vt) that is spent on ventilating the anatomic and physiological dead space (American Thoracic Society, American College of Chest Physicians 2003). The ratio Ve/VCO_2 is known as the ventilatory equivalent for CO_2. Ventilation is directly linked to Co2 production and in healthy individuals the Ve/VCO_2 relationship is linear. An increase in the ventilatory equivalent for CO_2 indicates ventilatory inefficiency (>Vd/Vt) or a lower set point for arterial PCO_2 (Johnson, Badr & Dempsey 1994). The two ventilatory derivatives; the ventilatory drive (Ve/VCO_2) and the ratio of dead space to tidal

volume (Vd/Vt) are elevated during all stages of exercise in CHF (Ingle 2008). This indicates progressive hyperventilation during all stages of exercise.

Patients with CHF have a low tidal volume at rest. This contributes to an elevated Vd/Vt at rest; however, the proportionate increase in tidal volume during exercise is normal in CHF (Sullivan, Higginbotham & Cobb 1988). Hence, a low tidal volume at rest alone does not explain the elevated Vd/Vt during exercise. There is a progressive increase in dead space during exercise. As the anatomical dead space is constant, the increase in dead space is a result of an increase in the physiological dead space. This is attributed to CHF related changes in the pulmonary parenchyma and ventilation perfusion defects (Collins, Clark & Brown 1975, Tattersfield, McNicol & Sillett 1972).

Acute increase in left ventricular pressure leads to reversible changes in the endothelial and epithelial lining of the alveolar capillary membrane. These changes predominantly include epithelial cell breakage and cause significant impairment of gas exchange (Elliott et al. 1992). A chronic increase in pulmonary pressure leads to irreversible deposition of type 4 collagen in the alveolar capillary membrane (Townsley et al. 1995, Kay, Edwards 1973). The thickened membrane is protective against excessive fluid leak across the increased pressure gradient of the lungs. However, it leads to impairment of gas exchange and contributes to increased physiological dead space in the lungs during exercise.
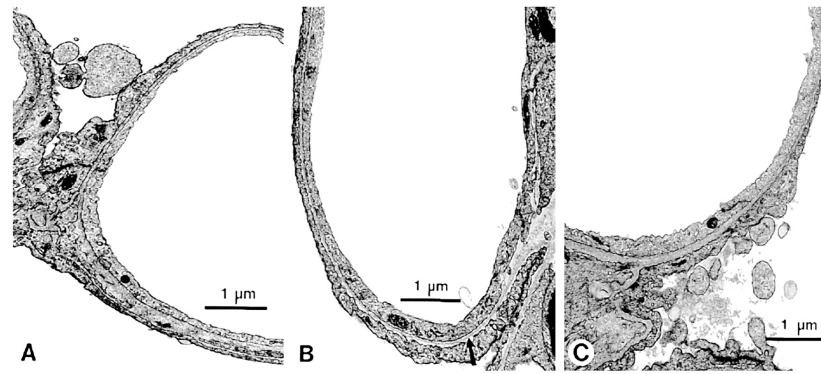
Figure 1.1   Electron micrograph images of the alveolar-capillary membrane in CHF

Image A is obtained from a dog (left, A) and after 4 weeks (middle, B) or 7 to 8 weeks (right, C) of long-term pacing therapy used to induce CHF. Taken from Guazzi M et al, Chest 2003.

An excessive ventilatory response to exercise

During exercise there is a progressive increase in both ventilation (Ve) and carbon dioxide production (VCO_2). Ventilatory, skeletal muscle and chemo receptors respond to increased arterial CO_2. The net result is an increase in Ve and VCO_2 output. In normal individuals the increase in Ve and VCO_2 is directly proportionate and maintains a linear increase. Patients however, require a greater increase in ventilation to balance carbon dioxide output. This increase in Ve is disproportionately higher than VCO_2, thus leading to tachypnea and the sensation of dyspnoea. The slope of Ve/VCO_2 is steeper in CHF and is indicative of the severity of the disease. Patients with severe mild, moderate and severe disease have a progressively greater increase in Ve and thus a steeper Ve/VCO_2 slope (Fig 2). Thus the Ve/VCO_2 slope is an indicator or morbidity and disease progression (Ingle 2008, Guazzi, Myers & Arena 2005, Ingle 2007).
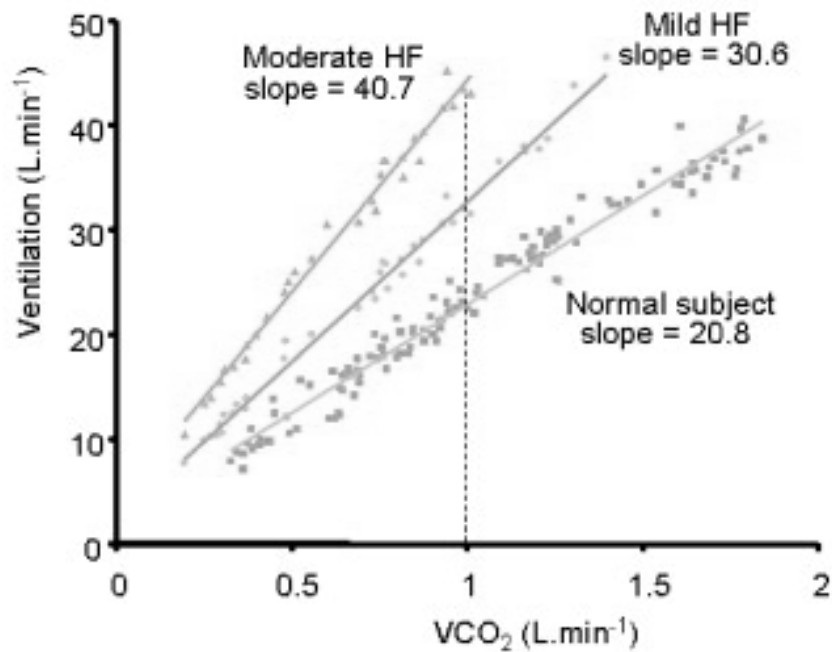
Figure 1.2   The VE/VCO2 slope in heart failure

The relation between ventilation (Ve) and carbon dioxide production (Vco_2) remains linear, but that the slope increases with worsening heart failure. For a VCO_2 of 1 L/min, a normal subject has to ventilate at 22 L/min, the patient with moderate heart failure ventilates at 42 L/min. Adapted from Lee Ingle, Heart Failure Review 2007.

When compared to other parameters of the disease, the slope of Ve/VCO$_2$ is directly related to VO$_2$ max and severity of disease. VO$_2$ max has traditionally been used as a useful prognostic indicator in patients with CHF. However, it has important technical limitations as the plateau phase may not be obtained and is dependent on the motivation of the patients (Robbins et al. 1999). Ve/VCO$_2$ overcomes these technical difficulties and is a suitable alternative to VO$_2$ max. A higher Ve/VCO$_2$ is directly related to mortality (Chua et al. 1997b) and inversely related to ventilatory dead space ratio Vd/Vt (Sullivan, Higginbotham & Cobb 1988) and VO$_2$ max (Chua et al. 1997b). The exact cause of this increased ventilatory drive is unclear. Increased ventilatory dead space (Sullivan, Higginbotham & Cobb 1988), heightened peripheral ventilatory stimuli (Clark, Poole-Wilson & Coats 1996) and slow

metabolic gas kinetics (Riley et al. 1994) have all been described as potential mechanisms.
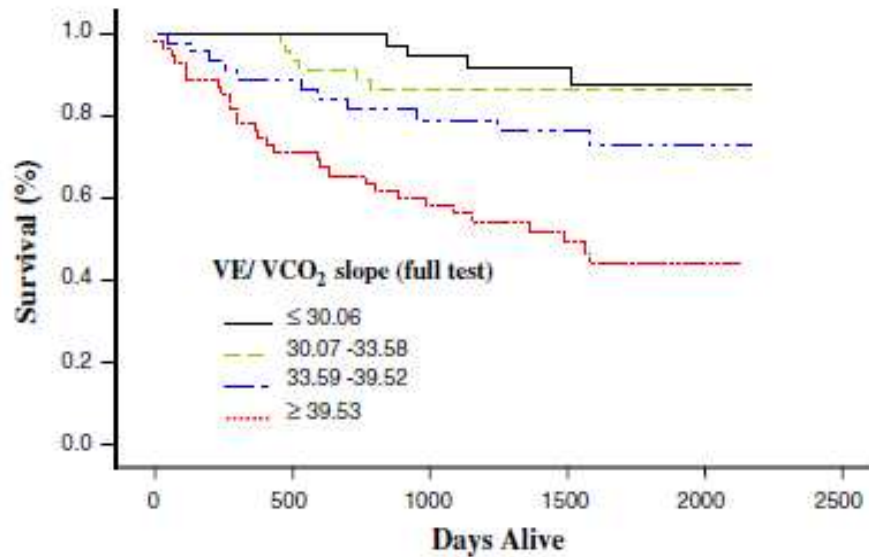
Figure 1.3   Kaplan Meier curve showing prognostic significance of Ve/VCO$_2$ slope in patients with CHF

Patients with well preserved exercise capacity and ventilation are at a low risk of death. Taken from Lee Ingle, Heart Failure Review, 2006.

Acute medical and surgical interventions have resulted in improved ventilatory response in patients with mild and severe CHF. Inhaled iloprost, a prostacyclin analog, is shown to act on pulmonary arterial pressures and improve symptoms in patients. Inhaled iloprost acts selectively on the pulmonary vasculature causing pulmonary vasodilatation. There is a resultant improvement in ventilatory response and exercise capacity (Wensel et al. 2000). Cardiac resynchronisation therapy and nasal continuous pressure ventilation therapy both lead to an improvement in the ventilatory response (Arzt et al. 2005, Abraham et al. 2004). However, the impact of these interventions on mortality is unknown.

Heightened ventilatory response to peripheral reflexes

The autonomous nervous system (ANS) adjusts circulation and respiration to maintain oxygen delivery to the tissue (Schmidt et al. 2005). CHF patients have chronically elevated sympathetic stimulation due to alterations in the autonomic balance (Francis et al. 1990). This imbalance contributes to the pathogenesis of dyspnea and exercise intolerance in CHF. Balance between the sympathetic and parasympathetic arm of the ANS is maintained by complex central and peripheral interacting reflexes. The central baroreflex, peripheral chemoreflex and skeletal muscle ergoreflex form the major reflexes that influence the cardiovascular response. The skeletal muscle ergoreflex is considered the major component of cardiovascular adjustments.

- Ergoreflex is defined as an over activation of nerve endings in the skeletal muscle by metabolic breakdown products leading to sympathetic overactivity (Scott et al. 2000, Smith et al. 2003). These nerve endings consist of group 3 and 4 neural afferents located in the skeletal muscle. They are activated by the skeletal muscle breakdown products that build up during exercise. In CHF the exercising skeletal muscle undergoes glycolytic metabolism rather than the oxidative leading to early acidosis and ATP depletion (Schmidt et al. 2005). Altered metabolic breakdown products including lactate, H^+, prostaglandins and bradykinin are produced in the exercising muscle. Afferent receptors present in the skeletal muscle are sensitive to these metabolic products (Scott et al. 2002). Their activation leads to increased ventilatory, haemodynamic and sympathetic response to exercise, causing exercise intolerance and dyspnea. Ergoreflex response is directly proportionate to objective exercise intolerance and subjective NYHA classification (Ponikowski et al. 2001). Prolonged periods of sympathetic overdrive lead to end organ damage in several

organs including the heart, blood vessels and skeletal muscle. Of significance is the effect of sympathetic overdrive on progressive ventricular dilatation and development of chronic heart failure (Pfeffer et al. 1988).

- Chemoreflex is defined as over-activation of the sympathetic system due to systemic hypoxia and hypercapnia. It is mediated via the central and peripheral arterial chemo-receptors (Schmidt et al. 2005). The systemic chemical stimulus (hypoxia and hypercapnia) exerts a major role on ventilation leading to dyspnea (Grassi et al. 1995, Grassi et al. 1995). Ventilatory rate (Ve), carbon dioxide output (VCO$_2$) and the ratio Ve/VCO$_2$ are used to calculate chemosensitivity. CHF patients have enhanced hypoxic and hypercapnic chemosensitivity at rest and during exercise, contributing to dyspnea. Hypoxic, central and peripheral hypercapnic chemosensitivity are all exaggerated in CHF, however, only central hypercapnic chemosensitivity correlates to NYHA class (Schmidt et al. 2005, Ponikowski et al. 1997).

- The baroreflex, a protective mechanism, is defined as the activation of the parasympathetic response to increased pressure in the carotid sinus. Arterial receptors are present in the carotid sinus. Activation of the baroreflex compensates for the increased sympathetic drive. It augments the vagal output leading to vasodilatation. Baroreflex is significantly impaired even in mild heart failure. (Grassi et al. 1995) A strong inverse relationship exists between baroreflex sensitivity and chemoreceptor drive. Due to the overriding significance of chemo and ergoreflex; baroreflex activation leads to minimal changes in the cardiovascular response (Ponikowski et al. 1997).

In CHF the ergoreflex and chemoreflex are overactive while the baroreflex response is diminished. Baseline autonomic parameters including heart rate, respiratory rate

and blood pressure are elevated. Exercise induces an exaggerated response due to over activation of the sympathetic reflexes. This leads to exercise intolerance and breathlessness.

<u>CHF and fatigue</u>

Fatigue is a major debilitating symptom in patients with CHF. Progression from inability to perform exercise to fatigue during activities of daily living highlights disease progression. Fatigue is predominantly due to a peripheral maladaptive response rather than central in CHF (Sullivan et al. 1989a, Drexler et al. 1992).

Aerobic muscular metabolism is predominant in the skeletal muscle during the initial stages of exercise. Cardiac output measured by oxygen uptake (VO_2) increases linearly with exercise. In healthy individuals the maximum cardiac output, measured as VO_2 max is reached at 85-95% of peak exercise (Clausen 1976). Beyond this stage anaerobic muscular metabolism predominates. This point is known as the anaerobic threshold and indicates a change in muscular metabolism. Unlike healthy individuals, the anaerobic threshold appears a lot earlier during exercise in CHF (Sullivan et al. 1989a). The cessation of aerobic muscular metabolism is early and abrupt in these patients and they are unable to exercise much beyond this point (Sullivan et al. 1989a). Cardiac reserve is not met and is not the limiting factor in the cessation of exercise (Coats 2001). Thus the predominant cause of fatigue in CHF is peripheral and attributed to changes in

- Skeletal muscle
- Vascular function

CHF and skeletal muscle changes

In CHF, skeletal muscle abnormalities are seen in its ultrastructure, oxidative capacity, metabolic function and blood flow. These changes are independent of the age of the patient. They correlate better with the functional status and exercise capacity than their cardiac systolic function (Sullivan et al. 1989a, Drexler et al. 1992, Wilson et al. 1984). This emphasises the hypothesis that exercise capacity and functional status in CHF is predominantly due to changes in the peripheral skeletal muscle and vasculature than in the central cardiac function.

The skeletal muscle is composed of myofibrillar protein type 1 (slow twitch), which is high in mitochondrial content and is dependent on oxidative metabolism, and type 2 (fast twitch) fibres, which is characterised by glycolytic metabolism and easy fatigue. The skeletal muscle in healthy individuals is composed of near quantities of both type 1 and type 2 fibres. Type 1 fibres are required for high endurance repeated exercises while type 2 fibres are required for short bursts of high strength exercises (Sullivan, Green & Cobb 1990, Lipkin et al. 1988). Ultrastructural studies of the skeletal muscle in CHF reveal a significant increase in the percentage of type 2 fibres comprising the skeletal muscle (Drexler et al. 1992, Sullivan, Green & Cobb 1990, Mancini et al. 1989). This change in muscle composition is independent of the aetiology of CHF (Drexler et al. 1992). Consequently the muscle is more susceptible to fatigue partly due to glycolytic metabolism causing acidification of the muscle bed. Lactic acid build up in the muscle leads to acidification of the muscle bed. However, onset or stimulus for conversion from type 1 to type 2 myofibrillar proteins in patients with CHF is unclear. The logistical difficulty in studying muscle ultrastructure in individuals prior to the onset of heart failure has lead to this understandable difficulty. However, changes in neuronal activity and protein

synthesis have been proposed. A change in neuronal stimulus either by repeated mechanical overload, endurance training, cross innervation by motor nerve fibres or electrical stimulation can lead to change in myofibrillar protein composition (Hood 2001, Jarvis et al. 1996). Neural studies have implicated a change in calcineurin signalling inhibiting slow fibre gene expression (Chin et al. 1998, Naya et al. 2000). Recent studies have also highlighted the significance of peroxisome-proliferator-activated receptor-γ co-activator 1 (PGC-1 γ), which is preferentially present in type 1 muscle fibres and serves as a target for calcineurin signalling (Lin et al. 2002). In CHF a lack of PGC-1 γ activity leads to inhibition of neural calcineurin activity and increased expression of type 2 myofibrillar proteins contributing to increased muscular fatigue.

Studies of the ultrastructure of skeletal muscle in CHF by Drexler et al revealed changes in skeletal muscle mitochondrial volume and its cytochrome oxidase activity (Drexler et al. 1992). The mitochondrial volume density was reduced, however, this reduction was greater in patients with severe heart failure (VO$_2$ max<16) than in those with moderate heart failure (VO$_2$ max >16). This reduction in mitochondrial volume indicating a decrease in its oxidative capacity is related to duration and severity of the disease and independent of age and aetiology of the disease. It is only present in the later stages of the disease and potentially reversible with exercise training and appropriate medical management. Cytochrome oxidase activity indicates the oxidative capacity of the muscle and is reduced in both moderate and severe heart failure. This reduction in cytochrome activity is independent of mitochondrial volume density.
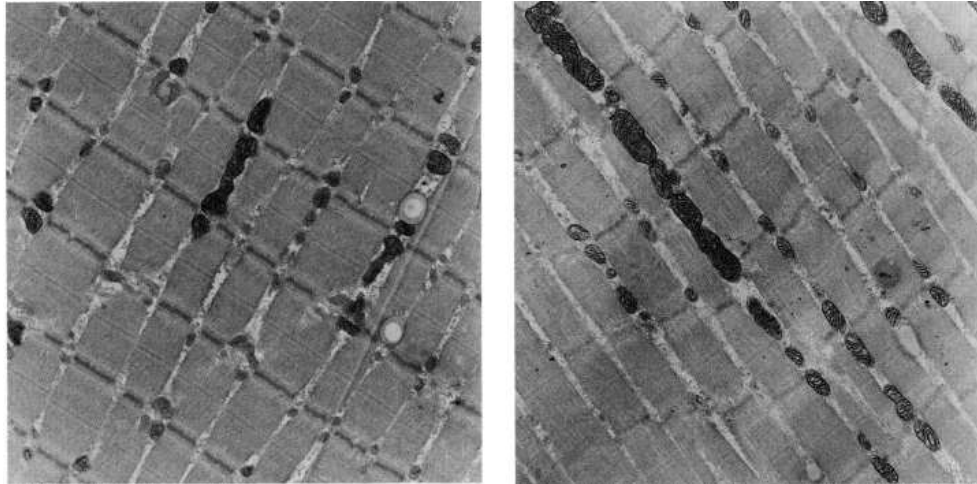
Figure 1.4　Electron micrograph of cytochrome c oxidase in a patient with severe heart failure (left panel) and in a normal subject (right panel)

Mitochondrial activity (black) is severely reduced in a patient with CHF (left) compared to healthy control (right). Taken from Drexler et al, Circulation, 1992.

Both mitochondrial volume density and cytochrome oxidase activity correlate with exercise capacity in CHF. These changes are reversible with exercise training and medical management. Cardiac transplantation did not lead to improvement in skeletal muscle structure and oxidative capacity even 1 year post surgery (Pierce et al. 2007).

Microvascular blood flow within the skeletal muscle is reduced, reflected by a reduction in the amount of blood capillaries per unit volume of muscle tissue. This reduction in capillary flow is linked to the mitochondrial volume and oxidative capacity of the muscle (Drexler et al. 1992). However, this reduction in capillary flow is not isolated to CHF and is also seen in deconditioning and diseases leading to reduced skeletal muscle oxidative capacity (Drexler et al. 1992, Ingjer 1979, Zumstein et al. 1983). It is unclear whether a reduction in blood flow to the muscle is the primary cause or the net result of its reduced oxidative state.

CHF and vascular function

Peripheral blood flow to the skeletal muscles is reduced in CHF (Wilson et al. 1984, Sullivan et al. 1989b). The abnormally low flow to the skeletal muscles is present both at rest and during maximal exercise (LeJemtel et al. 1986, Zelis, Mason & Braunwald 1968). However, studies by Weiner et al (Wiener et al. 1986) and Massie et al (Massie et al. 1987) did not demonstrate a reduction in the forearm blood flow during exercise. This highlights that peripheral blood flow during exercise is dependent on the muscle group involved.

Blood flow to the skeletal muscles is shunted to the non-exercising vital organs in CHF. This compensatory shunting is widened during exercise (Sullivan et al. 1989b). The resultant hypoperfusion causes nutritive deficiency and metabolic changes in the skeletal muscles leading to accelerated anaerobic metabolism (Wiener et al. 1986, Massie et al. 1987). Studies by Wilson et al (Wilson et al. 1984) suggest that the level of exercise tolerance is directly related to the degree of impairment of nutritive flow. Twenty three patients were divided into three groups depending on their maximal oxygen uptake (VO_2 max). A Swan-Ganz catheter was inserted in the pulmonary artery to measure cardiac output and a catheter was inserted in the femoral vein to measure leg flow and limb oxygen extraction. The results of the study indicated that there was a progressive decline in cardiac output and peripheral blood flow in the three groups. Oxygen extraction did not differ between the groups and exercise cessation correlated with critical level of muscle hypoperfusion suggesting muscular fatigue due to impaired nutritive flow.

In summary CHF presents with alterations in the skeletal muscle and its vascular flow both at rest and during exercise. These changes limit exercise tolerance and correlate to severity of the disease.

1.5   Current treatment modalities in cardiovascular disease

Current management of cardiovascular disease includes strategies aimed at both primary and secondary treatment. Behavioural changes including smoking cessation, dietary restriction of fat intake and regular exercise have all reduced the incidence of cardiovascular diseases. Medical management has chiefly focussed on minimizing the harmful effects of the sympathetic drive, platelet function, renin angiotensin system and elevated lipids.

Beta-blockers

Beta-blockers act by inhibiting the activation of beta-adrenoreceptors to prevent the harmful effects of chronic sympathetic overactivity. They have a negative chronotropic and inotropic effect on the heart thus delaying symptoms of angina. They are also indicated in the management of patients with well controlled CHF (Doughty et al. 1997). Earlier concerns regarding it negative chronotropic effect have been proven to be unfounded (Anonymous 1994b). Adequacy of treatment with beta-blockers is dose dependent. The dose is adjusted to achieve a resting heart rate between 50 and 60 beats/min. Management with beta blockers is shown to improve cardiac function and reduce mortality (Packer et al. 1996).

Angiotensin converting enzyme inhibitors (ACE)

ACE inhibitors prevent the conversion of angiotensin 1 to angiotensin 2 thus preventing the degradation of bradykinin. It modulates the renin angiotensin axis altering the balance of vasoconstriction, salt retention and cardiac remodelling

(Anonymous 1987b). The beneficial effect of ACE inhibitors in CHF is well documented; however, its role in stable ischaemic heart disease is unclear. It is shown to improve ventricular function, symptoms and reduce hospital admissions in CHF (Anonymous 1987a). Following a myocardial infarction, ACE inhibitors reduce the incidence of CHF and improve ventricular remodelling (Anonymous 1987a, Anonymous 1992).

Aspirin

The use of aspirin has been advocated for secondary prophylaxis of cardiovascular disease and primary prevention in high risk patients. It acts by preventing the adhesion of platelets to atherosclerotic plaques. It also reduces serum inflammatory markers in cardiovascular disease states and reduces inflammation in the vascular system (McAlister et al. 2006, Massie 2005).

Statins

Statins help reduce serum cholesterol levels above and in addition to diet control and lifestyle modification. Some statins have an additional beneficial effect on the lipid profile, lowering LDL cholesterol levels and raising the HDL cholesterol levels. The beneficial effects of lowering the serum cholesterol levels are seen within 6 months of therapy. It stabilises and causes regression of the atherosclerotic plaque (Jukema et al. 1995). A reduction in serum cholesterol levels translates into improved cardiovascular outcomes (Shepherd et al. 1995, Anonymous 1994a). There is a reduction in the number of ischaemic events and mortality in these patients (van Boven et al. 1996).

1.6   Role of oxygen in CHF

Oxygen, the third most abundant element, was discovered by Joseph Priestley in 1774. At standard temperature and pressure two oxygen atoms are chemically bound to each other to form a colourless and odourless dioxygen molecule, with the formula O_2. Then, in 1908, Pembrey MS used nasal oxygen therapy to successfully treat cardiovascular patients with central sleep apnoea.

Molecular oxygen is imperative for the functioning of the heart and vascular system. The human heart is an obligate aerobe and unlike skeletal muscle cannot produce sufficient energy through anaerobic metabolism (Das et al. 1987). The healthy heart consumes 8-15 ml/ O_2 /min/ 100 gm tissue at rest and can increase to more than 70 ml/ O_2 /min/ 100 gm tissue during vigorous exercise. Myocardial oxygen consumption is directly proportional to ventricular wall tension which in turn depends on the ventricular radius and wall tension (Baller, Bretschneider & Hellige 1981). Oxygen consumption in the thickened or dilated ventricles is higher due to mass effect (Malik et al. 1973). This situation is potentially exaggerated in coronary artery disease. However, the role of oxygen in cardiovascular disease is complex and transcends its significance in myocardial energetics. A comprehensive list of its significance in cardiovascular functioning includes:

- Myocardial energetics

- Myocardial gene expression

- Generation of reactive oxygen species (ROS)

Role of oxygen in myocardial energetics

The heart requires a large amount of energy to function. This energy is utilised to maintain both ionic homeostasis and cardiac contractility. ATP produced in the heart is the major source of energy. Oxygen is imperative for the generation of sufficient ATP in the cardiomyocytes. ATP production meets demand and the synthetic pathways need to respond quickly to meet changes in energy demand. This change in oxygen requirement is thus directly correlated to cardiac workload (Neely, Morgan 1974).

ATP required for cardiac metabolism is synthesised through oxidative metabolism and its synthesis can be categorised into 3 stages:

1) Substrate delivery: Though the heart can utilise a variety of fuels to produce energy, fatty acids are the preferred fuel. They account for 90% of the fuel utilised by the heart even in the presence of glucose. Lipids, carbohydrates and proteins are converted to acetyl-coenzyme A, a common precursor for the entry into the Krebs cycle, the next step in oxidative metabolism.

2) Krebs cycle: Acetyl-coenzyme A is metabolised in the Krebs cycle by β-oxidation to produce NADH and $FADH_2$. These then pass to the next step for oxidative phosphorylation.

3) Oxidative Phosphorylation: The reducing equivalents NADH and $FADH_2$ are oxidised by oxygen via cytochrome oxidase to form water (MITCHELL 1961). Three moles of ATP are produced per mole of NADH and 2 moles of ATP are produced per mole of $FADH_2$.

Patients with coronary artery disease have a restricted flow of nutrients and oxygen to the myocardium, while in CHF there is a chronic unmet demand. Ischemia is the

most common aetiology for the development of CHF. Increased cardiac workload leads to an influx of calcium into the mitochondria activating dehydrogenase and producing NADH, required for oxidative reduction. In an ischaemic state, oxidative phosphorylation slows with a resulting increase in NADH. Its reoxidation by lactate dehydrogenase is limited leading to further ischaemic injury.

Role of oxygen in myocardial gene expression

Cardiomyocytes require an adequate level of cellular oxygen tension for routine cardiac function. Oxygen concentrations are maintained within a narrow range due to the risk of metabolic demise from hypoxia and oxidative damage from hyperoxia (Hearse 1998). Changes in oxygen concentration represent a fundamental stimulus for cellular activation (Ladoux, Frelin 1993). The requirement for cellular oxygen is raised when either oxygen consumption (CHF) or delivery (coronary artery disease) is altered. Haemodynamic variations help balance the acute physiological change; however, alterations in genetic expression are also required. Hypoxia in both acute (coronary artery disease) and chronic hypoxic (CHF) conditions is a potent stimulus for change in genetic expression (Ladoux, Frelin 1993, Melillo et al. 1995). This change is seen immediately following a myocardial infarction and remains persistently elevated in CHF (Lee et al. 2000).

Genetic expression in the cardiomyocytes is altered primarily through gene translation of the hypoxia inducible translation factor (HIF-1). HIF-1 in turn regulates the expression of a variety of genes involved in angiogenesis, cellular apoptosis, erythropoiesis, NO secretion and vascular remodeling (Semenza 2004, Carmeliet et al. 1998). HIF-1 is a heterodimer composed of a 2 HIF subunits. A HIF-1β subunit is constitutively expressed, and a HIF-1 α subunit, the expression and

transcriptional activity of which are precisely regulated by the cellular O_2 concentration (Wang et al. 1995). The HIF-1 α subunit dimerizes with aryl nuclear translocase to bind with specific hypoxia response elements in the regulatory regions leading to transcriptional activation by the p300 coactivator. HIF-1 α subunit availability is the limiting factor in this process (Lee et al. 2000, Giordano 2005).

