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Determining whether plasma levels of KATP channels are related to vascular function

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Determining whether plasma levels of K_{ATP} channels are related to vascular function

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Declaration

“I declare that the content of this project report is my own work, unless otherwise stated, and has not previously been submitted for any other assessment. The report is written in my own words and conforms to the University of Dundee’s Policy on plagiarism and academic dishonesty. Unless otherwise indicated, I have consulted all of the references cited in this report”

Signature:_____ Date:_____

Abstract

Background: ATP-sensitive potassium channels are ATP sensitive pores that are found in vascular smooth muscle cells and play an important role in regulating vascular function. They are also expressed in blood but their relationship with vascular function is unknown. This study aims to investigate the previously unknown relationship between levels of K_{ATP} channels in blood and vascular function in normal healthy subjects.

Methods: Blood samples were obtained and vascular function was assessed by carrying out four non-invasive tests in 50 normal healthy subjects. The levels of K_{ATP} channels were determined by measuring levels of mRNA subunits, Kir6.1/SUR2B, using real time reverse transcription polymerase chain reaction (RT-PCR) test while vascular function was assessed using (i) iontophoresis with laser Doppler flowmetry (LDF); (ii) post-occlusive reactive hyperaemia test (PORH) in the skin microvessels; (iii) pulse wave analysis (PWA) for arterial stiffness and (iv) flow-mediated dilation (FMD) in the macrovessels.

Results: This study showed that Kir6.1 but not SUR2B, mRNA can be detected in human blood. In addition, acetylcholine (ACH) ($r=-0.579$, $p<0.001$, $n=40$) and PORH ($r= 0.366$ $p=0.020$, $n=40$) correlated with Kir6.1 mRNA levels. No correlation was noted in brachial artery FMD ($r=0.181$, $p=0.264$, $n=40$) with Kir6.1 mRNA levels. Further analysis demonstrated gender specific differences where males showed statistically significant correlation between Kir6.1 mRNA levels and ACH ($r=-0.561$, $p=0.005$, $n=23$) as well as PORH ($r= 0.418$, $p=$

0.047, n= 23) but in females significant correlation was only seen between Kir6.1 mRNA levels and ACH (r=-0.628, p=0.007, n=17) but not PORH.

Conclusion: The mRNA Kir6.1subunit levels in blood showed significant relationship with microvascular function. However, further studies are required to investigate the differences in direction of correlation between ACH and Kir6.1 mRNA levels (negative correlation) as well as PORH and Kir6.1 mRNA levels (positive correlation). Levels of K_{ATP} in blood could potentially be used as blood marker for vascular function *in vivo*.

Abbreviations

AIx- augmentation Index

AIx@75- augmentation index normalised to 75 beats per minute

ACH- acetylcholine

BH_4- tetrahydrobiopterin

CAM- calmodulin

CCD- charge-coupled device

cDNA- complementary deoxyribonucleic acid

cGMP- cyclic guanosine monophosphate

CIMT- carotid intima-media thickness

CRP- C-reactive protein

C_t- threshold cycle

CVD- cardiovascular disease

ECM- extracellular matrix

eNOS- endothelial NO synthase

FCVRS- Framingham Cardiovascular Risk Score

FMD- flow-mediated dilatation

FLPI- full field Laser Perfuser Imager

GTP- guanosine triphosphate

GPX1- glutathione peroxidase

HDL- high density lipoprotein

HsCRP- high sensitivity C-reactive protein

ICAM-1- inter-cellular adhesion molecule-1

IL- interleukin

K_{ATP} channel- ATP-sensitive potassium channel

K+(BK_{Ca}) channel- Big potassium calcium-activated channel

K_{Ca} channel- Ca^{2+}-activated potassium channel

K_{ir} channel- inwardly rectifying potassium channel

K_V channel- voltage-gated potassium channel

L-arginine/NO/cGMP- L-arginine/nitric oxide/cyclic guanosine monophosphate

LDF- laser Doppler flowmetry

LDL- low density lipoprotein

LDI- laser Doppler imaging

MLC- myosin light chain

MLCK- myosin light chain kinase

MPO- myeloperoxidase

mRNA- messenger ribonucleic acid

NO- nitric oxide

NO/cGMP/PKG- nitric oxide/cyclic guanosine monophosphate/protein kinase G

PAI-1- plasminogen activator inhibitor-1

PAT- peripheral arterial tonometry

PMLNs- polymorphonuclear neutrophils

PORH- post-occlusive reactive hyperaemia

PP- pulse pressure

P.U.- perfusion units

PWA- pulse wave analysis

PWV- pulse wave velocity

RNA- ribonucleic acid

RHI- reactive hyperaemia index

RT-PCR- reverse transcription polymerase chain reaction

SAA- serum amyloid A

sCD40L- soluble CD40 ligand

SEVR- subendocardial viability ratio

sGC- soluble guanylate cyclase

sICAM- soluble intercellular adhesion molecule

SNP- sodium nitroprusside

SUR- sulphonylurea receptor

TNFα- tumour necrosis factor α

TPA- tissue plasminogen activator

T_r- time of return of the reflected wave

VCAM-1- vascular cell adhesion molecule-1

VOP- venous occlusive plethysmography

VSM- vascular smooth muscle

VSMCs- vascular smooth muscle cells

WHO- World Health Organisation

ΔP- augmentation pressure

CHAPTER 1

1.1 Introduction

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality among adults in the UK and worldwide. The common occurrence of CVD in the UK is of particular concern since it is recognised that patients with CVD tend to have impaired quality of life with loss of independence, ultimately resulting in adverse social and economic implications.

While traditional risk factors for CVD are widely agreed as significant causes of CVD, numerous studies have demonstrated that abnormal CV risk factors are often absent in individuals who develop CVD. The National Cholesterol Education Program guidelines showed that among relatively young adults admitted into the hospital for acute myocardial infarction, their mean lipid levels were within the normal range and approximately 70% of these patients fell into the lowest CV risk categories (Cleeman and Grundy, 1997, Akosah et al., 2003). These research observations suggest that clinically significant CVD can occur without the presence of prior established CVD risk factors. For this reason, researchers have devised CV risk estimation systems to aid the detection of subclinical CVD and are constantly in search for novel markers for early presymptomatic CVD.

CV risk estimation systems/scores have been long developed and used by clinicians to assess and stratify CV risk in asymptomatic high-risk individuals who have not developed CVD. Nonetheless, it is generally agreed that there

exist limitations to CV risk estimation systems as they assume that all risk factors exert constant effects in all individuals of differing age. In addition, they are based on the assumption that different risk factors exert a similar “effect” which is not necessarily the case as it is recognised that certain combinations of risk factors may act synergistically leading to higher risks as the effect is more than additive (Faeh et al., 2013). In addition, serum markers such as total cholesterol (low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides) and C-reactive protein have been used in the attempt to stratify CV risk (Reiner et al., 2011, Keavney, 2011). Nonetheless, although it is shown that serum markers have been associated with CV risks, they are not believed to provide reliable measures or to substantially improve sensitivity of CV risk detection to justify their use in clinical practice (Francisco et al., 2006).

More recently, vascular function tests such as post-occlusive reactive hyperaemia (PORH) and flow-mediated dilatation (FMD) have been applied in CV risk assessment (Roustit and Cracowski, 2012, Peters et al., 2012). They have been shown to be reliable and can act as surrogate markers in predicting CV risks in asymptomatic individuals (Strain et. al, 2010, Peters et al. 2012). Despite its clinical relevance, these tests have their limitations. They are often technically demanding, time consuming and the reproducibility and quality of test results are to some extent dependent on the operator. This is particularly true for ultrasound assessment of FMD (Celermajer, 2008). Therefore, these tests are not generally used in the clinical setting but are used more extensively in the research arena. These techniques will be discussed in more detail under the “Assessment of vascular function in humans” in Chapter 4.

It is generally recognised that abnormalities in the vasculature play a key role in the pathophysiology of CVD. Given that vascular function evaluated in the peripheral vasculature has been shown to correlate with cardiovascular function (Kuvin et al., 2001) and is abnormal in patients at risk for CVD (Deanfield et al., 2007), an effort to search for a novel marker to detect and identify presymptomatic or subclinical CVD has focused on the vasculature, particularly in easily accessible peripheral arteries such as the brachial and carotid arteries.

The next section provides an outline of the proposed project followed by an overview of this thesis.

1.2 Project description

This project investigates the possibility of using the levels of ATP-sensitive potassium (K_{ATP}) channel in blood as an indicator of vascular function. Briefly, K_{ATP} channels has been shown to interact with a variety of endogenous and pharmacological vasodilators, such as calcitonin gene-related peptide, vasoactive intestinal polypeptide, prostacyclin and adenosine, which all exert their effects directly on the vasculature (Isomoto et al., 1997, Quayle et al., 1997). They are known to be composed of the protein, Kir6.1 and SUR2B subunits (Standen et al., 1989, Clapp and Gurney, 1992, Beech et al., 1993) and under pathophysiological conditions and in disease states, these channels are activated and may play a pivotal role in regulating vascular function (Wang et al., 2003). In a preliminary study by Jovanovic and Du (unpublished), it has been shown in that K_{ATP} channels are expressed in blood cells of mice. This observation raises a very interesting question- can the levels of K_{ATP} channels

in blood be a reflection of those expressed on vascular smooth muscle cells (VSMCs), and if that is the case, can the levels of K_{ATP} channels in blood be a surrogate marker of vascular function? The general features, structure and function of K_{ATP} channels will be explored, in more detail in Chapter 3 but briefly the role of K_{ATP} channels can be explained as follows: K_{ATP} channels play an important role in coupling cell metabolism to membrane potential and are crucial in the physiology and pathophysiology of many tissues. They were first identified in cardiac myocytes (Noma, 1983, Trube and Hescheler, 1984) and subsequently in other tissues such as pancreatic β-cells (Ashcroft et al., 1984, Cook and Hales, 1984), skeletal muscle (Spruce et al., 1985, Spruce et al., 1987), central neurones (Ashford et al., 1984, Ashford et al., 1988) and smooth muscles (Standen et al., 1989, Beech et al., 1993, Kajioka et al., 1991, Kamouchi and Kitamura, 1994) . In these tissues, they have the role to, respectively, shorten the duration of the action potential in cardiac myocytes (Noma, 1983), regulate insulin release in pancreatic β-cells (Cook and Hales, 1984), regulate the excitability of skeletal muscle (Spruce et al., 1985), regulate neuronal excitability, regulate neurotransmitter release (Kawano et al., 2009) and control the tone of vascular smooth muscle (Standen et al., 1989). K_{ATP} channels expressed in the VSMCs are of particular interest in this project.

1.3 Overview of thesis

The thesis is organised as follows: Chapter 2 begins with a brief overview of the normal human vasculature which includes the structure and function of the endothelium and VSMCs. This is followed by a discussion on the mechanisms involve in the regulation of vascular tone by VSMCs and the underlying

pharmacology involved in this vasoregulatory mechanism. The second part of Chapter 2 provides a summary of CVD and the importance of vascular dysfunction. Chapter 3 gives an overview of the general features of K_{ATP} channels and provides evidence of their involvement in controlling vascular tone. In Chapter 4, the different modalities available to assess vascular function, invasively and non-invasively, are summarised. The aim and objectives of this project are listed towards the end of Chapter 4. This is followed by Chapter 5 explains the experimental setup for data acquisition. The statistical analysis used for interpreting the data collected is summarised at the end of Chapter 5. Chapter 6 shows the results of this project. The discussion about the findings in this project, the project limitations, conclusions and recommendations for future research are found in Chapter 7.

CHAPTER 2

The Human Vasculature

2.1 Background

In this section, I will discuss the normal structure and function of the endothelium and the vascular smooth muscle. Both components form the wall of the blood vessel and plays a central role in regulating vascular function.

2.1.1 The endothelium

The endothelium was originally thought to be a passive, inert semipermeable barrier between the circulating blood and the vascular smooth muscle cells of the tunica media. However, over the last four decades, extensive research has shown that the endothelium is indeed a highly dynamic organ which involves actively and reactively in various vasoregulatory processes.

Structure

Structurally, the endothelium consists of a monolayer of flat cells which line the innermost layer of all blood vessels (Figure 2.1). In adults, the endothelium weighs approximately 1kg, comprises about $1X10^{13}$ cells and has a surface area between 1-7m^2 (about 7-8 tennis courts) (Nematbakhsh et al., 2008). Individually, the endothelial cell has a length of between 25-50μm, a width of between 10-15μm and a depth of up to 5μm and has a large nucleus which often protrudes into the blood vessel lumen.

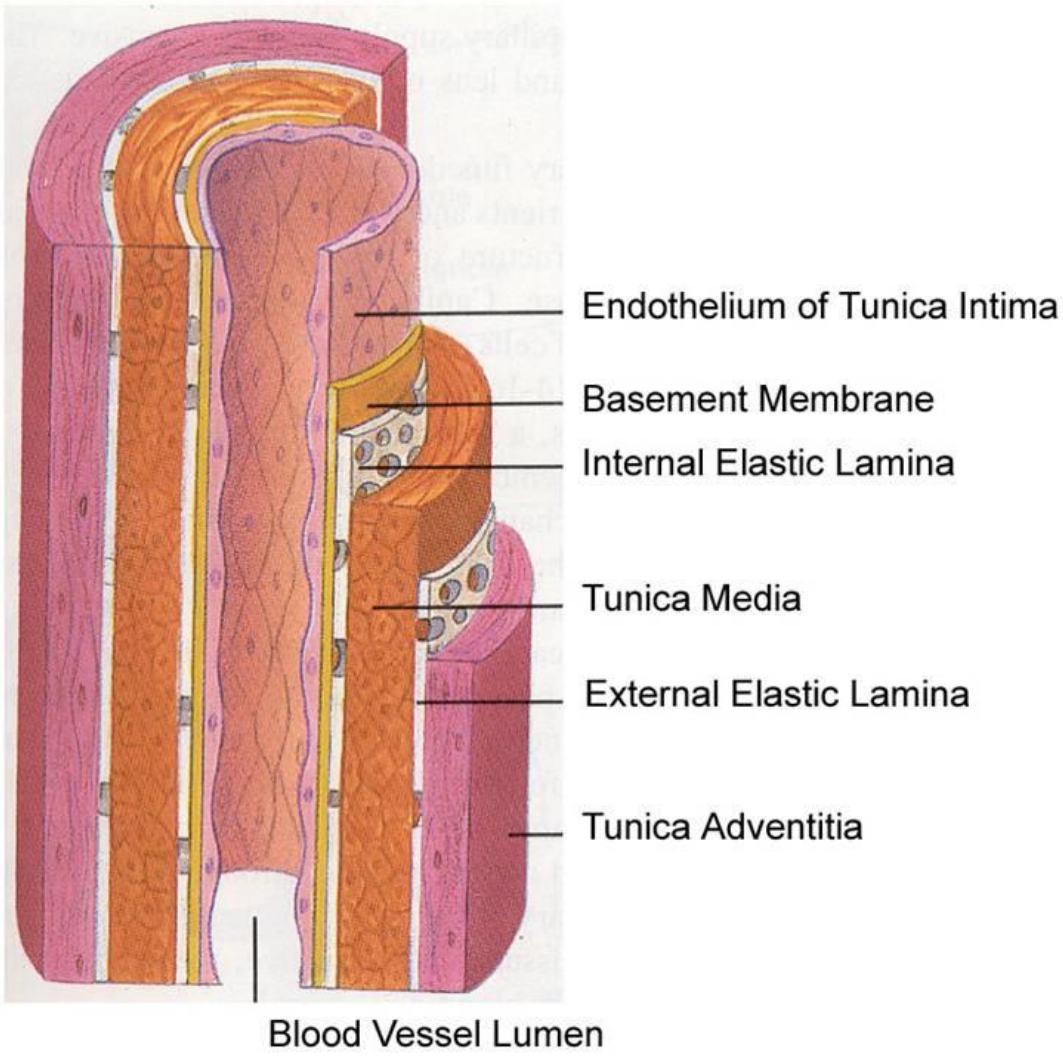

Figure 2.1 Schematic diagram of the layers of blood vessel wall. (Adapted from http://bme.ccny.cuny.edu/faculty/jtarbell/SMC%20images%20for%20web%20site/vessel%20wall%20schematic.jpg (25-10-2013))

Function

In its strategic location i.e. at the interface between blood and vessel wall, the endothelium does not only act as a physical anatomical barrier but it also serves as a gate keeper against the effect of various cellular and neurohumoral mediators. As a key regulator of vascular homeostasis, the endothelium plays an important role in maintaining the balance between blood vessel vasodilatation and vasoconstriction by secreting various vasoactive agents such as nitric oxide (NO) and prostacyclin (vasodilators) as well as endothelin and platelet activating factor (vasoconstrictors) (Vita, 2011). The endothelium plays a crucial role in thrombogenesis by favoring aggregation of platelets and formation of clots through organised induction of procoagulant/prothrombotic factors and suppression of anticoagulant mechanisms and fibrinolysis by releasing tissue-type plasminogen activator and urokinase (Gross and Aird, 2000). The endothelium also participates in the stimulation and inhibition of VSMC proliferation and migration (Sumpio et al., 2002). In addition, the endothelium directs and regulates inflammatory processes by expressing an array of adhesion molecules, such as inter-cellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and selectins which allow the binding of platelets, leukocytes and monocytes onto the endothelial surface (Sumpio et al., 2002). Furthermore, it proves to be an indispensible participant in infection, haemostasis and immune responses. In terms of vascular pharmacology, the endothelium mainly controls underlying vascular smooth musculature hence vascular tone via the L-arginine/NO/cyclic guanosine monophosphate (L-arginine/NO/cGMP) pathway. In this pathway, nitric oxide (NO) is synthesised in the endothelial cells from L-arginine by the enzyme,

endothelial NO synthase (eNOS or NOS3) with the presence of important cofactors such as calmodulin (CAM) and tetrahydrobiopterin (BH_4) etc. This signaling cascade is activated initially in response to blood flow shear stress or to receptor agonists such as acetylcholine, bradykinin and adenosine triphosphate (Figure 2.2). The endothelial-derived NO then diffuses directly into the adjacent VSMCs where it activates the enzyme soluble guanylate cyclase (sGC) which increases the intracellular concentrations of cGMP substantially from guanosine triphosphate (GTP). Sequentially, cGMP activates the cGMP-dependent kinase I which in turn increases the open probability of Ca^{2+}-activated K^+(BKCa)-channels, leading to membrane hyperpolarisation as a result of an efflux of K^+ and relaxation of the VSCMs.

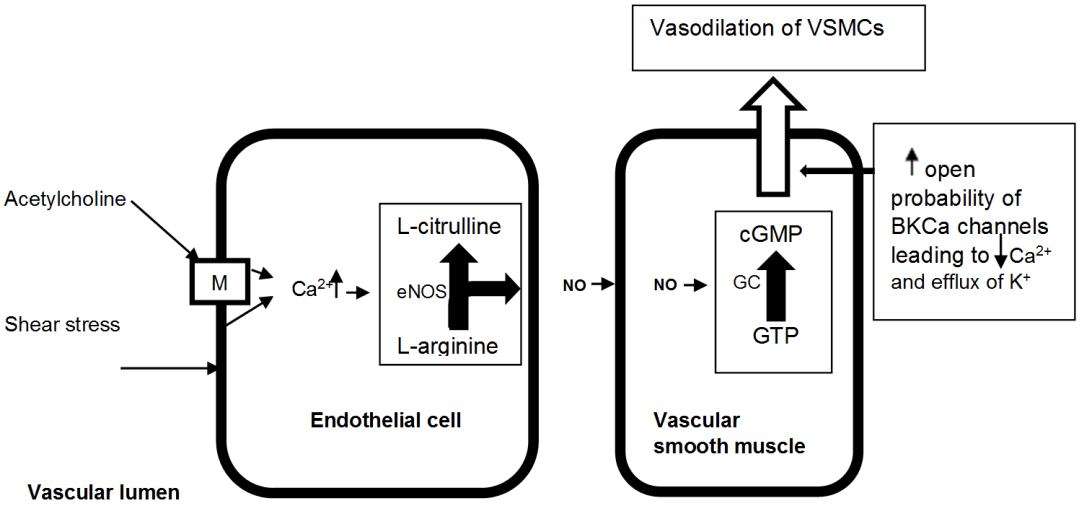

Figure 2.2 The L-arginine/NO/cGMP pathway (cGMP- cyclic guanosine 3',5'-monophosphate; eNOS- endothelial nitric oxide synthase; GC- guanylate cyclase; GTP- guanosine 5'-triphosphate; M- muscarinic receptor; NO- nitric oxide).

2.1.2 Vascular smooth muscle

VSMCs are mechanically active effectors of the vasculature and can be found in all blood vessels except the capillaries. By contraction or relaxation, the VSMCs change the distensibility of the vasculature which consequently results in vasoconstriction and vasodilatation of the blood vessels. It is recognised that VSMCs are not only controlled by the autonomic nervous system, specifically the sympathetic nervous system, but are also regulated by neurohormonal mechanisms such as circulating catecholamines e.g. adrenaline and local mechanisms such as locally produced metabolites which act directly on the VSMCs (Table 1.1).

Table 2.1 Mechanisms involved in regulating vascular tone (Golan et al., 2012).

Mechanisms involved	**Effect on VSMCs**
Autonomic nervous system	Activation of α1-adrenergic receptors on VSMCs results in vasoconstriction whereas activation of β_2-adrenergic receptors on VSMCs causes vasodilatation
Neurohumoral mechanisms	Circulating catecholamines secreted from the adrenal glands regulate vascular tone via α1-adrenergic receptors and β_2-adrenergic receptors on VSMCs. Other neurohumoral mediators include angiotensin II which acts on angiotensin II receptor subtype I leading to vasoconstriction of arterioles
Local mechanisms	Includes autoregulation of VSMCs (by vasoconstriction or vasodilatation) in response to changes in perfusion pressure and regulation of vascular tone by locally producing substances such as H^+, CO_2, O_2

Structure

VSMCs typically have a diameter of 5 to 10 µm and a length which varies from 50 to 300µm. They make up the main stromal portion of the blood vessel wall and have the role of producing the extracellular matrix (ECM) which provides the arterial wall with high capability to withstand high pressure of circulating blood (Clark and Pyne-Geithman, 2005). Structurally, the cell membrane of VSMC contains caveolae (small invaginations) which increase the cell surface area significantly and within the cell, contractile proteins such as actin and myosin filaments are connected by dense bands and dense bodies (Figure 2.3).

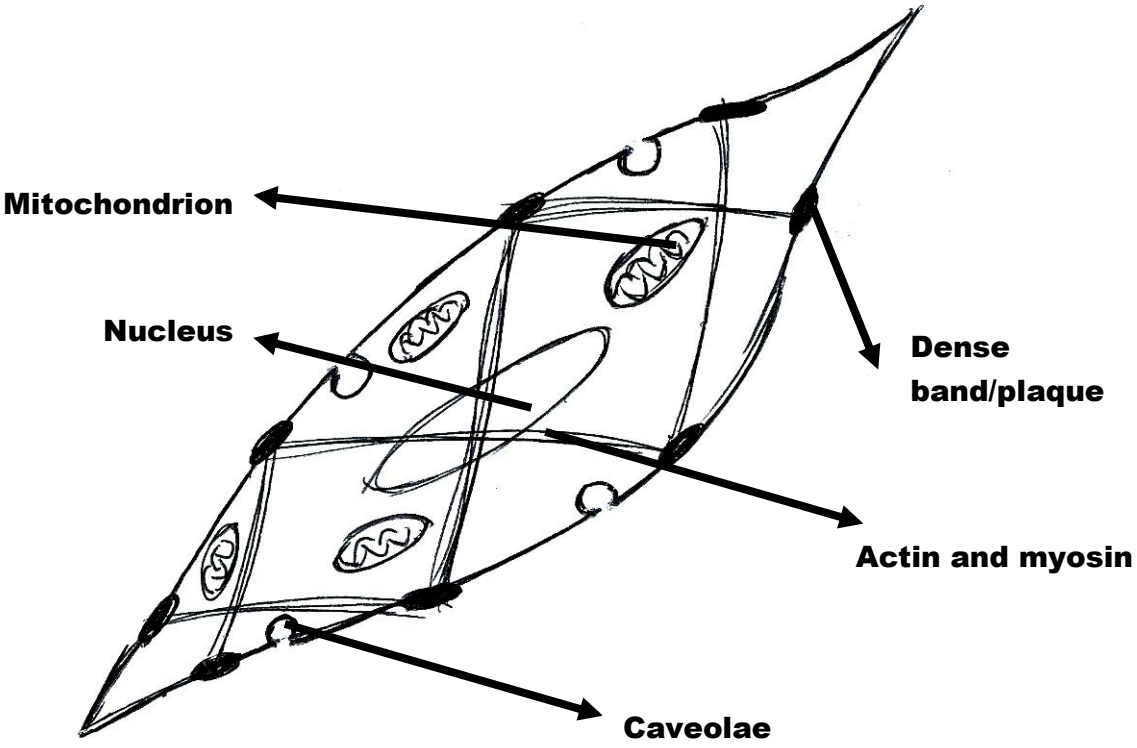

Figure 2.3 Structure of a vascular smooth muscle cell.

Function

Smooth muscles can be categorised according to their function: (i) tonic or (ii) phasic contracting smooth muscle (Fisher, 2010). VSM displays characteristics of tonic smooth muscle and they are generally thought to be continuously contracted. Conversely, visceral smooth muscles, such as those found in the urogenital and gastrointestinal system, are phasic smooth muscle which displays rhythmic contractile activity (Fisher, 2010).

VSM constitutes most of the intermediate layer (tunica media) of the blood vessels and their contraction and relaxation play an important role in the maintenance of blood pressure and the regulation of vascular resistance which are crucial in ensuring adequate blood flow to the surrounding tissues (Creager et al., 2006). Abnormal VSM contractions are associated with complications such as ischaemia, infarction and hypertension. In addition, VSM plays a role in blood vessel remodeling in physiological conditions e.g. exercise and pregnancy or after blood vessel injury (Owens et al., 2004). Under these circumstances, VSM increase their proliferation and migration and produce large quantities of extracellular matrix components. Hence, VSM is not only involved in short term regulation of vascular tone but are also involved in long term adaptation via structural remodeling of the vasculature.

Regulation of vascular smooth muscle function

VSM contraction and relaxation occurs as a direct result of the phosphorylation and dephosphorylation respectively of the myosin light chain (MLC). VSM contraction is initiated by an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) due to an influx of Ca^{2+} from the extracellular space into the VSMC through voltage-gated

Ca^{2+} channels in the plasma membrane and the release of Ca^{2+} into the cytosol from the smooth sarcoplasmic reticulum intracellular stores down the concentration gradient. Ca^{2+} binds to CAM to form the Ca^{2+}-CAM complex which then activates myosin light chain kinase (MLCK) and causes phosphorylation of MLC, interaction between actin and myosin and subsequently VSM contraction (Figure 2.4) (Walsh, 1994). VSM relaxation, on the other hand, is initiated by a decrease in intracellular Ca^{2+} ($[Ca^{2+}]_i$) due to efflux of Ca^{2+} via the plasma membrane Ca^{2+} pump and Na^{+}-Ca^{2+} exchanger and the reuptake of Ca^{2+} by the smooth sarcoplasmic reticulum Ca^{2+} pump. The fall in $[Ca^{2+}]_i$ leads to Ca^{2+}-CAM complex dissociation and followed by the dephosphorylation of the phosphorylated MLC by MLC phosphatase (Walsh, 1994).

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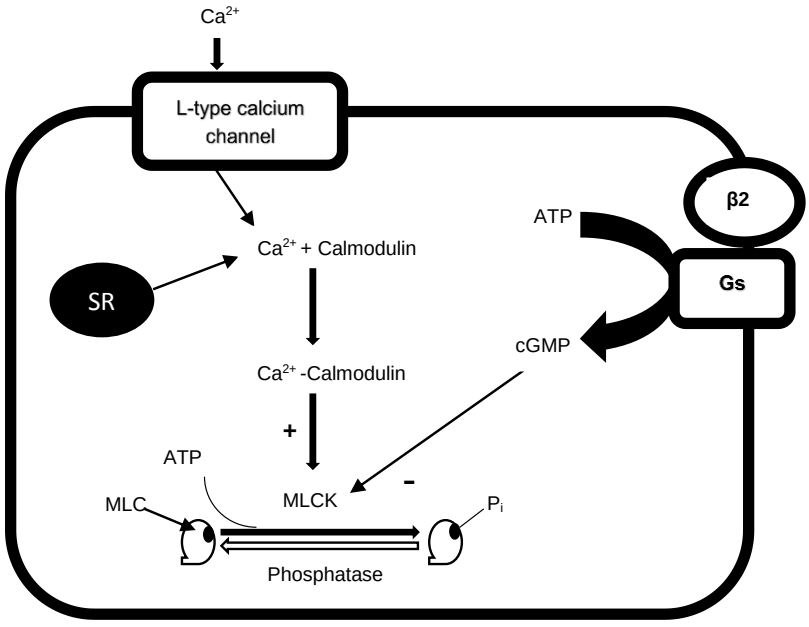


Figure 2.4 Mechanism behind vascular smooth muscle contraction. Phosphorylated MLC leads to cross-bridge formation between actin filaments and myosin heads lead to VSMC contraction.

2.2 Cardiovascular Disease and Vascular Dysfunction

2.2.1 Epidemiology of cardiovascular disease

According to the World Health Organisation (WHO), CVD is the leading cause of global death and disability, accountable for one-third (17.3 million) of all annual deaths (WHO, 2012). WHO projected that by 2030, CVD will remain as the main cause of global mortality affecting 23.6 million people (WHO, 2012). In the United Kingdom in 2008, similar trends were observed with CVD being the leading cause of mortality, responsible for around one-third of all deaths (191000) annually (BHF, 2012). In UK in 2009, it was estimated that CVD costs the healthcare system approximately £8.6 billion annually (BHF, 2012). In addition, non-health care costs such as production losses due to CVD death and illness in the current workforce, cost the UK over £3 billion per annum (BHF, 2012). In view of the huge economic burden of treating already established CVD, it would be of wise to implement the more cost-effective cardiovascular prevention strategies.