HIF-1 plays a crucial role in the development of CHF. Immediately following an ischaemic event, the development of cardiac hypertrophy forms an adaptive response to maintain cardiac workload. However, the development of hypertrophy leads to a mismatch in the number of capillaries supplying nutrients to the cardiomyocytes (Tomanek 1990). HIF-1 plays a significant role in cardiac angiogenesis and ventricular remodelling. However, prolonged hypoxia leads to a fall in HIF-1 levels. Recent animal studies by Sano et al highlighted this variable role of HIF-1 in the development of CHF. Cardiac hypertrophy leading to heart failure was induced in mice by severe transverse aorta constriction. There was an acute rise in HIF 1 levels in response to hypertrophy leading to increased levels of VEGF and angiogenesis. However, chronic hypoxia developed after 14 days. This was documented using a hypoxyproble in the cardiomyocytes. Chronic hypoxia lead to a serial fall in HIF-1 levels after 14 days and this coincided with the development of heart failure. Further analysis highlighted the role of tumour suppressor p53 in inhibiting HIF-1 (Sano et al. 2007). These findings were confirmed in human studies by Zolk et al. HIF-1α mRNA expression was low in the failing LV myocardium and autoregulatory mechanisms were impaired leading to a further attenuation of the HIF pathway (Zolk et al. 2008).

Role of oxygen in the formation of Reactive Oxygen Species (ROS)

Oxygen is vital for energy production and cardiovascular function; however, paradoxically the formation of ROS is also deleterious to cellular existence. Over 95% of inhaled oxygen undergoes tetravalent reduction in the mitochondria to form water. It acts as a terminal electron acceptor via the cytochrome oxidase pathway. The complete reduction of oxygen leads to the formation of water, a harmless compound. However, the pathway of electron donation in the mitochondria also leads to an electron leak leading to the formation of ROS (Giordano 2005, Davies 1995, Chance, Sies & Boveris 1979). Under normal conditions oxygen undergoes a tetravalent reduction by cytochrome oxidase at the end of the mitochondrial electron transport chain. The non enzymatic reduction of oxygen leads to several intermediates. Firstly, a one electron reduction of oxygen generates superoxide O_2^-. Addition of a second electron forms the highly reactive hydrogen peroxide (H_2O_2). Addition of the third electron generates the highly reactive hydroxyl radical ($^.OH$). It is estimated that about 2% of the total oxygen consumption in the mitochondria leads to the formation of ROS. In a 60 kg man this is estimated to be 160 to 320 mmol of superoxide per day while in an 80 kg man would produce 215 to 430 mmol of superoxide per day (Davies 1995).

While the role of ROS in the ageing process remains debatable it plays an important role in the various cellular biological processes. They are essential for the oxidative burst reaction by phagocytes in response to bacterial infections. They also act as vital secondary messengers for TGF-β1, endothelin and PDGF (Hensley et al. 2000, Nishida et al. 2000, Griendling, FitzGerald 2003, Machida et al. 2003). In the heart they play a vital role in the development and progression of coronary artery disease and CHF. ROS activity in the endothelium of the coronary artery leads to the

formation of oxidised LDL and the development and progression of atherosclerosis (Witztum, Steinberg 1991). In vivo studies have also highlighted their role in plaque destabilisation. ROS secreted by foam cells react with vascular interstitium leading to matrix degradation and areas of high oxidative stress and plaque rupture (Rajagopalan et al. 1996). Post myocardial infarction, ROS play a significant role in tissue necrosis and reperfusion thus limiting infarct size and influencing ventricular remodelling (Chen et al. 1996, Asimakis, Lick & Patterson 2002, Yoshida et al. 2000). However, animal studies targeting the superoxide dismutase (SOD) gene to limit the production of ROS have yielded mixed results. They suggest that ROS does not play an isolated role in the progression of cardiac failure (Giordano 2005, Asimakis, Lick & Patterson 2002, Yoshida et al. 2000).

Role of inspired oxygen in CHF

Acute inspired oxygen is shown to improve exercise performance and modify ventilatory response to exercise (Bernardi et al. 1998, Clark, Coats 1992, Moore et al. 1992). Studies done at our institution and by others have shown that domiciliary nocturnal home oxygen improves exercise performance and quality of life in CHF (Shigemitsu et al. 2007, Sasayama et al. 2009, Paul, Joseph & De Pasquale 2008). Oxygen treatment is also shown to decrease ventricular arrhythmias in CHF (Suzuki et al. 2006). The pathogenesis of this beneficial effect of oxygen in unclear. Supplemental oxygen increases arterial oxygen saturation (Moore et al. 1992). However, oxygen does not improve the cardiac function or pulmonary artery pressures in CHF (Paul, Joseph & De Pasquale 2008, Krachman et al. 2005). The improved arterial saturation might help reduce the chronic tissue hypoxia seen in CHF and correct the autonomic imbalance.

1.7   Role of folic acid and tetrahydrobiopterin in CHF

Folic acid (C19H19N7O6) a water-soluble B vitamin was discovered in 1931 by Lucy Wills for the treatment of macrocytic anemia (Wills 1991). Humans cannot synthesize folic acid de novo and solely rely on sufficient dietary intake to maintain required levels. Rich sources of dietary folic acid include dark green leafy vegetables, citrus fruits and whole grains. 5-methyl tetrahydrofolate (5-MTHF) the active form of folic acid, is the primary form of folic acid entering into circulation from the intestinal cells. It has recently gained attention for its beneficial effects on endothelial function in different conditions such as ischaemic heart disease, cigarette smoking, diabetes, and hypercholesterolemia (Verhaar et al. 1999, Mangoni et al. 2005, Chambers et al. 2000, Mangoni et al. 2002, van Etten et al. 2002).

Serum folate levels have been inversely related to cardiovascular mortality. In the largest of its kind study done by Morrison et al, patients with no prior cardiovascular events were followed up for 15 years. Cardiovascular mortality was significantly higher in patients with low serum folate levels (<3ng/L) (Morrison et al. 1996). These findings were re-emphasised by Loria et al even amongst patients without diabetes. Cardiovascular mortality was inversely related to folate levels amongst patients without prior vascular disease or diabetes (Loria et al. 2000). Conversely moderate to high levels of serum folate are known to be protective and are associated with a reduction in all-cause and cardiovascular mortality (Voutilainen et al. 2000, Ford, Byers & Giles 1998).

Traditionally, the endothelial effects of folic acid have been ascribed to its homocysteine lowering effects (Mangoni, Jackson 2002). (Fig 5) Folic acid and homocysteine play a major role in the metabolic pathways of the amino acid

methionine. Their plasma concentrations are negatively correlated (Mangoni, Jackson 2002). Homocysteine is a highly-reactive non-proteinogenic amino acid which is an intermediary in sulphur amino acid metabolism. There is extensive evidence that acute and chronic hyperhomocysteinaemia causes endothelial dysfunction (Mangoni, Jackson 2002). The mechanisms responsible are very complex, as they involve every major component of eNOS signalling, synthesis of hydrogen peroxide, generation of superoxide anion radicals, decreased release of NO, increased leukocyte-endothelium interactions by up-regulation of cell adhesion molecules, and alterations of the intracellular redox state, thus eliciting a stress response (Mangoni, Jackson 2002).

However, recent evidence suggests that the effects of folic acid on endothelial function are independent of its homocysteine lowering ability (Mangoni et al. 2005, Doshi et al. 2002, Mangoni et al. 2002). Changes in folic acid and homocysteine concentrations were put into a regression model to determine which of these factors described the enhancement of endothelial function in chronic smokers and diabetic patients (Mangoni et al. 2005, Mangoni et al. 2002). Only changes in folic acid concentration turned out to be a strong and independent predictor of the endothelial effects. Similar results have also been obtained in other studies (Doshi et al. 2002). Therefore, the "endothelial" effects of folic acid seem to be largely independent of the well-known homocysteine lowering effect. In other words, folic acid may exert "direct" effects on endothelial function.

BH4 is an essential cofactor for all three isoforms of Nitric oxide synthase (Klatt et al. 1994, Stuehr 1997). In its fully reduced form it combines with eNOS to produce NO resulting in vasodilatation (Moens, Kass 2006). A close relationship exists

between the availability of BH4 and NO synthesis. BH4 deficiency influences vascular damage in patients with diabetes, hypercholesterolemia, hypertension and smokers (Alp, Channon 2004). BH4 deficiency leads to structural changes in eNOS termed uncoupling of NOS. Uncoupling of NOS leads to superoxide production causing vascular damage. Recently tetrahydrobiopterin deficiency has also been shown to play a major role in cardiac ischaemic/reperfusion injury, hypertrophy and remodeling (Moens, Kass 2006).

Recent studies have focused on changes in myocardial blood flow during acute intra-coronary administration of tetrahydrobiopterin. Intra-coronary infusion of BH4 in high-risk patients (early coronary artery disease) significantly improved coronary blood flow (Setoguchi et al. 2001). In patients with late coronary artery disease tetrahydrobiopterin prevented acetylcholine induced vasoconstriction of angiographically normal coronary arteries and improved coronary flow volumes (Maier et al. 2000).
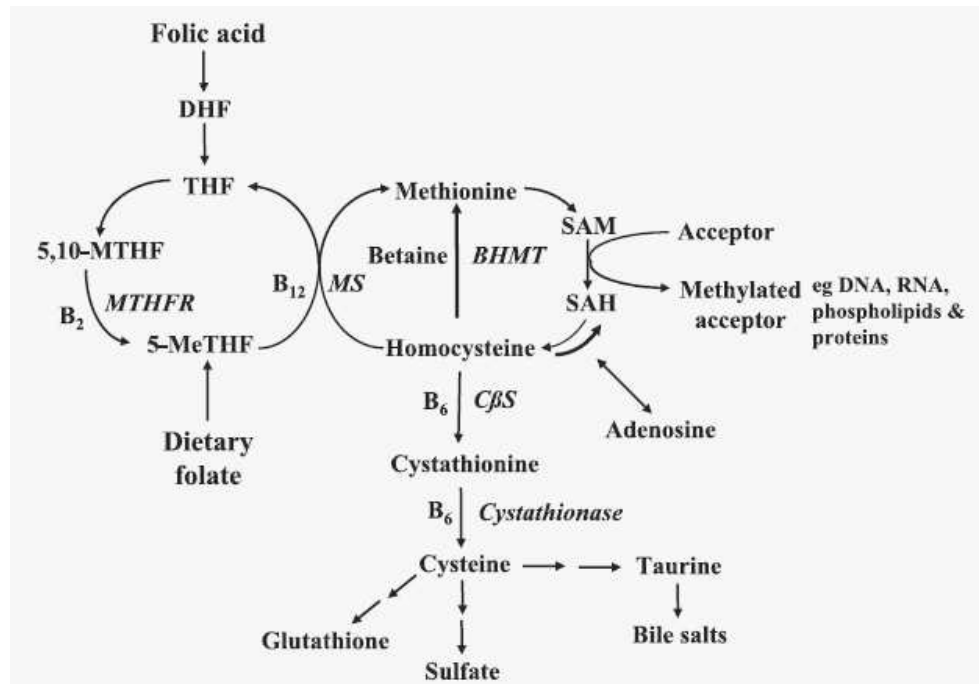
Figure 1.5   Metabolism of folate and homocysteine

SAM, Sadenosylmethionine; SAH, S-adenosylhomocysteine; CβS, cystathionine β-synthase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; BHMT, betaine-homocysteine methyl transferase; DHF, dihydrofolate; THF, tetrahydrofolate; 5,10-MTHF, 5,10-methylenetetrahydrofolate; 5-MeTHF, 5-methyltetrahydrofolate; B2, vitamin B2; B6, vitamin B6; B12, vitamin B12.Taken from Moens et al, Am J Physiol Heart Circ Physiol, 2008.

In vitro studies using computational modelling have demonstrated that 5-methyltetrahydrofolate (5-MTHF), the active metabolite of folic acid, binds the active site of eNOS thus enhancing NO production and endothelial function (Hyndman et al. 2002). Therefore, acute administration of folic acid could significantly improve endothelial function and reduce arterial stiffness in CHF patients, potentially providing significant cardiovascular protection and improving outcome.

The exact mechanism of action of folic acid in CHF remains unclear; however potential mechanisms of action have been postulated.

- Elevated plasma homocysteine induces endothelial dysfunction and has been linked to coronary artery disease. Folic acid reduces plasma homocysteine levels and restores endothelial function. However; the beneficial effects of folic acid are independent of its plasma homocysteine lowering ability (Tawakol et al. 2005).

- Structural changes in eNOS induced by tetrahydrobiopterin deficiency leads to the production of superoxide that damage the endothelium. 5-MTHF in high doses scavenges superoxides and reduces vascular damage. The scavenging property of 5 MTHF is about 20 fold lower than that of vitamin C and hence may not completely explain its beneficial effects.

- Folic acid is an essential in the production of NO by NOS. It enhances the binding of BH4 to NOS and enhances regeneration of BH4 from inactive BH2 (Kaufman 1991). Folic acid did not enhance nitric oxide production in the absence of tetrahydrobiopterin (Stroes et al. 2000). However studies done in tetrahydrobiopterin-deficient fructose fed rats' show that folic acid and tetrahydrobiopterin bind to the same active site on eNOS and improve vasodilatation.

- Folic acid supplementation in rats decreased the expression of endothelial adhesion molecules VCAM-1. Though studies in humans are lacking; folic acid might exert an anti-thrombotic effect on the vascular endothelium (Li et al. 2006).
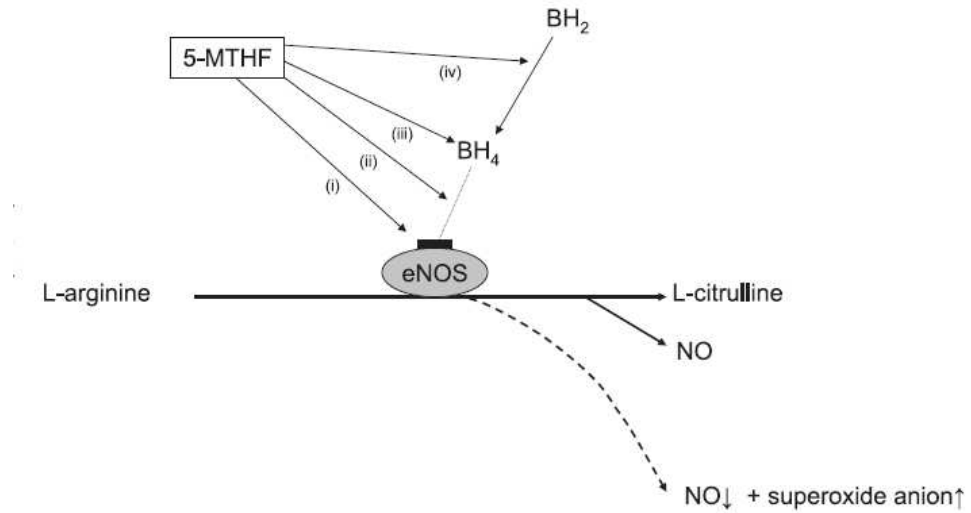
Figure 1.6   Interaction of folic acid and tetrahydrobiopterin with eNOS

5-Methyltetrahydrofolate (5-MTHF) is capable of directly interacting with eNOS (i) Folic acid also restores the bioavailability of BH4 by ameliorating the binding affinity of BH4 to eNOS, (ii) by chemically stabilizing BH4, (iii) and by enhancing the regeneration of BH4 from the inactive form BH2. (iv) Oxidative stress-induced BH4 depletion leads to an imbalance between NO production and the generation of free radicals. Taken from Moens et al, Am J Physiol Heart Circ Physiol, 2008.
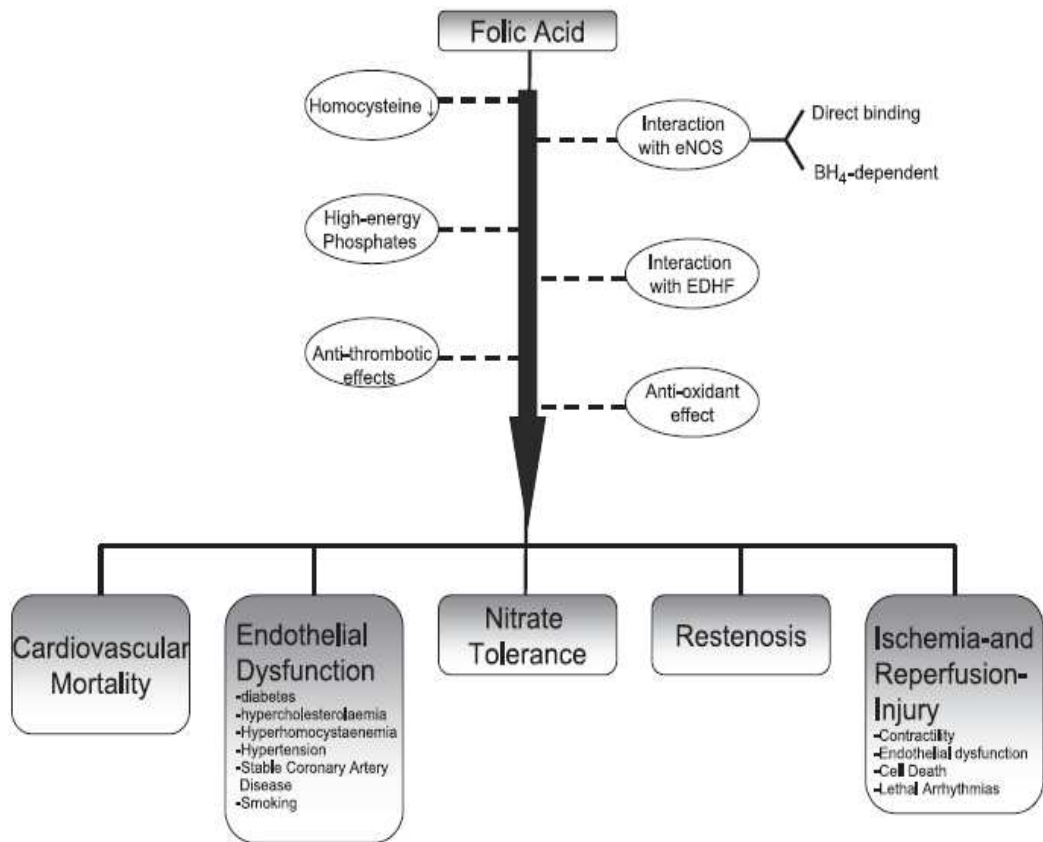
Figure 1.7   Role of folic acid in cardiovascular disease

eNOS, endothelial nitric oxide (NO) synthase; EDHF, endothelium-derived hyperpolarizing factor; BH4, tetrahydrobiopterin. Taken from Moens et al, Am J Physiol Heart Circ Physiol, 2008.

1.8   Objectives of the thesis

The aim of the thesis is to assess the effects of multiple interventions on the automnomic and endothelial function in patients with well established cardiovascular disease. Three interventions namely, 5MTHF, the active form of folic acid, tetrahydrobiopterin and oxygen were selected based on their suitability, low cost, non toxic potential and current evidence.

The effects of folic acid, tetrahydrobiopterin and oxygen were studied in patients with coronary artery disease and heart failure. Folic acid and tetrahydrobiopterin have a beneficial effect on endothelial function while the regional sympathetic nervous activity lowering effects of oxygen may improve regional vascular blood flow. Our previous experiences with these agents in patients with CHF lead to the selection of these agents for the thesis. Our experiements analysed the effects of oxygen on autonomic function and its concurrent effects on peripheral resistance and blood flow while the combined effects of folic acid and tetrahydrobiopterin on the endothelium were studied.

The first study analysed the influence of supplemental oxygen on peripheral autonomic reflexes during sub maximal exercise in patients with established CHF. Autonomic function was assessed by analysing the cardiopulmonary exercise reflex during sub maximal exercise. Patients were divided into two groups; those with impaired and preserved ejection fraction. The parameters analysed included

- Peripheral skeletal muscle receptor reflex; the ergoreflex.
- Vascular blood flow in the non exercising limb.

These parameters were recorded both on air and oxygen in a double blinded fashion, one week apart. This is the first study to have analysed the effect of supplemental oxygen on autonomic function during sub maximal exercise in patients with established CHF.

The second study analysed the influence of supplemental oxygen on central autonomic parameters during maximal exercise testing. The ventilatory and haemodynamic response to maximal exercise was analysed. This study has added to existing knowledge by demonstrating the influence of inspired oxygen on ventilatory

efficiency and haemodynamic response to maximal exercise. Parameters analysed included:

- Ventilatory parameters at rest and during incremental exercise.

- Non-invasive haemodynamic parameters.

Ventilatory parameters recorded included Ve, VCO_2 and VO. Data from these parameters was used to derive ventilatory efficiency and incremental ventilatory drive. Haemodynamic parameters including heart rate and blood pressure were recorded non-invasively using a task force monitor.

The third study analysed the effects of 5 MTHF on endothelial function in patients with end stage CHF. This is the first study to analyse the effects of folic acid on endothelial function in patients with established CHF. Direct assessment of endothelial function was performed using Pulse Wave Analysis. Nitric oxide inhibitors, namely serum asymmetrical dimethylarginine (ADMA) and symmetrical dimethylarginine (SDMA) were also measured. Studies were conducted in a randomised double blinded fashion with a control group. CHF patients had an established diagnosis based on Framingham criteria and a recent (<6 month) echocardiogram.

The fourth study was a technical analysis of the short term reproducibility of the pulse wave analysis technique during pharmacological interventions. Pulse wave analysis is a well established technique used to measure endothelial function in both healthy and diseased conditions. However, existing literature only supports the reproducibility of this technique for baseline studies. The reproducibility of this technique during acute pharmacological interventions is unknown. Our study is the

first to provides an indepth analysis of the reproducibility this technique for both baseline and post pharmacological interventions.

The fifth study was a pilot study conducted to analyse the effects of the combined administration of folic acid and BH4 on endothelial function in patients with well established CAD. Endothelial function in the large (aorta) and small (brachial) artery was studied using cardiac magnetic resonance imaging (CMR). Baseline studies were performed to analyse aortic distensibility and brachial artery FMD. These patients were then randomised in a double blinded fashion to receive folic acid and BH4 versus a placebo for 14 days. Repeat studies were conducted 2 weeks later to study the effect of supplementation on these parameters.

In conclusion the thesis was aimed to analyse the effects of various interventions on established abnormal parameters in patients with well established cardiovascular disease. It has analysed the effect of folic acid, tetrahydrobiopterin and oxygen on endothelial function and central and peripheral autonomic reflexes in these patients.

CHAPTER 2

2.1   Introduction

This thesis aims to build on the current understanding of cardiovascular disease. The primary focus of the thesis is:

- endothelial function in CHF and CAD

- neuroendocrine reflexes in CHF

In keeping with the aims of the thesis this section on methodology is divided to explain the procedures used to study these parameters. The techniques used include:

- neuroendocrine reflexes

    1) cardiopulmonary exercise testing

    2) ergoreflex assessment

- endothelial function

    1) pulse wave analysis

    2) flow mediated dilatation (MRI brachial artery technique)

    3) venous plethysmography

2.2   Cardiopulmonary exercise testing

Cardiopulmonary exercise testing (CPET) is considered the gold standard for cardiovascular assessment involving an integrated exercise response from the pulmonary, cardiovascular and skeletal muscle systems. Cardiopulmonary capacity at rest cannot reliably predict a patients` functional capacity and exercise tolerance (Palange et al. 2007). Thus CPET is increasingly being used in clinical practise to identify a patients` level of intolerance to exercise. Exercise intolerance is defined as

the inability to successfully complete a physical task that a healthy individual would find tolerable. It is also used to estimate severity of chronic heart failure by assessment of autonomic responses in CHF. CPET involves measurements of respiratory oxygen uptake (VO_2), carbon dioxide production (VCO_2) and ventilatory parameters. These parameters are used to measure peak exercise capacity, defined by the maximum ability of the cardiovascular system to deliver oxygen to the exercising muscle and of the skeletal muscle to extract oxygen from the blood (Albouaini et al. 2007). The test also provides important information regarding the pathophysiology of exercise limitation and risk stratification.

The physiology of CPET

Exercise capacity is determined by three interconnected factors: cardiac output, pulmonary gas exchange and skeletal muscle metabolism. Cardiac output increases linearly with exercise. This increase is achieved at the initial stages of exercise by an increase in stroke volume and heart rate while at the later stage is predominantly by an increase in heart rate (Andersen 1968, Saltin 1969). At peak exercise levels 90% of maximal cardiac output is utilised by the exercising skeletal muscle. Variations in skeletal muscle metabolism including a shift from aerobic to anaerobic metabolism lead to an increase in oxygen extraction. It can increase fourfold during exercise, from 5ml/dl to 20 ml/dl during peak exercise (Ingle 2008).

The primary function of the cardiovascular system is to supply oxygen and remove carbon dioxide from the exercising muscle. These gases are exchanged at the alveolar-capillary membrane in the lungs. Hence, the expired oxygen and carbon dioxide are equivalent to the cellular consumption and production of these gases. CPET accurately measures the inspired and expired gases during exercise. Oxygen

consumption is proportional to calorie expenditure at the cellular level and both increase in a linear fashion with exercise. Measurement of expired gases thus provides an accurate assessment of cellular function. Total oxygen consumption approximates calorie expenditure and provides the best possible assessment of cardiovascular reserve. CPET remains the sole modality of determining exercise tolerance (Ingle 2008, Wasserman, Hansen & Sue 2005a).
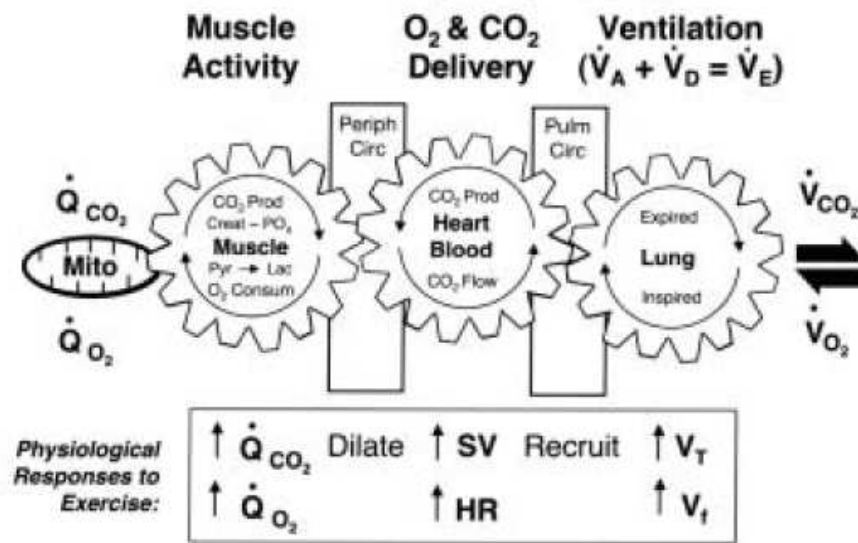
Figure 2.9   Gas transport mechanism during exercise

Circ = circulation; CO_2 = carbon dioxide; Consum = consumption; Creat = creatine; Lac =lactate; HR = heart rate; Mito = mitochondria; PO4 = phosphate; O_2= oxygen; Periph = peripheral; Prod =production; Pulm = pulmonary; Pyr = pyruvate; $Q \cdot CO_2$ = carbon dioxide production; $Q \cdot O_2$ = oxygen utilization; SV = stroke volume; $V \cdot A$ = minute alveolar ventilation; $V \cdot D$ = minute dead space ventilation; $V \cdot E$ =minute ventilation; V_f = breathing frequency; V_T = tidal volume; $V \cdot CO_2$ = carbon dioxide output; $V \cdot O_2$ = oxygen uptake.Adapted from principles of exercise testing and Interpretation 3rd edition.

The coupling between cardiac output and tissue extraction is explained by the Fick

equation (Albouaini et al. 2007). At rest it is defined as stroke volume times the

arterial minus venous oxygen content.

$$VO_2 = (SV \times HR) \times (CaO_2 - CvO_2)$$

Where VO_2 is the oxygen uptake, SV: stroke volume, HR: heart rate, CaO_2: arterial

oxygen content and CvO_2: venous oxygen content.

At peak exercise each variable of the equation can be modified to reflect changes at

peak exercise level.

Thus

$$VO_2max = (SV \ max \times HR \ max) \times (CaO_2 \ max - CvO_2 \ max)$$

VO_2 max serves as the gold standard for the measurement of peak cardiovascular function. In healthy individuals VO_2 increases in a linear fashion with exercise until a plateau is reached near maximal exercise capacity. This plateau phase represents peak VO_2 max. Exercise intolerance is identified as an abnormally low VO_2 max. Any one of the four variables in the Fick equation can influence exercise tolerance. In CHF a reduction in stroke volume response along with low arterial minus venous oxygen content is primarily responsible for exercise intolerance (Albouaini et al. 2007, Milani et al. 2006).

<u>CPET protocols</u>

CPET involves the measurement of respiratory gases during an incremental exercise protocol. The test is conducted in a temperature controlled environment. The instruments and computer algorithms used for the study and gas mixtures require calibration prior to each study. Repeat studies on the same individual should be conducted at the same time of the day of avoid diurnal variations in results. Vigorous exercises should be avoided for 24 hours prior to the study. Patients are advised to refrain from caffeine products for 4 hours prior. The tests should be conducted in the presence of a medical officer available to supervise the test protocol. It is a safe procedure in patients with dyspnoea with between 2 and 5 deaths per 100,000 tests performed (Gibbons et al. 2002). Patients with unstable angina, uncontrolled heart failure and aortic stenosis are unsuitable for the study.

The exercise protocol can involve either treadmill walking or bicycle ergometry. For most patients treadmill walking is more familiar and utilises a larger group of

antigravity muscles. Consequently VO$_2$ max measured on a treadmill is 5-10% higher than on a bicycle (Gibbons et al. 2002). The bicycle exercise protocols are easier to record with less signal interference due to a stationary position. This is especially useful in patients with CHF who require frequent ECG and BP measurements. At similar exercise workloads dyspnoea is the limiting factor for treadmill walking while fatigue limits bicycle ergometry.

Various exercise protocols are used to test functional capacity during CPET (Albouaini et al. 2007). The recommended total duration of exercise is 8 – 17 minutes to achieve between 5 to 8 stages of exercise. The increments in workload used for each stage are arbitrary (Buchfuhrer et al. 1983). On an electronically braked bicycle the baseline workload is adjusted at 20-25 W and increased by 10 W/min. The modified Bruce protocol was initially used during treadmill walking exercise protocols. It required an initial belt speed of 2.7 km/hr on the treadmill and unequal increments every three minutes rendering it difficult for CHF and elderly patients. The modified Naughton protocol was thus recommended for patients with CHF using the treadmill (Naughton, Sevelius & Balke 1963). This protocol involved modest increments every 2 minutes to accommodate a predictable increase in workload.

Respiratory gases and ventilatory parameters are measured during each breath and analysed at 15 or 30 second intervals. Air/oxygen is inspired through a tight fitting mask attached via a non-rebreathing valve to prevent the mixing of inspired and expired gases. Inspiratory and expiratory gases are analysed to measure oxygen and carbon dioxide levels while the flows are measured to estimate volumes. The measured gases are plotted on a computerised metabolic chart to follow trends. Flow

volumes are derived by multiplying flow rates by respiratory rate. These are also plotted on the metabolic chart to obtain trend patterns.

Figure 2.10   CPET using a bicycle ergometry
Cardiopulmonary exercise testing using the cycle ergometer. Taken from Albouaini et al, Heart 2007.

Parameters measured during CPET

CPET is the pivotal modality for assessment of patients with CHF. The study helps evaluate patients with dyspnoea and understand its pathophysiology. In CHF, left ventricular ejection fraction does not correlate well with symptoms or prognosis. The VO_2 max serves as the best prognostic marker and is a reliable guide for heart transplant.

VO_2 max

VO_2 max is defined as the VO_2 achieved near peak exercise. In healthy individuals it is the plateau phase achieved near peak exercise levels. Cardiac output cannot increase beyond this level. It is a reliable and reproducible measure of

cardiopulmonary function. VO_2 max measurements depend on the weight of the person and hence are required to be standardised to the body mass index (BMI) for comparison. Men record a 15% higher VO_2 max as compared to women. This is because of higher muscle mass, haemoglobin concentration and stroke volume. VO_2 max levels declines with increasing age. The rate of decline is 10% per decade in sedentary individuals and 5% in the healthy individuals. VO_2 max is reduced if it is lower than 85% of predicted for age, sex and BMI.
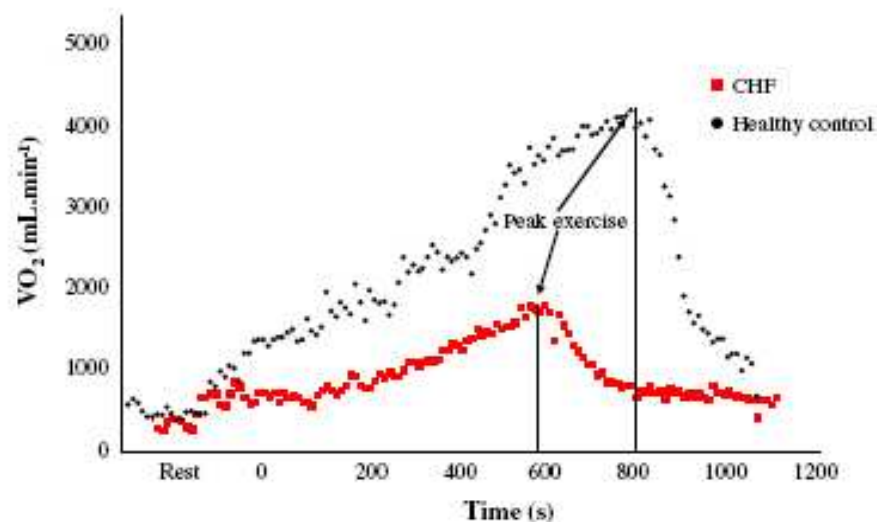
Figure 2.11 VO_2 uptake in CHF and healthy individuals

Oxygen uptake response to incremental exercise in a CHF patient compared to a healthy control. Adapted from Lee Ingle, Heart Failure Review 2007.

<u>Ventilatory efficiency (VE/VCO$_2$)</u>

Carbon dioxide is produced during cellular respiration. It is transported by the circulatory system and exchanged at the alveolar-capillary membrane. The ratio of ventilation to carbon dioxide provides a measure of ventilatory efficiency in clearing products of cellular respiration. Ventilatory efficiency depends on various parameters including the alveolar-capillary membrane, acid-base balance and chemoreceptor sensitivity (Johnson 2000, Milani et al. 1996). During mild exercise there is an initial improvement in ventilatory efficiency due to improved perfusion and ventilation perfusion match. With moderate exercise, there is a linear increase in ventilation to match the increased CO_2 production. Ventilatory efficiency thus remains constant. With severe exercise, there is a build up in metabolic acidosis leading to a steeper rise in ventilation. The resulting increase in VE/VCO$_2$ reduces ventilatory efficiency (Milani et al. 2006). It is typically reduced in patients with CHF and chronic pulmonary diseases (Milani et al. 2006).

In addition to VO$_2$ max, ventilatory efficiency (VE/VCO$_2$) serves as a powerful prognostic marker in CHF. Patients with CHF are characterised by an increased ventilatory response during exercise and VE/VCO$_2$ is inversely related to VO$_2$ max (Chua et al. 1997a, MacGowan et al. 2000).
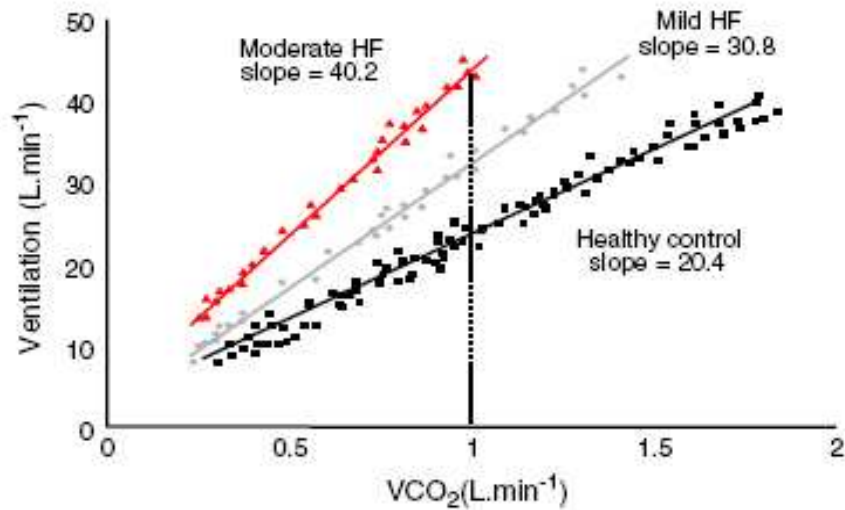
Figure 2.12   Ventilatory efficiency as a marker of severity of disease

Ventilatory efficiency slope in patients with mild and moderate CHF compared to healthy controls. Adapted from Heart 2005.

Respiratory exchange ratio (RER)

Respiratory exchange ratio is defined as the ratio of carbon dioxide output to oxygen uptake (VCO_2/VO_2). RER is a guide to metabolic events at the cellular level. It is an indicator of the type of fuel used for cellular metabolism. An RER < 1 is indicates aerobic metabolism. Carbohydrates are the chief substrate of metabolism at this stage. An RER > 1 indicates anaerobic metabolism and fats are predominantly metabolised by the cell. An RER > 1 is used during CPET to indicate an adequate level of exercise (American Thoracic Society, American College of Chest Physicians 2003).

Conclusion

CPET serves as a useful and non-invasive tool for the evaluation of patients with CHF. It aids in the understanding of the pathophysiology of dyspnoea in patients presenting with undifferentiated symptoms. The crucial role of CPET is to serve as a prognostic marker in CHF. Parameters measured including VO_2 max and VE/VCO_2

is powerful prognostic tools for disease progression. However, the lack of standardisation of the study protocol is a major drawback for CPET to be used as a diagnostic tool for CHF.

2.3   Autonomic function testing in CHF

Ergoreflex

Exercise limitation is a major disability for patients with CHF. The exaggerated ventilatory response to exercise correlates to exercise limitation and severity of disease (Buller, Poole-Wilson 1990a). Additionally, patients also have various abnormalities of the skeletal muscle that limit exercise tolerance (Massie et al. 1987). Afferent nerve fibres arising from the skeletal muscle modulate the haemodynamic and ventilatory response to optimize exercise performance (Piepoli, Clark & Coats 1995). These receptors are overactive in CHF contributing to an exaggerated ventilatory and haemodynamic response to exercise in CHF (Piepoli et al. 1996).The ergoreflex is defined as the elevated neuroendocrine reflex due to over activation of receptors in the skeletal muscle seen in patients with CHF. It explains the vital link between the peripheral skeletal muscle changes and elevated central cardiopulmonary parameters.

Methodology

The subjects refrain from strenuous physical activity for at least 24 hours before the study. All exercise tests are performed at the same time of day with the patient supine and arms extended. First, a maximal voluntary handgrip contraction is measured as the greatest of the peak forces produced by three brief maximal handgrip contractions.

The evaluation of the ergoreflex activity includes two exercises, performed in random order: (1) a control handgrip exercise using repetitive finger flexion by pulling a lever or inflated bulb of a dynamometer at 50% of the predetermined maximal contraction (this is done with the non dominant arm at the rate of 40 squeezes per minute until exhaustion); (2) the same protocol followed by, from 10 seconds before the end of exercise, 3 minutes of circulatory (venous and arterial) occlusion by forearm tourniquet inflation to +30 mm Hg above systolic pressure (PH-RCO). After the cuff is inflated, the subject is instructed to relax. Thus, the contribution of the muscle ergoreceptors is evaluated by trapping of the metabolites in the exercising muscle after exercise. Thirty minutes separate each bout of handgrip exercise. (Piepoli et al. 1996, Piepoli, Clark & Coats 1995).
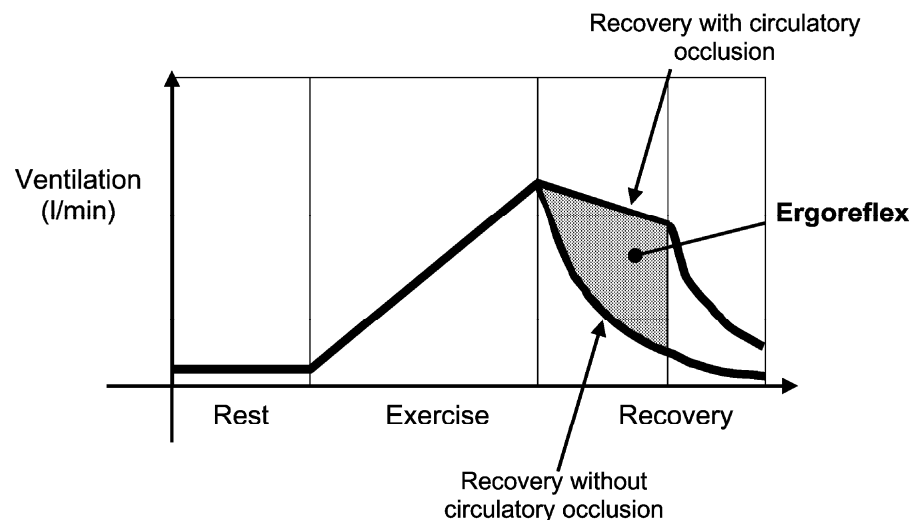
Figure 2.13   Determination of ergoreflex by hand grip exercise

A schematic representation of the measurement of ergoreflex; the grey area represents the ventilator component of ergorelfex, calculated as the difference in ventilation between the recovery with PHRCO and control recovery without. Taken from Scot et al, The European Journal of Heart Failure 2003.

The following parameters are measured to assess the effect of ergoreflex

- Arterial blood flow in the non exercising limb

- Haemodynamic parameters

- Ventilation

Arterial blood flow in the non exercising limb

Blood flow in the right leg is measured to assess for changes in blood flow to the skeletal muscle during exercise. This is done with a mercury-in-polymeric silicone (Silastic) strain-gauge plethysmograph with the venous-occlusion technique. The strain gauge is placed ~5 cm below the popliteal crease. Blood flow (ml/min/100 ml) is calculated from the rate of increase in volume, whereas venous return from the leg is prevented by a cuff inflated on the thigh. The pressure in the venous occlusion or congesting cuff on the thigh is 40 mm Hg. Circulation to the feet is arrested by a cuff inflated around the ankle. The ankle cuff is inflated before the determination of leg blood flow and kept inflated throughout the measurements. Leg vascular resistance is calculated by dividing the mean arterial pressure by the leg blood flow. An average of four flow measurements made at 15-s intervals is used for analysis (Greenfield, Whitney & Mowbray 1963).

Haemodynamic measurements

The task force monitor was used for non-invasive beat to beat haemodynamic measurements. Heart rate, systolic, diastolic and mean blood pressure, stroke volume, cardiac output and total peripheral resistance were measured non-invasively. The system consists of a patient biosignal electronic system (PBES) which is a self calibrating, non-invasive instrument for ECG, impedance cardiography (ICG) and continuous blood pressure.

Continuous blood pressure if recorded from the small digital arteries using the patented Finapress device. This measures the beat to beat change of continuous blood pressure change at the finger. The measurements are corrected to the ascillometric blood pressure values obtained from the contralateral upper arm. The continuous blood pressure recording is validated technique used for continuous blood pressure recording (Fortin et al. 2006).

Stroke volume and cardiac output was calculated using impedence cardiography technique. ICG is a safe and non-invasive tool that measures cardiac output. It detects and measures changes in thoracic electrical bioimpedence over time in relation to the cardiac cycle. A low voltage, high amplitude, alternating current is introduced through the outermost sensors of paired electrodes placed on the roof of the neck and the base of the thorax. The conducted voltage is sensed through the immermost sensor pads and the drop in voltage (difference between what is introduced and what is sensed) is used to determine resistance (impedance) to the current (Fortin et al. 2006).

Stroke volume was calculated from the measured left ventricular ejection time and base impedance using the calculation of Sramek et al.

$SV = Vth \times LVET \times (dZ/dT) \max / Zo$

Zo is the base impedance and V_{th} the electrically participating thoracic volume calculated as

$V_{th} = (0.17 \, H)^3 / 4.2$

where H is the body height.

Cardiac output is calculated as the product of SV and heart rate (HR)

The data acquisition (DAQ) system is a personal computer using Lab Windows/CVI. This software controls the calibration routines, detecting the beat-to-beat haemodynamic parameters, computing the sliding power spectra and calculating the baroreceptor reflex sensitivity. The data are visualized in real-time on the screen and finally an automatic report of the investigation is printed.

<u>Ventilatory data</u>

Subjects breathe air through a mouthpiece with a nose clip in place, and continuous on-line ventilation and expiratory gas data are collected. The analysis is performed throughout the different stages of the study for a given subject. It evaluates ventilation ($\dot{V}$, inspiratory flow) and expiratory CO_2 and O_2 concentrations to derive O_2 consumption ($\dot{V}O_2$) and CO_2 production ($\dot{V}CO_2$) from standard formulas. The gas meters are calibrated against gases of known concentrations before each test. The $\dot{V}$, $\dot{V}CO_2$, and $\dot{V}O_2$ is calculated on line at every breath by a fuel cell JAGER (Oxycon, USA)

The JAGER (Oxycon) hardware consists of a compact fast response differentil paramagnetic oxygen analyser and an infrared carbon dioxide analyser. The device is connected to the mouth via a tight fitting mask. Volume measurements are performed by a lightweight bi-directional digital volume transducer placed at the mouth. This device is resistant to water vapour and breathing gases. The dead space is calculated at 30 mls. The device is calibrated before each study. Breath by breath analysis is calculated for respiratory parameters using the computer interpretation software IntelliSupport (JAGER, USA).

The device is used for routine gas analysis in CPET, sleep diagnostic and therapeutic studies and exercise assessments (Sinha et al. 2004). It is a valid and reliable technique for gas analysis (Huynh et al. 2006, Attinger et al. 2006).

2.4 Assessment of endothelial function

The endothelium, a single layer of cells lining all the vessels of the body, is the main regulator of vascular tone and vessel wall homeostasis. Vascular tone is maintained by the release of various vasodilator and vasoconstrictor agents from the endothelial cells. These act upon the underlying smooth muscle to maintain vascular tone (Vane, Anggard & Botting 1990). A formal assessment of endothelial function incorporates measurement of vascular wall tone (reactivity) and plasma levels of its secretions. The recent incorporation of both these measurements has helped us understand the mechanisms and potential sites of endothelial dysfunction (Deanfield et al. 2005). Tesferamariam and colleagues identified endothelium derived vasoconstrictors, TXA2 and PGH2 in diabetics with endothelial dysfunction (Tesfamariam, Brown & Cohen 1991, Tesfamariam et al. 1990). These secretions lead to vasoconstriction and cellular apoptosis (Jariyawat et al. 1997).

Pulse wave velocity is a non-invasive method of measuring vascular resistance. Both arterial stiffness and endothelial dysfunction coexist in patients at increased risk of cardiovascular disease leading to the hypothesis that increased vascular resistance may be exerted by endothelial dysfunction. (Oliver, Webb 2003). The ratio of endothelium dependent to endothelium independent vasodilatation is measured using these techniques. Various agents are used to assess this differential effect on the vessel. These agents act either on the endothelial cells or on the smooth muscle cells leading to vasodilatation. Agents such as salbutamol and acetylcholine act on the

endothelial cells leading to vasodilatation. These agents act on the endothelium leading to secretion of nitric oxide, the key endothelium derived relaxing factor (Furchgott, Zawadzki 1980). Endothelium dependent vasodilation is impaired in patients with atherosclerosis (Verma, Anderson 2002). This is compared to the endothelium independent vascular response to glyceryl trinitrate, which acts directly on the vascular smooth muscles and leads to maximum vasodilatation. The effects of these agents on vascular tone is measured by

1) change in arterial pulse waveform (Pulse Wave Analysis)
2) change in diameter of the vessel (Flow-mediated dilatation)
3) change in blood flow (Venous plethysmography)

Haematological assessment of endothelial function includes the assessment of its secretions. Under pathological conditions the endothelium secretes prothrombotic mediators that rapidly convert it from a non-thrombogenic surface to a procoagulant one. Hypercholesterolemia and disruption of endothelial surface are known to precipitate this change (Flavahan 1992). Additionally, the endothelium also forms the target surface of these prothrombotic mediators and hence a haematological assessment of these agents forms a useful tool in the assessment of endothelial function. Both pro-thrombotic agents (tissue plasminogen activator (t-PA) and PAI-1) and pro-inflammatory agents (C-reactive protein, interleukin-8 and monocyte chemoattractantprotein-1), down regulate NO release (Padro et al. 1997, Thiruvikraman et al. 1996). These agents serve as important markers of endothelial dysfunction and levels measured predict disease progression (Barac, Campia & Panza 2007).

Pulse wave analysis

Introduction

Non invasive recording of the pulse wave form was first performed by Marey in 1860 in Paris using a sphygmograph (Lawrence 1978). The sphygmograph was taped to the wrist of the patient. It had one lever which rested on the radial artery to sense the pulse waveform while the other end scratched a trace on to a smoked paper. The science of pulse wave analysis was further developed by Mackenzie who described waveform changes in his general practise patients and Mahomed who studied vascular changes in hypertensive patients.
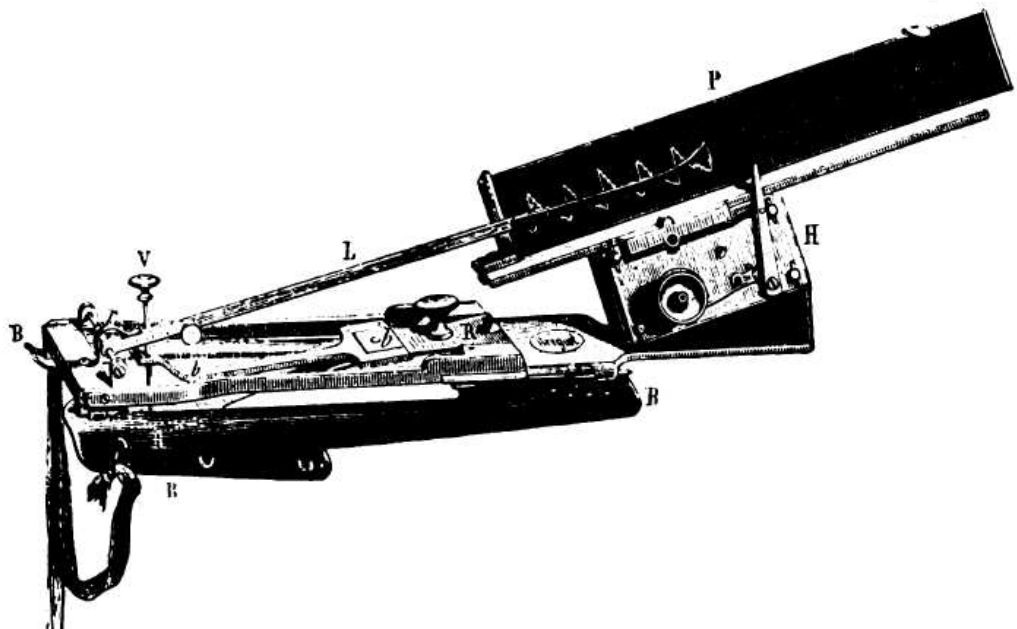
Figure 2.1 Marey's original sphygmograph

Marey's original illustration of the sphygmograph. The sphygmograph was taped to the wrist by a belt (B), while tracings were recorded on a smoked paper (P). Adapted from Paris: E Thunot et Cie, 1860.

The Principle

Pulse wave analysis is the study of arterial stiffness by analysis of the blood pressure waveform. During systole the left ventricle ejects blood into the aorta, generating the central aortic pulse waveform. An arterial wave form is comprised of its pressure amplitude and waveform pattern. Both these components are progressively and predictably altered as it travels down the arterial tree towards the periphery.

The pulse pressure amplitude increases progressively in the smaller vessels (Kelly et al. 1989a). This increase is more pronounced in young adults with pliable arteries. With increasing age the initial central aortic waveform amplitude is higher than in younger individuals (Fuster et al. 2008, Nichols, O'rourke & Kenney 1991). However, the change in amplitude as the wave passes distally is less pronounced (Mills et al. 1970). The pulse pressure is also dependent on heart rate. At rest the pressure in the brachial artery is 20%-50% higher than in the aorta; however at peak heart rate it is three times the central aortic pressure (Mills et al. 1970, Kelly et al. 1989b). An understanding of the pulse waveform and amplitude is central to the pulse wave analysis.

The pulse waveform and amplitude is recorded at a peripheral artery, either the radial or carotid. This waveform is then used to derive the central aortic wave. A mathematically derived transfer function incorporates the site of recording to derive the central aortic waveform (Nichols, O'rourke & Kenney 1991, Chen et al. 1997).
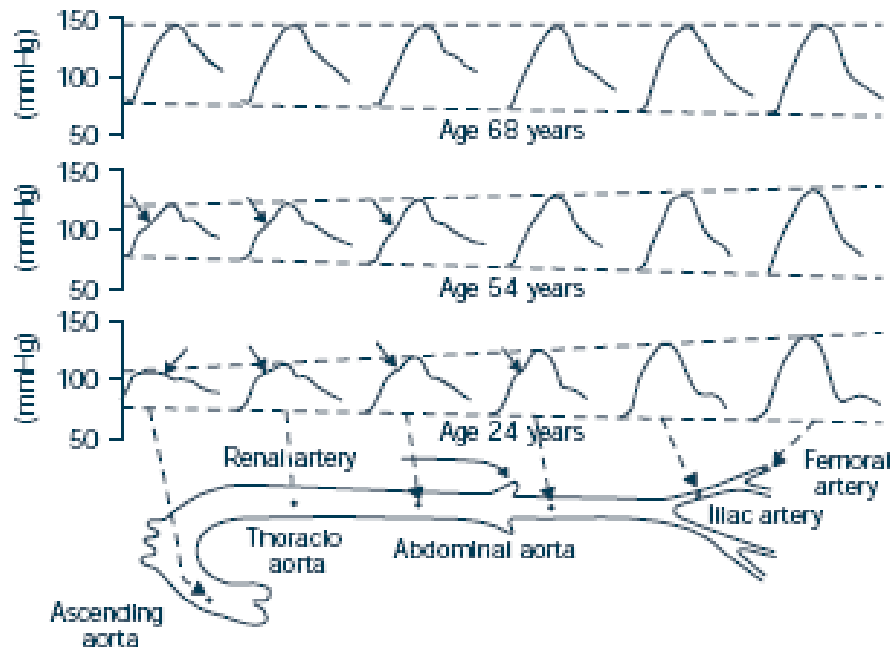
Figure 2.2   Change of pressure wave amplitude and wave pattern from central to peripheral arteries at different ages

The central aortic pressures are higher in elderly patients. The progression of wave amplitude and pattern is more pronounced in young individuals. Adapted from Nichols et al, Theoretical, experimental and clinical principals; 1998 (4th edition).

Applanation tonometry

A device called the tonometer is used to detect the peripheral pulse waveform by placing it on the artery to measure changes in its diameter. It is a high fidelity transducer that responds to dynamic changes in the underlying arterial segment. It detects changes in force and volume due to changes in blood flow within the artery. This principle of non-invasive recording of intra-arterial pressure changes is known as applanation tonometry (Drzewiecki, Melbin & Noordergraaf 1983).

The instrument used to record these intra-arterial changes is a tonometer and it consists of a pencil shaped probe. The probe incorporates a high fidelity

micromanometer which is connected to a computer through an amplifier. The sensor has a stiff ceramic tip that connects through a double arm wheat-stone bridge circuit to a preamplifier. Recordings are taken from superficial arteries overlying bony structures. The carotids, femoral and radial are usual sites for recordings. Once the superficial artery overlying a bony structure is identified, the point of maximal impulse is located. The tip of the tonometer is placed at the point of maximal impulse and pressed down against the underlying bone thus flattening the curved surface of the artery (Kelly et al. 1989a, Nichols, O'rourke & Kenney 1991), (Kelly et al. 1990). Once the artery is flattened; the circumferential stresses in the wall are balanced and a true intra-arterial pressure is registered by the sensor (MACKAY, MARG & OECHSLI 1960). Recordings are taken when reproducible high amplitude signals are obtained. The technique is difficult to use in patients with excessive amount of overlying tissue or weak pulse. In the study conducted by Kelly R and colleagues, arterial pressure waves were recorded from the carotid, femoral and radial arteries of 1005 normal out patient subjects. These large superficial arteries with minimal overlying tissue were found be optimal for measurement of arterial pulse (Kelly et al. 1989b).
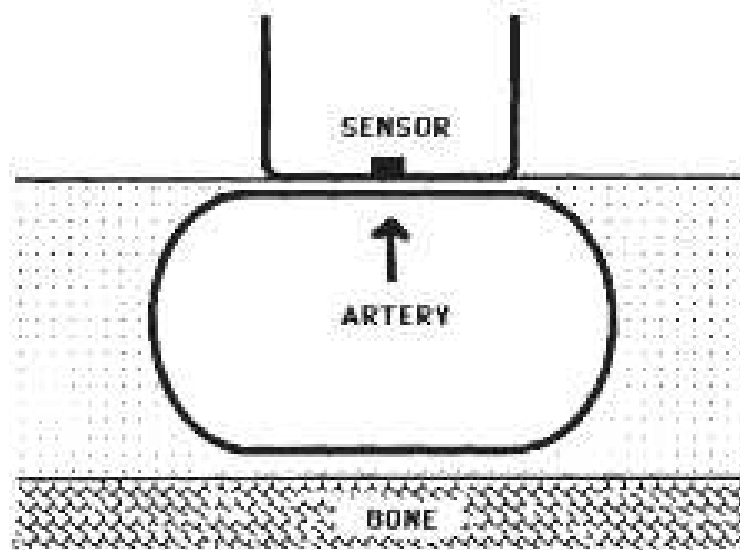
Figure 2.3   Applanation of the tubular artery against underling bone permits accurate measurement of intra-arterial pressure

The applanation tonometry process; flattening of the curved pressure containing structure allows accurate registration of transmitted pressure. Taken from Kelly et al, Circulation 1989.

Augmentation index

As the pulse waveform travels along to the periphery, a reflected wave from the previous ventricular ejection, superimposes on it (Karamanoglu et al. 1993). The reflected wave accentuates the peak systolic waveform produced by the ventricular ejection. This increase is known as augmentation index. Thus augmentation index is defined as $\Delta P = P2-P1$. (Fig 2.4). It is defined in two ways, relative to P1 ($\Delta P/P1$) or pulse pressure (Pulse Pressure/P1).

Augmentation index is a quantitative index of systemic arterial stiffness (Safar, London 2000). The AIx is a measure of the contribution made by the reflected pressure wave to the ascending aortic pressure waveform (O'Rourke, Kelly 1993) and provides a measure of systemic arterial stiffness (Safar, London 2000) Augmentation Index is closely and positively correlated to Pulse Wave Velocity. However, it is influenced by heart rate, blood pressure and gender (Yasmin, Brown

1999, Marchais et al. 1993). The augmentation index for women is higher than for men (London et al. 1995).