2.2.2 Risk factors of cardiovascular disease and cardiovascular risk score

Throughout the years, the Framingham Heart Study which started in 1948 has identified numerous risk factors associated with CVD. These risk factors include age, sex, cigarette smoking, systolic blood pressure, diabetes mellitus, electrocardiogram-based left ventricular hypertrophy, total cholesterol and HDL cholesterol. Based on these risk factors, researchers have constructed risk scoring systems such as the well-established Framingham Cardiovascular Risk Score (FCVRS) and more recently in the UK, the QRISK®2 CV Risk Score

(Table 2.1 and Table 2.2). In Scotland, the Scottish Heart Health Extended Cohort develop a new CV risk score, ASSIGN to minimise the potential unfairness in the FCVRS when applied across different social groups in the same population. These efforts are important as it enables clinicians to identify and predict the CV risk of individual within a specified period hence allowing effective primary prevention against CVD. Current guidelines in the UK recommend that all adult aged 40 or more, and adults at any age who have a first-degree relative with hereditary lipid disorders and premature CVD to undergo at least every five yearly CV risk assessment (NICE, 2007). This means that adults younger than 40 years and/or those without CV risk factors or family history may be perceived as “healthy” and are misleadingly regarded as having no CVD risk when they may actually do. Moreover, it is uncertain whether these scoring systems are equally accurate at predicting CV risk across certain subgroups. It is recognised that the FCVRS have a tendency to overestimate risk in low and medium risk groups and underestimate risk for certain subgroups such as British Asians and people of African origin, people with Type I diabetes mellitus or Type 2 diabetes with nephropathy, people with hereditary lipid disorders, those with a strong family history of premature CVD etc (Brindle et al., 2006). The FCVRS also underestimated the true level of CVD in men with lower socioeconomic status (Woodward et al., 2007). Conversely, although FCVRS is widely used in the general population to stratify CV risk, the value of this risk score is less clear in younger adults and women.

Table 2.2 Predictors for Framingham Cardiovascular Risk Score (FCVRS). FCVRS predicts the chance of a person having CVD in the next 10 years and only applicable to adults aged 20 and older who do not have CVD, intermittent claudication and diabetes mellitus

Age (years) Total cholesterol (mg/dL) HDL cholesterol (mg/dL)	Smoking status Systolic blood pressure (mmHg) Treatment for hypertension

Table 2.3 Predictors in the QRISK®2 CV Risk Score. Validation studies have concluded that QRISK®2 CV Risk Score is better calibrated to the UK population as it provides better risk estimates than Framingham risk score in identifying patients with high CVD risk in the UK (Hippisley-Cox et al., 2008)

Age Sex Ratio of serum cholesterol to HDL levels Systolic blood pressure	Body mass index Family history of premature CVD Smoking status Townsend deprivation score Treatment for hypertension

2.2.3 Risk stratification of cardiovascular disease with vascular function tests

It is not uncommon for patients to present with sudden unexpected death as the first manifestation of CVD (Priori et al., 2001). Annually, it is estimated that 1 in 1000 in the general population dies of sudden cardiac death (Straus et al., 2004). Since the mortality rates from out-of hospital cardiac arrests are distressingly high, it is therefore fundamental to identify alternate approaches to estimate CV risks especially in asymptomatic patients (Roger et al., 2011).

Over the past few decades, vascular endothelial function has been extensively researched and this is reflected from the numerous publications over the years

which showed the effectiveness and accuracy of vascular function as a surrogate marker of CV health. Fichtlscherer et al. demonstrated that systemic vascular dysfunction predicts the recurrence of cardiovascular events in patients with acute coronary syndromes (Fichtlscherer et al., 2004). Gokce et al. found that impaired large vessel function is an independent predictor of long-term CV events in patients with peripheral arterial disease whereas Shimbo et al. noted the same observation in a multi-ethnic population who previously had no history of cardiovascular diseases (Gokce et al., 2003, Shimbo et al., 2007). Furthermore, much research work carried out in the past has shown that most if not all, cardiovascular risk factors are associated with vascular dysfunction (Schächinger et al., 2000, Suwaidi et al., 2000, Halcox et al., 2002).

2.2.4 Vascular dysfunction

It is understood that the healthy human vasculature is pro-vasodilatator, anti-inflammatory and exerts antithrombotic and antiplatelet properties which are central to prevent thrombotic events (Davignon and Ganz, 2004, Flammer and Lüscher, 2010). The endothelium- derived vasodilatator effect is known to be primarily mediated by the major vasodilator agent, NO. In endothelial dysfunction, there exists a disruption in balance between vasodilatator and vasoconstrictor agents resulting in an endothelium which promotes vasoconstriction, thrombosis, impairment of coagulation, platelet activation and leukocyte adherence as well as VSMC proliferation and migration (Verma and Anderson, 2002) (Figure 2.5). Under these circumstances, endothelium-dependent vasoconstriction predominates due to the release of vasoconstrictor agents such as endothelin-1 and angiotensin II. In addition, the concomitant lack of NO bioavailability results in a reduction of blood vessel vasodilatation

and a disruption in the vascular growth remodeling mechanism favouring all stages of atherogenesis. The lack of NO bioavailabilty could be explained by the following: (i) a reduction in eNOS expression; (ii) inadequate substrate L-arginine or co-factors for eNOS or the presence of antagonists such as asymmetric dimethyl arginine; (iii) impaired activation of eNOS and (iv) an increased NO degradation (Faulx et al., 2003).

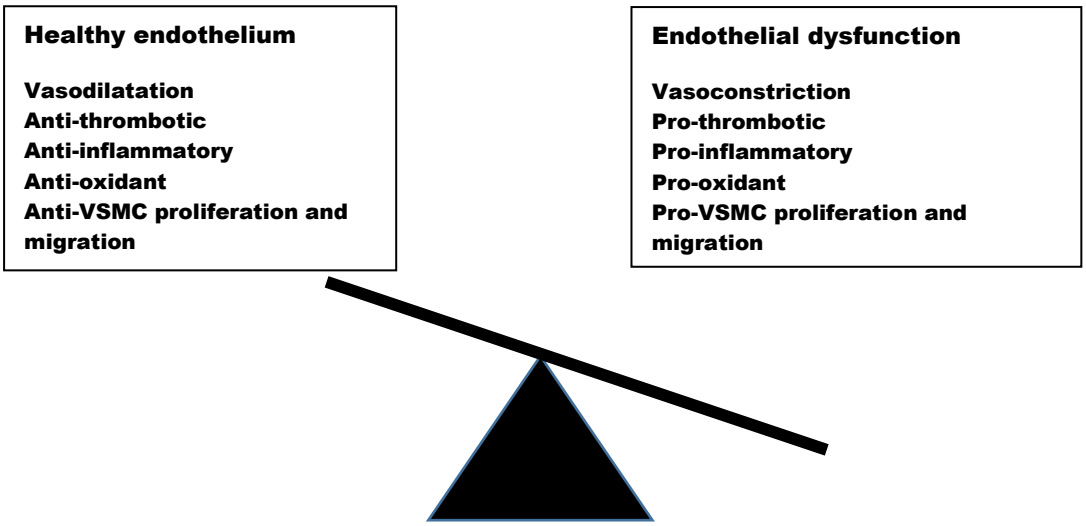


Figure 2.5 Phenotypic characteristics of healthy and diseased endothelium

It is known that endothelial lining injury coupled with endothelial dysfunction appears to be an early pivotal event in atherogenesis and has been demonstrated to herald the development of clinically detectable atherosclerotic plaques in the coronary arteries (Mano et al., 1996, Ross, 1999, Glass and Witztum, 2001). Considering the fact that controlling and treating cardiovascular risk factors such as hypercholesterolaemia, hypertension, hyperglycaemia and stopping smoking, leads to the reversal of endothelial dysfunction, endothelial dysfunction can therefore be used as a surrogate marker for predicting the risk of developing CVDs in addition to being a primary therapeutic target in preventing atherosclerotic disease.

2.2.5 Pathophysiology of vascular dysfunction

Under homeostatic conditions, the maintenance of vascular tone and blood fluidity is tightly regulated by the endothelium. In addition, proinflammatory factors such as tumour necrosis factor-α (TNFα) and C-reactive protein (CRP) are not or minimally expressed in the vasculature. However, when both traditional and novel cardiovascular risk factors such as hypercholesterolaemia, hypertension, hyperglycaemia, smoking, advancing age, and a family history of premature atherosclerotic disease are present, a chronic inflammatory process is initiated which results in alteration in endothelial function. This alteration can be seen as reduction in antithrombotic factors and an increase in vasoconstrictor and prothrombotic products (Widlansky et al., 2003). It is also now recognised that risk factors including obesity, raised C-reactive protein and chronic systemic infection are related with endothelial dysfunction (Widlansky et al., 2003).

Since it is clear that peripheral vascular function is predictive of CV function, surrogate markers of vascular function in the peripheries could potentially be indicators of CV risk. One of these potential surrogate markers could be potassium channels which can be found in VSMCs.

The next chapter will discuss about the role of K_{ATP} channels in peripheral vasoregulation and how these channels can potentially be surrogate markers of vascular function.

CHAPTER 3

ATP-sensitive potassium channels (K_{ATP} channels)

3.1 Background

The importance of potassium channels and their role in vivo are gaining recognition among researchers. Amidst the different potassium channels expressed in vivo, the least understood are the K_{ATP} channels in endothelium and VSMCs (Köhler and Ruth, 2010). Not much is known about these channels, however, some effort has been made in the past, using genetically manipulated mice models to allow a greater understanding of the physiological and pathophysiological roles of these K_{ATP} channels (Seino and Miki, 2003).

The endothelium and vascular smooth muscle cells express four different classes of potassium ion channels: (1) voltage-gated potassium channels (K_V); (2) Ca^{2+}-activated potassium channels (K_{Ca}); (3) ATP-sensitive potassium channels (K_{ATP}) and (4) inwardly rectifying potassium channels (Kir) (Hibino et al., 2010). Of particular interest to this study are the K_{ATP} channels in endothelium and vascular smooth muscle cells since they have been shown to be downregulated in disease states such as diabetes (Mayhan, 1994, Zimmermann et al., 1997, Miura et al., 2003) and hypertension (Sobey, 2001) where vascular dysfunction is the hallmark of these diseases (Puddu et al., 2000, Calles-Escandon and Cipolla, 2001). It would be of interest to examine the feasibility of assigning a quantitative measure to vascular dysfunction by assessing the levels of K_{ATP} channels.

3.2 General features of K_{ATP} channels

K_{ATP} channels are potassium-ATP-sensitive pores. They were first identified in heart muscles and since then were found to be expressed in a variety of tissues such as the pancreatic β-cells, skeletal muscle, the brain, vascular smooth muscle and endothelium (Noma, 1983). In the heart, activation of K_{ATP} channels was shown to play a cardioprotective role against cardiac failure (Yamada et al., 2006). In the pancreatic β-cells, they regulate glucose-stimulated insulin secretion whereas in skeletal muscles, they are key in controlling insulin-dependent glucose uptake into these muscles (Aguilar-Bryan et al., 2001, Lin et al., 2005, Ashcroft and Gribble, 1999, Seino et al., 2008). On the other hand, K_{ATP} channels activation in the brain has a neuroprotective effect against metabolic challenge (Zeng et al., 2007). In the endothelium and VSMCs, K_{ATP} channels have been reported to play an important role in regulating vascular tone (Standen et al., 1989).

The K_{ATP} channels provide a link between the cellular metabolism and membrane electrical excitability. One of the characteristics of these channels is that their open-state probability depends on the concentrations of intracellular ATP, ADP and other nucleotides. In other words, these channels are regulated by intracellular ATP and ADP. Furthermore, they are targets which can be activated pharmacologically by chemicals such as K^+ channel openers e.g. iptakalim and be inhibited by sulphonylurea derivatives e.g. glibenclamide. It is not exactly clear what role K_{ATP} channels play in vascular dysfunction but it was widely agreed that they are responsible for the maintenance of the resting

potential of endothelial cells and the regulation of release of vasoactive compounds (Wang et al., 2007).

K_{ATP} channels are targets of endogenous vasodilators, primarily NO, and vasoconstrictors, such as endothelin-1 (Quayle et al., 1997). Previous studies have shown that activation of K_{ATP} channels using K^+ channel openers such as nicorandil results in membrane hyperpolarisation, a reduction in voltage-dependent Ca^{2+} channel activity and ultimately vasodilatation of VSMCs (Jackson, 1998, Brayden, 2002). On the other hand, blocking K_{ATP} channels, both in vitro and in vivo, leads to membrane depolarisation and vasoconstriction of VSMCs (Kubo et al., 1997). In disease states such as hypertension and diabetes, the activity of the K_{ATP} channels appear to be downregulated (Zimmermann et al., 1997, Miura et al., 2003). In animal models, disruption of K_{ATP} channels in VSMCs results in coronary arteries vasospasm and sudden cardiac death (Miki et al., 2002, Chutkow et al., 2002). These observations not only demonstrate the importance of K_{ATP} channels in regulating vascular tone in animal models but also raises some important questions- could these channels possess similar function in humans? If this is the case, could K_{ATP} channels potentially act as blood biomarker of vascular dysfunction in humans? This research project will attempt to address these questions.

Structurally, K_{ATP} channels are octamers (Clement et al., 1997, Shyng and Nichols, 1997) which consist of: 4 α-subunits [the pore-forming inwardly rectifying potassium channels (Kir6)] and 4 β-subunits [the ATP-binding sulphonylurea receptors (SUR)]. To date, two Kir6.x genes (*KCNJ8* for Kir6.1 and *KCNJ11* for Kir6.2) and two SUR genes (*ABCC8* for SUR1 and *ABCC9* for

SUR2A and Sur2B) are identified (Inagaki et al., 1995, Inagaki et al., 1996, Standen, 2003, Shi et al., 2012). Both Kir6 and SUR components are required to form a complete active K_{ATP} channel giving a 1:1 stoichiometry (Aguilar-Bryan et al., 1998). In the vascular smooth muscle, the molecular identities of these K_{ATP} channel isoforms have not been determined to the same degree of certainty as the other K_{ATP} channels such as those found in the pancreatic β-cell (Kir6.2/SUR1) and cardiac muscle (Kir6.2/SUR2A) (Standen, 2003, Teramoto, 2006). Two types of channels namely Kir6.2/SUR2B channels (Isomoto et al., 1996) and Kir6.1/SUR2B channels (Yamada et al., 1997) have been cloned and identified in the VSMCs. Nonetheless, there is good evidence that Kir6.1/SUR2B channel plays a more important role in vascular smooth muscle (Figure 3.1) (Suzuki et al., 2001).

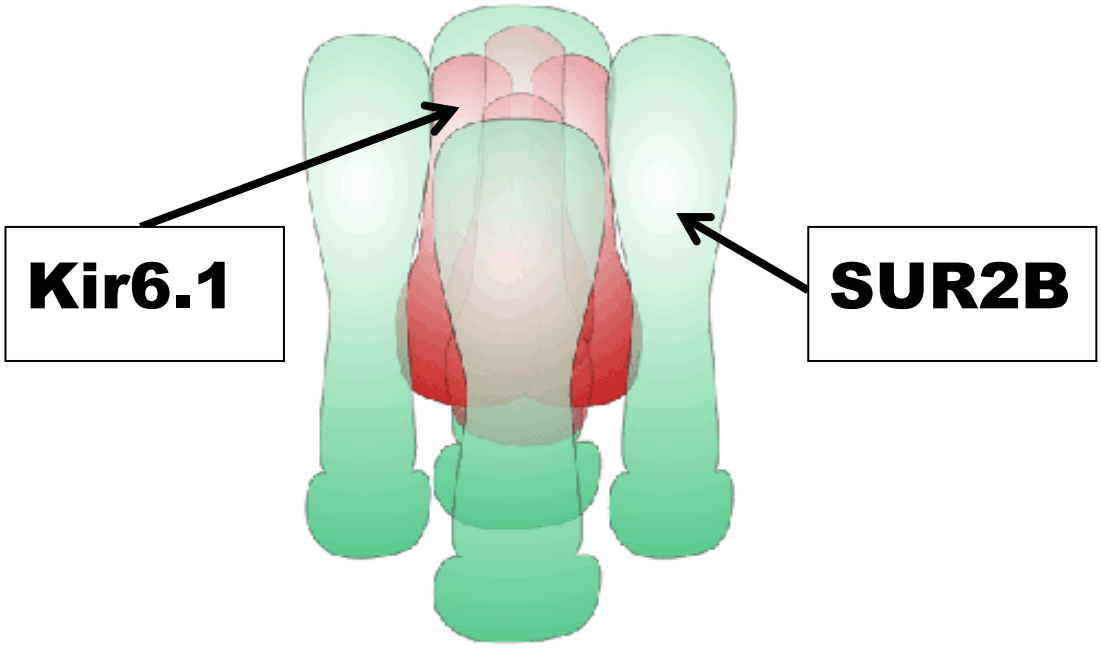


Figure 3.1 The structure of the vascular smooth muscle KATP channels. Kir6.1 is the pore-forming subunit whereas SUR2B is the regulatory subunit.