Augmentation index is derived from the central aortic waveform as invasive measurements are difficult. Peripheral arterial waveform patterns are recorded at the radial or femoral artery. They are analysed by computer to mathematically derive central aortic waveform patterns. Chen-Huan Chen and colleagues demonstrated a significant similarity for the amplitude and phase configuration of aortic and radial pressures both within and between patients (Chen et al. 1997). In this study the aortic pressure was recorded in 20 patients using a micromanometer placed inside the lumen of a pigtail catheter and placed in the ascending aorta. The radial pressure waves were recorded using an automated tonometry device. Vascular compliance and the timing of the reflected wave influenced both aortic and radial pressures in this study. Takayuki and colleagues studied the correlation between invasive intra-arterial blood pressure monitoring and non-invasive technique using applanation tonometry. Twenty normotensive healthy volunteers and 10 patients with uncomplicated essential hypertension were included in this study. An analysis of the pressure waveform recording revealed a good correlation between the applanation tonometry technique and the intra-arterial recording except in the early systolic phase. Beat-to-beat variability and postural variability were well correlated by applanation tonometry (Sato et al. 1993). Endothelium-dependent and -independent vasodilatation is assessed by measuring the maximum changes in AIx during a 20-min period following the administration of inhaled salbutamol (400μg) via a spacer and sublingual nitroglycerin (GTN) (150μg), respectively. Both salbutamol and GTN induce a significant reduction in AIx in healthy subjects {{293 Wilkinson,I.B. 2002}}. The salbutamol-mediated changes in AIx have been reported to correlate

well with the changes in forearm arterial blood flow during infusion of acetylcholine, an endothelial dependent vasodilator while GTN produces arterial dilatation, independent of endothelial function.

Conclusion

In conclusion, pulse wave analysis is the study of arterial stiffness by analysis of the blood pressure waveform. A high fidelity tonometer is used to derive the peripheral pulse waveform and central waveform is derived by using a mathematical formula. The augmentation index, derived from the analysis of the arterial waveform is a quantitative index of systemic arterial stiffness.
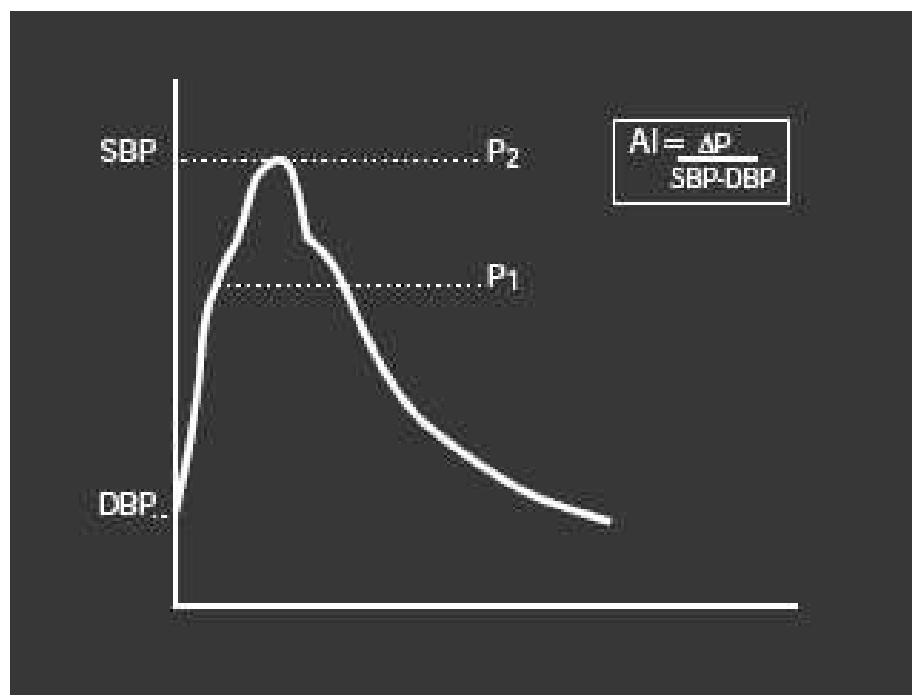
Figure 2.4   Augmentation Index = ΔP=P2-P1

The increase in pressure, augmentation index, is predominantly due to the reflected component of the original pressure pulse generated by ventricular ejection. Adapted from Murgo et al, Circulation 1980.

2.4.B   Flow mediated dilatation

Introduction

Flow mediated dilatation (FMD) is defined as a measure of the relaxation of peripheral conduit arteries in response to increased sheer stress exerted by increased blood flow through them. This phenomenon was first studied in the femoral artery and demonstrated by Schretzenmayer in 1933 (Rubanyi, Romero & Vanhoutte 1986). FMD is impaired in patients with atherosclerosis and underlying cardiovascular disease (Benjamin et al. 2004a, Moens et al. 2005). The FMD in healthy individuals is 7 -10% of the baseline diameter. However, in patients with cardiovascular disease FMD is impaired or absent with values ranging between 0 - 5% (Moens et al. 2005). In the Framingham heart study, a community based analysis, the mean FMD was 2.4% in males and 3.3% in females. Increasing age, smoking, systolic blood pressure and BMI were associated with a lower FMD% in the community (Benjamin et al. 2004b). Initial studies to measure changes in flow mediated dilatation were invasive. They required invasive monitoring of change in vasodilatation following intra-arterial pharmacological stimulation with specific endothelium dependent vasodilators such as acetylcholine and bradykinin. They act primarily via the endothelial muscarinic membrane receptor-mediated stimulation of nitric oxide synthase (Quyyumi 2003). This invasive nature made this technique unsuitable for studying asymptomatic healthy individuals. Non-invasive measurement of changes in arterial diameter in the superficial conduit arteries extended the scope of flow mediated dilatation to large population studies.

Physiology of flow mediated dilatation

An intact endothelium, forming a single layer of cells lining all the blood vessels, is crucial for the relaxation of arteries in response to increased shear stress (Pohl et al.

1986, Smiesko, Kozik & Dolezel 1985). Endothelial cells lining the large conduit arteries differ significantly from those lining the venous system. They react differently to haemodynamic stress exerted by changes in blood flow through them (Franke et al. 1984, van Grondelle et al. 1984). *In vitro*, there is a flow dependent increase in the secretion of prostacyclin (PGI2) by endothelial cells obtained from the arteries. These cells also contain straight actin filament. Following exposure to increased sheer stress there is a significant increase in the quantity and myofibrillar staining of these actin filaments. However, the exact amount of stress required to induce these changes in undefined (Franke et al. 1984). Endothelial denudation leads to an abolition of stress induced arterial vasodilatation. Acetylcholine infusion, an endothelial dependent vasodilator, did not lead to vasodilation in the denuded segment. However, these denuded segments responded to nitro-glycerine, an endothelium independent vasodilator (Pohl et al. 1986).

In vivo, there is a pulsatile increase in arterial shear stress corresponding to the cardiac cycle. As blood flow is laminar and unidirectional in conduit arteries it is possible to estimate shear stress in these vessels. It is directly related to the velocity and viscosity but inversely related to the diameter of the vessel and is calculated by the formula:

Shear stress = velocity × viscosity/vessel diameter (Gnasso et al. 2001)

The endothelium in the conduit arteries responds to this increase in shear stress by generating a trigger for vasodilatation. It does so by releasing various vasodilators such as prostaglandins, nitric oxide and endothelial-derived hyperpolarising factor. Importantly, nitric oxide is the chief vasodilator released by these cells. Its release opens calcium activated potassium channels leading to hyperpolarisation of the

endothelial cells, which in turn activates endothelial nitric oxide synthase (eNOS) (Feletou, Vanhoutte 2000). Though it is initiated by the opening of potassium channels, the hyperpolarisation is sustained by capacitative calcium entry triggered by the depletion of intracellular calcium stores in the endoplasmic reticulum. Endothelial hyperpolarisation is transmitted through gap junctions to the underlying smooth muscle leading to vasodilatation (Feletou, Vanhoutte 2000, Griffith, Chaytor & Edwards 2004).

Measurement of Flow Mediated Dilatation

Measurement of flow mediated dilatation was first described by Celermajer in 1992 (Celermajer et al. 1992). Patient preparation is crucial to arterial measurements. Patients are advised to fast for 4 hours prior to the procedure. Caffeine and alcohol are to be avoided for 24 hours prior. Physical activity is restricted to avoid strenuous exercise for 24 hours prior. On the day of the procedure the patient is laid in a quiet, temperature controlled environment for 10 minutes prior to measurement.

Large superficial conduit arteries including the femoral, carotid and brachial are suitable to be studied. The arterial diameter is measured at a fixed distance from an anatomical marker, such as a bifurcation. Traditionally arterial measurements are conducted using a two dimensional ultrasound probe. The diameter of the target artery is recorded using two dimensional ultrasound images, with a 7.0 MHz linear array transducer. The arterial flow velocity is measured by a pulsed Doppler signal at a 70 degrees angle to the vessel in the centre of the artery. The femoral artery is scanned just below the bifurcation of the common femoral artery and the brachial artery is scanned 2-15 cms above the elbow in the longitudinal section. Arterial measurements are taken to coincide with the R wave on ECG.

Following baseline measurements, the arterial diameter is measured during endothelial dependent and endothelial independent dilatation. Endothelium dependent vasodilation is induced by the vascular endothelium in response to increased sheer stress ie. reactive hyperaemia. Endothelium independent vasodilatation occurs when nitric oxide in sourced by an exogenous donor and not from the endothelium. The exogenous nitric oxide acts directly on the smooth muscle to cause vasodilatation.

Endothelium dependent vasodilation is measured by inflating a pneumatic cuff in the forearm for brachial artery measurements or in the upper thigh for femoral artery measurements to supra-systolic pressures for 3-5 minutes and then released. The release of the cuff leads to increased flow through the artery in response to peripheral ischemia. The repeat scan is taken 45-60 seconds after release of the cuff to measure endothelium derived vasodilatation. Glyceryl trinitrate (GTN) is used to measure endothelium independent vasodilation. GTN acts directly on the smooth muscle and causes vasodilation independent of endothelial function. GTN (400 mcg) is administered sublingually and arterial diameter is measured 3 minutes after its administration. 15 minutes is allowed for vessel recovery before a repeat scan is performed.

Ultrasound imaging is known to provide a reliable and cost effective method of measuring arterial wall thickness (Wendelhag et al. 1991). With a 7.0 MHZ transducer the coefficient of variation for inter observer measurements of arterial wall anatomy and diameter is 1-3% (Celermajer et al. 1992). Brachial artery FMD is widely used in clinical research due to its non-invasive nature. It can be used effectively for the serial evaluation of arterial response in young adults. This presents

the distinct advantage of assessing the artery in an early preclinical stage, when the disease process is reversible. Studies by Woo and colleagues demonstrated its serial use in young obese children to study the effect of diet and exercise (Woo et al. 2004). It has also been used to study endothelial dysfunction in other disease states such as hypercholesterolemia (Creager et al. 1990a), diabetes (Lee et al. 2007), hyperhomocysteinemia (Celermajer et al. 1993a) and smoking (Celermajer et al. 1993b). The technique, however, presents practical challenges to its routine use in clinical practise. A high degree of operator training and standardization of equipments is required for its use (Deanfield, Halcox & Rabelink 2007). Small variations in the placement of the transducer or placement of the cuff can alter the final measurements (Esper et al. 2006).

Magnetic resonance imaging (MRI) – brachial artery endothelial function

The recent development of MRI to assess endothelial function has significantly added to the non-invasive nature of vascular testing. The major advantage of MRI is its ability to study both peripheral and central (coronary and aortic) vascular measures at the same time. Early studies have highlighted its concurrence with the ultrasound technique (Leeson et al. 2006). The difference between CMR and ultrasound for FMD was 0.14 +/- 6.8%. The correlation between these modalities for FMD was 0.62, p = 0.01 and for measurement of the area of artery was 0.87, p = < 0.0001. Inter-study reproducibility was also similar. The coefficient of variation (CV) for FMD was 0.3 for CMR and ultrasound each. CMR and ultrasound show good agreement for quantitative measures of vascular structure and function with good reproducibility for both modalities. CMR offers the unique advantage of being able to assess both vascular structure and physiology in a single study (Wiesmann et al. 2004a). An integrated measurement of the brachial artery can enable measurement

of pulse wave velocity, arterial distensibility and flow mediated dilatation in a single study. In addition to the assessment of superficial vessels it can also provide extensive coverage of large arteries such as the aorta (Lee et al. 2007). It has been extensively validated for assessment of vessel wall parameters and vascular elasticity (Mohiaddin, Firmin & Longmore 1993, Mohiaddin et al. 1989).

The superficial brachial artery is the best to be imaged by MRI technique. It is chosen from a three-dimensional angiographic pilot scan in order to align the imaging plane perpendicular to the artery. Cross-sectional area of the brachial artery is measured by manual delineation of inner vessel boundaries using CMR image post-processing software. Magnetic resonance measurement of brachial artery area is performed at baseline and 1 min after reactive hyperaemia induced by release of a forearm cuff inflated to supra-systolic pressure for 5 min. For assessment of endothelial-independent brachial artery dilation, magnetic resonance data acquisition is repeated before and 3 min after sublingual application of 400 μg glyceryl trinitrate. Cardiac-gated TrueFISP cine images of the brachial artery are acquired with the following parameters: TR/TE 56/3 ms, flip angle 66°, FOV 117 × 77 mm, matrix 384 × 252, 16 segments, 11 to 19 phases depending on heart rate.

The aim of FMD analysis is to detect a change in arterial diameter from baseline. This is dependent on the baseline diameter of the artery and time to peak flow. Interpersonal variation leads to a significant difference in the arterial diameter (Celermajer et al. 1992). Small arteries dilate to a larger extent than large arteries. Therefore, an analysis of FMD should include the percentage change in arterial diameter to overcome this baseline variation. Another important factor is the time to peak flow in the brachial artery. Repeated measurements can be taken to identify the

peak flow. The advantage with the MRI technique is the availability of recorded cine images which help identify the peak flow with relative ease. Peak flow is identified by scrolling through the cine images to identify the point of maximum flow. Maximal brachial artery diameter is identified from the cine images to record the point of maximum flow and measure change in flow.

FMD serves as a useful tool for the assessment of systemic arterial disease. It is also a surrogate marker of coronary artery disease and shows good correlation with early coronary atherosclerotic disease and cardiovascular pathology (Anderson et al. 1995, Takase et al. 1998). Studies by Anderson and co demonstrated a close relationship between coronary and brachial artery vasodilatation. In this study 50 patients with suspected coronary artery disease underwent cardiac catheterization for evaluation of coronary vasomotor response and brachial artery FMD for evaluation of brachial artery vasodilatation. The study identified the positive predictive value of abnormal brachial artery dilatation (<3%) as 95% in predicting coronary endothelial dysfunction (Anderson et al. 1995). Additionally it provides prognostic information about future cardiovascular events in patients with mild coronary artery disease indicating its role in the development of coronary atherosclerosis. (Suwaidi et al. 2000).
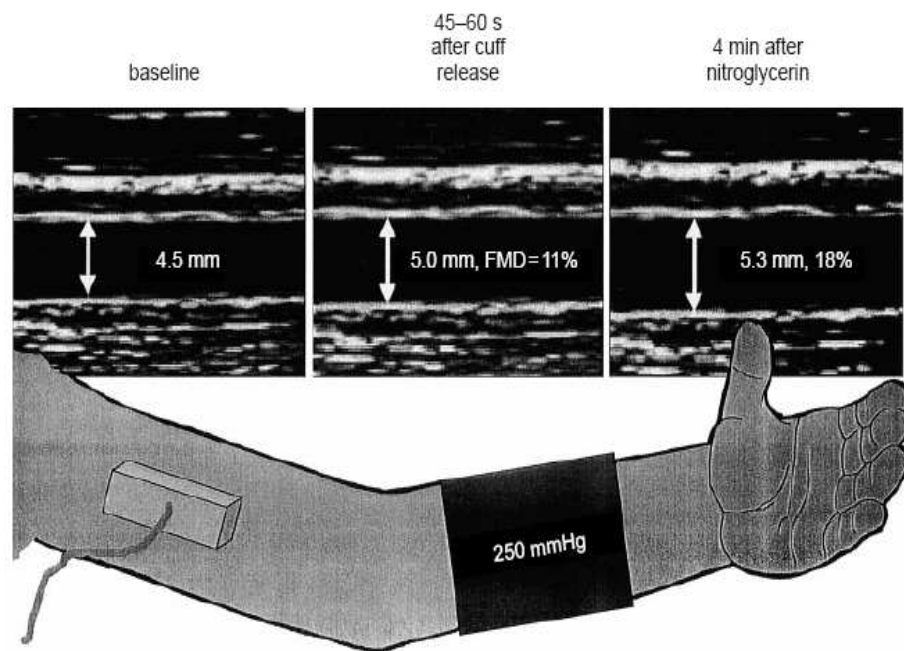
Figure 2.5   FMD measurement in the brachial artery

Brachial artery diameter is measured during three conditions; baseline (after at least 10 min supine rest), during reactive hyperaemia and finally after the administration of sublingual glyceryl trinitrate. Taken from Olli et al, Research methods in Human Cardiovascular Pharmacology 2000.
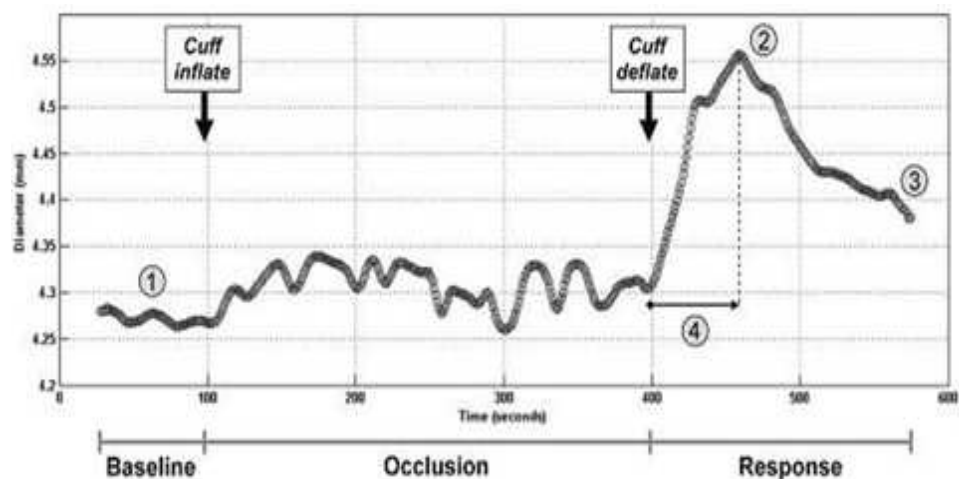
Figure 2.6   Endothelium dependent vasodilation in the femoral artery

Graphical representation of the change in arterial diameter in response to reactive hyperaemia Adapted from Echocardiography, Martin Stout, 2009.

2.4.C   Venous plethysmography

The technique of venous plethysmography was first described in 1909 by Hewlett to measure blood flow in the limb. This technique has served as the gold standard for measurement of blood flow in vivo. It has served as a useful technique to study the effects of drugs and interventions on focal vascular regions without its systemic administration. This is achieved by the infusion of drugs in the brachial or femoral artery followed by the measurements of blood flow in the limb. The advent of mercury-in-silastic gauges and computerised analysis of measurements has greatly enhanced the reliability and reproducibility of the technique. This technique utilises mercury filled silastic tubing wrapped around the limb. Changes in the diameter and length of the guage correspond to variations in limb circumference and are measured by the computer software. Newer techniques using automatic calibration without the use of mercury have also lately been described (Christ et al. 2000).

Physiology and methodology

The basic principle of venous plethysmography is to isolate and differentiate the arterial and venous flows (Wilkinson, Webb 2001). The limb to be studied is identified and placed above the level of the heart to assist in venous drainage. A blood pressure cuff is placed above the elbow for arm measurements and above the knee for leg measurements. The cuff is inflated to 40 mm Hg to obstruct venous drainage. Arterial inflow is uninterrupted. Arterial inflow continues until the pressure in the arm/leg is above the systolic pressure. The resulting increase in arm/leg volume is directly proportionate to the arterial inflow. Changes in volume are measured by the silastic-mercury-strain guage using computer software.

The majority of blood flow in the limbs is directed towards the skeletal muscle, while the remainder is supplied to the skin and soft tissue. An increase in limb volume is

linear to arterial blood flow. However, blood flow in the hand and foot does not follow a linear increase. This is due to the large number of arterio-venous shunts in them. The ratio of skin surface area to skeletal muscle is also large. Blood flow in the skin is variable and dependent on surface temperature. The hand and foot are excluded during blood flow measurements in the limb. This is achieved by inflating a second cuff at the wrist or ankle to 40 mm Hg above systolic pressure at least 60 seconds before taking measurements.

The study should be conducted in a temperature controlled environment. All patients should refrain from coffee and tea for 4 hours prior to the study. Vigorous exercises should be avoided for 24 hours prior. The arm/leg to be studied is placed on foam pads and supported by pillows. This is done to elevate it above the level of the heart. Two cuffs are placed, one above the elbow/knee and the other at the wrist/ankle. Cuff inflation pressure and timings are automated and computer controlled. The cuffs are inflated for 10 sec and then deflated for 5 secs. This provides for adequate venous drainage from the limb. Change in limb volume is measured by the silastic mercury strain guage. These are placed at the widest part of the forearm or leg. Changes in the circumference of the limb are measured at 15 second intervals. These are recorded sequentially and plotted using computer software.

Venous plethysmography is a reliable technique to measure blood flow changes in the limb. There is a marginal difference between measurements taken in the forearm and leg. At rest the coefficient of variation in the arm is 10% while in the leg is 11% at rest and 13% post exercise (Roberts, Tsao & Breckenridge 1986). Blood flow measurements following the infusion of intra arterial drugs is better expressed as a ratio to the flow at baseline (Petrie et al. 1998).

Figure 2.7   **Forearm venous plethysmography**

A cuff is inflated above the elbow to occlude venous drainage, while the second is placed at the wrist to exclude circulation to the wrist. A mercury-strain guage located at the widest part of the forearm measures the change in forearm volume. Taken from Ian Wilkinson et al. Research Methods in Cardiovascular Pharmacology 2001.
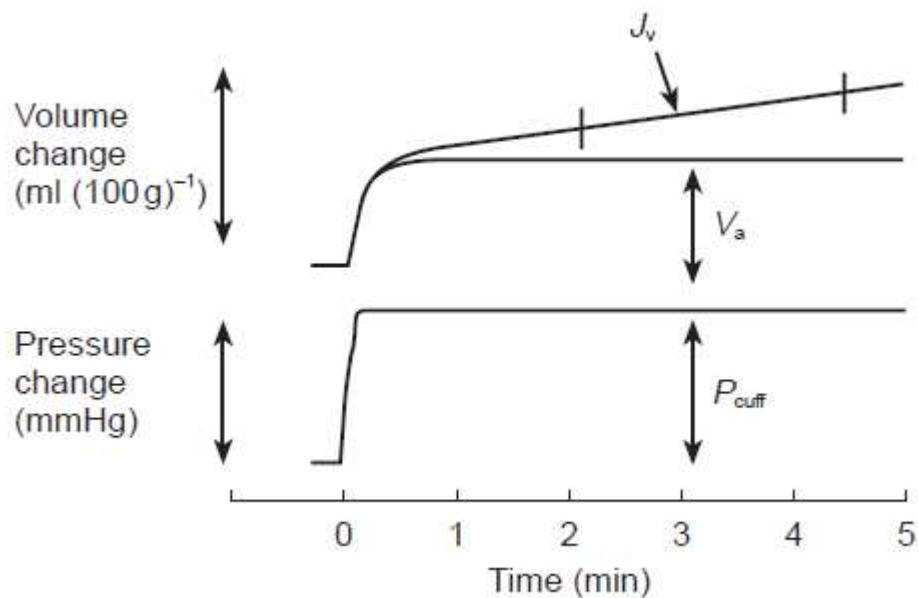
Figure 2.8   **A simulated response to inflation of upper arm cuff**

There is a rapid increase in forearm volume V_a followed by a slower continued rise J_v following inflation of the upper arm cuff P_{cuff}. Adapted from British Journal of Clinical Pharmacology. Ian B WIlkinsin et al 2001.

2.5   Cardiac MRI

Cardiac MRI (CMR) is a recent technique used to analyse aortic distensibility and brachial artery endothelial function. The added benefit of this technique is the ability to analyse the cardiac anatomy and myocardial perfusion in a single study.

MRI - aortic distensibility

ECG gated MRI (1.5 T Siemens Sonata) measurements of arterial areas were determined using a TrueFISP (fast imaging with steady state free precession) cine sequence with off-line analysis (Image-Pro Plus, MediaCybernetics, USA). Cross-sectional measurement of aorta were undertaken at 3 separate locations: the ascending aorta (AA), the proximal descending aorta (PDA) at the crossing of the pulmonary artery, and the distal descending aorta (DDA) within the first 10cms below the diagram. Aortic stiffness was assessed using the following equation-

$$\text{Aortic Distensibility} = A_s \ (mm^2) - A_d \ (mm^2)/ \ BPP \ (mmHg) \ x \ A_d(mm^2)$$

[A_s : Area of aorta at end systole, A_d : Area of aorta at end diastole, BPP : Brachial Pulse]

Aortic distensibility using MRI has been used to evaluate patients with both early and late cardiovascular disease (Wiesmann et al. 2004b, Hundley et al. 2001). Studies by Lehman and co have demonstrated that aortic distensibility measured using MRI is a valid and reproducible technique. (Lehmann, Hopkins & Gosling 1994).

Brachial artery endothelial function

CMR using a surface coil was used to study the local brachial artery endothelial function. The brachial artery at the elbow on the right arm was used for the study. The imaging position for the brachial artery was chosen from a three-dimensional angiographic pilot scan in order to align the imaging plane perpendicular to the

artery. The surface coil was placed above the elbow and a pressure cuff was placed on the forearm distally. High resolution MRI scans were aligned perpendicular to the brachial artery at the elbow. Cross-sectional area of the brachial artery was measured manual delineation of the inner vessel boundaries using CMR tools image post-processing software. Magnetic resonance measurement of brachial artery area was performed at baseline and 1 min after reactive hyperemia induced by release of a forearm cuff inflated to suprasystolic pressure for 5 min. For assessment of endothelial-independent brachial artery dilation, magnetic resonance data acquisition was repeated before and 3 min after sublingual application of 400 μg glyceryl trinitrate. Cardiac-gated TrueFISP cine images of the brachial artery were acquired with the following parameters: TR/TE 56/3 ms, flip angle 66°, FOV 117 × 77 mm, matrix 384 × 252, 16 segments, 11 to 19 phases depending on heart rate.

Conclusion

In conclusion CMR is a recent, well established and reproducible technique used to study both central and peripheral artery endothelial function in the same study.

CHAPTER 3

INFLUENCE OF OXYGEN ON ERGOREFLEX AND PERIPHERAL BLOOD FLOW IN CHRONIC HEART FAILURE

3.1   Introduction

Chronic heart failure (CHF) is characterised by dyspnoea and exercise intolerance. Patients have increased minute ventilation during exercise despite intact ventilatory control. Though the exact mechanism of this ventilatory overdrive is unclear; the ergoreflex, defined as the activation of nerve endings in the skeletal muscle during sub maximal exercise leading to sympathetic overactivity is believed to be a major contributor (Scott et al. 2000, Smith et al. 2003). These nerve endings are stimulated by the build up of altered skeletal muscle breakdown products (Scott et al. 2002). The circulatory insufficiency seen in CHF leads to reduced clearance and build up of skeletal muscle metabolites during exercise (Guazzi et al. 2008).

Recent studies have demonstrated that nearly half of all patients with heart failure have preserved systolic function. The importance of heart failure with preserved (EF>40) ejection function (HFpEF) is increasingly being recognised (Bhatia et al. 2006). Exercise intolerance, dyspnea and mortality at 1 year is similar to those with impaired systolic function (Bhatia et al. 2006, Lenzen et al. 2004). However, the role of skeletal muscle ergoreflex and peripheral blood flow in these patients is unclear.

3.2   Autonomic reflexes in CHF

The autonomic nervous system (ANS) plays a major role in balancing circulation and respiration to maintain oxygen delivery (Schmidt et al. 2005). This balance is maintained by complex central and peripheral interacting reflexes. In CHF, patients

have chronic sympathetic over activity and the skeletal muscle ergoreflex forms the major reflex that influences cardiovascular and ventilatory response to exercise (Schmidt et al. 2005, Grieve et al. 1999). Baseline autonomic parameters including heart rate, respiratory rate and blood pressure are elevated. Exercise induces an exaggerated sympathetic response due to over activation of the ergoreflex causing exercise intolerance and dyspnoea (Scott et al. 2000, Scott et al. 2002).

3.3   Role of oxygen in CHF

Acute inspired oxygen improves exercise performance and modifies the ventilatory response to exercise (Bernardi et al. 1998, Clark, Coats 1992, Moore et al. 1992) while domiciliary nocturnal home oxygen improves exercise performance and quality of life in CHF (Paul, Joseph & De Pasquale 2008). Oxygen does not improve cardiac function and the pathogenesis of this beneficial effect of oxygen in unclear (Paul, Joseph & De Pasquale 2008). Supplemental oxygen increases arterial oxygen saturation (Moore et al. 1992). It is unclear whether the increased arterial oxygen saturation might influence the peripheral ergoreflex during sub maximal exercise.

We aimed to study the acute effects of low flow oxygen (4L/min) on the ergoreflex and peripheral blood flow by analysing its effects on ventilatory and haemodynamic parameters during sub maximal exercise. Patients were subdivided into 2 groups; those with preserved and impaired ejection fraction. We hypothesize that oxygen will improve the peripheral ergoreflex and blood flow in patients with heart failure regardless of their ejection fraction.

3.4 Methodology

The methods used to analyse this study include

- Measurement of ergoreflex (detailed in section 2.3)

- Measurement of ventilatory parameters (detailed in section 2.3)

- Measurement of blood flow in the non exercising limb (detailed in section 2.2)

<u>Subjects</u>

Twenty eight patients including ten age matched healthy controls were recruited for the study. Eighteen consenting consecutive patients were recruited from the heart failure out patient clinic at a tertiary level hospital. The diagnosis of CHF was confirmed in each patient during a hospital admission for pulmonary oedema in the preceding 24 months. Their cardiac status was deemed to be stable (no change in cardiac drugs for the preceding 3 months) with a recent (<6 month old) echocardiogram. Patients with significant valvular disease, renal failure (GFR<30mls), underlying malignancies or chronic lung disease (FEV< 1 L/min) were excluded from the study. Control subjects were recruited through local advertisements.