3.3 Evidence that K_{ATP} channels exist in the human blood cell

It was thought by Du and Jovanovic (unpublished results) and several other researchers that both Kir6.1/SUR2B subunits of K_{ATP} channels would be present in human blood cells as well, since they are expressed in mice blood cells (specifically leukocytes) (Du and Jovanovic, unpublished results, Da Silva-Santos et al., 2002, Pompermayer et al., 2005, Zanardo et al., 2006). Da Silva-Santos *et al.* (2002) established that inflammatory neutrophil migration was mediated by K_{ATP} channels in mice. They showed that glibenclamide, a K_{ATP} channel blocker, decreases the exudation and neutrophil influx whereas minoxidil, a K_{ATP} channel opener, increases the exudation and neutrophil influx (Da Silva-Santos et al., 2002). Zanardo *et al.* (2006) demonstrated that hydrogen sulphide, an endogenous vasodilator, down-regulated leukocyte adherence via the activation of K_{ATP} channels which were found on the leukocytes of rats themselves (Zanardo et al., 2006). This is strongly supported by more recent studies by Dal-Secco *et. al.* (2008) and Spiller *et. al.* (2010) (Dal-Secco et al., 2008, Spiller et al., 2010). Both respectively showed that neutrophil migration during an inflammatory and infection response rely on a K_{ATP}-dependent mechanism.

3.4 Understanding of the pathophysiological roles of K_{ATP} channels from genetically manipulated mice models

Genetic manipulation of the K_{ATP} channels mRNA subunits in animal models such as mice have clarified some of the many pathophysiological roles of K_{ATP} channels in the vasculature. Two studies of animal models, Kir6.1 knockout

mice and SUR knockout mice, have demonstrated that both knockout mouse models do not exhibit K_{ATP} channel activity in the smooth muscle cells of the aorta and that there was no pinacidil-induced vasodilation observed (Miki et al., 2002, Chutkow et al., 2002). These findings suggest that both Kir6.1 and SUR2 mRNA subunits are required to form a fully functioning K_{ATP} channel and that the activation of Kir6.1/SUR2 channels has an important role in mediating the vasodilator effects of potassium channel openers in VSMCs (Seino and Miki, 2003).

3.5 Role of K_{ATP} in vascular dysfunction

Various studies have been conducted and showed that activation of K_{ATP} channels in the vasculature results in the protection against vascular dysfunction. One such study found that iptakalim, a new K_{ATP} channel opener, protects endothelial cells against vascular dysfunction through the activation of K_{ATP} channels which inhibits the release and synthesis of endothelin-1 and increases NO release and endothelial NO synthase activities (Wang et al., 2007). This finding was supported by Long *et*. *al*. (2008) and Pan *et*. *al*. (2010) where they reported that the activation of K_{ATP} channels by iptakalim protected vascular function against hypertension (Pan et al., 2010) and renal injury (Long et al., 2008). On the other hand, Date *et*. *al*. (2005) showed that nicorandil, also a K_{ATP} channel opener, is capable of preserving vascular function by inhibiting endothelial cell apoptosis through mitochondrial K_{ATP} channels (Date et al., 2005).

The exact mechanism as to how K_{ATP} channel activation leads to the protection against vascular dysfunction is unclear. However, three possible pathways have

been implicated. They are: (i) NO/cGMP pathway, (ii) prostaglandin/cAMP pathway and (iii) adenosine/cAMP/protein kinase A (Nelson and Quayle, 1995, Kleppisch and Nelson, 1995, Quayle et al., 1997, Standen and Quayle, 1998). There is accumulating evidence that K_{ATP} channels expressed in different tissues can be modulated by NO. In the heart, Shinbo and Iijima showed that NO potentiates the action of K^+ channel openers on the K_{ATP} channels via an unknown mechanism (Shinbo and Iijima, 1997). This finding was validated by subsequent studies and it was suggested that the possible mechanism by which NO exerts its effect on K_{ATP} channels is through the NO/cGMP/PKG pathway (Baker et al., 2001, Han et al., 2002, Cuong et al., 2006). In pancreatic β-cells, K_{ATP} channels activation by NO is likely due to decreased production of ATP as a result of impaired glucose metabolism (Tsuura et al., 1994), while in VSMCs, activation of K_{ATP} channels by NO was suggested via a cGMP-mediated pathway leading to blood vessel vasodilatation (Kubo et al., 1994, Murphy and Brayden, 1995).

Only one study to date was carried out to determine the relationship between levels of K_{ATP} channels in blood cells and vascular function in humans (Choong, 2010 (unpublished results)). In the current project the aim is to investigate this relationship and examine the feasibility of using K_{ATP} channels levels in blood cells as a possible biomarker of vascular dysfunction.

In the following chapter, the different modalities available to assess vascular function invasively and non-invasively will be described and discussed.

CHAPTER 4

Assessment of vascular function in humans

4.1 Background

The assessment of vascular function includes the measurement of endothelial function and arterial stiffness. When assessing endothelial function, interventions such as administration of vasoactive agents like acetylcholine; or mechanical stimulus such as shear stress, are applied onto the vessel in question (Verma et al., 2003). In the healthy vascular endothelium, endothelium-dependent vasodilatation predominates primarily through the release of endothelial-derived NO. Conversely, in vascular endothelial dysfunction, an impaired endothelium-dependent vasodilation or paradoxical vasoconstriction can be observed. Vascular endothelial function can be assessed either invasively or non-invasively in the coronary or peripheral circulations (Hadi et al., 2005). Some techniques are summarised in Table 4.1. Doppler Echocardiography, PET and phase contrast MRI will not be discussed in this section as they are out with the scope of the study. The vasculature can also be subdivided into macrovessels or microvessels. The assessment of macrovessels or microvessels overlaps with the assessment of the coronary and peripheral circulations as stated in Table 4.1. Briefly, macrovessels are conduit arteries that play an important role in distributing blood flow from the heart to the different organs in the body. In addition, it helps to reduce the load on the heart and increase blood pressure during diastole by providing

compliance to each stroke volume produced by the heart. On the other hand, microvessels have the role of regulating local vascular resistance in response to local demand as well as to control blood flow to tissues and organs. Arterial stiffness measurement is another domain of vascular function assessment. This can be measured using the Pulse Wave Analysis test as illustrated below.

Table 4.1 Methods for assessing vascular function.

Central circulation		**Peripheral circulation**	
Invasive	**Non-invasive**	**Invasive**	**Non-invasive**
Intracoronary agonist infusion with quantitative coronary angiography and intracoronary Doppler*	Doppler Echocardiography*	Intrabrachial infusion of agonist with Venous Occlusive Plethysmography (VOP)^	Carotid Intima-Media Thickness (CIMT) on Ultrasound*
	Positron Emission Tomography*		Brachial Artery Flow-mediated Dilatation (FMD)*
	Phase contrast MRI*		EndoPAT^
			Pulse Wave Velocity (PWV)*
			Pulse Wave Analysis (PWA)*^
			Iontophoresis with Laser Doppler Flowmetry (LDF)^
			Post-occlusive Reactive Hyperaemia with

			Full-field Laser Perfuser Imager (FLPI)^
PWV and PWA measure arterial stiffness; the other tests measure vascular endothelial function. *Assessment of macrovessels ^Assessment of microvessels			

4.1.1 Intracoronary agonist infusion with quantitative coronary angiography and intracoronary Doppler

Intracoronary agonist infusion with quantitative coronary angiography is one of the earliest methods used to evaluate coronary endothelial function in response to intracoronary infusion of an agonist such as acetylcholine or increase in blood flow (Ludmer et al., 1986, Cox et al., 1989, Hadi et al., 2005). To measure coronary blood flow velocity in response to the agonist, a Doppler catheter is positioned into the studied epicardial coronary artery, such as the left anterior descending artery, using a guide catheter (Nabel et al., 1990). Coronary blood flow can be calculated from the velocity and the cross sectional area of the blood vessel which can be obtained by quantitative coronary angiography (Figure 4.1) (Newby, 2000). Infusion of the agonist into the coronary artery is through the Doppler catheter. Angiography is performed after the bolus injection within a specific time. This is followed by recovery and intracoronary infusion of nitroglycerin to test endothelium-independent vasodilatation. In healthy vascular endothelium, an increased in blood flow velocity as a result of endothelium-dependent vasodilatation can be observed. In endothelial dysfunction, there is an abnormal vasomotor response whereby the vessel fails to dilate in response to the agonist.

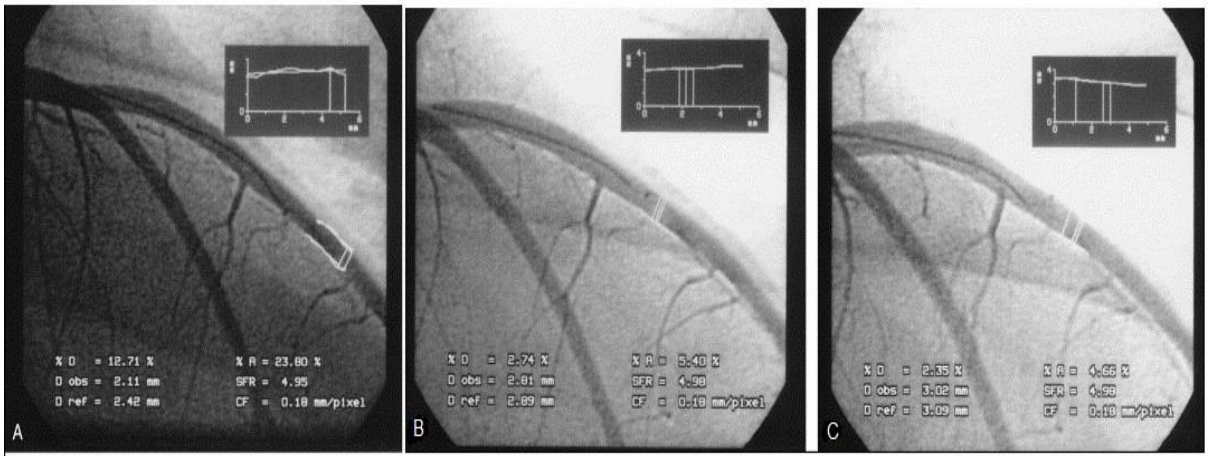


Figure 4.1 Changes in coronary artery cross-sectional diameter obtained by quantitative coronary angiography (a) Baseline; (b) ACH infusion; (c) Nitroglycerin administration (Adapted from Richartz et al., 2001).

4.1.2 Intrabrachial infusion of agonist with venous occlusive plethysmography

This method involves the infusion of endothelium-dependent agonists such as acetylcholine into the brachial artery and the subsequent measurement of the vasomotor responses of forearm resistance vessels using venous occlusion plethysmography (VOP) (Widlansky et al., 2003b). Unlike the study of coronary circulation, this method allows the evaluation of vascular endothelial function in a more accessible vascular bed. Since vascular dysfunction is recognised as a diffuse pathology, peripheral vascular abnormalities have been shown to correlate significantly with coronary vascular abnormalities, therefore, allowing peripheral vascular testing to be an indirect surrogate of coronary vascular function (Kuvin et al., 2007).

VOP is a widely used method for assessing forearm blood flow (Figure 4.2). It was first described by Hewlett and Zwaluwenburg in 1909 and has remained essentially the same since then (Hewlett and Zwaluwenburg, 1909). The

underlying principle involves occlusion of venous outflow from the forearm but not arterial inflow which results in swelling of the forearm due to an increase in forearm volume over time (Alam et al., 2005). The forearm blood volume can be measured by voltage-dependent strain-gauge and it is a direct indicator of forearm arterial blood inflow (Wilkinson and Webb, 2001). VOP allows forearm vascular resistance to be tested and by manipulating intrabrachial infusion of vasoactive agents, changes in forearm vascular resistance can be a good reflection of normal vascular endothelial function (Alam et al., 2005). Studies that have utilised VOP to assess vascular function include those that are carried out by Panza et. al. (1900) and Creager et. al.(1900) (Panza et al., 1990, Creager et al., 1990). Panza et. al. (1990) showed impaired endothelium-mediated vasodilatation in response to acetylcholine in a group of patients with essential hypertension (Panza et al., 1990). Using intrabrachial artery infusions of methacholine and nitroprusside with VOP, Creager et. al. (1990) demonstrated, on both occasions, blunted vasodilator responses in humans with hypercholesterolaemia (Creager et al., 1990).

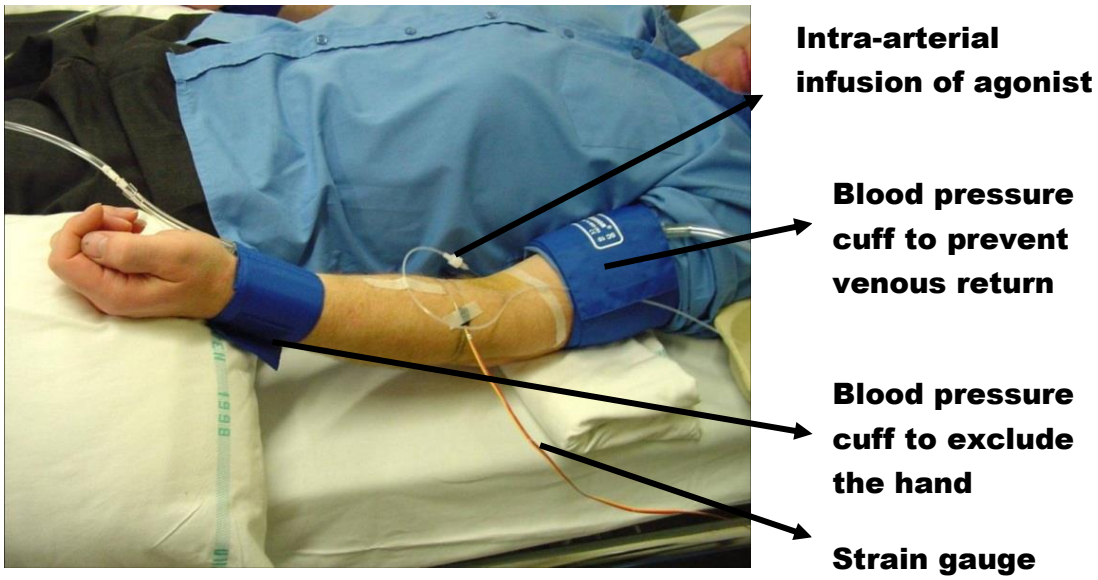


Figure 4.2 Assessment of forearm blood flow using venous occlusion plethysmography (Adapted from http://arwatch.co.uk/2011/11/allopurinol-to-reduce-cad-mortality/ (2013)).