<u>Sample size</u>

The sample size was derived from a previous study on the change in the respiratory component of the ergoreflex in patients with impaired ejection fraction (Piepoli et al. 1996). In a one-way ANOVA, a total sample size of 30 subjects from 3 groups achieves 84% power to detect a non-zero contrast of the means versus the alternative that the contrast is zero using an F test with a 0.05 significance level. The value of the contrast of the means is 20 between heart failure patients with normal and impaired ejection fraction. The common standard deviation within a group is

assumed to be 17. The sample size consists of 9 patients in each group of heart failure considering a 10% drop out rate and 10 controls. The total sample size was 28.

Statistical analysis

Subject characteristics are summarized as mean (SD). Comparison between groups was performed using Student unpaired t test. Serial measurements for respiratory parameters, haemodynamic measurements and blood flow were recorded at baseline and regular increments during ergoreflex measurement. The ergoreflex was measured by calculating the difference of the average of the second and third minute of recovery from baseline. The difference between the two readings calculated without and during inflation of the cuff was determined as the ergoreflex component. Comparison between repeated measurements in the same group was done using the student t test. Changes in ergoreflex in response to oxygen were analysed using repeated measures ANOVA for the three subgroups. SPSS (version 13.0) was used for all the analyses. A $P<0.05$ was taken to be significant, and all of the tests were 2-tailed.

3.5   Study protocol

The study was approved by the local ethics committee. Patients were provided with written and verbal information about the study. Upon request patients were taken to the cardiopulmonary exercise laboratory and cardiac unit to acquaint them with the procedures involved. Informed consent was obtained from all patients prior to the study. Baseline studies included a clinical assessment, ECG, blood pressure, body mass index (BMI) and an assessment of the NYHA class.

Studies were conducted in a temperature controlled environment on two separate days a week apart. Patients refrained from alcohol, tea and coffee for 12 hours prior to the study. They were also advised to avoid high intensity work 24 hours preceding the study. Studies were conducted at 10 AM in the morning and 2 PM in the afternoon. Each patient was booked for the same study time a week apart to avoid diurnal variations. The ergoreflex and arterial blood flow in the right leg was measured simultaneously.

On the day of the study patients were rested in a quiet room and a cannula was placed in the non-dominant arm for collection of blood. Patients were randomised to receive either air or oxygen (4 L/min) in a randomised double blinded fashion. They were rested for 15 minutes, breathing through a tight fitting Hudson mask. Baseline readings were then obtained. The maximum voluntary handgrip contraction was first measured as the greatest peak force produced from three brief maximal handgrip contractions. After a 10 minute interval patients underwent repetitive forearm exercise at 50% of the predetermined maximal contraction. This was continued until exhaustion. Following cessation of exercise the recovery period was recorded for 3 minutes. The patients rested for 30 minutes after the recovery period before repeating the same protocol. During the second (PH-RCO) exercise a cuff was inflated to + 30 mmHg above systolic pressure to trap metabolites in the exercising muscle. The cuff was inflated 10 seconds before the end of exercise and was followed by 3 minutes of recovery. Ergoreflex response as described in section 2.3 was measured in the non-dominant arm. The test involved two exhaustion limited handgrip exercises. During the first test the patients recovered normally (PH), while during the second a regional circulatory occlusion was induced in the exercising arm (PH-RCO). Thus, the contribution of the muscle ergoreceptors was evaluated by trapping of the

metabolites in the exercising muscle after exercise. The ergoreflex was quantified as the difference in ventilation during the recovery periods (PH-RCO – PH).

Arterial blood flow (ABF) as described in section 2.2 was measured in the right leg by venous occlusion mercury strain gauge plethysmography. Heart rate and blood pressure were measured non-invasively using the task force monitor in the dominant arm.

The second phase of the study was conducted one week later at the same time of the day. A similar protocol to day 1 was followed with the patient breathing air/oxygen in a randomised fashion. Air/oxygen was randomly allocated using a computer generated algorithm in the cardiopulmonary laboratory. Ergoreflex and arterial blood flow were assessed as on day 1.

3.6   Results

Patient characteristics are described on Table 1. Patients and controls were matched for age, sex and body mass index (BMI). The duration of hand grip exercise was higher in controls (P=0.001) The majority of patients were identified to have NYHA class 2 symptoms and the aetiology of heart failure was judged to be coronary artery disease (90%) and idiopathic dilated cardiomyopathy (10%). Patients were divided into two subgroups based on their ejection fraction.

Table 3.1   Patients demographics

	CHF (impaired EF) [n=9]	CHF 2 (preserved EF) [n=9]	Controls [n=10]	P
Age (years)	69.6 ± 6	67 ± 9	67.8 ± 6	0.75
Male, n (%)	9 (100)	8 (89)	8 (80)	

BMI	29.1 ± 8.1	32.7 ± 9	24.8 ± 2.9	0.08
EF	25.9 ± 7.9	70 ± 11.6		0.001*
Grip Strength (PSI)	11.4 ± 4.0	13.1 ± 3.9	14.5 ± 3.5	0.27
Grip Duration (seconds)	128.3 ± 63.4	240.0 ± 117.7	358.5 ± 116.6	0.001[†]
Diabetes	4	3		
Duration of heart failure (years)	8 ± 7.2	9 ± 8.2		
Medications				
Aspirin	5	8		
Nitrates	3	5		
Beta-blockers	5	5		
Ca-channel blockers	2	4		
Diuretics	8	6		
ACE inhibitors	6	8		
Warfarin	5	2		

Values are mean ± SD unless indicated otherwise. BMI indicates Body Mass Index;

EF indicates ejection fraction, PSI indicates pound-force per square inch.

*P<0.05 vs CHF (impaired EF)

[†]P<0.05 vs controls

Effect of ergoreflex on ventilatory parameters

The effect of ergoreflex on respiration was assessed for minute ventilation, breathing frequency, VO_2 and VCO_2. VO_2 was not measured during the concurrent use of inhaled oxygen. A single inlet on the Hudson mask was used to administer additional oxygen during exercise. The proximity of the gas analyser to the oxygen inlet leads to high VO_2 readings during its administration. Hence VO_2 measurements during the concurrent administration of oxygen were not analysed.

There was a significant increase in ventilatory parameters during sub maximal exercise in patients' while breathing room air. They displayed a significant ventilatory overdrive during ergoreflex. Ventilation (P=0.03), VCO_2 (P=0.03) and VO_2 (P=0.02) but not breathing frequency (P=0.85) were raised. (Table 3.2).

Inhaled oxygen reduced the elevated ventilatory parameters. Breathing frequency (42%), ventilation (20%) and VCO_2 (31%) were lower during sub maximal exercise. This drop reversed the ventilatory overdrive seen previously while breathing room air and narrowed the difference between controls and patients. Breathing frequency (P=0.06), ventilation (P= 0.32) and VCO_2 (P= 0.11) did not differ from controls while breathing oxygen. (Table 3.2).

Patients with impaired and preserved EF heart failure did not differ during the inhalation of air and oxygen. (Table 3.3).

Table 3.2   Respiratory and haemodynamic contribution to ergoreflex

	Air			Oxygen		
	Control	CHF	P	Control	CHF	P
BF (breaths/min)	1.6 ± 4.7	2.0 ± 6.2	0.85	6.7 ± 9.1	1.2 ± 6.1	0.06
VE mL/min	-0.1 ± 2	4.5 ± 6.3*	0.03	0.9 ± 3.5	3.6 ± 8	0.32
VCO_2 mL/min	-18.5 ± 63.5	108.8 ± 171.6*	0.03	-22.8 ± 139.3	74.5 ± 158.4	0.11
VO_2 mL/min	-19.9 ± 93.7	103.1 ± 146.1*	0.02			
Haemodynamic Parameters						
HR (beats/min)	6.3 ± 8	0.8 ± 8.1	0.10	6.7 ± 8.1	1.4 ± 11.2	0.16
sBP (mmHg)	24.2 ± 30.9	8.8 ± 30.1	0.23	5.8 ± 27.2	23.6 ± 41	0.19
dBP (mmHg)	20.3 ± 29.5	1.9 ± 22.8	0.12	10.2 ± 22	18.6 ± 36	0.46
mBP (mmHg)	23.5 ± 29.7	4.0 ± 22.7	0.10	8.5 ± 23.1	20.2 ± 37.9	0.33
SV (ml)	-1. 8 ± 13.2	2.0 ± 10.1	0.47	6.2 ± 18	1.6 ± 7	0.51
SI (ml/beat/m^2)	-0.9 ± 6.7	1.0 ± 5.1	0.46	3.1 ± 9	0.7 ± 3.5	0.49
CO (ml/min)	0.6 ± 1.7	0.2 ± 1	0.60	0.8 ± 1.1	0.1 ± 0.5	0.13
CI (ml/min/m^2)	0.3 ± 0.9	0.1 ± 0.5	0.62	0.4 ± 0.6	0.1 ± 0.3	0.13
TPR (mmHg/ml/min)	181.9 ± 393.9	266.8 ± 763.2	0.74	-141.8 ± 482.4	29.1 ± 429.7	0.44
TPRI (mmHg/ml/min/m^2)	358.4 ± 783.6	534.8 ± 1524	0.73	-283.1 ± 963.1	68.8 ± 865.4	0.43

Values are mean ± SD unless indicated otherwise. BF indicates breathing frequency, VE indicates ventilation, VCO_2 indicates ventilatory carbon dioxide, VO_2 indicates ventilatory oxygen, HR indicates heart rate, sBP indicates systolic blood pressure, dBP indicates diastolic blood pressure, mBP indicates mean blood pressure, SV indicates stroke volume; SI indicates stroke index; CO indicates cardiac output; CI indicates cardiac index; TPR indicates total peripheral resistance; TPRI indicates total peripheral resistance index.

Table 3.3   Ergoreflex in CHF patients with impaired and preserved ejection fraction

	Air			Oxygen		
	IEF	PEF	P	IEF	PEF	P
Ventilation						
BF (breaths/min)	1.9 ± 4.5	2.1 ± 7.5		2.1 ± 6.2		
VE mL/min	5.1 ± 6.4	4.0 ± 6.5		2.7 ± 4.7	0.4 ± 6.2	
VCO$_2$ mL/min	147.8 ± 201.8	76.8 ± 144.6		56.4 ± 98.8	90.8 ± 202.2	
VO$_2$ mL/min	130.1 ± 182.9	80.9 ± 112.2				
Haemodynamic Parameters						
HR (beats/min)	2.3 ± 3.5	0.8 ± 8.4	0.43	2.8 ± 6.1	1.4 ± 11.2	0.60
sBP (mmHg)	-1.9 ± 30.4	8.8 ± 30.1	0.15	26.17 ± 52.86	23.6 ± 41	0.80
dBP (mmHg)	-1.1 ± 26	1.9 ± 22.8	0.61	20.08 ± 48.5	18.6 ± 36	0.86
mBP (mmHg)	-1.7 ± 24.4	4.0 ± 22.7	0.32	21.19 ± 51.6	20.2 ± 37.9	0.91
SV (ml)	1.3 ± 1.6	2.0 ± 10.1	0.82	0.92 ± 4.3	1.6 ± 7	0.75
SI (ml/beat/m^2)	0.8 ± 0.8	1.0 ± 5.1	0.85	0.35 ± 2.3	0.7 ± 3.5	0.76
CO (ml/min)	0.2 ± 0.2	0.2 ± 1	0.86	0.01 ± 0.3	0.1 ± 0.5	0.50
CI (ml/min/m^2)	0.10.1	0.1 ± 0.5	0.86	0.01 ± 0.15	0.1 ± 0.3	0.41
TPR (mmHg/ml/min)	228.7 ± 685.3	266.8 ± 763.2	0.90	-95.32 ± 555.8	29.1 ± 429.7	0.40
TPRI (mmHg/ml/min/m^2)	463.0 ± 1369.8	534.8 ± 1524	0.90	-190.30 ± 1109.5	68.8 ± 865.4	0.38

Values are mean ± SD unless indicated otherwise. BF indicates breathing frequency, VE indicates ventilation, VCO$_2$ indicates ventilatory carbon dioxide, VO$_2$ indicates ventilatory oxygen, HR indicates heart rate, sBP indicates systolic blood pressure,

dBP indicates diastolic blood pressure, mBP indicates mean blood pressure, SV indicates stroke volume; SI indicates stroke index; CO indicates cardiac output; CI indicates cardiac index; TPR indicates total peripheral resistance; TPRI indicates total peripheral resistance index.

Effect of ergoreflex on haemodynamic parameters

The haemodynamic effect of ergoreflex was assessed on heart rate, systolic, diastolic and mean blood pressure, stroke volume, stroke index, cardiac output and total peripheral resistance. The haemodynamic component of the ergoreflex did not differ significantly between patients and controls both on air and oxygen. Patients with impaired and preserved ejection fraction had similar haemodynamic response to exercise. (Table 3.3).

Blood flow

Blood flow to the non exercising lower limb was obtained at 15 second intervals and averaged for 1 minute. Blood flow was calculated at baseline, 50% and peak exercise and for 3 minutes of recovery. Flow was calculated in the non exercising limb concurrently during and without inflation of cuff in the exercising upper arm.

Blood flow to the non-exercising lower limb remained unchanged during ergoreflex. Administration of oxygen did not influence blood flow to the non-exercising lower limb in either group. (Table 3.4).

Table 3.4   Blood flow in the non-exercising limb during ergoreflex on air and oxygen

		Baseline	50% Peak	Peak	Recovery (3 min)
Controls	Air	-0.6 ± 1.2	-0.28 ± 0.89	-0.16 ± 0.94	0.02 ± 1.55
	Oxygen	0.59 ± 1.5	0.2 ± 1.7	0.64 ± 2.09	0.61 ± 1.42
	P value	0.07	0.46	0.28	0.38
CHF (IEF)	Air	0.54 ± 2.4	0.89 ± 2.3	0.51 ± 2.5	1.05 ± 2.83
	Oxygen	-0.58 ± 1.4	0.23 ± 0.84	-0.55 ± 2.31	-0.68 ± 1.86
	P value	0.27	0.49	0.37	0.14
CHF (PEF)	Air	-0.31 ± 0.44	-0.15 ± 0.38	-0.33 ± 1.07	-0.11 ± 0.72
	Oxygen	-0.27 ± 0.67	0.6 ± 0.93	1.11 ± 3.44	-0.2 ± 0.79
	P value	0.88	0.56	0.27	0.8

Values are mean ± SD unless indicated otherwise. Blood flow values are in

$ml*min^{-1}*100\ ml^{-1}$ unless otherwise noted.

3.7   Discussion

The study analysed the effect of acute inhaled oxygen on the peripheral ergoreflex in patients with impaired and preserved heart failure. The chief findings of the study are:

1) Patients had a significantly elevated ventilatory but not haemodynamic contribution to ergoreflex.

2) Administration of oxygen reversed the elevated ventilatory component of ergoreflex in CHF. Haemodynamic parameters remained unaffected.

3) Patients with impaired and preserved systolic function had a similar ventilatory and haemodynamic response to ergoreflex.

Exertional dyspnoea is characteristic of CHF and indicates poor prognosis. This subjective symptom is objectively identified as an increased ventilatory overdrive during exercise. There is a disproportionate rise in ventilatory parameters limiting exercise tolerance. The ergoreflex defines the peripheral skeletal muscle contribution to this ventilatory overdrive. Though the exact mechanism is unclear; stagnation of altered skeletal muscle metabolites such as lactate, H+ ion and prostaglandins are believed to stimulate nerve endings in the skeletal muscle.

Nearly half of all patients with CHF have preserved ejection fraction. However, they have similar symptoms and prognosis as patients with impaired ejection fraction. Marco Gauzzi et al compared the two forms of heart failure with cardio-pulmonary exercise testing and found similar ventilatory response to maximum exercise (Guazzi, Myers & Arena 2005). Our study extends to these findings to confirm the similarity in peripheral ergoreflex in the two subgroups. as well as similar ventilatory, haemodynamic and blood flow.The chief finding of our study was the

influence of ergoreflex in patients with CHF. Ergoreflex contributed to an increase in ventilation but not altered haemodynamics or blood flow during sub-maximal exercise. These findings are similar to those by Grieve et al. who demonstrated an increased ventilatory response to exercise in CHF (Grieve et al. 1999). However, unlike the findings by Grieve et al our study did not demonstrate an increase in the systolic and diastolic blood pressure. The lack of haemodynamic influence could be due to the different muscle groups that were studied. Unlike the previous study wherein ankle dorsiflexion was studied our patients were studied for hand grip strength. As the forearm muscles are smaller it might have lead to the differential influence on blood pressure. However, it is unlikely to have altered the ventilatory parameters as both these muscle groups are shown to have a reproducible effect on ventilation (Scott et al. 2003).

Our study demonstrates for the first time that low flow oxygen reverses the ventilatory overdrive seen during exercise. However, inhaled oxygen did not influence haemodynamic parameters or blood flow. The beneficial effect of inhaled oxygen might have been due to its peripheral effect on the skeletal muscle or a central pulmonary influence. Oxygen is known to improve skeletal muscle energy metabolism due to its effect on creatinine phosphate and creatinine concentrations (Jakobsson, Jorfeldt 1995). An improvement in ventilation could have been due to the effect of oxygen at the alveolar capillary membrane. Increased dead space, alveolar oedema and pulmonary congestion lead to reduced ventilatory efficiency in CHF. Increased alveolar oxygenation may help improve the ventilatory efficiency in CHF.

We have previously demonstrated the lack of effect of inhaled oxygen on central cardiac parameters such as ejection fraction. The current study adds further to demonstrate the lack of effect of oxygen on peripheral skeletal muscle blood flow. Reduced vascular elasticity is a feature of CHF and acute interventions have not proven successful in improving blood flow. Improved arterial oxygenation may however have improved tissue hypoxia.

3.8 Conclusion

CHF patients have a similar autonomic response to sub maximal exercise regardless of their central cardiac function. This heightened autonomic response primarily affects the ventilatory parameters. Inhaled oxygen therapy reduces the elevated ventilatory response to exercise without influencing haemodynamic and blood flow parameters. Further studies are required to study the influence of oxygen on skeletal muscle metabolism and its contribution to the ventilatory overdrive in CHF.

CHAPTER 4

IMPROVEMENT IN EXERCISE VENTILATION BY ACUTE OXYGEN THERAPY IN CHRONIC HEART FAILURE PATIENTS

4.1   Introduction

Chronic heart failure (CHF) is a major clinical and public health problem. Over the last 25 years mortality associated with cardiovascular disease has declined; however, its prevalence has increased, particularly in the elderly patients (Senni et al. 1998). Fatigue and exertional dyspnea are the most common symptoms of CHF. (Garg et al. 1993, Rector, Cohn 1992, Mayou et al. 1991, Dracup et al. 1992). In the Framingham study exertional dyspnea was identified as a major criterion for the diagnosis of CHF (McKee et al. 1971).

The cardiovascular system transports oxygen and carbon dioxide between the pulmonary and musculoskeletal system. Increased musculoskeletal demand during exercise is matched by a concurrent increase in cardiac output and ventilatory efficiency in healthy individuals. The hallmark of CHF is impaired cardiac output and ventilatory inefficiency during exercise leading to exertional fatigue and dyspnoea. (Balady et al. 2010). Cardiopulmonary exercise testing (CPET) provides a unique and clinically useful tool for the assessment of these symptoms in CHF (Albouaini et al. 2007, Balady et al. 2010). It measures the haemodynamic and pulmonary parameters during increamental stages of exercise providing an objective assessment of the interplay of each system during exercise. It is also used to prognosticate and assess drug efficacy in CHF. (Hunt, American College of Cardiology & American Heart Association Task Force on Practice Guidelines

(Writing Committee to Update the 2001 Guidelines for the Evaluation and Management of Heart Failure) 2005).

Supplemental oxygen increases arterial oxygen saturation during exercise in patients with CHF (Moore et al. 1992). Moreover, acute inspired oxygen improves exercise performance in patients with CHF. (Bernardi et al. 1998, Clark, Coats 1992, Moore et al. 1992). Previous trials conducted at our institution have demonstrated that domiciliary nocturnal home oxygen improves exercise performance and quality of life in CHF (Paul, Joseph & De Pasquale 2008). However, the influence of supplemental nasal oxygen on cardiac, pulmonary and musculoskeletal system during maximal exercise is not known. We aim to study the acute effects of low flow oxygen (4L/min) on each of these parameters using CPX testing and hypothesize that oxygen will lead to an improvement in these parameters during exercise.

4.2   Methodology

Patient Selection

A prospective randomised double blinded study was conducted after approval from the local ethics committee. Thirty consecutive patients including 20 patients with NYHA class 2 and 3 heart failure and 10 age matched control subjects were recruited. Patients were recruited from the heart failure out patient clinic at a tertiary level hospital and controls were recruited through local advertisement. Informed consent was obtained from all patients prior to the study. Patients were diagnosed with heart failure using the Framingham criteria during a hospital admission in the preceding 24 months. Patients with a recent (<3 months) myocardial infarction, corpulmonale and inability to exercise on a bicycle were excluded. All patients underwent a detailed history, physical examination and baseline electrocardiogram.

Cardiopulmonary exercise testing

Patients were encouraged to visit the cardiopulmonary lab and familiarised with the exercise test protocol prior to the study. CPET was conducted as described in section 2.2. On the studt day patients received air/oxygen at 4 L/min starting 20 minutes prior and continued during the exercise test. Oxygen and air were supplied from unmarked cylinders in a randomised double blinded fashion. A second CPX test was conducted one week later using inspired gas from the second cylinder. Studies were conducted at the same time of the day to avoid diurnal variations. Exercise tests were performed on an electrically braked cycle ergometer (Jaeger, Germany) under the supervision of a physician. Patients first pedalled for 3 minutes at 55 rpm without added load. The work rate was then increased by 15 W/min up to their symptom-limited maximum. Heart rate was recorded continusously using a task force monitor.

Ventilatory data

Patients breathed through a tight fitting mask (Hans Rudollph, Kansas City, USA) and continuous on-line ventilation and expiratory gas data was collected. The analysis was performed throughout the different stages of the study. Ventilation ($\dot{V}$, inspiratory flow) and expiratory CO_2 and O_2 concentrations were measured to derive O_2 consumption ($\dot{V}O_2$) and CO_2 production ($\dot{V}CO_2$) using standard formulas. The gas meters were calibrated against gases of known concentrations before each test. The $\dot{V}$, $\dot{V}CO_2$, and $\dot{V}O_2$ was calculated on line at every breath by a fuel cell JAGER (Oxycon, USA) Data was averaged at 15 second intervals and analysed at baseline and increments of 25% to peak exercise.

Data analysis

In sample characterization, the student t test was used to compare the mean values of quantitative variables in patients and controls. Serial measurements were recorded at

25% increments during cardio-pulmonary testing and repeated analysis of variance was used to assess the effect of air and oxygen on cardiopulmonary parameters. Comparison between the two groups was conducted using between group analysis of variance for repeated measurements.

4.3   Results

Ventilatory and haemodynamic evaluations were performed in 30 individuals including 3 (10%) studies that were rejected and repeated due to technical reasons. There was 1 individual whose data on oxygen could not be recorded due to inability in obtaining a tight facial seal.

Characteristics of the study sample are outlined in Table 1. Patients with CHF were characterised by reduced work loads and lower VO_2 max at peak exercise. Maximum work load achieved was significantly higher in controls both on air (140.5 ± 32.78 vs 87.5 ± 30.2, P= 0.001) and oxygen (142 ± 41.37 vs 86.1 ± 27.73, P=0.002). The heart rate reserve (defined as the difference in peak and resting heart rate) was significantly lower in CHF [P = 0.004 (air), P = 0.01 (oxygen)]. At peak exercise the RR interval was significantly higher in CHF indicating a tachycardic response to lower work load. [P = 0.01 (air), P = 0.02 (oxygen)]. Table 1 outlines the metabolic, haemodynamic and ventilatory differences in controls and patients both on air and oxygen.

Table 4.1   Patient characteristics

		Control (n=10)	CHF (n=20)	P
Age (years)		67.8 ± 6	68.3 ± 4	0.80
Male n (%)		8 (80)	17 (85)	
Arterial Blood gas (Air)	Ph	7.415 ± 0.01	7.418 ± 0.03	0.84
	pCO_2 (mmHg)	33.92 ± 5.5	38.31 ± 4.11	0.11
	pO_2 (mmHg)	87.67 ± 18.97	89.87 ± 9.77	0.76
	S lactate (mmol/L)	0.9 ± 0.29	0.98 ± 0.38	0.70
HR Reserve (beats/min)	Air	67.5 ± 19.48	40.6 ± 25.38	0.004*
	Oxygen	62.97 ± 23.87	35.6 ± 24.6	0.01*
Work (max) (W/min)	Air	140.5 ± 32.78	87.5 ± 30.2	0.001*
	Oxygen	142 ± 41.37	86.11 ± 27.73	0.002*
RR Interval (peak) (mm)	Air	443.82 ± 56.25	550.56 ± 162.09	0.01*
	Oxygen	462.06 ± 80.7	566.43 ± 155.48	0.02*
VO_2 Max (mL/min)	Air	1936.3 ± 526.55	1324.1 ± 540.25	0.009*
VO_2/HR	Air	14.27 ± 4	11.97 ± 5.9	0.24
VCO_2 (peak) (mL/min)	Air	2202.9 ± 633.05	1428.84 ± 682.76	0.006*
	Oxygen	2112.3 ± 486.31	1426.77 ± 440.77	0.002*

Values are mean ± SD unless indicated otherwise.

*P<0.05 vs controls.

Cardiac and pulmonary parameters during maximal exercise

Table 4.2 presents the haemodynamic and ventilatory parameters during the incremental stages (rest, 50% peak and peak) of exercise. Results on air and oxygen are analysed individually for each incremental stage.

CHF patients had a significant increase in VE [P=0.001 (air), P=0.001 (oxygen)], VO_2 [P=0.001] and VCO_2 [P=0.001 (air), P=0.001 (oxygen)] during exercise. However, this did not correlate with an improvement in ventilatory efficiency (VE/VCO_2) during the incremental stages of exercise [P = 0.58 (air), P = 0.62 (oxygen)].

The oxygen pulse (VO_2/HR), defined as the amount of oxygen extracted per heart beat did not improve in CHF during increamental stages of exercise (P=0.25).

Effect of oxygen on ventilatory parameters during exercise

Table 4.3 shows the effect on oxygen on ventilatory parameters during maximal exercise testing. Data was analysed at rest, 50% peak exercise and peak exercise. The effect of oxygen was analysed for controls and patients at each of these exercise levels. Oxygen did not influence ventilatory parameters in either patients or controls.

Metabolic parameters

Nasal oxygen supplementation significantly increased arterial oxygen saturation (89 ± 12 vs 129 ± 23, P=0.001) However, serum Ph (7.40 ± 0.02 vs 7.41 ± 0.02, P = 0.11), serum lactate (1 ± 0.32 vs 0.91 ± 0.32, P = 0.25) and plasma CO_2 (37.96 ± 5.35 vs 37.09 ± 4.93, P = 0.21) did not differ (Table 1).

Table 4.2   ANOVA analysis for ventilatory parameters during exercise

Control				
	Rest	Half	Peak	P
Ve/VCO$_2$ (air)	34.94 ± 3.36	29.27 ± 2.2	31.6 ± 3.61	0.02*
Ve/VCO$_2$ (oxy)	34 ± 8.36	28.28 ± 2.36	30.6 ± 2.9	0.06
VO$_2$/Hr	5.71 ± 2.17	11.4 ± 2.18	14.27 ± 4	<0.001*
VCO$_2$ (air)	320.8 ± 109.42	1034.9 ± 169.95	2202.9 ± 633	<0.001*
VCO$_2$ (oxy)	386.7 ± 107	1064 ± 223.62	2112.3 ± 486.31	<0.001*
Ve (air)	11 ± 3.2	30.3 ± 5.35	70 ± 23.71	<0.001*
Ve (oxy)	13.2 ± 3.15	30.4 ±6.75	65 ± 18.09	<0.001*
VO$_2$	391.1 ± 139.57	1206.3 ± 187.44	1936.3 ± 526.55	<0.001*
CHF Patients				
Ve/VCO$_2$ (air)	37.7 ± 8.86	34.85 ± 7.63	35.66 ± 9.6	0.58
Ve/VCO$_2$ (oxy)	36.23 ± 6.36	33.87 ± 6.47	34.94 ± 8.61	0.62
VO$_2$/Hr	8.97 ± 6.14	11.68 ± 5.64	11.97 ± 5.95	0.25
VCO$_2$ (air)	606.89 ± 428.32	923.73 ±417.66	1428.84 ± 682.76	<0.001*
VCO$_2$ (oxy)	522.77 ± 243	845.16 ± 325.78	1426.77 ± 440.77	<0.001*
Ve (air)	20.68 ± 10	30.31 ± 8.47	47.73 ± 16.6	<0.001*
Ve (oxy)	18.44 ± 7.38	27.55 ± 8.8	48.22 ± 14.78	<0.001*
VO$_2$	675.44 ± 430.9	1014 ± 416.3	1324.1 ± 540.25	0.001*

Values are mean ± SD unless indicated otherwise. VE (ml/min) indicates ventilation, VCO$_2$ (ml/min) indicates ventilatory carbon dioxide, VO$_2$ (ml/min) indicates ventilatory oxygen, HR (beats/min) indicates heart rate.