4.1.3 Carotid intima-media thickness using ultrasound

Carotid intima-media thickness (CIMT) is a method widely used as a proxy end point in observational studies and research trials to predict CVD risk (Bots et al., 1997, Chambless et al., 1997, Hodis et al., 1998, O'Leary et al., 1999). The relationship between CIMT and CVD has been well established and CIMT has been shown to be an independent predictor of future CVD (Chambless et al., 1996, O'Leary et al., 1996, Chambless et al., 1997, O'Leary et al., 1999, Lorenz et al., 2007). This method also provides a measure of sub-clinical atherosclerosis, its progression and the degree of success in therapeutic intervention for preventing atherosclerosis. In addition, it is approved by the US Food and Drug Administration (FDA) as a marker of subclinical atherosclerosis. In brief, CIMT involves the measurement of the thickness of the intimal and medial layers of the carotid artery using B-mode ultrasound (Hurst et al., 2007) (Figure 4.3). Based on the Manheim Intima-media Thickness Consensus Panel,

a plaque is defined as a focal structure that builds up in the inner surface of the arterial lumen of at least 0.5mm or 50% of the surrounding IMT value or demonstrates a thickness > 1.5mm as measured from the tunica media-to-tunica adventitia interface to the tunica intima lumen interface (Touboul et al., 2007). Since CIMT is highly reproducible, simple, safe, noninvasive yet relatively inexpensive technique, it appears to be an attractive approach to the investigation of the cumulative effect of atherosclerosis risks.

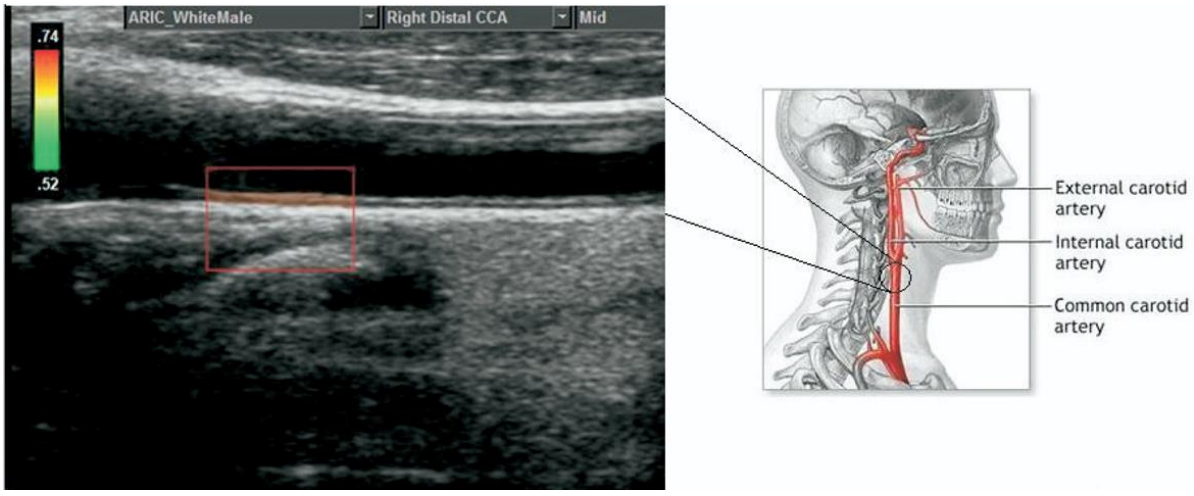


Figure 4.3 Image of the carotid artery obtained using ultrasound in carotid IMT (Adapted from Hurst et al., 2007).

4.1.4 Brachial artery flow-mediated dilatation

Flow-mediated dilatation (FMD) reflects endothelium-dependent vasodilatation of an artery in response to an increase in luminal blood flow (reactive hyperaemia) and internal-wall shear stress (Pyke and Tschakovsky, 2005, Thijssen et al., 2011). By measuring the luminal diameter of the artery before and after ischaemic cuff occlusion, vascular endothelial function can be estimated as FMD percentage change which represents the increase in artery diameter from resting conditions to maximum artery vasodilatation during reactive hyperaemia. FMD was first developed by Celermajer et. al. in 1992 and

since then, FMD has been extensively used as a non-invasive technique to measure macrovessel function in peripheral arteries such as the brachial artery, radial and femoral artery (Celermajer et al., 1992). The FMD of these peripheral arteries are often taken to represent the response of the central circulation (Anderson et al., 1995, Takase et al., 1998). In addition, FMD has been shown to be a reliable indicator of vascular function (Schroeder et al., 1999, Herrington et al., 2001, Meirelles et al., 2007, Simova et al., 2008, Pala et al., 2009). However, it is recognised that the reliability of FMD is dependent on various factors such as cuff placement site, duration of occlusion, addition of ischaemic handgrip exercise and the experience of the sonographer (Pyke and Tschakovsky, 2005). Furthermore, the coefficients of variation for brachial artery FMD have been reported to vary from as little as 1.5% to 50% in others (Sorensen et al., 1995, Andrews Md et al., 1997, de Roos et al., 2001). However, it is largely agreed that a normal brachial artery vasodilates about 5 to 15% from resting diameter post cuff release (Berry et al., 2000, Vogel, 2001). To address the disparities in FMD techniques across different research centres, guidelines for FMD procedures have been published to support standardisation of FMD measurements (Mullen et al., 2001, Coretti et al., 2002). Since FMD is non-invasive, well-tolerated, it can be safely used as a tool of measuring macrovessel endothelial function in studies involving large patient populations over-time. However due to technical difficulties, dependence on costly equipment and large interindividual differences, FMD has not been utilised in daily clinical practice.

The underlying physiological mechanism of FMD has been extensively investigated but it is not well understood. It is proposed that the mechanisms

mainly responsible for the observed FMD response may change over time when there is a prolonged shear stress stimulus (Pyke and Tschakovsky, 2005). Mullen et. al. (2001) suggested the possibility of sequential recruitment mechanisms i.e. prolonged shear stress will eventually lead to another mechanism taking over as the one that is primarily responsible for the FMD response (Mullen et al., 2001). However, it is generally agreed that the immediate FMD response is mainly mediated by NO (Pyke and Tschakovsky, 2005). During FMD, high resolution Doppler ultrasonography is used to scan the conduit artery e.g. the brachial artery under resting condition and post cuff release (reactive hyperaemia phase) which are induced by the inflation and deflation of the blood pressure cuff. The cuff is normally placed around the forearm distal to the site of ultrasound scan of the artery. The resultant shear stress caused by an increased in blood flow following ischaemic cuff released induces endothelium-dependent NO release, which in turn results in vasodilatation of the artery. Hence, based on previous studies, brachial artery FMD reflects NO bioavailability and its impairment signify conduit artery dysfunction which has been shown to correlate with the presence and severity of coronary artery disease (Nabel et al., 1990, Anderson et al., 1995, Neunteufl et al., 1997, Kaku et al., 1998, Neunteufl et al., 2000). In addition, brachial artery FMD was reported to predict restenosis in patients undergoing percutaneous coronary revascularisation (Patti et al., 2005) and ischaemic episodes in patients with ischaemic heart disease and hypercholesterolaemia (Søndergaard et al., 2002). Several studies have also found that FMD was predictive of CV events in high CV risk subjects (Gokce et al., 2002, Gokce et al., 2003, Akcakoyun et al., 2008). Gokce et. al. (2002). Nonetheless, FMD

responses should be interpreted with care as most recent FMD study by Wray et al. (2013) suggests that these responses should be viewed as endothelium-dependent vasodilatation but not necessarily NO-dependent vasodilation (Wray et al., 2013).

4.1.5 Endo-peripheral arterial tone

A more convenient way of assessing endothelial function is by using peripheral arterial tonometry (EndoPAT). The EndoPAT device consists of a fingertip plethysmograph which has the ability to detect volume changes in the fingertips with every arterial pulsation (Figure 4.4) (Axtell et al., 2010). This test involves placing finger probes on the index fingers of both hands and measuring digital pulse amplitude in the fingertip on one hand both at rest and after inducing reactive hyperaemia. Reactive hyperaemia is induced by inflating the blood pressure cuff to suprasystolic pressure and then rapidly deflating the cuff on the arm, temporarily decreasing blood flow to the fingers. The changes in digital pulse amplitude in response to reactive hyperaemia are expressed as reactive hyperaemia index (RHI). It is calculated as the ratio of the average pulse wave amplitude of the PAT signal over one minute, starting one minute after cuff release, divided by the average pulse wave amplitude at baseline i.e. 3.5-min time period before cuff inflation (Syvänen et al., 2011). This measurement reflects the local peripheral arterial tone. Measurements are also taken simultaneously in the contralateral arm to act as control for vascular tone changes which are independent of the endothelium (Figure 4.4). The precise technique involved in performing the EndoPAT test can be found in the paper published by Axtell et al (2010) (Axtell et al., 2010).

It has been shown that PAT correlated with traditional CV risk factor such as higher cholesterol, body mass index, smoking and diabetes and has been validated for the detection of impaired coronary microvascular function (Bonetti et al., 2004). The RHI score is also recognised to exhibit an excellent association with measures of coronary and peripheral vascular dysfunction (Kuvin et al., 2007).

The EndoPAT is a relatively rapid (15-30 minutes) and operator-independent method of assessing the effects of CV risk factors on endothelial function. Table 4.2 summarises the advantage and disadvantages of this technique. In addition, EndoPAT is a validated method that can be used to determine the effects of therapeutic interventions and lifestyle changes on vascular function (Moerland et al., 2011)

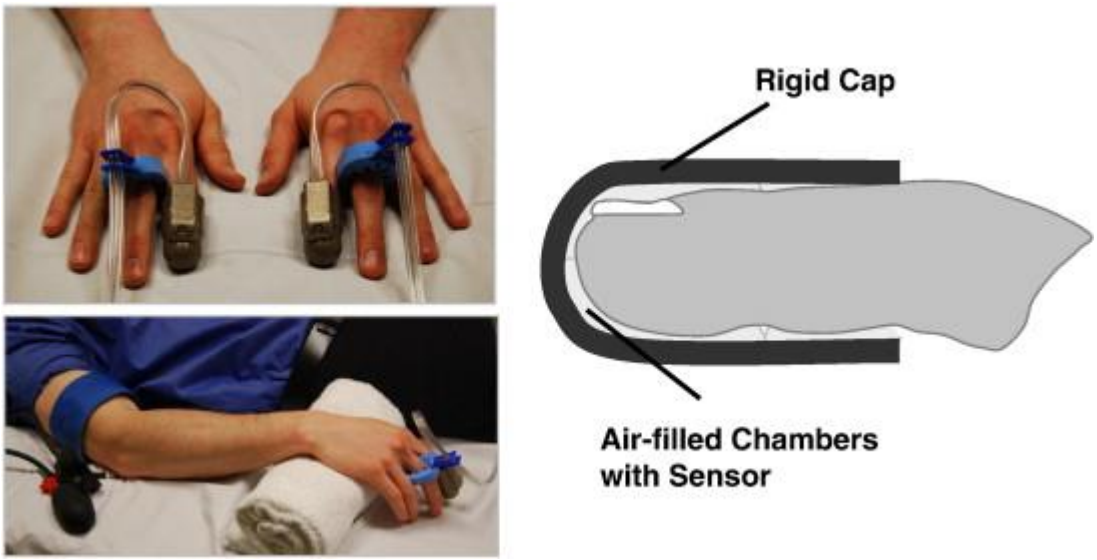


Figure 4.4 On the top left corner- the PAT device, which is a plastic cap with a closed end, is placed on an index finger of each hand. Tubes running from the finger probes connect the EndoPAT device to a recording unit that conveys data directly to a computer for analysis. On the right- a diagrammatic illustration of the device showing a longitudinal section of the finger probes. The device contains air-filled chambers with sensors which detect changes in finger volume with each arterial pulsation. On the bottom left corner- the normal setup for a research study is demonstrated (Adapted from Axtell et al., 2010).

Table 4.2 Advantages and disadvantages of EndoPAT.

Advantages	**Disadvantages**
• Easy to perform • Non-invasive • Non operator-dependent with comprehensive automatic analysis	• Expensive • Difficult to interpret as increased vasoreactivity in vasculature in the finger

4.1.6 Pulse Wave Velocity

Pulse wave velocity (PWV) is a direct marker of arterial stiffness that can be measured noninvasively. It is now well recognised that PWV is an independent predictor of cardiovascular mortality and morbidity (Benetos et al., 2002, Abraham et al., 2010). The standard method of measuring PWV is to record pressure waves between arterial segments at two different sites on the arterial tree. Measurement of PWV between the common carotid and femoral artery is the gold standard technique due to its reproducibility, accuracy, relatively easy to measure and low costs (Laurent et al., 2006, Joly et al., 2009). However, more importantly, much research has shown that the carotid-femoral PWV is correlated with the incident of CVD, independent of traditional CV risk factors and in different populations (Blacher et al., 1999, Laurent et al., 2001, Cruickshank et al., 2002, Shokawa et al., 2005, Sutton-Tyrrell et al., 2005, Mattace-Raso et al., 2006, Choi et al., 2007).

There are several different methods of measuring PWV noninvasively (Joly et al., 2009):

(i) Simultaneous measurements (Complior device)- measures the pulse transit time between the common carotid and femoral artery using mechanotransducers (Figure 4.5) and simultaneously records the pressure pulses in the form of pressure waves. The mechanotransducers are applied directly onto the skin with the first probe positioned at the base of the neck on the common carotid artery (the central detection site) and the second probe placed over the femoral artery. The pulse transit time is measured from the beginning of each waveform where the steep rise starts. PWV can be derived with the following formula:

$PWV = \frac{\Delta x}{\Delta t}$ where Δx is the distance traveled and Δt is the time for the wave to travel that distance

(i) Sequential or non-simultaneous measurements- applanation tonometry (SphymoCor, AtCor Medical) is another method used to measure carotid-femoral PWV (Figure 4.6). This technique involves sequential or non-simultaneous detection and recording of carotid and femoral pressure waveforms using sensitive tonometers. Tonometers are instruments used to measure pressure and tension (Salvi et al., 2008). At both sites, an ECG trace is also recorded. PWV is calculated according to the formula above with the distance divided by the time delay measured between pressure upstroke at carotid and femoral artery site (Joly et al., 2009).

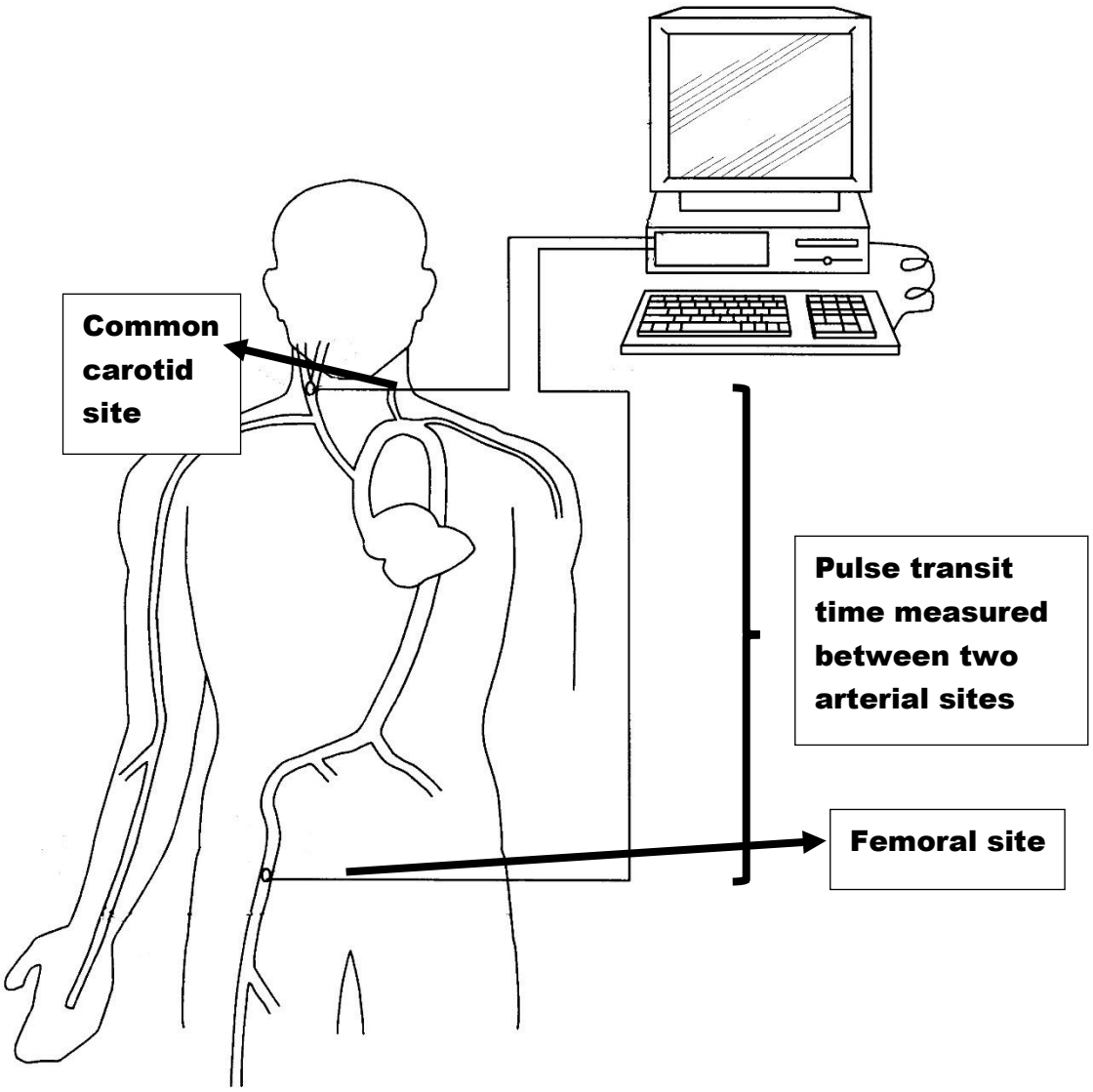


Figure 4.5 Pulse transit time are measured between two arterial sites i.e. the carotid and the femoral artery (Adapted from http://www.freepatentsonline.com/6511436.html (25-10-2013)).

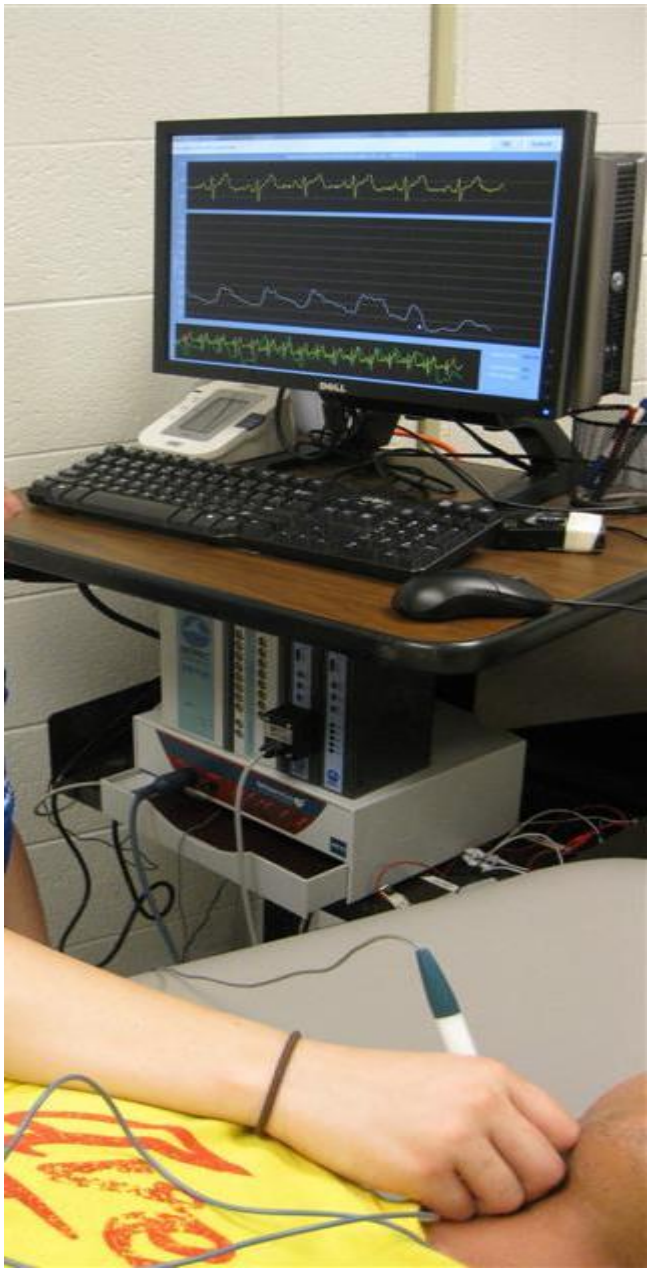


Figure 4.6 Sequential measurements of carotid-femoral PWV.

4.1.7 Pulse Wave Analysis

Pulse wave analysis (PWA) is a technique which is used to assess arterial stiffness indirectly. Using applanation tonometry, PWA allows accurate recording of radial (peripheral) pressure waveforms and subsequent generation of the corresponding aortic (central) waveform using a generalised transfer function (Figure 4.7) (O'Rourke et al., 2001). This allows the derivation of the augmentation index (AIx) (an index of arterial stiffness) and central pulse pressure. Higher values of AIx suggest increased PWV (due to increased arterial stiffness) and vice versa. The PWA technique is described in more detail in Chapter 4.

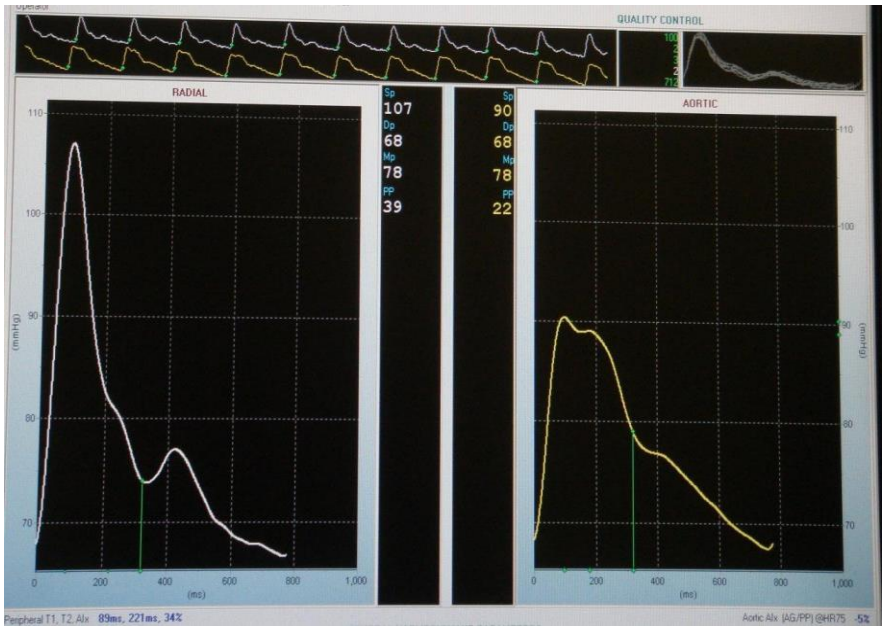


Figure 4.7 On the left - the peripheral or radial pulse waveform obtained through applanation tonometry. One the right- central or aortic pulse waveform derived from the peripheral waveform after the application of a generalised transfer function.

Several studies have reported associations between AIx and certain clinical conditions such as advanced age, hypertension, chronic kidney disease and CVDs such as myocardial infarction and coronary artery disease (Kelly et al.,

1989, Mitchell et al., 1997, Chae Cu, 1999, London et al., 2001, Safar et al., 2002, Williams et al., 2006). AIx may therefore be a useful therapeutic tool to monitor disease changes in selected patient populations. It has been shown that in hypertensive patients, AIx change in response to antihypertensive therapy was superior to conventional cuff measurement in predicting left ventricular mass change (Hashimoto et al., 2007). In addition, the Sixth International Workshop on Structure and Function of the Vascular System produced a consensus document which supports the notion that central haemodynamics measured by applanation tonometry has a robust predictive value for CV events (Agabiti-Rosei et al., 2007). This mounting evidence suggest that the central pressure and indices obtained from PWA could prove to be relatively straightforward, noninvasive and useful tool to predict CV risk in patients in the clinical setting.

4.1.8 Laser Doppler flowmetry with iontophoresis

This is a noninvasive method commonly used to study the dynamics of skin microcirculation providing insight to endothelial function. This technique allows the delivery of vasoactive substances, such as acetylcholine and sodium nitroprusside, to the skin and provides a continuous, sensitive and real time assessment of cutaneous blood flow.

Laser Doppler flowmetry

LDF is based on the principle of Doppler effect whereby the emitted laser light travels through skin and interacts with both static tissue and moving objects such as red blood cells. The Doppler effect is the relative frequency change between the moving source and the static detector. The wavelength of the light

that is backscattered from stationary tissue remains the same frequency whereas the wavelength of the light that is reflected from moving red blood cells differs slightly depending on the speed of the moving red blood cells. When this difference is detected and analysed, information about the density of the moving red blood cell and flux can be obtained. Flux, *F*, is the product of RBC density (*N*, the number of RBC in the sample) and their velocity,*s* (Murray et al., 2004).

$F=ksN$ where k is an arbitrary constant.

Since it is recognised that there is a linear correlation between the RBC flux and skin blood flow, the RBC flux is taken as an approximation of blood flow. This is the reason why flux signals are not absolute values and are usually expressed in arbitrary perfusion units (P.U.)

The Doppler effect was first utilised by Stern to monitor blood flow (Stern, 1975). Using a helium-neon laser, he demonstrated the difference in fingertip blood perfusion under normal blood flow conditions and under brachial artery occlusion. Since then, the method of LDF has undergone continuous improvement over the last 30 years. The LDF, which is a non-invasive technique, is a single-point perfusion monitoring system (Figure 4.8(a)) widely used both in research and as a diagnostic tool in the clinical setting to aid measurement of blood flow in the microcirculation. However, since skin circulation is known to be heterogeneous, laser Doppler imaging (LDI) (Figure 4.8(b)) was developed to allow blood flow data to be collected over a larger skin area. The fundamental principle underlying LDF and LDI is demonstrated in Figure 4.8.

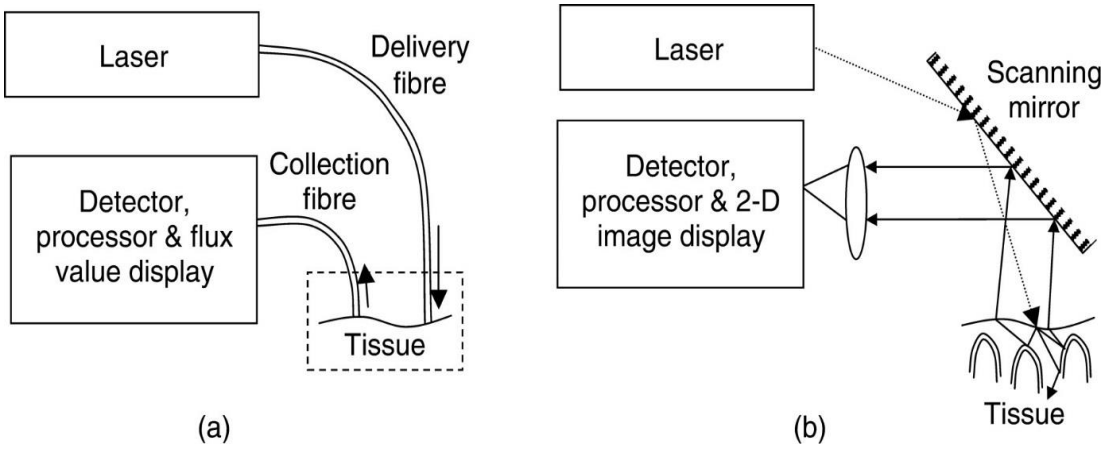


Figure 4.8(a) Schematic diagram representing LDF which is a single-point probe consisting of an emitting delivery fiber with the source of laser light and a separate collection fiber that serve as receivers; (b) Schematic diagram representing LDI (Adapted from Murray et al., 2004).

The depth of penetration of the emitted laser light varies among tissue: for skin, it is approximately 0.5-1mm. In view of the anatomical organisation of skin microcirculation, the LDF is an ideal method for capturing blood flow signals from the superficial nutritional to deeper thermoregulatory blood vessel.

In research, it has been shown that patients with increased risk for CVD are characterised by impaired cutaneous ACH-induced vasodilatation (Ijzerman et al., 2003). In addition, Khan et. al. (2008) demonstrated that cutaneous vasodilatation to ACH correlates with coronary microvascular function (Khan et al., 2008). Since it is understood that coronary microvascular dysfunction is part of a systemic microvascular process, iontophoresis with LDF serves to be an attractive method to stratify cardiovascular risk (Brocx and Drummond, 2009, Turner et al., 2008, Ijzerman et al., 2003).

Iontophoresis

The term iontophoresis refers to the delivery of charged pharmacological substances through biological membrane using an externally applied low-density electric current (Holowatz et al., 2008, Turner et al., 2008, Roustit and Cracowski, 2012). This technique was first popularised by Leduc, one of the scientists who pioneered this technique at the beginning of the 20th century as a result of his animal research work (Chien and Banga, 1989, Rawat et al., 2008).

Iontophoresis is a technique based upon the general principle that similar charges repel each other while opposite charges attract each other. An electric current is applied externally to increase the penetration rate of the pharmacological substances through the biological membrane such as the skin. When a positively charged substance is to be delivered transdermally, it is placed in the surrounding of the positively charged electrode (anode) from which it is repelled (Figure 4.9). The positively charged substance is subsequently attracted towards the negative electrode placed on another area of the body.

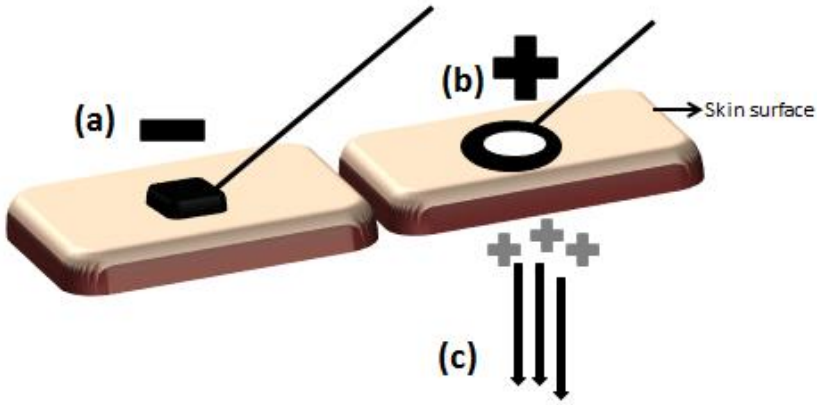


Figure 4.9 A schematic diagram illustrating the set-up of the electrode system on the skin surface. (a) Negatively charged electrode. (b) The iontophoresis chamber containing positively charged pharmacological substances with the attached electrode. (c) By applying a positive current to the electrode in (b), the positively charged pharmacological substances are repelled from the electrode and forced through the surface of the skin.