*P<0.05 ANOVA analysis for rest vs half vs peak

Table 4.3   Paired T test for influence of oxygen on ventilatory parameters

	Control		
	Rest	Half	Peak
Ve (air)	11 ± 3.26	30.3 ± 5.35	70 ± 23.71
Ve (oxy)	13.2 ± 3.15	30.4 ± 6.75	65 ± 18
P value	*0.24*	*0.95*	*0.16*
VCO (air)	320.8 ± 109.42	1051.11 ± 171.86	2202.9 ± 633.05
VCO (oxy)	386.7 ± 107	1064 ± 223.62	2112.3 ± 486.31
P Value	*0.27*	*0.86*	*0.40*
Ve/VCO (air)	34.85 ± 3.56	29.15 ± 2.3	31.6 ± 3.61
Ve/VCO (oxy)	34 ± 8.36	28.28 ± 2.36	30.6 ± 2.9
P Value	*0.77*	*0.13*	*0.28*
	CHF Patients		
Ve (air)	19.77 ± 9.47	29.77 ± 8.38	47.66 ± 17
Ve (oxy)	18.44 ± 7.38	27.55 ± 8.85	48.22 ± 14.78
P Value	*0.33*	*0.20*	*0.83*
VCO (air)	584.5 ± 429.14	919 ± 429.26	1441.88 ± 700.12
VCO (oxy)	522.77 ± 243	845.16 ± 325.78	1426.77 440.77
P Value	*0.36*	*0.33*	*0.89*
Ve/VCO (air)	37.79 ± 9.11	34.58 ± 7.75	35.36 ± 9.83
Ve/VCO (oxy)	36.23 ± 6.36	33.87 ± 6.47	34.94 ± 8.61
P Value	*0.27*	*0.52*	*0.72*

Values are mean ± SD unless indicated otherwise. VE (ml/min) indicates ventilation, VCO_2 (ml/min) indicates ventilatory carbon dioxide, VO_2 (ml/min) indicates ventilatory oxygen, HR (beats/min) indicates heart rate.

4.4   Discussion

This study analysed the effects of low flow oxygen on the dynamic cardiopulmonary parameters using CPX testing. Participants identified to have stable heart failure were recruited from the heart failure clinic of a tertiary level hospital.

The main findings of our prospective double blinded study are

- Heart failure patients were characterised by a combination of inefficient ventilation and reduced cardiac capacity. Ventilatory efficiency (VE/VCO$_2$) and oxygen pulse (VO$_2$/HR) failed to improve during incremental exercise.

- Inhaled oxygen did not improve ventilatory efficiency during maximal exercise.

- Cardiovascular and metabolic parameters were not influenced by the oxygen supplementation.

Our study provides a unique insight into cardiopulmonary exercise dynamics in CHF by correlating both pulmonary and cardiac parameters during incremental exercise. Previous studies have demonstrated an abnormally heightened ventilatory rate and minute ventilation during peak exercise in these patients (Sullivan, Higginbotham & Cobb 1988, Buller, Poole-Wilson 1990b, Reddy et al. 1989). Minute ventilation rose disproportionately to CO$_2$ production leading to reduced ventilatory efficiency, which is measured as an increase in ventilatory equivalent for carbon dioxide (Ve/Vco$_2$) (Sullivan, Higginbotham & Cobb 1988, Franciosa et al. 1984, Rajikin 1980). Our findings are in keeping with previous findings and confirm that increasing workloads during exercise is not matched by improved ventilatory efficiency limiting exercise tolerance.

Ventilatory efficiency is an important prognostic marker in patients with CHF (Ingle 2008). Though the exact pathogenesis is unclear, various abnormalities are believed

to contribute to ventilatory inefficiency in CHF. Structural pulmonary vascular changes, abnormalities of the alveolar membrane (Puri et al. 1994), altered ventilatory drive by stimulation of peripheral muscular ergoreceptors (Clark, Coats 1994), and ventilation-perfusion mismatch (Buller, Poole-Wilson 1990b, Uren et al. 1993) are reported to be contributing factors.

Our study also demonstrates a lack of increase in the mean oxygen pulse during incremental exercise. The oxygen pulse (VO_2/HR) reflects the amount of oxygen extracted per heart beat. It provides an indirect assessment of the stroke volume during exercise (Jones 1988, Wasserman, Hansen & Sue 2005b). Our findings are in agreement with studies by Sullivan et al who demonstrated that peak Ve/Vco2 is inversely correlated to cardiac output in heart failure (Sullivan, Higginbotham & Cobb 1988). During exercise reduced cardiac output worsens ventilation perfusion mismatch contributing to ventilatory inefficiency.

Controversy exists over the benefit of nasal oxygen therapy in CHF. Nasal oxygen therapy is shown to improve exercise performance and prevent progression of symptoms in chronic heart failure (Shigemitsu et al. 2007, Paul, Joseph & De Pasquale 2008, Kojima et al. 2001, Sasayama et al. 2006, Toyama et al. 2009). Studies by Russell et al, however, did not demonstrate an improvement in exercise capacity with its use in patients with stable heart failure. The influence of oxygen on haemodynamic and ventilatory parameters during exercise is unknown. In our study oxygen supplementation did not improve ventilatory efficiency during exercise. Oxygen is known to reduce pulmonary artery pressure and increase right ventricular ejection fraction during exercise (Olvey et al. 1980, Pitton et al. 1998, Wright et al. 1983). This improvement is likely to reduce the elevated alveolar capillary

membrane resistance seen in patients with heart failure (Puri et al. 1994). However, in our study this previously demonstrated benefit did not translate to an improvement in ventilatory efficiency at peak exercise.

4.5 Study Limitations

Our study aimed to study the influence of oxygen on cardiopulmonary exercise parameters in CHF. However, further studies are required to analyse the influence of oxygen on capillary permeability and ventilation perfusion mismatch. Our study did not analyse the peripheral effects of oxygen during peak exercise. Further studies are required to study the influence of oxygen on this important afferent limb.

CHAPTER 5

ACUTE EFFECTS OF 5-METHYLTETRAHYDROFOLATE ON ARTERIAL WAVE REFLECTION AND ENDOTHELIAL FUNCTION IN PATIENTS WITH CHRONIC HEART FAILURE

5.1   Introduction

The beneficial effect of folic acid on endothelial function in pro-atherosclerotic conditions is well estalished. Both intravenous and oral folic acid administration leads to an acute and chronic improvement in endothelium-dependent vasodilatation in vascular disease states such as diabetes, coronary artery disease and hypercholesterolemia (Mangoni et al. 2005, Chambers et al. 2000, Mangoni et al. 2002, van Etten et al. 2002). The beneficial effects of folic acid on endothelial function are predominantly mediated by its direct effects on the enzyme endothelium nitric oxide synthase (eNOS) (Doshi et al. 2002, Hyndman et al. 2002).

Impairment of endothelium-dependent vasodilatation is a common feature in patients with chronic heart failure (CHF) (Katz et al. 1993, Morgan et al. 2004, Nakamura et al. 2004, Sakane et al. 2000) This abnormality is evident in the peripheral as well as in the coronary and pulmonary circulation (Elkayam et al. 2002, Takahashi et al. 2005) It results from impaired endothelial release of nitric oxide, abnormal production of cyclooxygenase-dependent vasoconstricting factor and decreased vascular smooth muscle responsiveness to cyclic GMP-mediated vasodilatation. Endothelial dysfunction is characteristic of progression of atherosclerosis (Gonzalez, Selwyn 2003). Furthermore, it has major clinical implications as it is independently associated with increased cardiovascular mortality in CHF (Katz et al. 2005).

An improvement in endothelial function in pro-atherosclerotic conditions is associated with improved survival (Gupta 2004, Kiliszek et al. 2007, Wilkinson, McEniery 2004). This highlights the significance of mediators that influence endothelium-dependent vasodilatation.

Nitric oxide (NO), an endogenous vasodilator produced in the endothelium by the enzyme nitric oxide synthase (eNOS), is the chief modulator of endothelial function and arterial stiffness in humans (Wilkinson et al. 2002). In pro-atherosclerotic conditions eNOS production shifts from NO to superoxide production due to the relative deficiency of tetrahydrobiopterin (BH4) in endothelial cells (eNOS 'uncoupling') (Pritchard et al. 1995). The superoxides produced by the uncoupled eNOS degrade endothelial NO and lead to vascular injury and inflammation (Celermajer et al. 1993c, Johnstone et al. 1993, Stroes et al. 1995). Folic acid has been shown to interact directly with eNOS to enhance NO production in vascular disease states by;

1) replacing the natural cofactor tetrahydrobiopterin (BH4) and

2) actively scavenging superoxides and thus preventing vascular damage (Hyndman et al. 2002, Shirodaria et al. 2007). However, the effects of folic acid on endothelial function in CHF are largely unknown.

Our study tested the hypothesis that the acute intravenous administration of 5-methyltetrahydrofolate (5-MTHF), the metabolically active form of folic acid, enhances endothelial function and reduces arterial wave reflections in CHF patients by NO-dependent mechanisms.

We also tested the influence of 5-MTHF on serum asymmetrical dimethylarginine (ADMA) and symmetrical dimethylarginine (SDMA), known inhibitors of nitric oxide. ADMA is known to be an independent risk factors in the progression of atherosclerotic disease (Hov et al. 2007, Busch et al. 2006).

Role of ADMA

Traditional risk factors do not help explain the onset of cardiovascular events in a large cohort of patients (Zoccali 2000). Attention was drawn to endogenous pro-atherosclerotic substances to help explain the onset in these patients. The discovery of N monomethly-L-arginine (L-NMMA) and asymmetrical dimethylarginine (ADMA), both L arginine analogs which selectively inhibit NO synthesis, heralded the role of endogenous pro-atherosclerotic agents. Vallence et al first described these guanidino-substituted analogs of L-arginine to be endogenous inhibitors of NO (Vallance et al. 1992). Elevated levels of L-NMMA and ADMA result in vasoconstriction and platelet activation and thus forming the major contributor. Unlike ADMA its regioisomer symmetric dimethylartheginine (SDMA) does not inhibit NO.
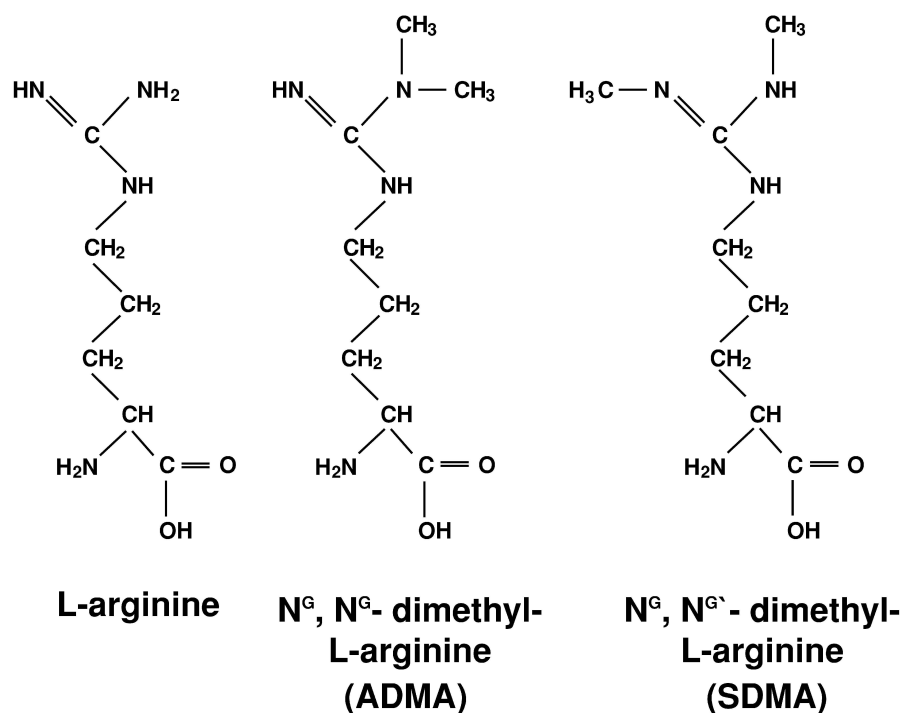
Figure 5.1   Chemical structures of L-arginine, asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA)

L-Arginine is the natural substrate for NO synthase, ADMA is a competitive inhibitor of NO synthase, whereas SDMA is biologically.

An understanding of the biosynthesis and excretion of ADMA is essential to understand its role in NO inhibition. Both ADMA and SDMA are produced during the degradation of methylated proteins (Boger 2003). These proteins undergo proteolytic breakdown to release ADMA and SDMA into plasma. However, proteolytic breakdown of methylated proteins is not the only source of dimethylarginine. They are also produced in the endothelial cells where they act as an autocrine regulator for NO synthesis (MacAllister et al. 1996). This synthesis of ADMA in endothelial cells is accelerated in the presence of LDL cholesterol further adding to vascular injury (Ito et al. 1999).

Both ADMA and SDMA are excreted by the kidneys. Their levels are elevated in chronic renal failure; forming part of the syndrome of uraemia. This was first

demonstrated by Vallance et al in nine patients with end stage renal disease (Vallance et al. 1992). ADMA contributes to the characteristic endothelial dysfunction seen in patients with end stage renal disease. Haemodialysis eliminates these toxins from the blood and concomitantly improves endothelial function but peritoneal dialysis is not shown to aid in its excretion (Cross et al. 2001).

In addition ADMA is also metabolised by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). In the presence of DDAH, ADMA undergoes hydrolytic degradation to form citrulline and dimethylamine. Hence DDAH plays a vital role in the metabolism of ADMA. Elevated levels of serum homocysteine depress the DDAH activity leading to increased ADMA levels (Stuhlinger et al. 2001). Ito et al demonstrated that oxyLDL and TNF-α depress DDAH activity without affecting its protein expression (Ito et al. 1999). Nitric oxide production in endothelial cells is increased by all-trans retinoic acid. This is primarily medicated by inducing DDAH (Achan et al. 2002). Two isoforms of DDAH exist. DDAH-1 is present in neuronal tissue while DDAH-2 is chiefly present in endothelial cells (Leiper et al. 1999).

5.2   Methods

Twenty two CHF patients (70±9, M:F 16:6) and 22 healthy controls (53±18, M:F 11:11) were prospectively enrolled into the study. CHF was confirmed by a cardiologist using the Framingham heart failure guidelines. Patients with well controlled NYHA class 2 symptoms and an ejection fraction<40 on 2-D echocardiography within the preceding 12 months were included. Patients with moderate-severe chronic renal failure (GFR<30ml/min), uncontrolled diabetes

(HBA$_1$C>7) and uncontrolled hypercholesterolemia (LDL-C>3.5mmol/L) were excluded from the trial.

The study was approved by the local ethics committee and consent was obtained from all patients prior to the commencement of the study.

5.3   Study protocol

All experiments were performed in a quiet room kept at a constant temperature of 22°C to 24.5°C. Patient refrained from caffeine on the day of the study. None of the study subjects were active smokers. Beta-blockers were held on the day of the study. Two intravenous cannulas were placed, one on each arm. The first cannula was used for treatment/administration whereas the second cannula was used for blood sampling.

After a 20-min resting period in supine position CHF patients and control subjects were allocated to one of the following 3-step treatments (1 hour each, 3 hours total) in a parallel group, randomised, placebo-control trial 1) active treatment [N saline (1 mL/min), 5-MTHF (1μg/min) (Merck Eprova, Schaffhausen, Switzerland), and 5-MTHF (1μg/min) + the eNOS inhibitor L-NMMA (400 μg/min)]; and 2) placebo [N saline (1ml/min) x 3]. The allocation sequence was generated by software located in the Pharmacy Department of our institution. Both the investigators and the subjects were blinded to the treatment.

Arterial wave reflection and endothelial function

Pulse wave analysis (PWA) was used to measure arterial wave reflection and endothelial function as described in section 2.2.A. Pressure waves were non-invasively recorded from the radial artery using the SphygmoCor device (AtCor

Medical, Sydney, Australia) as previously described by Ronnback et. Al (Ronnback et al. 2007). The radial artery was flattened (applanated) against a rigid underlying bone using a tonometer to obtain a digital recording of the radially directed intra arterial pressure waveform. A generalised transfer function was used to generate a corresponding central (ascending aortic) waveform. The augmentation index (AIx) was calculated as the augmentation of the aortic systolic blood pressure by the reflected pulse wave, expressed as a percentage of the aortic pulse pressure. AIx was adjusted for a standard heart rate of 75 beats/min in order to minimize the influence of heart rate Aix (Wilkinson et al. 2000) Only high quality readings, defined as a quality index > 80% were included in the analysis. An average of 5 recordings was taken for each reading. This technique is shown to be highly reproducible for repeated studies (Papaioannou et al. 2007).

Endothelial function was studied using PWA according to an established protocol (Wilkinson et al. 2002). Briefly, endothelium-dependent and -independent vasodilatation was assessed by measuring the maximum changes in AIx during a 20-min period following the administration of inhaled salbutamol (400μg) via a spacer and sublingual nitroglycerin (GTN) (150μg), respectively. Salbutamol and nitro-glycerin were administered after 20 and 40 minutes respectively during each infusion.

Haemodynamic parameters

Systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP), and heart rate (HR) were measured by using an automatic device (DinamapTM, DRE Inc, Louisville, KY, USA).

Biochemical parameters

Serum folate

Serum folate was measured by automated competitive protein-binding chemiluminescence assay on a Modulator Analytic E170 analyser using instrument conditions and reagents supplied by the manufacturer (Roche Diagnostics, Mannheim, Germany). The between run CV of this method was 9.6% at a value of 8.1 nmol/L (n=256) and 6.1% at a value of 20.3 nmol/L (n=249).

Homocysteine (Hcy)

Plasma samples were prepared for reduction and derivatisation as previously described by Ubbink and colleagues (Ubbink, Hayward Vermaak & Bissbort 1991). High performance liquid chromatography (HPLC) was employed for the assay of Hcy using Primus PPQ auto-analyser (Primus® Diagnostics, Kansas City, MO). The fluorescence spectrophotometer was operated at an excitation wavelength of 385 nm and an emission wavelength of 515 nm. This method had a between run CV of 8.7% at a value of 9.2 μmol/L (n=437) and 5.3% at a value of 18.4 umol/L (n=432).

Arginine, asymmetrical dimethylarginine, symmetrical dimethylarginine, and N^G-monomethyl-L-arginine.

L-arginine and its mono- and di-methylated forms (L-NMMA, ADMA, and SDMA) were measured in serum by liquid chromatography - tandem mass spectrometry of the butyl esters on an API-Sciex 3200 Q-trap instrument (Applied Biosystems Asia Pacific, Bangkok, Thailand) (Schwedhelm et al. 2005). Deuterated internal standards (98 atom% 2H isotopic purity) were purchased from Cambridge Isotope Laboratories (Andover, MA), and were L-[2H7]-arginine for arginine quantitation and 2,3,3,4,4,5,5-2H7-ADMA for ADMA, SDMA, and L-NMMA analyses. In 6 analytical runs on different days, the between run CV for arginine, ADMA, SDMA

and L-NMMA were 3.3, 4.0, 11.9 and 32.3% at levels of 102, 0.48, 0.50 and 0.07 μmol/L respectively.

Statistical analysis

Data is presented as means ± SD and ratios. Comparison of baseline clinical characteristics between the CHF patients and controls was performed using chi-square test for categorical data and *t* tests for continuous data. ANOVA for repeated measurements was used to test the hypothesis that folic acid and L-NMMA had a significant influence on vascular function and biochemical parameters. Statistical analysis was conducted using SPSS for Windows 14.0 (SPSS Inc, Chicago, IL, USA). A P value of ≤0.05 was considered to be statistically significant.

5.4   Results

Baseline clinical and biochemical characteristics

Table 1 described the baseline clinical characteristics of healthy controls and CHF patients. Although total cholesterol did not differ between the two groups, CHF patients had a significantly lower LDL-C (P=0.04). Blood sugar level (P<0.01), serum creatinine (P<0.01), serum homocysteine (P<0.01), S ADMA (P=0.03) and S SDMA (P<0.01) levels were all significantly elevated in CHF patients. However, serum folate (P=0.67) and ARG (P=0.75) did not differ between the two groups.

Baseline arterial function and haemodynamics

Baseline vascular characteristics are described in Table 1. Heart rate (P=0.81), systolic blood pressure (P=0.27), diastolic blood pressure (P=0.68) and augmentation index (P=0.71) did not differ between the CHF patients and healthy controls. However, the ejection duration was significantly lower in CHF patients (308±30) than among healthy controls (339±18) P<0.01.

Salbutamol-mediated endothelial dependent vasodilatation although lower in patients with CHF (-5.6±9%) than among controls (-9±9%), did not differ significantly. (P=0.17). However, the GTN mediated endothelial independent response was significantly attenuated in patients with CHF patients (-23±8%) than amongst healthy controls (17±8%) P=0.02 .

Table 5.1   Clinical and biochemical characteristics when classified by healthy controls and CHF patients

	Healthy Controls	CHF Patients	P value
Systolic Blood Pressure (mmHG)	129±18	123±20	0.27
Diastolic Blood Pressure (mmHg)	67±10	66±12	0.68
Heart Rate (beats/min)	63±8	63±9	0.81
Augmentation Index (%)	22±9	23±10	0.71
Ejection Duration (msec)	339±17	308±30	<0.01*
Total Cholesterol (mmol/L)	4.8±1	4.2±1	0.08
Triglyceride (mmol/L)	1.7±0.9	1.7±0.6	0.90
HDL-C (mmol/L)	1.5±0.3	1.3±0.6	0.17
LDL-C (mmol/L)	2.3±0.6	1.8±0.7	0.04*
Serum Folate (nmol/L)	30±16	33±19	0.67
Serum B12 (pmol/L)	367±214	285±133	0.16
Serum Creatinine (μmol/L)	71±20	102±30	<0.01*
Homocysteine (μmol/L)	9±3	16±6	<0.01*
CRP (mg/L)	3±2	3±3	0.84
ADMA (μmol/L)	0.6±0.1	0.7±0.1	0.03*
SDMA (μmol/L)	0.5±0.1	0.7±0.2	<0.01*
Arginine (μmol/L)	215±46	210±48	0.75

Values are mean±SD unless otherwise indicated. HDL-C indicates High density lipoprotein cholesterol, LDL-C indicates Low density lipoprotein cholesterol, CRP indicates C - reactive protein, ADMA indicates serum asymmetrical dimethylarginine, SDMA indicates serum symmetrical dimethylarginine.

* Statistical significance at P≤0.05 vs healthy controls

Effects of 5MTHF

Arterial function and haemodynamics

Administration of 5 MTHF lead to a rise in serum folate after one (P=0.06) and two hours (P=0.006). This lead to a concurrent reduction in serum ADMA (P=0.05) but not serum SDMA (P=0.41)

Administration of 5 MTHF lead to a reduction in systolic (P=0.03) and diastolic (P=0.06) blood pressure without affecting the augmentation index (P=0.49). 5 MTHF infusion had no significant influence on endothelial dependent (P=0.95) or endothelial independent vasodilatation (P=0.52)

The mean endothelial dependent response after 5 MTHF was 7±9%, and there was better in healthy control (10±12%) than amongst CHF patients (3±2%) P=0.05. The endothelial independent response did not differ between healthy subjects (17±9%) and CCF patients (12±5%) P=0.11.

Effects of 5MTHF + L-NMMA

Arterial function and haemodynamics

Co-administration of L-NMMA with 5 MTHF did not obliterate the reduction in systolic blood pressure (P=0.02). Augmentation index (P=0.81) and diastolic blood pressure (P=0.43) did not differ between the two groups.

Endothelial dependent (P=0.22) and endothelial independent (P=0.81) vasodilatation did not differ after co-administration of L-NMMA.

Co-administration of L-NMMA lead to a lower endothelial dependent vasodilatation in CHF patients than amongst the healthy subjects (3±5% vs. 9±10%, P=0.06).

However, endothelial independent vasodilatation did not differ between the two groups. (14±7% vs. 18±7%, P=0.18)

Biochemistry

Co-administration of L-NMMA did not significantly affect the reduction in ADMA (P=0.03).
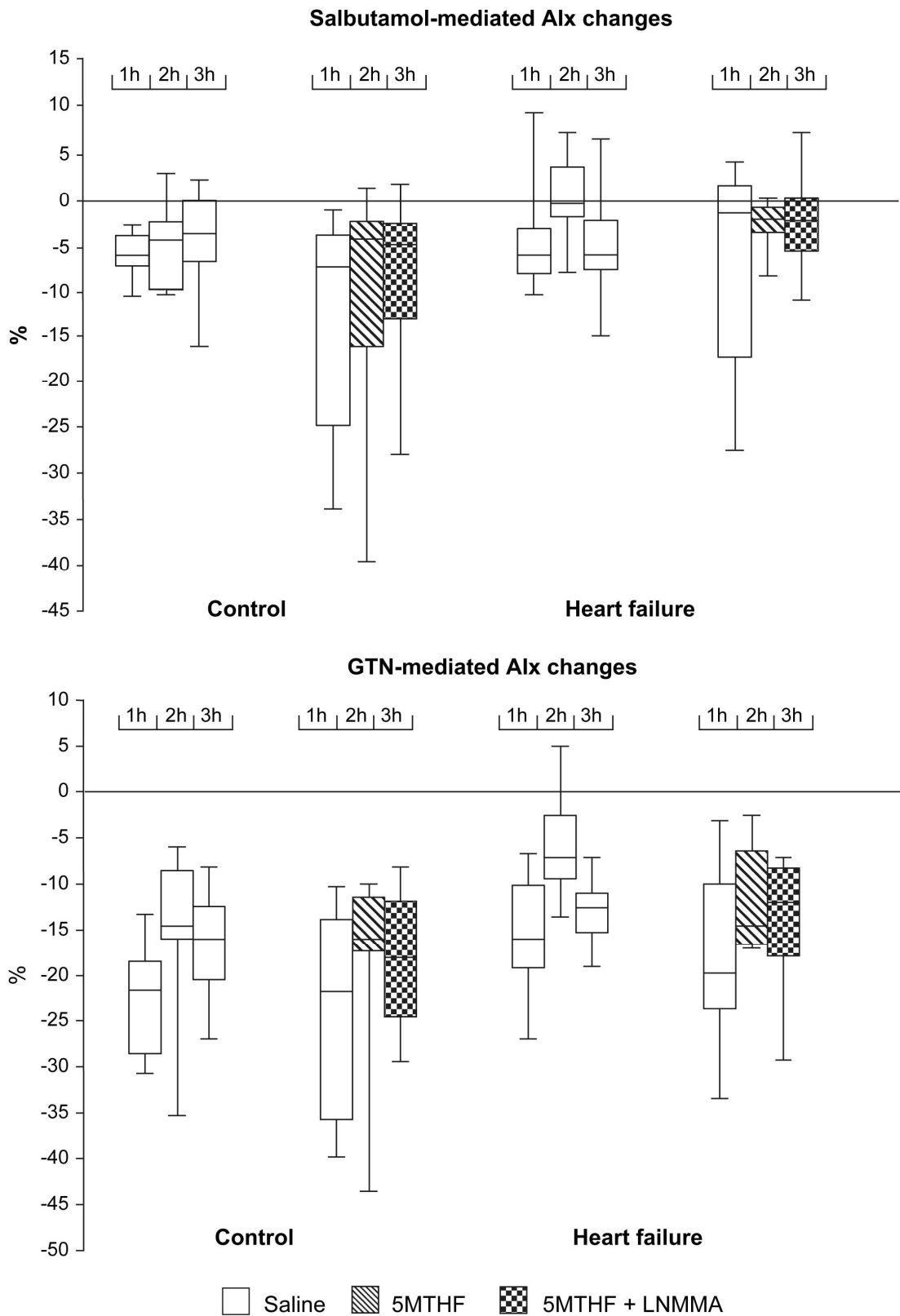
Figure 5.2 **Salbutamol (endothelial dependent) and GTN (endothelial independent) mediated changes in augmentation index when characterised by healthy controls and CHF patients receiving intervention and placebo**
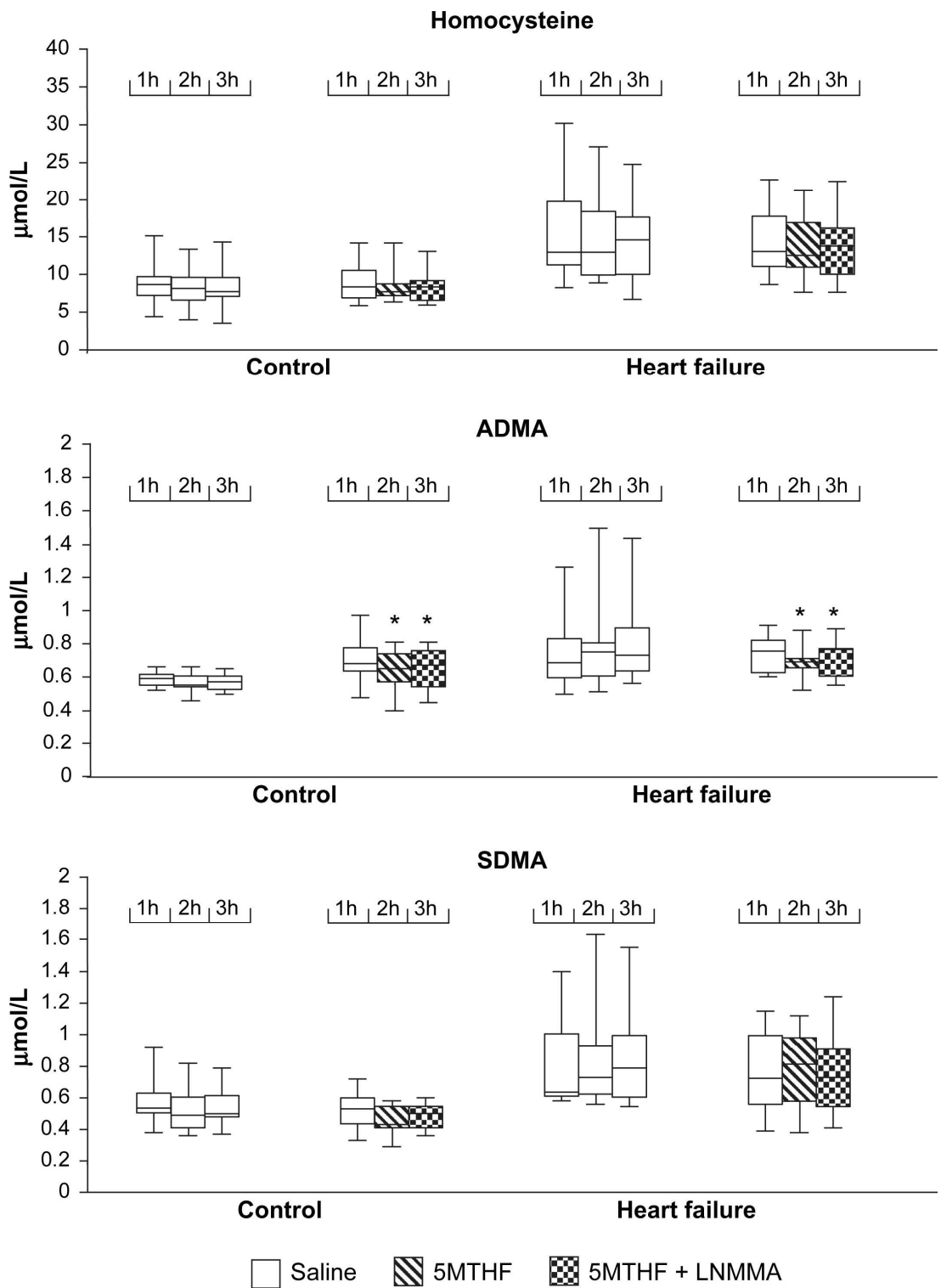
Figure 5.3 **Changes in homocysteine, ADMA, SDMA levels when characterised by healthy controls and CHF patients receiving intervention and placebo**

5.5   Discussion

The chief findings of this prospective randomised study are:

- Administration of 5-MTHF, the active form of folic acid lead to a significant reduction in systolic and diastolic blood pressure without influencing the endothelial dependent and independent vasodilatation.