Iontophoresis is an effective drug delivery system with many advantages. Since it is noninvasive, this technique avoids the risks of invasive procedures such as bleeding during intra-arterial puncture. It also prevents first pass metabolism by the liver therefore increases therapeutic efficacy of the pharmacological substance. In addition, iontophoresis minimises the risk of overdosing or underdosing as it provides a constant delivery of the drug. However, iontophoresis has its disadvantages (Turner et al., 2008). It is recognised that the exact quantity of drug such as ACH and SNP delivered in iontophoresis cannot be determined precisely, therefore, providing a less accurate blood flow response. In terms of side effects, the most commonly seen are minor reactions such as redness, itching and irritation of the skin surface exposed to the

iontophoretic delivery system. Rarely, some drugs may cause skin pigmentation after iontophoresis. Major side-effects are very rare.

In clinical research, iontophoresis has been used in conjunction with laser Doppler flowmetry to study the role of the endothelium in the regulation of cutaneous blood flow. An example of such studies includes the assessment of skin microcirculation function in patients with Type I and II diabetes mellitus (Caballero et al., 1999, Lim et al., 1999, Khan et al., 2000, Beer et al., 2008). In these studies, cutaneous blood flow was assessed by measuring vascular responses to transdermal iontophoresis of acetylcholine (ACH) (endothelium-dependent vasodilatation) and sodium nitroprusside (SNP) (endothelium-independent vasodilatation). A reduction of vascular responses to both ACH and SNP were observed hence suggesting an impairment in the vasodilatory response (Caballero et al., 1999, Lim et al., 1999, Khan et al., 2000, Beer et al., 2008).

4.1.9 Post-occlusive reactive hyperaemia with full-field laser perfuser imager

Reactive hyperaemia is defined as a transient compensatory increase in blood flow after a brief period of limb occlusion. In this condition, an increased shear stress against the blood vessel wall as a result of an increase in blood flow leads to the production of vasodilator metabolites, such as adenosine and NO, which dilates the microvasculature as a result of limb ischaemia.

In research, PORH is used to assess the function of skin microcirculation. During this procedure, blood perfusion is measured at the forearm, before, during and after limb occlusion, therefore, allowing blood flow changes to be

recorded concurrently upon cuff released. When the FLPI is used to detect blood flow changes in response to PORH, the PORH tracing demonstrates a decrease in blood perfusion from the resting flux level to biological zero level on limb occlusion and after a period of time (normally 5 minutes), the occlusion is released and blood perfusion returns to the resting flux level but with a brief overshoot. The significance of this overshoot (peak response) has been shown to correlate with microcirculation function in some animal studies but generally the total area under the curve post 2 minutes recovery has been shown to correlate with vascular function in humans.

The FLPI utilises a 780nm laser beam to provide real time microvascular perfusion that is proportional to the average velocity of red blood cells (Briers, 2007). A charge-coupled device (CCD) video camera records the differences in motion of these red blood cells into speckled patterns. When there is fast blood flow, low contrast images are produced and inversely, when there is low blood flow, high contrast images are produced (Boas and Dunn, 2010). The contrast image is then processed and based on speckle contrast, a colour-coded image is produced. FLPI mainly measures superficial skin blood flow to a maximum depth of about 1mm.

In healthy normal vasculature, blood flow rapidly increases above normal levels immediately after cuff release and is followed by a gradual drop in blood flow to the initial resting flux level. This so-called reactive hyperaemia occurs because during cuff occlusion, tissues become hypoxic and there is a buildup of vasodilatory mediators and metabolites which act to relax surrounding vascular smooth muscle cells, leading to vasodilatation of blood vessels (Kontos et al.,

1965). In addition, decreased intravascular pressure in the arterioles due to arterial occlusion leads to myogenic mediated vasodilation which contributes to reactive hyperaemia (Lombard and Duling, 1977).

Since NO bioavailability correlates with vascular function, it is important that a solely NO-dependent hyperaemic response is elicited as part of the assessment. Studies examining the contribution of NO in reactive hyperaemia, however, have yielded inconsistent findings with some showing that only immediately after arterial occlusion is the vasodilatory response most NO-dependent (Raff et al., 2010, Tagawa et al., 1994) while others have demonstrated respectively the peak (Dakak et al., 1998, Yvonne-Tee et al., 2005) and late phase (Tagawa et al., 1994, Joannides et al., 1995) of reactive hyperaemic depends most on NO bioavailability. In this project, I will investigate the relationship between the levels of K_{ATP} channels in blood and the different phases of the reactive hyperaemia response.

4.2 Blood markers of vascular function

In addition to the vascular function tests mentioned above, there exist blood biomarkers that are predictive of risk for CVD. However, full details will not be provided here as they are not within the scope of my thesis. They have been reviewed in other journals and some has been listed below.

Table 4.3 Blood biomarkers predictive of CVD.

Biomarker	**Articles**
Inflammatory markers:	

• CRP	Clearfield (2005); Blankenberg et al.(2010)
• sICAM-1	Jager et al. (2000)
• IL-6	Volpato et al. (2001); Kanda & Takahashi (2004); Danesh et al. (2008)
• IL-18	Blankenberg et al. (2002); Jefferis et al. (2011)
• SAA	Johnson et al. (2004)
• MPO	Nicholls & Hazen (2005); Schindhelm et al. (2009)
• sCD40L	Schonbeck et al. (2001); Heeschen et al. (2003)
• Oxidised LDL	Meisinger et al. (2005)
• GPX1 activity	Blankenberg et al. (2003)
• Homocysteine	Homocysteine (2002)
• Cystatin-C	Koenig et al. (2005); Luc et al. (2006)
• Natriuretic peptides	Galvani et al. (2004); Kragelund (2005); Blankenberg et al. (2010); 50, 86,91, 298-305
Blood vulnerability	
(i) Hypercoagulable	
• Fibrinogen	Fibrinogen (2005)
• D-dimer	Danesh et al. (2001)
(ii) Decreased fibrinolysis	

• TPA/PAI-1	Lowe et al. (2004)
(iii) Increased coagulation factors	
• Von Willebrand factor	Whincup et al. (2002)
Endothelial function markers	
• E-selectin	Gearing et al. (1992); Hwang et al. (1997)
• VCAM-1	Richter et al. (2003)
• ICAM-1	Hwang et al. (1997)

CRP, C-reactive protein; sICAM, soluble intercellular adhesion molecule; IL, interleukin; SAA, serum amyloid A; MPO, myeloperoxidase; sCD40L, soluble CD40 ligand; GPX1, glutathione peroxidase; TPA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor-1.

Having discussed the importance of early detection of subclinical CVD in preventing future CV events, there exists a need to identify novel markers which could potentially act as reliable surrogate indicators for vascular function. Up until now, vascular function has been assessed using different vascular function tests and by measuring levels of different blood biomarkers (Table 4.2). More recently, it has been recognised that K_{ATP} channels could potentially act as surrogates for vascular function as they play key roles in VSMCs in regulating peripheral vascular tone. In addition, there is a possibility that the levels of K_{ATP} channels in VSMCs are reflective of those found in blood.

4.3 Aim and objectives

The **aim** of this study, therefore, is:

- To investigate in normal healthy subjects whether the levels of K_{ATP} channel expressed in blood samples are related to the function of microvessels and macrovessels

The **objectives** of this study is:

- To determine whether there is a correlation between plasma levels of K_{ATP} channels and microvascular function, assessed using LDF with iontophoresis and post-occlusive reactive hyperaemia with FLPI, and macrovascular function assessed using FMD and pulse wave analysis

The hypothesis of this study is there is a relationship between the levels of K_{ATP} channels in blood and vascular function.

CHAPTER 5

Methods and materials

5.1 Subjects

Fifty young healthy volunteers (28 males, 22 females) aged between 19 and 27 years were recruited for the study. All subjects were enrolled between 18th October 2010 and 31st October 2011. None of the subjects were smokers, used any medication or had a history of any symptomatic vascular disease(s). Subject characteristics are shown in Table 5.1. Ethnicity of subjects is summarised in Table 5.3. No formal power calculation has been performed to attain the sample size for this pilot study as it was carried out to obtain preliminary data that can be utilised for further planning of definitive studies. The study protocol was approved by the Tayside Committee on Medical Research Ethics and written informed consent was obtained from each subject before participation in the study. All fifty subjects attended at least one visit lasting up to three hours in which a blood sample was taken and vascular function was assessed using the tests discussed in the previous chapter. Blood samples were analysed for levels of K_{ATP} channels and markers of cardiovascular risk i.e. lipid profile. Vascular assessments were carried out in a blood flow laboratory at a temperature of 23 °C after 10 minutes of acclimatisation. Subjects were asked to refrain from any food and drink for at least 2 hours beforehand and also to refrain from physical activity for one day

before their visit. All subjects had their blood pressure, height and weight measured before vascular function tests were carried out.

Table 5.1 Subject characteristics (n=50; 28 males, 22 females). Data are presented in mean ± SD.

Subject characteristics	**n**	**Mean ± SD**
Age (years)	50	22.0 ± 1.7
Weight (kg)	50	65.2 ± 8.7
Height (m)	50	1.7 ± 0.1
BMI (kg/m^2)	50	22.2 ± 2.6
Brachial SBP (mmHg)	50	116 ± 12
Brachial DBP (mmHg)	50	68 ± 8
Ethnicity:		
White European males	12	
White European females	14	
South Asians males	7	
South Asians females	3	
Chinese males	9	
Chinese females	5	

5. 2 Real time RT-PCR

Five millilitres of venous blood was collected into a heparinised vacutainer from a vein in the upper arm of each subject. The vacutainer was placed in a sealed transport plastic bag containing ice and sent immediately to the laboratory for determination of the levels of K_{ATP} channels by real-time RT-PCR test. The time for transport took approximately 5-10 minutes. The real time RT-PCR was performed using well-established and characterised primers as described by Jovanović et. al. (2008) (Jovanović et al., 2008). In brief, once the blood samples are received by the laboratory technician, the process involved total ribonucleic acid (RNA) extraction from the human whole blood using TRIZOL reagent (Invitrogen, Paisley, UK) according to manufacturer's instructions. The extracted RNA was further purified with RNeasy Mini Kit (Qiangen, Crawley, UK). The specific primers for human K_{ATP} channel genes (i.e. Kir6.1 and SUR2B) were designed using Beacon Designer 3.0 software and are described in Jovanović et al., 2008 (Jovanović et al., 2008) and Du et al., 2010 (Du et al., 2010).The reverse transcriptase (RT) reaction was carried out with ImProm-II Reverse Transcriptase (Promega, Southampton, UK). A final volume of 20 µl of RT reaction containing 4µl of 5X buffer, 3mM MgCl2, 20U of RNasin® Ribonuclease inhibitor, 1U of ImProm-II reverse transcriptase, 0.5mM each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and thymidine triphosphate, 0.5µg of oligo(dT), and 1µg of RNA was incubated at 42˚C for 1h, then inactivated at 70˚C for 15min. The resulting complementary deoxyribonucleic acid (cDNA) was used as a template for real-time RT-PCR. A SYBR Green I system was used for the RT-PCR test and the 25µl reaction mixture contained: 12.5µl of iQTM SYBR® Green Supermix (2X),

7.5nM each primers, 9µl of double distilled water, and 2 µl of cDNA. In principle, the conditions for thermal cycling were as follows: an initial denaturation at 95˚C for 3 minutes, 15s of annealing at 56˚C, and 30s of extension at 72˚C. The real-time PCR was performed in the same wells of a 96-well plate in the iCycler iQTM MultiColor Real-time Detection System (Bio-Rad, Hercules, CA, USA). Following each cycle, data were collected and shown graphically (iCycler iQTM Real-time Detection System Software, version 3.0A, Bio-Rad).

Primers were tested for their ability to produce no signal in negative controls by dimer formation and then with regard to the efficiency of the PCR reaction. Efficiency is evaluated by the slope of the regression curve obtained with several dilutions of the cDNA template. Melting curve analysis tested the specificity of primers. Threshold cycle values (C_t), PCR efficiency (examined by serially diluting the template cDNA and performing PCR under these conditions) and PCR specificity (by constructing the melting curve) were determined by the same software (Jovanović et al., 2008, Du et al., 2010). Each cDNA sample was duplicated; the corresponding no-RT mRNA sample was included as a negative control (blank) (Jovanović et al., 2008).

5.3 Assessment of microvascular function

5.3.1 Laser Doppler flowmetry with acetylcholine iontophoresis

LDF with iontophoresis was tested in 50 subjects (28 males, 22 females). Vascular function was assessed with the subjects lying supine on a bed with both arms comfortably beside the body (Figure 5.1). The response to acetylcholine (ACH) iontophoresis in this test was measured on the volar surface of the right forearm. Flux recording was started after subjects acclimatised to the room temperature for 10 minutes, during which the forearm skin was prepared for measurements. The sites were cleaned by first using an adhesive tape to remove the superficial dead layer of the skin and washed gently with alcohol and then wiped gently dry. The iontophoresis chamber (Moor Instruments Ltd., Axminster, UK) is a Perspex ring of diameter 20 mm with a wire electrode running round its inner surface, and this was fixed to the skin with adhesive tape. The hydrochloride salt of ACH (Miochol, Novartis), which is an endothelium-dependent vasodilator, was dissolved in de-ionised water to a concentration of 10 g/l (1%), and 2 ml of this solution was used to fill the chamber. The positive lead of a current source was connected to the chamber, and the negative lead attached to a conductive hydro-gel pad on the wrist, which served as the reference electrode. The subsequent changes in skin perfusion were assessed using laser Doppler imaging (moorLDI, Moor Instruments Ltd., Axminster, UK), which produces a colour image representing the distribution of skin blood flow (Figure 5.2). A 2mW helium-neon laser scans the surface of the skin, and light back scattered from moving erythrocytes is shifted in frequency by an amount proportional to their velocity. For each scan,

the computer builds up a colour-coded image representing skin blood perfusion over the scan area giving a relative measure called the laser Doppler flux (recorded in perfusion unit, P.U.). After two baseline recordings, ACH was delivered using an incremental anodal current of 10μA, 20μA and 50μA with iontophoretic stimulation repeated four times each. Two recordings were made at 100μA (Figure 5.2). For each dose response, the mean of the two highest stable flux values was calculated.