- 5-MTHF leads to a significant reduction in serum ADMA, which was not influenced by co-administration of L-NMMA.

Folic acid ($C_{19}H_{19}N_7O_6$) is a water-soluble B vitamin. 5-MTHF, the circulating form of folic acid has a beneficial effect on endothelial function in pro-atherosclerotic conditions. However, its vascular effects in CHF a condition characterized by end stage vascular dysfunction, is unknown. Our study adds significant clinical insight into the acute vascular and biochemical effects of folic acid in CHF.

Vascular function as assessed by augmentation index, endothelial dependent and independent vasodilatation did not differ significantly between the CHF patients and healthy controls. This is in contrast to previous studies that have indicated an abnormal vascular function in patients with CHF (Morgan et al. 2004, Elkayam et al. 2002, Katz et al. 2005). Pharmacological interventions are known to significantly improve vascular function in CHF (Jeserich et al. 1995). Patients recruited to our study were optimally controlled for cardiac function and serum cholesterol levels. This may have contributed to the normal baseline vascular function in these patients.

Administration of 5MTHF lead to a significant rise in serum folate levels (P=0.006). This was associated with a significant reduction in systolic and diastolic blood pressure. Though the blood pressure lowering effects of folic acid have been

previously demonstrated in healthy controls and high risk subjects (Williams et al. 2005, Schutte et al. 2004, van Dijk et al. 2001); our study demonstrates similar vascular effects in patients with CHF. This effect may help reduce cardiac afterload and ventricular strain in these patients (Kawaguchi et al. 2003). However, a recent study did not demonstrate a beneficial effect of folic acid supplementation on plasma NT pro-Brain Natriuretic Peptide and severity of CHF (Herrmann et al. 2007)

Vascular function as assessed by endothelial dependent and independent vasodilatation was not influenced by the acute administration of folic acid. This is in keeping with previous studies which did not show a beneficial effect of folic acid in end stage vascular conditions. In the elderly low dose folic acid did not lead to an improvement in vascular function (Carlsson et al. 2004). Similarly, low dose folic acid supplementation in patients with renal failure and heart failure associated with hyperhomocysteinemia did not lead to a significant improvement in vascular function (Carlsson et al. 2004, De Vriese et al. 2002). Our data confirms that acute supplementation of high-dose folic acid dose not improve vascular function in CHF. This suggests that the vascular changes in CHF are predominantly irreversible and unaffected by the acute intervention. However, we cannot exclude the possibility that optimal pharmacological management of these patients may have masked the benefits of the intervention. Age is linked to both vascular function and cardiovascular structure and arterial compliance is not altered by folic acid in these patients (Lind et al. 1999).

ADMA plays an important role in arterial compliance by inhibiting NO synthesis. It may play an important role in the progression of atherosclerosis and increase the risk of cardiovascular events (Cooke 2004). Plasma ADMA levels are independent of

serum Hcy levels (Doshi et al. 2005). Pharmacotherapy with metformin and rosiglitazone in diabetics and angiotensin converting-enzyme inhibitor or angiotensin receptor antagonist in hypertensives have shown to reduce plasma ADMA levels and have a beneficial effect on disease progression (Asagami et al. 2002, Delles et al. 2002). Our data suggests that folic acid significantly reduces ADMA levels in CHF. Further studies are required to evaluate its effects on disease progression.

In conclusion, the present data suggests that acute supplementation of high dose folic acid does not improve vascular function in CHF. However, it reduces plasma ADMA levels and may have long term beneficial effects in CHF patients.

CHAPTER 6

ANALYSIS OF SHORT-TERM REPRODUCIBILITY OF ARTERIAL VASOREACTIVITY BY PULSE-WAVE ANALYSIS AFTER PHARMACOLOGICAL CHALLENGE

6.1   Introduction

The pulse-wave analysis (PWA) technique is used extensively for the assessment of arterial wave reflections and the indirect assessment of central blood pressure in humans (O'Rourke, Pauca & Jiang 2001). Pulse-wave analysis has been used successfully in both epidemiological and interventional studies (London et al. 2001, Weber et al. 2005, Chirinos et al. 2005, Mullan et al. 2004, Nurnberger et al. 2002, Smith et al. 2002, Williams et al. 2006). More recently, the publication of studies demonstrating the tonic control of the endothelium on arterial stiffness has expanded the use of PWA to the assessment of arterial vasoreactivity. Hayward *et al*. (Hayward et al. 2002) and Wilkinson *et al*. (Wilkinson et al. 2002) examined the effects of inhaled salbutamol, an endothelium-dependent vasodilator enhancing nitric oxide (NO) production, and sublingual glyceryl trinitrate (GTN), an endothelium-independent vasodilator, on radial artery waveforms and the augmentation index (AIx; calculated as the difference between the second and first systolic peaks, expressed as a percentage of the pulse pressure). The salbutamol-mediated changes in AIx correlated well the changes in forearm arterial blood flow during infusion of acetylcholine and were abated by co administration of the endothelial NO synthase inhibitor *N*G-monomethyl-l-arginine, suggesting the validity of this methodological approach to assess endothelial function in humans.(Wilkinson et al. 2002, Hayward et al. 2002)

Any valid technique used for the measurement of physiological parameters must also be reproducible and repeatable. A high hour to hour, day-to-day and week-to-week reproducibility of baseline AIx has been reported in both healthy controls and patients with vascular disease states (Papaioannou et al. 2007, Wilkinson et al. 2002, Filipovsky, Svobodova & Pecen 2000, Papaioannou et al. 2004, Savage et al. 2002, Siebenhofer et al. 1999, Wilkinson et al. 1998, Frimodt-Moller et al. 2008, Liang et al. 1998). However, the reproducibility of the effects of GTN and salbutamol on AIx, using appropriate statistical methods, is largely unknown. The assessment of endothelial function by PWA is theoretically appealing because it provides a simple and non-invasive tool to assess endothelial function. This could be useful in acute studies examining the effects of pharmacological agents, similar to other established techniques such as venous occlusion plethysmography (Wilkinson, Webb 2001). The aim of the present study was to assess the short-term reproducibility of GTN- and salbutamol-mediated changes in AIx in healthy controls and in patients with cardiovascular disease. Specifically, we sought to address whether:

1) the reproducibility of changes in AIx following pharmacological challenge is as good as baseline AIx; and

2) there are any differences in reproducibility between healthy and vascular disease states.

6.2   Methods

Population

Reproducibility was assessed in 22 healthy subjects (mean (± SD) age 52 ± 13.4 years; 9 men, 13 women) and 11 elderly patients with stable mild–moderate chronic heart failure (CHF; mean age 73.1 ± 8.7 years; two men, nine women). Healthy

subjects had normal blood pressure (< 140/90 mmHg), no history of diabetes, obesity or renal disease and no family history of ischaemic heart disease. The CHF patients had well controlled New York Heart Association class II symptoms and ejection fraction < 40% on two-dimensional echocardiography within the preceding 12 months. None of the CHF patients had moderate–severe chronic kidney disease (glomerular filtration rate < 30 mL/min), uncontrolled diabetes (glycosylated haemoglobin < 7%) or uncontrolled hypercholesterolaemia (low-density lipoprotein–cholesterol < 3.5 mmol/L). Medication use among CHF patients included beta-blockers (64%), angiotensin-converting enzyme inhibitors (72%), angiotensin receptor blockers (24%), statins (52%), diuretics (64%) and nitrates (16%). None of the study subjects was an active smoker, had a history of alcohol abuse or was receiving vitamin supplementation. The study was approved by the local ethics committee and consent was obtained from all patients prior to the commencement of the study.

Table 6.1   Baseline measurements of repeated vascular and haemodynamic assessments in the two study groups

	Controls (n=22)			CHF (n=11)		
	First hour	Second hour	P value	First hour	Second hour	P value
SBP (mmHg)	126 ± 17	120 ± 17	0.02*	113 ± 12	119 ± 16	0.04*
Δ SBP GTN (mmHg)	-5 ± 10	-2 ± 12	0.21	-4 ± 7	-3 ± 10	0.89
Δ SBP salbutamol (mmHg)	-6 ± 9	0 ± 11	0.11	0 ± 6	-2 ± 5	0.35
DBP (mmHg)	69 ± 8	65 ± 7	0.06*	61 ± 12	64 ± 11	0.10
Δ DBP GTN (mmHg)	-3 ± 6	-5 ± 8	0.54	-1 ± 7	-2 ± 7	0.70
Δ DBP salbutamol (mmHg)	0 ± 7	-1 ± 6	0.74	0 ± 9	-1 ± 6	0.56
HR (b.p.m.)	63 ± 10	61 ± 9	0.04	63 ± 12	62 ± 14	0.37
Δ HR GTN (b.p.m.)	3 ± 3	4 ± 4	0.11	1 ± 5	-1 ± 5	0.34
Δ HR salbutamol (b.p.m.)	0 ± 6	1 ± 3	0.46	0 ± 2	0 ± 3	0.63
AIx (%)	12.7 ± 11.9	13 ± 11.3	0.80	12.1 ± 10.9	15.3 ± 9	0.06
Δ AIx GTN (%)	-19.6 ± 8.8	-18.6 ± 7.2	0.55	-6.2 ± 5.6	-12.9 ± 3.1	0.006*
Δ AIx salbutamol (%)	-7.1 ± 3.7	-5.4 ± 6.9	0.29	-0.1± 4.6	-5.6 ± 5.8	0.06

*Bonferroni adjusted P value vs first hour in the same category

CHF, chronic heart failure; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; AIx, augmentation index; GTN, glyceryl trinitrate.

6.3   Study protocol

Subjects were studied in the morning after an overnight fast. Studies were conducted in a quite, temperature-controlled room (22–24ºC). Beta-blockers and vasodilators were withheld on the day of the study in CHF patients. Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were measured by using an automatic device (Dinamap; DRE, Louisville, KY, USA). Subjects rested supine for 20 min before vascular and haemodynamic assessments. PWA and

haemodyanmic assessments were measured for 20 minutes after the administration of both salbutamol and GTN. The patients rested for a further 40 minutes before assessments were repeated. Both PWA and haemodynamic assessments were repeated after 1 h by the same investigator.

Pulse-wave analysis

Arterial wave reflections and endothelial function were assessed by PWA from the radial artery using a SphygmoCor device (AtCor Medical, West Ryde, NSW, Australia) (Wilkinson et al. 2002, O'Rourke, Pauca & Jiang 2001). An average composite radial waveform was obtained with a high-fidelity applanation tonometer. The pressure calibration of the tonometer was performed by assuming that the SBP and DBP measured by the device are equal to the SBP and DBP measured from the brachial artery, in agreement with previous studies using this method (Wilkinson et al. 2002). The central aortic pressure wave was derived using a validated transfer function algorithm (Karamanoglu et al. 1993, Wilkinson et al. 2002, O'Rourke, Pauca & Jiang 2001). The AIx, a measure of the effect of wave reflection on the second systolic peak (i.e. a measure of the additional load to which the left ventricle is subject as a result of wave reflection), was calculated as the increment in pressure from the first shoulder in the ascending aortic pressure wave to the peak of this wave, expressed as a percentage of the peak ascending aortic pressure wave (Wilkinson et al. 2002, O'Rourke, Pauca & Jiang 2001). The AIx was adjusted for a standard heart rate of 75 b.p.m. in all measurements (AIx@75) (Gatzka et al. 2001). Baseline AIx, measured during the last 5 min of resting conditions, was obtained by averaging five repeated measurements. Then, endothelium independent and dependent vasodilatation was assessed by measuring the maximum changes in AIx during a 20 min period following the administration of sublingual GTN (150 μg) and inhaled

salbutamol (400 µg via a spacer), respectively, according to an established protocol (Wilkinson et al. 2002). The AIx was measured every 2 min during the 20 min period following pharmacological challenge. Only high-quality readings, defined as a quality index > 80%, were included in the analysis. Both GTN and salbutamol induce a significant reduction in AIx in healthy subjects (Wilkinson et al. 2002). The dose of GTN used in the present study was smaller than that used in other studies because we observed symptomatic hypotension in some CHF patients in preliminary studies. Brachial artery SBP and DBP were also measured every 2 min in order to allow synchronized calibration with PWA measurements.

Statistical analysis

Data are expressed as the mean ± SD. Differences between two repeated measurements were assessed by paired t-test or Wilcoxon signed-rank test. A Bonferroni correction was applied for the two comparisons (control vs CHF group) performed for each variable when the original P was ≤ 0.05. A two-sided $P ≤ 0.05$ after Bonferroni correction indicated statistical significance.

Reproducibility of the measurements was assessed by measuring the intraclass correlation coefficient (ICC), the between-subject coefficient of variation (CV) and the Bland–Altman method. The ICC assesses the reproducibility of measurements by comparing the variability of different measurements within the same subject to the total variation across all measurements and all subjects. The ICC is calculated according to the formula

$$S_b{}^2 / (S_b{}^2 + S_w{}^2)$$

where S_b and S_w are the between- and within-subject standard deviations of the measured variable, respectively. Values > 0.70 indicate substantial reproducibility

(Giraudeau, Mary 2001). The between-subject CV is calculated according to the formula CV = 100 * sw/x, where sw is the standard deviation of the absolute difference of repeated measurements and x is the mean value of measurements within subjects (Bland, Altman 1986). The Bland–Altman method is widely used for validation of reproducibility. The differences between two repeated measures d = (x1 – x2) are plotted against their mean value m = (x1 + x2)/2 (Bland, Altman 1986). We used a paired t-test to test for fixed bias and simple linear regression to test for proportional bias for each of the outcomes. Analysis was performed using spss for Windows version 11.0 (SPSS, Chicago, IL, USA) and Analyse-it Software (Leeds, UK).

6.3 Results

Haemodynamic variables

A significant reduction in SBP and HR between the first and second hours was observed in the control group (Table 1). In contrast, a significant increase in SBP was observed in the CHF group. The changes in blood pressure and HR following pharmacological challenge were similar between the two measurements.

Pulse wave analysis

Absolute bias

No significant differences in baseline AIx or GTN and salbutamol mediated changes in AIx between the first and second hours were observed in the control group (Table 1). Similarly, baseline AIx and salbutamol-mediated changes in AIx did not change significantly in the CHF group. However, there was a statistically significant difference in GTN-mediated changes in AIx in the CHF group.

Intraclass correlation coefficient and CV

The ICC values for baseline AIx were well above 0.85 in both groups, indicating very good reproducibility (Table 6.2). In contrast, the ICC values for GTN and salbutamol mediated changes in Aix were substantially lower, particularly in the CHF group. Calculations of CV yielded similar results, with relatively low CV values for baseline AIx measurements and higher CV values after pharmacological challenge with both GTN and salbutamol (Table 6.2).

Table 6.2   Intraclass correlation coefficients of repeated measurements of augmentation index and its changes following pharmacological challenge with glyceryl trinitrate (endothelium-independent vasodilatation) and salbutamol (endothelium-dependent vasodilatation).

	Controls (n=22)		CHF (n=11)	
	ICC	CV (%)	ICC	CV (%)
Baseline AIx	0.90	25.0	0.87	22.5
Δ AIx GTN	0.58	22.3	0.17	59.8
Δ AIx salbutamol	0.18	45.1	0.04	184.0

CHF, chronic heart failure; ICC, intraclass correlation coefficient; CV, coefficient of variation; GTN, glyceryl trinitrate.

Bland–Altman analysis

Bland–Altman analysis revealed poor reproducibility for changes in AIx following either GTN or salbutamol (Figs 1–3). The limits of agreement were beyond either +10% or −10% for baseline Aix measurements for both control and CHF patients and beyond either +15% or −15% for measurements following GTN and salbutamol for both controls and CHF patients. There was also significant fixed bias for both GTN ($6.8 \pm 5.9\%$; $P = 0.003$) and salbutamol ($5.5 \pm 7.3\%$; $P = 0.03$) measurements among CHF patients, with measurements in the second hour tending to be lower than those in the first. A significant proportional bias existed for the AIx in response to salbutamol among control subjects, with a unit increase in Aix indicating a likely greater difference in the second response compared with the first ($b = 0.94$ (95% confidence interval 0.30, 1.58); $P < 0.006$).
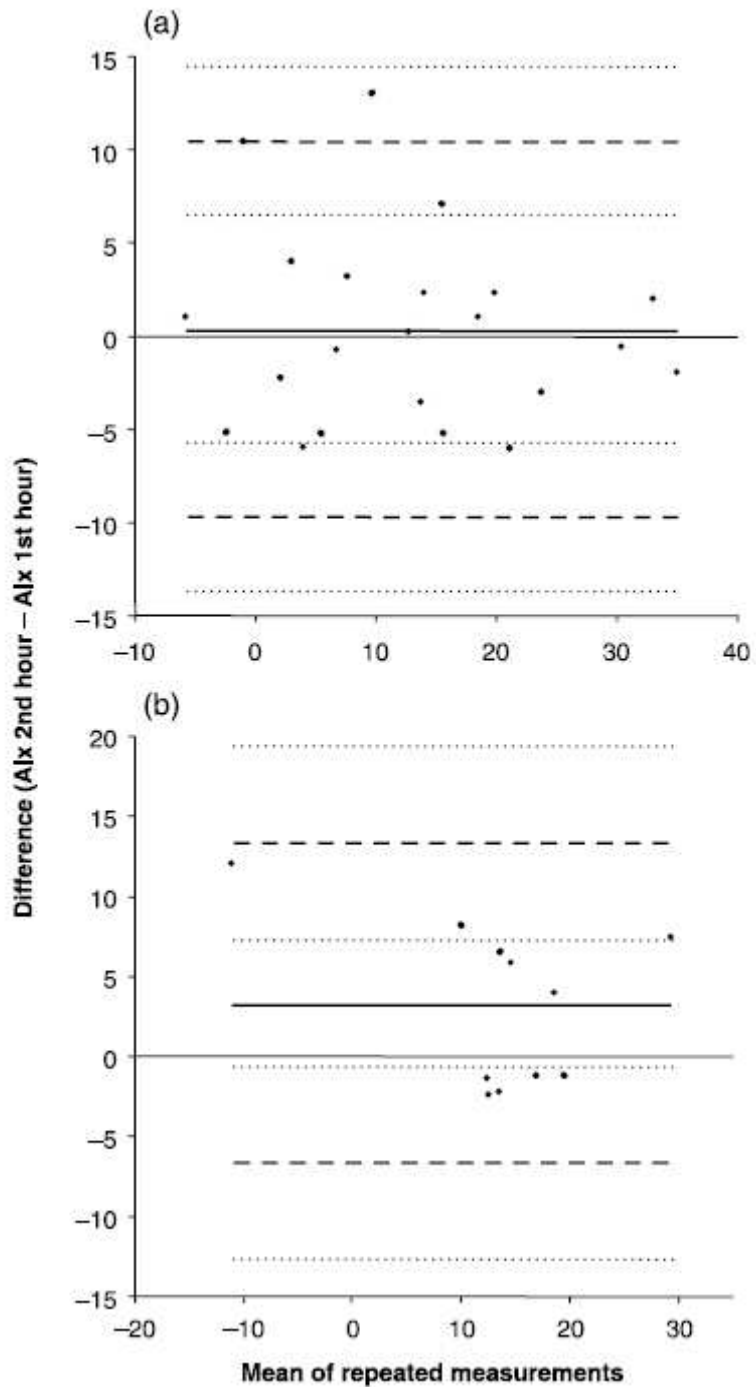
Figure 6.1   Bland- Altman plots of baseline augmentation index (AIx) for hour-to-hour repeated measurements

(____), mean difference; (----), limits of agreement, 95% confidence intervals for limits of agreement.
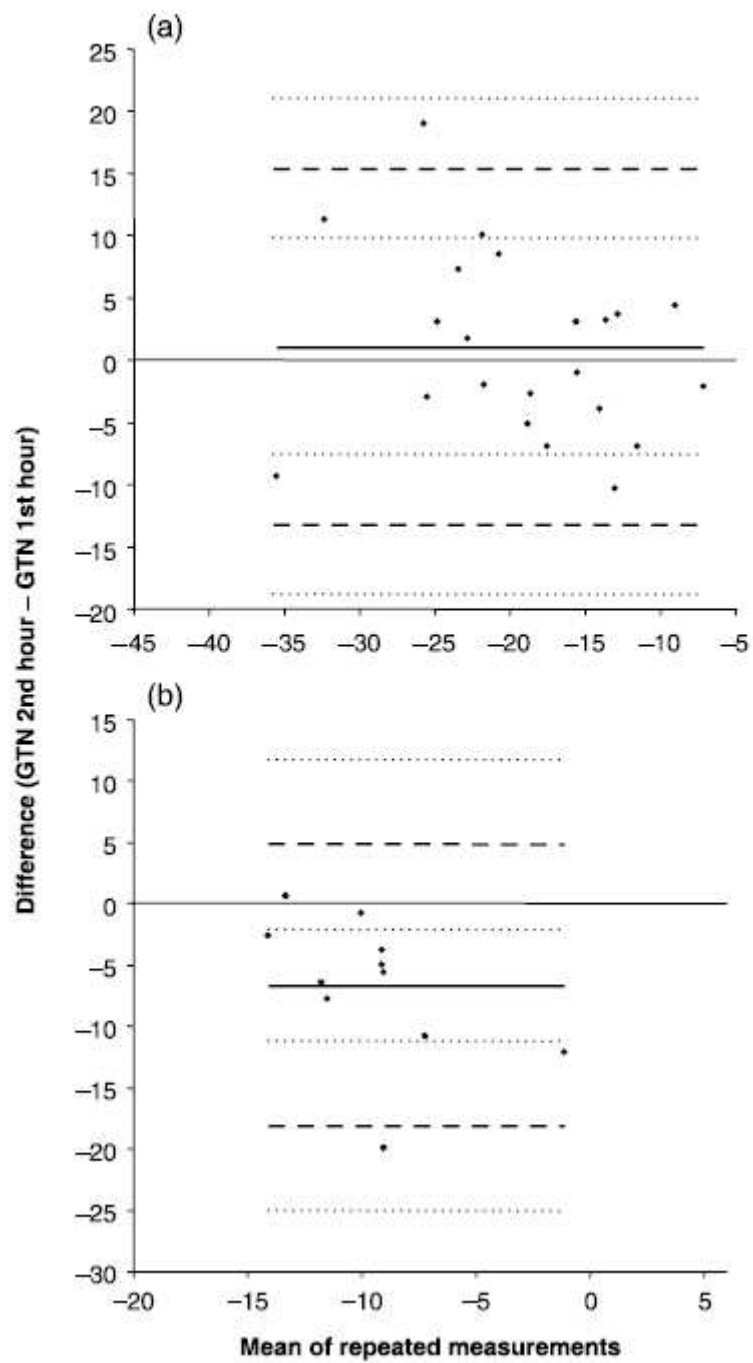
Figure 6.2   Bland- Altman plots of sublingual GTN mediated changes in augmentation index (AIx) for hour-to-hour repeated measurements

(____), mean difference; (----), limits of agreement, 95% confidence intervals for limits of agreement.
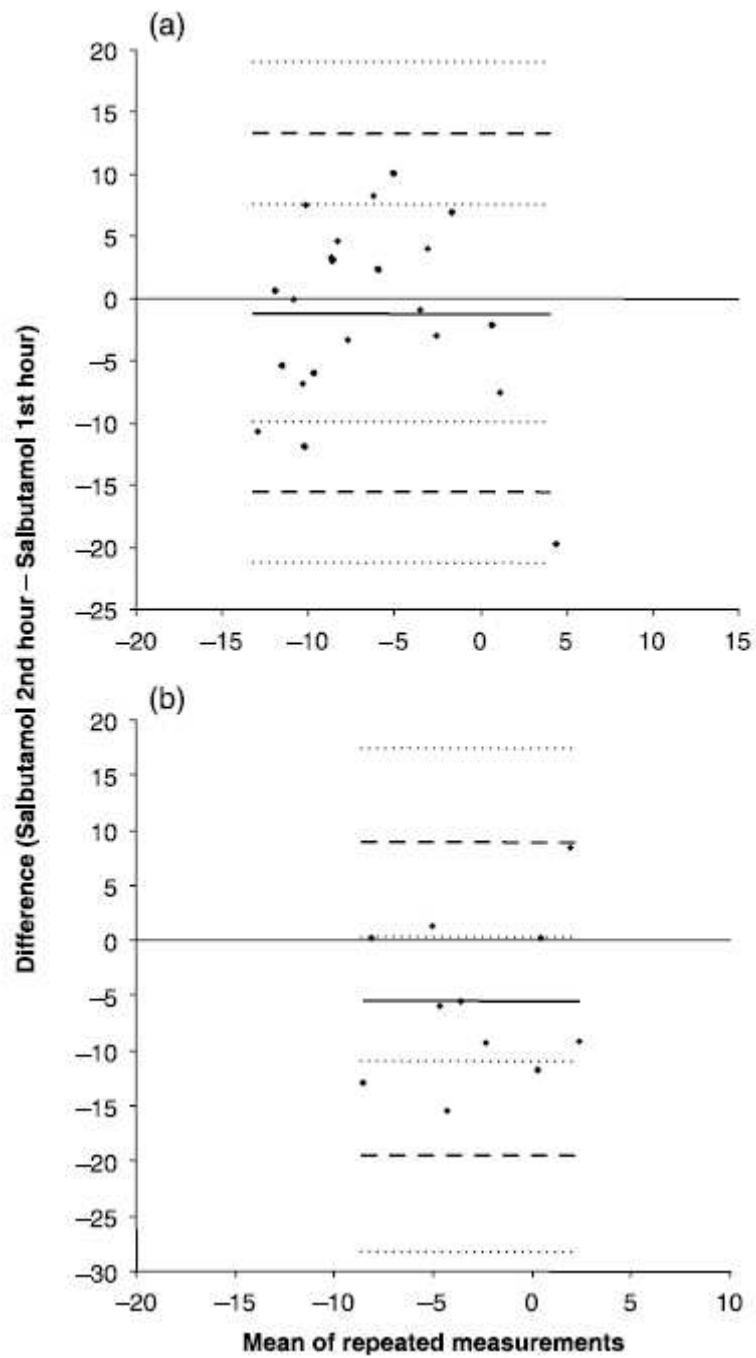
Figure 6.3   **Bland- Altman plots of salbutamol- mediated changes in augmentation index (AIx) for hour-to-hour repeated measurements**

(_____), mean difference; (----), limits of agreement, 95% confidence intervals for limits of agreement.

6.5   Discussion

The results of the present study show a poor short-term reproducibility of arterial vasoreactivity assessments following pharmacological challenge by using PWA. Although baseline AIx measurements were highly reproducible, as confirmed by previous studies, both GTN- and salbutamol-mediated changes in AIx showed a relatively high variability in both control subjects and patients with heart failure. The latter group showed a relatively higher variability in changes in AIx compared with the control group. Our results on baseline AIx are in agreement with previous studies assessing the short-term reproducibility of this parameter (Papaioannou et al. 2007, Wilkinson et al. 2002, Filipovsky, Svobodova & Pecen 2000, Papaioannou et al. 2004, Savage et al. 2002, Siebenhofer et al. 1999, Wilkinson et al. 1998, Frimodt-Moller et al. 2008, Liang et al. 1998). In these studies, the reproducibility was assessed by the mean ± SD and the Bland and Altman analysis was generally reported to show good reproducibility (Filipovsky, Svobodova & Pecen 2000, Papaioannou et al. 2004, Savage et al. 2002, Siebenhofer et al. 1999, Wilkinson et al. 1998, Liang et al. 1998). However, it is questionable that the limits of agreement presented in these studies (−10% and +10% and beyond (Papaioannou et al. 2007, Filipovsky, Svobodova & Pecen 2000, Papaioannou et al. 2004, Savage et al. 2002, Wilkinson et al. 1998, Frimodt-Moller et al. 2008) are acceptable in order to detect significant differences in interventional placebo-controlled studies. More recently, Papaioannou et al. measured ICC values from hour-to-hour AIx measurements (Papaioannou et al. 2007). The ICC obtained in their study (0.90) is very similar to the ICC values obtained in the present study. In contrast, there was a substantial difference between the CV of AIx measurements in the study by Papaioannou et al.(Papaioannou et al. 2007) and the present study (47.3 vs 25.0%, respectively).

However, it should be noted that the measurement of CV may be inappropriate when having a measurement scale that crosses zero and a mean that is therefore quite small relative to the SD (Papaioannou et al. 2007, Wilkinson et al. 1998). To our knowledge, this is the first report assessing the short-term reproducibility and repeatability of GTN- and salbutamol-mediated changes in AIx by PWA using established statistical approaches. Wilkinson et al. have previously assessed the repeatability of these parameters (Wilkinson et al. 2002). However, they presented the mean ± SD of the differences after GTN and salbutamol without assessing ICC, CV or using Bland–Altman analysis (Wilkinson et al. 2002). Hayward et al. presented the reproducibility of GTN- and salbutamol-mediated changes in AIx by means of correlation coefficients and Bland–Altman plots (Hayward et al. 2002). However, assessments of reproducibility by calculation of correlation coefficients may lead to data misinterpretation and there were no limits of agreement in the Bland–Altman plots (Bland, Altman 1986). Several factors could explain the high variability of changes in Aix following the administration of GTN and salbutamol. First, arterial vasoreactivity after pharmacological challenge, assessed with other established techniques, demonstrates a significant diurnal variability (Shaw et al. 2001). Second, the PWA technique for assessing endothelium dependent and -independent vasodilatation relies on the absorption into the systemic circulation of inhaled salbutamol and sublingual GTN. Although in the present study both drugs were administered by trained staff, it is theoretically possible that variations in the absorption rate between the two different study sessions may have affected the maximum changes in AIx. Differences in absorption rates have been observed in previous pharmacokinetic studies on repeated drug administration. Tomlinson et al. determined the reproducibility of urinary salbutamol measurements following

salbutamol inhalation in 15 healthy subjects (Tomlinson, Corlett & Chrystyn 2003). The reported CV values after one, three and five doses was 10.5, 10.1 and 9.4%, respectively (Tomlinson, Corlett & Chrystyn 2003). A high intra individual variability in the absorption rate of sublingual GTN has been reported, with CV values as high as 66% for GTN bioavailability (Noonan, Benet 1985). One limitation of our study was the lack of measurements of plasma salbutamol and nitroglycerin to confirm or refute this hypothesis. Third, the differences in blood pressure, HR and changes in AIx between the first and second studies may be explained by a carry-over pharmacodynamic effect of either GTN and/or salbutamol from the first study assessment instead of diurnal variability. However, in our opinion this is unlikely because the HR tended to be lower and baseline AIx tended to be higher in the second versus first measurement. We would have expected a higher HR and a lower AIx in case of a carry-over effect because both GTN and salbutamol exert vasodilatory effects accompanied by a tachycardic response (Wilkinson et al. 2002). How does the reproducibility of arterial vasoreactivity assessment by PWA compare with other techniques? Studies on the short-term reproducibility of measurements of flow-mediated dilatation of the brachial artery have reported CV values between 1 and 84% and ICC values between 0.60 and 0.92 (Liang et al. 1998, De Roos et al. 2003, de Roos et al. 2002, de Roos, Bots & Katan 2001, de Roos et al. 2001, Herrington et al. 2001, Sorensen et al. 1995, Hale et al. 2002, Berry, Skyrme-Jones & Meredith 2000, Hashimoto et al. 1995, Lind, Hall & Johansson 2002, Uehata et al. 1997, Welsch, Allen & Geaghan 2002, Hijmering et al. 2001, Malik et al. 2004, Harris et al. 2006, Peretz et al. 2007). Reproducibility studies using forearm occlusion plethysmography to assess post ischaemic blood flow or flow responses to vasodilator agents have reported CV values between 6.1 and 40% and ICC values

between −0.22 and 0.82 (Thijssen et al. 2005, Vanmolkot, de Hoon 2005). The results of these studies indicate a wide range of reproducibility that may reflect differences in study populations, operators and techniques, as well as variability in arterial vasoreactivity over time. In conclusion, the present study has demonstrated a poor short term reproducibility of endothelium-dependent and independent vasodilatation with the PWA technique. Similar findings reported previously using other established methods cast doubts on the validity of these approaches for the acute assessment of interventions on arterial vasoreactivity.

A RANDOMISED, DOUBLE-BLINDED TRIAL OF THE EFFECT OF TETRAHYDROBIOPTERIN AND FOLIC ACID ON AORTIC DISTENSIBILITY AND PERIPHERAL VASOREACTIVITY USING CARDIOVASCULAR MAGNETIC RESONANCE

7.1   Introduction

Ischaemic heart disease refers to the state of inadequate supply of oxygen and metabolic substrates to the underlying myocardium. It includes a heterogenous group of clinical disorders varying from stable angina to acute myocardial infarction. Disease progression is a dynamic interplay between the pathological progression and physiological balances leading to progressive regional ischemia. Central to the pathological disease process is the endothelium (Vane 1994, Vane, Anggard & Botting 1990). It forms a single layer of cells lining the vascular wall and is central to maintaining vascular tone and haemostasis. Disease progression is evident by the progressive constriction of the endoluminal surface to acute disruption of the endothelial layer and thrombus formation leading to MI (Fuster, Fayad & Badimon 1999, Virmani et al. 2000). Nitric oxide (NO) secreted by the endothelial nitric oxide synthase (eNOS) and prostacyclin produced by cyclooxygenase are important endothelium-derived vasodilator (Palmer, Ferrige & Moncada 1987, Moncada, Vane 1978). Vasodilatation occurs through hyperpolarisation of the vascular smooth muscle cells, which involves the opening of ATP sensitive potassium channels (K-ATP)(Feletou, Vanhoutte 2000). NO can activate the K-ATP channels in either a cyclic-GMP dependent or cyclic-GMP independent manner (Feletou, Vanhoutte 2000). Endothelial dysfunction is characterised by an imbalance in this activity and leads to vasoconstriction and pro-inflammatory and pro-coagulant changes in the

endothelium. Both coronary and peripheral endothelial dysfunction is strong predictors of cardiovascular events (Fichtlscherer, Breuer & Zeiher 2004, Targonski et al. 2003).

Cardiac Magnetic (CMR) imaging is recently identified as a multi-dimensional investigation tool. It provides the option of using a single non invasive investigational modality to assess central (aortic distensibility) and peripheral (brachial artery) vasoreactivity. It is a well described and validated technique to study endothelial dysfunction (Fayad et al. 2000, Mohiaddin, Firmin & Longmore 1993, Sorensen et al. 2002). Recent studies have also demonstrated the sensitivity and significance of CMR in detecting myocardial ischemia (Nagel et al. 2009).

Tetrahydrobiopterin is essential cofactor in the synthesis of endothelial NO. Folic acid is known to exert its beneficial effects on the endothelium by various methods. It reduces plasma homocysteine levels, scavenges endothelial superoxides and decreases the expression of endothelial adhesion molecules. Additionally, it prevents BH_4 oxidation and eNOS uncoupling. BH4 is essential for the vascular effects of folic acid. However, studies have also shown an independent beneficial effect of folic acid on endothelial function (Moat et al. 2006). Computer modeling suggests that folic acid and tetrahydrobiopterin bind to NOS in a similar fashion (Hyndman et al. 2002). Explained in detail in section 1.6.

Studies on the combined effects of folic acid and BH4 have not been done. It is unclear whether folic acid and BH4 would have an additive effect on the vascular endothelium.

We aim to conduct a pilot study to analyse the sub-acute effects of oral folic acid and tetrahydrobiopterin supplementation on central (aortic distensibility) and peripheral

(brachial artery) vasoreactivity using CMR in patients' with underlying myocardial ischemia. We initiated a randomized double blind placebo controlled trial to test the hypothesis that folic and BH4 supplementation would result in improved vascular outcomes in patients with myocardial ischemia. The study was a proof of concept study aimed to study the aortic and brachial endothelial effects using CMRI and use the data for sample size calculation for further studies.

7.2   Methodology

Patients were recruited from the outpatient services of the cardiology departments at two tertiary levels hospitals. Inclusion criteria for the trial were as follows: a recent angiogram (<24 months) with at least 75% narrowing in one coronary artery, intermittent symptoms despite medical management but unsuitable for surgical intervention and normal ventricular function. Patients with uncontrolled diabetes (HbA1C>7), recent (<6 weeks) addition of anti-anginal medications and those with metal implants rendering them unsuitable for MRI were excluded from the trial. Thirteen successive patients' were recruited in the pilot phase of this study.

Study protocol

The study protocol was approved by the local ethics committee and informed consent was obtained from all patients prior to the study. Patients were provided with information regarding the trial by written leaflets and personal interviews. They were screened and clinical parameters recorded included heart rate, blood pressure, body mass index and medication list.

On the day of the study a cannula was inserted in the non-dominant arm. Study subjects underwent CMR for assessment of cardiac parameters including left ventricular ejection fraction, left ventricular mass, left and right atrial area. Vascular

analysis included aortic distensibility at the ascending, proximal and distal descending aorta. Brachial artery studies were done with a surface coil to analyze endothelium dependent vasodilatation. This was performed according to the technique described in section 2.4.

Patients were randomized to receive treatment with folic acid plus tetrahydrobiopterin versus placebo for 14 days. Folic acid 5 mg/day and BH4 20 mg/kg/day was administered to the treatment group. High dose folic acid (30 mg/day) has been used in patients with coronary artery disease without adverse events. Tetrahydrobiopterin has been used extensively in infants and children with Phenylketonuria. Dose used range from 1 to 20 mg/kg/day. Oral, intravenous, topical and intradermal administration using 30 mg/kg was studied on animals without significant side effects. The tablets were prepared in the pharmacy department and randomized using a computer generated randomization code in the pharmacy department. Patients received the treatment/placebo tablets in a container with clear instructutions. Compliance was assessed by weekly phone calls. Patients were also asked to bring the drug containers at the end of the trial to ensure compliance.

A repeat MRI study was performed after 14 days to assess for changes in aortic distensibility and brachial artery endothelial function.

Statistical analysis

Data is presented as mean ± SD. Baseline measurements of the two groups are compared using the Student's unpaired *t*-test. The effects of folic acid and tetrahydrobiopterin were compared using the Mann-Whitney U test.

7.3   Results

The baseline patient characteristics are described in Table 1. The two groups were matched for all measurements at baseline, including cardiac parameters and haemodynamic measurements.

Table 7.1   Patient characteristics

	Controls (n=5)	Treatment group (n=8)	P Value
Age (years)	67.4 ± 6.5	63.8 ± 11.08	0.47
Heart rate (beats/min)	55 ± 1.4	66 ± 12.34	0.21
sBP (mmHg)	149.6 ± 20.6	137.3 ± 26.2	0.34
dBP (mmHg)	91.6 ± 10.3	77.8 ± 22.9	0.17
BMI (kg/m^2)	33.1 ± 5.6	27.6 ± 4.4	0.06
LV EF (%)	65.2 ± 11.7	69.7 ± 5.2	0.37
LV ESV (ml)	53 ± 27.8	44.6 ± 9.7	0.46
LV EDV (ml)	143.6 ± 29.4	146 ± 23.9	0.86
SV (ml)	90.6 ± 5.4	101.4 ± 19	0.17
CO (L/min)	5.12 ± 1.9	6.3 ± 1	0.17
LV mass (gm)	164.8 ± 30	139 ± 26.4	0.11
LV mass (g/m^2)	81.6 ± 12.6	70.4 ± 10	0.09
LA area (mm)	29.4 ± 5	26.6 ± 2.4	0.20
RA area (mm)	24.8 ± 2.5	26.7 ± 3.8	0.29

Values are mean ± SD unless otherwise indicated. sBP indicates systolic blood pressure; dBP indicates diastolic blood pressure; BMI indicates body mass index; LV EF indicates left ventricular ejection fraction; LV ESV indicates left ventricular end systolic volume; LV EDV indicates left ventricular end diastolic volume; SV indicates stroke volume; CO indicates cardiac output; LV mass indicates left ventricular mass; LA area indicates left atrial area; RA area indicates right atrial area.

Brachial artery FMD

The baseline brachial artery cross-sectional area was acquired at end-diastole. It was similar in controls and treatment group (23.4 ± 5 mm^2 vs 25.3 ± 7 mm^2, P = 0.6). Intragroup analysis did not indicate a change in the FMD % in either the control or treatment group after 14 days (4.71 ± 4.58 vs 3.87 ± 3.75, P=0.96 and 4.1 ± 5.34 vs 5.34 ± 5.46, P = 0.64) (Table 7.2). In an intergroup analysis the Δ FMD % (change in FMD % after treatment) did not differ between the two groups (-0.84 ± 6.01 vs 1.29 ± 0.63, P = 0.63) (Table 3). In the treatment group; oral folic acid and BH4 did not improve FMD (P=0.63).

Aortic distensibility

Cross-sectional areas were measured at three different sites in the aorta. These included the ascending aorta (AA), proximal descending aorta (PDA) and distal descending aorta (DDA). Cross-sectional areas and distensibility at each of these sites and their average is shown in Table 7.2. In an intragroup analysis, the average aortic distention did not differ after 14 days in either the control or treatment group (1.17 ± 0.47 vs 1.38 ± 0.55, P = 0.39 and 1.88 ± 1.24 vs 2.19 ± 1.32, P = 0.30) (Table 7.2). The intervention did not improve aortic distensibility in the ascending (P=0.61), proximal descending (P=0.61) or distal descending aorta (P= 0.48) (Table 7.3).

Table 7.2   Aortic distensibility and FMD% in the two groups before and after treatment

	Control group (n=5)			Treatment group (n=8)		
	Day 1	Day 14	P value	Day 1	Day 14	P Value
AA d (mm^2)	11.24 ± 0.66	11.93 ± 0.82	0.06	9.26 ± 1.56	9.66 ± 1.52	0.12
AA s (mm^2)	11.58 ± 0.51	12.56 ± 0.86	0.03*	9.75 ± 1.59	10.46 ± 1.61	0.08
AA distention (10^{-3} mmHg^{-1})	0.50 ± 0.44	0.97 ± 0.74	0.18	0.98 ± 0.65	1.66 ± 1	0.51
PDA d (mm^2)	6.47 ± 1.6	6.96 ± 1.33	0.88	5.68 ± 1.31	6.01 ± 1.17	0.80
PDA s (mm^2)	6.90 ± 1.36	7.66 ± 1.49	0.57	6.28 ± 1.25	6.64 ± 1.22	0.74
PDA distention (10^{-3} mmHg^{-1})	1.23 ± 0.97	1.68 ± 0.92	0.68	2.01 ± 1.39	2.10 ± 1.08	0.98
DDA d (mm^2)	5.39 ± 1.36	6.12 ± 1.37	0.40	4.77 ± 0.97	4.92 ± 0.78	0.73
DDA s (mm^2)	5.91 ± 1.37	6.65 ± 1.36	0.27	5.42 ± 1	5.59 ± 0.87	0.30
DDA distention (10^{-3} mmHg^{-1})	1.77 ± 0.92	1.49 ± 0.53	0.37	2.65 ± 2.09	2.81 ± 2.15	0.24
Avrg A distention (10^{-3} mmHg^{-1})	1.17 ± 0.47	1.38 ± 0.55	0.39	1.88 ± 1.24	2.19 ± 1.32	0.30
FMD %	4.71 ± 4.58	3.87 ± 3.75	0.96	4.1 ± 5.34	5.34 ± 5.46	0.64

Values are mean ± SD unless otherwise indicated. AAd indicates Ascending Aorta diastole; AAs indicates Ascending Aorta systole; PDAd indicates Proximal descending Aorta diastole; PDAs indicates Proximal descending Aorta systole; DDAd indicates Distal descending Aorta diastole; DDAs indicates Distal descending Aorta systole; Avrg A distention indicates the Average Aortic distention for ascending, proximal and distal descending aorta; FMD(%) indicates the percentage change in flow mediated dilatation.

P<0.05 vs control in the selected category

Table 7.3   Comparative analsysis of aortic distensibility

Δ values	Control group (n=5)	Treatment group (n=8)	P value
AA (10^{-3} mmHg^{-1})	0.46 ± 0.87	0.67 ± 0.58	0.61
PDA (10^{-3} mmHg^{-1})	0.44 ± 1.7	0.09 ± 0.67	0.61
DDA (10^{-3} mmHg^{-1})	- 0.28 ± 1.27	0.16 ± 0.98	0.48
FMD (%)	-0.84 ± 6.01	1.29 ± 0.08	0.63

Δ values indicate the difference in day 14 and day 1 readings; AA indicates Ascending Aorta; PDA indicates Proximal descending Aorta; DDA indicates distal descending aorta; FMD (%) indicates the percentage change in flow mediated dilatation.

7.4   Discussion

Impaired endothelial dependent vasodilatation is characteristic of patients with IHD. Furthermore, it is a strong predictor of future cardiovascular events in patients with CAD (Fichtlscherer, Breuer & Zeiher 2004). Endothelial dysfunction is defined as reduced nitric oxide bioavailability. Folic acid and tetrahydrobiopterin play a vital role in the synthesis of nitric oxide from L-arginine. Recent studies have demonstrated that acute administration of folic acid and tetrahydrobiopterin improve coronary endothelial function (Moens, Kass 2006, Setoguchi et al. 2001, Maier et al. 2000, Tawakol et al. 2005). Our study aimed to study the combined effect of these agents on endothelial function in the large (aortic) and small (brachial) arteries.

The chief findings of our study are:

- Folic acid and BH4 did not improve aortic distensibility

- Folic acid and BH4 did not improve brachial artery vasoreactivity

Aortic distensibility, a marker of aortic wall sclerosis is lower in patients with coronary artery disease (Bogren et al. 1989, Matsumoto et al. 1996). It is well established that aortic distensibility is associated with aging and glycemic control (Stacey et al. 2010). Therapy with beta blockers and ACE inhibitors are shown to improve aortic compliance in young patients with pre-hypertension (Celik et al. 2008). Our study is to the best of our knowledge, the first to study the effect of folic acid and BH4 on aortic distensibility in patients with established CAD. The combined oral administration of folic acid and BH4 did not detect a change in aortic distensibility, measured by magnetic resonance imaging. Our study is in agreement with the study by Carlsson et al that folic acid does not improve vasodilator response in patients with CAD (Carlsson et al. 2004). Our study further demonstrates that the addition of BH4 to folic acid does not alter aortic distensibility in these patients.

Recent studies have demonstrated aortic distensibility to be a resistant marker for CAD. Studies by Little et al did not find an improvement in aortic distensibility following 16 weeks of treatment with alagebrium chloride, a thiazolium derivative which breaks glucose cross-links that develops between collagen and elastin, in patients with diastolic heart failure (Little et al. 2005). This failure in improvement was despite its beneficial effects of reducing left ventricular mass and left ventricular diastolic filling. The concomitant use of anti-hypertensive agents in patients may also have obscured any beneficial effect due to the intervention. It is also possible that 2 weeks of therapy is not sufficient to alter the chronic arterial characteristics.

Our study did not demonstrate folic acid and BH4 to have a beneficial effect on peripheral endothelial function in patients with CAD. The limited sample size of our study might have been a limiting factor. This study was conducted as a pilot study

and hence had a limited sample population. The results from this study will be used to calculate an appropriate sample size for a larger study. The results of this study would hence not be confirmatory due to the limited sample size. However, previous studies have used similar sample sizes (Setoguchi et al. 2001, Tawakol et al. 2005). Patients included in our study had advanced CAD with resulting extensive arteriosclerosis which may not be easily amenable to endothelium based treatment. Finally, folic acid fortification is mandatory in Australia. Ingestion of fortified food products may have resulted in the lack of benefit seen during the trial. Further large scale studies are required to confirm the findings of our study and evaluate the role of endothelial modifiers in CAD.

CHAPTER 8

CONCLUSIONS

Cardiovascular disease has gained epidemic proportions and is the healthcare challenge of the current generation. Current trends reveal a reduction in disease specific mortality but an increase in its prevalence. Coupled with the increasingly ageing population this has lead to poor quality of life in a large number of patients. The aim of this experimental work was to assess the effects of multiple non toxic and cost effective therapeutic agents on autonomic function and endothelial functio in patients with well established end stage cardiovascular disease states. Folic acid, tetrahydrobiopterin and oxygen were selected due to their low cost, current evidence and their potential for low toxicity. Folic acid and tetrahydrobiopterin have a beneficial effect on the endothelium. The beneficial effects of folic acid are independent of its homocysteine lowering effect and tetrahydrobiopterin is an essential cofactor in the syntheis of nitric oxide. Their combined effect on the endothelium has not been studied. Nasal oxygen supplementation is not known to have a beneficial effect on the endothelium and is not known to have a synergistic effect with folic acid or tetrahydrobiopterin. Our study did not provide any evidence of a synergistic interaction between oxygen and folic acid/tetrahydrobiopterin.

The primary focus of the the thesis is to study the endothelial and autonomic effects of pharmacological interventions. They play a major role in the early diagnostic and prognostic stratification of cardiovascular patients. A complete evaluation of the endothelial effects required the study of both peripheral and central arterial endothelial function. While PWA provides a non-invasive strategy for the study of

peripheral endothelial function; CMRI provides a reliable method of the study of peripheral and central endothelial function.

Autonomic function is impaired in CHF. Progression of the disease is clinically evident by worsening symptoms at rest and minimal exertion. The study of autonomic function at both submaximal and maximal exercise provided a comprehensive analysis of autonomic function in CHF. Submaximal exercise was used to analyse the role of peripheral skeletal muscle ergoreflex while maximal exercise was used to study the role of cardiopulmonary reflexes.

The main conclusions gathered from the experiments are as following:

1) Peripheral autonomic (ergoreflex) response to sub maximal exercise is raised in CHF and is independent of the central cardiac ejection fraction. Supplemental inhaled oxygen therapy significantly reduced the ventilatory overdrive seen in these patients during sub-maximal exercise. Haemodynamic (heart rate and blood pressure) and peripheral blood flow to the non exercising limb were unchanged. Inhaled oxygen did not influence these central and peripheral vascular parameters.

   Our findings of heightened ventilation in patients with CHF are similar to those by Grieve et al and Scott et al (Grieve et al. 1999, Scott et al. 2003). Our study adds further by comparing these autonomic parameters in patients with impaired and preserved heart failure and demonstrating the effects of oxygen on each of these sub groups. Studies done previously by ourselves and others have shown oxygen to improve exercise performance and quality of life in CHF patients (Shigemitsu et al. 2007, Paul, Joseph & De Pasquale 2008, Guzzetti et al. 2008). This study for the first time demonstrates the role of oxygen in reducing the

ventilatory overdrive during sub-maximal exercise. This improvement is independent and despite its lack of effect on central and peripheral vascular parameters.

2) During maximal exercise CHF patients were characterised by the combination of inefficient ventilation and a lack of increase in cardiac output to match increasing exercise loads. However, oxygen did not influence either of these parameters during peak exercise. Furthermore we did not detect an improvement in metabolic parameters during the inhalation of oxygen. The lack of improvement during peak exercise is probably reflective of the severity of reduced cardiac and pulmonary reserves in CHF. Structural pulmonary vascular changes and abnormalities of the alveolar membrane (Puri et al. 1994) combined with ventilation-perfusion mismatch (Uren et al. 1993) reduce peak exercise capacity in these patients. Our results are in agreement with those of Uren et al and Buller et al that exercise capacity is limited by reduced pulmonary capacity. Our study adds to these findings in demonstrating that nasal oxygen therapy does not improve pulmonary reserves or metabolic parameters to improve peak exercise performance.

3) Acute intravenous administration of 5-MTHF, the active form of folic acid significantly reduced the systolic and diastolic blood pressure in patients with CHF. It also significantly reduced the serum ADMA level, a surrogate marker of cardiovascular events. Studies by Cooke et al demonstrate that serum ADMA levels inhibit NO synthesis and increase the risk of cardiovascular events (Cooke 2004). However, it did not acutely improve endothelial function in these patients. Our study did not demonstrate an improvement in endothelial function with acute folic acid infusion; however, it did demonstrate a reduction in serum ADMA

levels indicating a possible benefit with its long term use. Folic acid may play a vital role in reducing serum ADMA levels and reducing cardiovascular events. Long term studies would be required to analyse its effects on disease progression. The acute administration did not improve endothelial function indicating the advanced nature of endothelial dysfunction and its irreversibility in CHF. Our findings are in agreement with others that did not show an improvement in endothelial function following folic acid supplementation (Carlsson et al. 2004, De Vriese et al. 2002).

4) Pulse wave analysis is used to assess central aortic pressures. Recently it has also been used to assess endothelial function by analysing the character of the pulse wave. Our study demonstrates good reproducibility for baseline measurements with this technique, however, poor short-term reproducibility following acute pharmacological interventions with salbutamol and GTN. Our study is the first to demonstrate this disparity in short term reproducibility with the use of PWA. Our studies differ from those by Papaioannou et al in highlighting this difference (Papaioannou et al. 2007). However, our study presents a more comprehensive review of the technique with an analysis of the intraclass correlation coefficient, the between subject coefficient of variation and the Bland-Altman analysis. Our findings have since been validated by recent studies by Dawson et al and Phillips et al (Dawson et al. 2009, Phillips, Avolio & Grunstein 2009).

5) The combined use of folic acid and tetrahydrobiopterin did not improve endothelial function in the large (aorta) and small (brachial) artery of patients with CAD. Our study is the first to analyse the effects of combined

administration of these agents. The possible explanations for this lack of effect include

- The resistant nature of endothelial damage in patients with well established CAD.

- A possible masking of the effects of folic acid and tetrahydrobiopterin due to the widespread fortification of food with folic acid in Australia.

Our studies demonstrate the role of oxygen, folic acid and tetrahydrobiopterin in patients with end stage cardiovascular disease and add vital information about their therapeutic role in these patients. A significant proportion of patients continue to suffer the debilitating symptoms of dyspnoea, fatigue and persistent angina despite maximal medical therapy. Surgical treatment in these patients is limited due to the advanced nature of the disease, comorbidities and advanced age. These patients indicate a strong preference for better symptom control to improve their quality of life. Our study focussed on patients with advanced cardiovascular disease as there is an unmet need for further therapeutic agents in this group.

Oxygen is not standard therapy for the management of patients with CHF. It does not have a beneficial effect on the central or peripheral cardiovascular parameters. However, previous studies have demonstrated a benefit in exercise tolerance and quality of life with its use. Our study demonstrates that this improvement could result from its beneficial effects during sub maximal exercise. However, this benefit is nulled during maximal exercise demonstrating its limitation in patients with advanced CHF. This beneficial influence of oxygen highlights its possible role as a therapeutic agent in the control of symptoms during submaximal exercise in CHF. It

may help patients tolerate their activities of daily living and improve qulity of life. The influence of oxygen on mortality and disease progression would be essential to advocate its widespread use in CHF.

Folic acid and BH4 did not have an effect on endothelial function in patients with advanced CHF and CAD. Initial studies indicated a promising therapeutic role for folic acid in patients with cardiovascular risk factors. However, our studies demonstrate that endothelial dysfunction is advanced and irreversible in these patients. Interestingly our study did not demonstrate a significant difference in endothelial function in patients on maximal medical therapy and control subjects. Our studies highlight the role of current medical agents in improving endothelial function and do not advocate the use of folic acid or BH4 in these patients. However, the beneficial effect of folic acid on serum ADMA levels woud requires further evaluation. Our findings may have been confounded by the use of PWA, a technique demonstrated to have limited value in the assessment of endothelial function. The technique does not accurately assess endothelial dependent and endothelial independent response to salbutamol and GTN. PWA was used for our study based on then current evidence and our analysis of the technique was conducted after our initial study in CHF. However, the PWA is a reliable technique for assessment of baseline endothelial function and we did not detect an improvement in baseline endothelial function with folic acid. Our fndings using the PWA were also substantiated in patients with CAD using the CMR technique. Folic acid and BH4 did not improve endothelial function in these patients.

The thesis also raises several important questions for future research;

1) Does oxygen supplementation prevent disease progression and reduce mortality in CHF? Oxygen is known to reduce mortality in patients with chronic obstructive pulmonary disease (Cranston et al. 2005). However, the survival benefit is seen only in patients with prolonged periods of hypoxia. Patients with CHF are known to have nocturnal hypoxia which identifies them as high risk for subsequent cardiac death (Lanfranchi et al. 1999). Oxygen therapy has improved exercise performance and quality of life in these patients. Our study demonstrates that this improvement is due to its effects on peripheral rather than central autonomic reflexes. However, there are no studies currently being undertaken to study the effect of oxygen on mortality and disease progression.

2) Folic acid and tetrahydrobiopterin do not improve endothelial function in central and peripheral vasculature in patients with established cardiovascular disease. The lack of effect of these agents highlights the irreversible nature of endothelial dysfunction. However, folic acid and tetrahydrobiopterin reduce blood pressure and disease specific surrogate markers. Studies conducted by BioMarin Pharmaceuticals and Emory University are currently underway to analyse the effects of tetrahydrobiopterin in hypertension and peripheral vasculare disease. Recently, studies have indicated a possible benefit of these agents in proximal coronary perfusion. However; its effect on myocardial perfusion (distal coronary) is not known. Further studies to demonstrate the effect of these agents on myocardial perfusion and disease related mortality in cardiovascular disease would provide conclusive evidence regarding their role.

Answers to these questions are expected for the next few years.

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