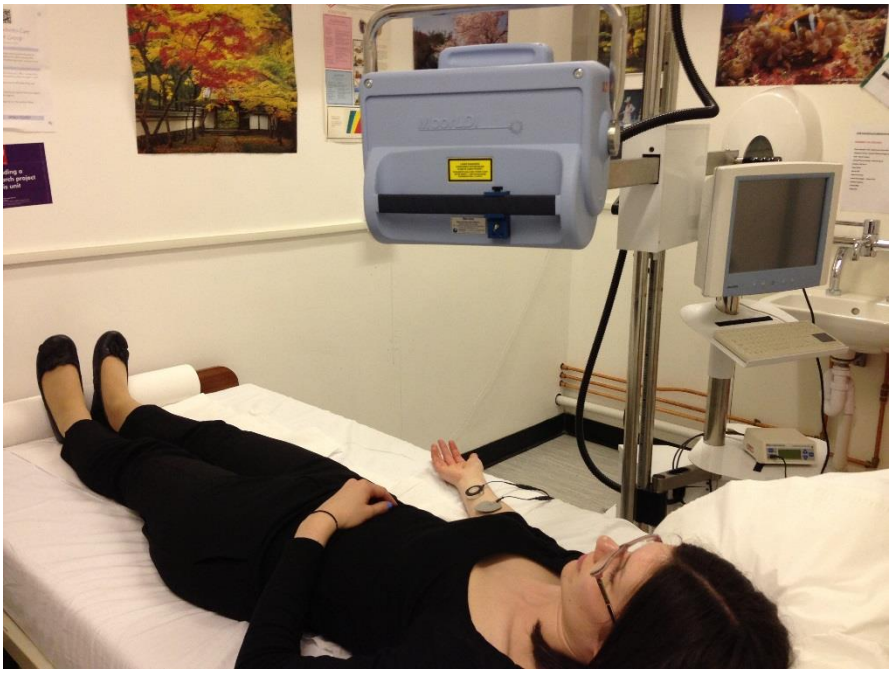


Figure 5.1 Research set up for laser Doppler flowmetry with iontophoresis.

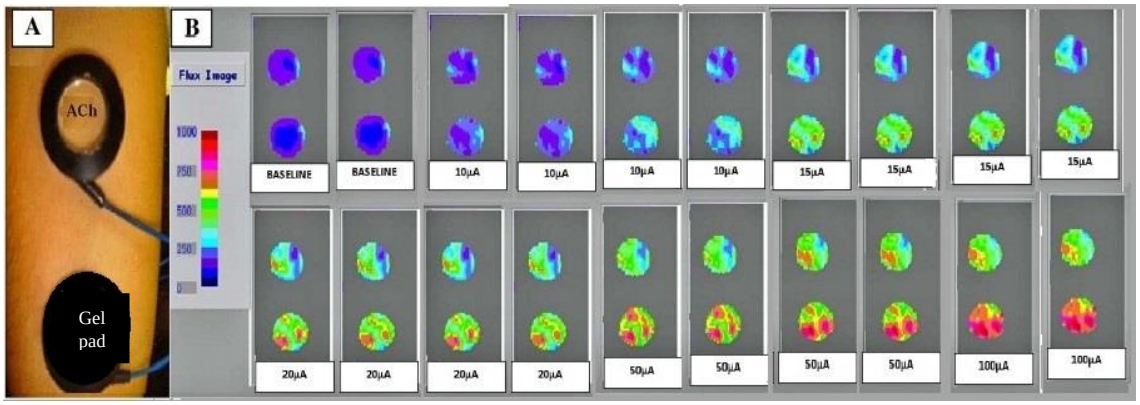


Figure 5.2 (a) A close-up view of the iontophoresis ACH drug chamber and the gel pad (reference electrode) on a subject's forearm. (b) Colour-coded perfusion map showing increased skin perfusion after concurrent iontophoresis of ACH at increasing currents of 10µA, 20µA, 50µA and 100µA (Adapted from Turner et al., 2008).

5.3.2 Post-occlusive reactive hyperaemia

PORH was tested in 50 subjects (28 males, 22 females). Vascular function was assessed with the subjects lying supine on a bed. The left forearm was rested at heart level and the skin microcirculation was measured at the volar aspect using a full field laser perfusion imager (moorFLPI, Moor Instruments Ltd., Axminster, UK) (Figure 5.3 (a)). A low-power laser beam was directed by the moorFLPI onto the skin surface of the forearm and a video frame of 10 images per second of blood perfusion in the skin microvasculature was obtained. Superficial microvascular perfusion was measured continuously from five individual regions of interest over an area of approximately 30cm^2 and was recorded simultaneously in the form of a line graph. It is recognised that five regions are sufficient to be representative of the response by previous studies carried out in our department. Data collected from the five regions of interest were averaged to provide an overall response in arbitrary perfusion units. A blood pressure cuff was positioned over the upper arm and a baseline measurement of skin perfusion was obtained over 2 minutes. The cuff was then

inflated to a suprasystolic pressure (200mmHg), thus, occluding any blood perfusion distal to the cuff for 5 minutes. After 5 minutes of occlusion, the cuff was deflated immediately resulting in an increase of blood through the skin microcirculation distal to the occlusion, termed PORH. The peak perfusion post-cuff release was measured and the average perfusion over 2 minutes after cuff release was determined (i.e. the response over 2 minutes of recovery in reactive hyperaemia) (Figure 5.3(b))

(a)

(b)

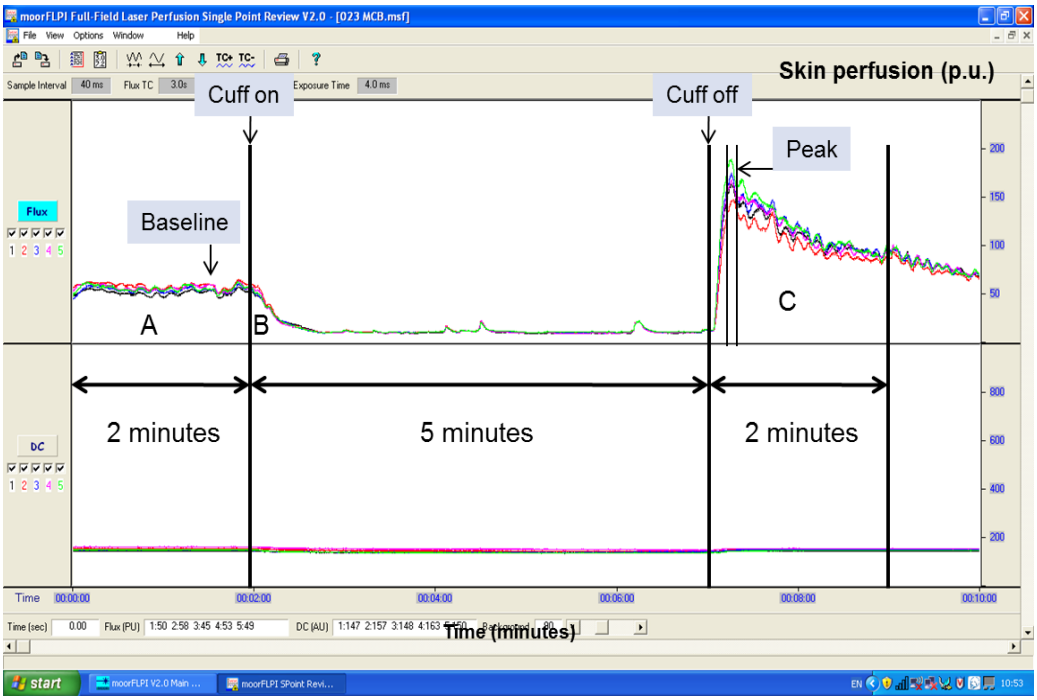


Figure 5.3 (a) Research setting of PORH with FLPI; (b) Tracing of PORH showing baseline, peak and average perfusion i.e. area under the curve, C.

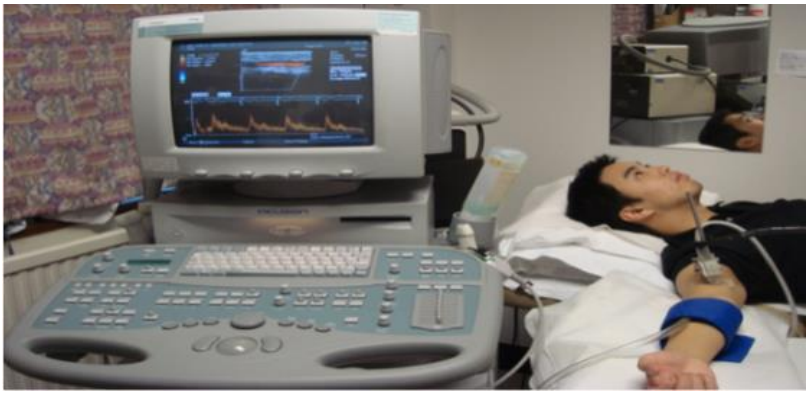
5.4 Assessment of macrovascular function- brachial artery flow-mediated dilatation

Brachial artery FMD was tested in 50 subjects (28 males, 22 females). Vascular function was assessed according to standard guidelines with the subjects lying on a bed in a supine position and their right arm rested at heart level (Figure 5.4(a)) (Coretti et al., 2002). Using high-resolution ultrasound imaging (Acuson Sequoia 512, Siemens Medical Solutions, Berkshire, UK), the brachial artery was imaged at the volar aspect above the antecubital fossa in the longitudinal plane. The ultrasound system is equipped with the vascular software for two dimensional imaging, a high-frequency vascular transducer, spectral and colour Doppler and an internal electrocardiogram (ECG) (to provide a trigger for the R wave, therefore, not a diagnostic ECG) (Coretti et al., 2002).

A snake-arm clamp was used to hold the ultrasound probe, therefore, allowing a stable image of the artery to be obtained throughout the study (Figure 5.4(b)). The ischaemic stimulus was produced by placing a blood pressure cuff above the antecubital fossa and inflating the cuff to suprasystolic pressure of around 200mmHg for 5 minutes. After cuff release, reactive hyperaemia produces a transient increase in blood flow through the brachial artery, resulting in an increase in shear stress and dilatation of the brachial artery (Barac et al., 2007). Two dimensional images of the brachial artery were acquired at baseline for 1 minute and post cuff release for 2 minutes. The precise diameter of the brachial artery on both occasions (baseline and post cuff released) were measured by automated edge-detection software (Figure 5.5). FMD was calculated as the

maximum percentage change in diameter post reactive hyperaemia relative to the baseline diameter (Hardie et al., 1997).

(a)



(b)

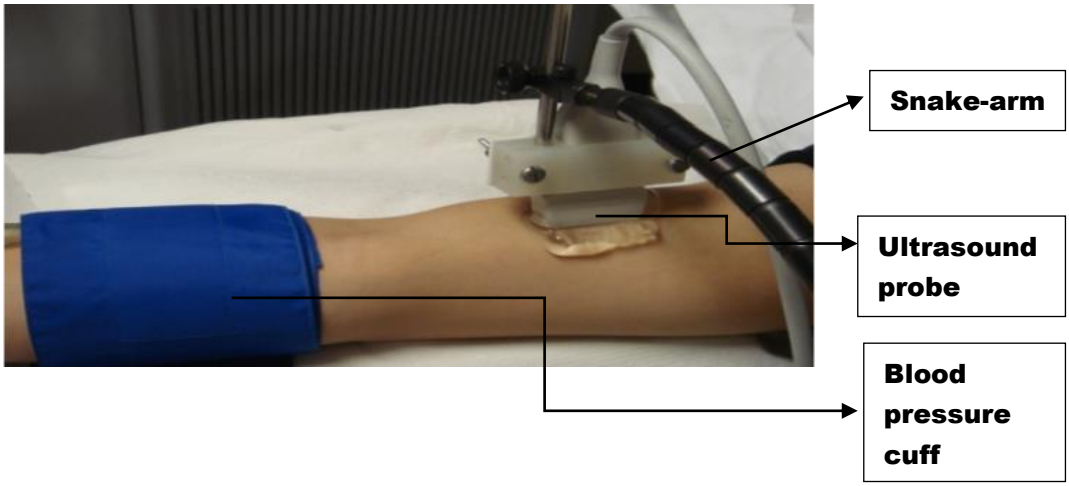


Figure 5.4 (a) Brachial artery FMD in the research set up. (b) A close up view of the placement of the ultrasound probe and the blood pressure cuff on a subject's arm.

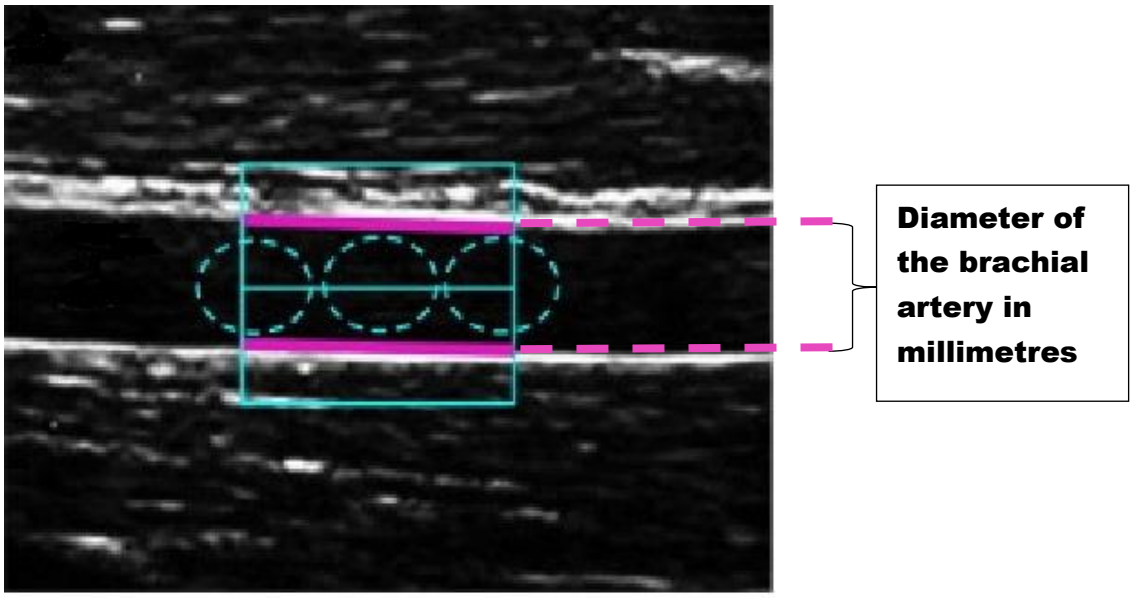


Figure 5.5 Measurement of the diameter of the brachial artery (mm) using automated edge-detection software. The three circles are placed in the lumen of the brachial artery to permit correct alignment of the edge detection software (the two outer circles can be adjusted depending on the angle of the region of interest of the artery). Once the correct position is obtained, the circles disappear and the diameter is being tracked by the lines on the edge of the vessel.

5.5 Assessment of arterial stiffness- pulse wave analysis

Subjects were rested in a supine position for at least 10 minutes. This is followed by triplicate blood pressure measurement using an automated blood pressure monitor (Omron 705 CPII). The degree of arterial stiffness was non-invasively assessed using the AIx, which provides an estimation of systemic arterial and muscular stiffness, using the validated SphygmoCor pulse waveform analysis system (Scanmed Medical Instruments, Moreton-in-Marsh, UK). Peripheral pressure waveforms were recorded at the radial artery by applanation tonometry using a high fidelity micromanometer (Miller Instruments) (Figure 5.6). The peripheral waveform is calibrated against the systolic and diastolic blood pressure of the brachial artery as there is a negligible amplification of pressure between both sites. At least fifteen consecutive high

quality peripheral pressure waveform recordings were taken, from which an averaged peripheral pressure waveform is generated and a corresponding central (aortic) pressure waveform was derived automatically with the use of a validated generalised transfer function (Figure 5.7). From the averaged aortic pulse wave, the following parameters were calculated:

- Aortic AIx (percentage), which is calculated as augmentation pressure divided by central pulse pressure multiply by 100. It is the difference between the first and second systolic peaks expressed as a percentage of the pulse pressure (Figure 5.8 and Figure 5.9). Larger values of AIx suggest increased PWV as a result of increased arterial stiffness and vice versa. Augmentation pressure represents the summation of the forward propagating incident wave generated by the left ventricular ejection and the backward propagating reflected wave.
- Pulse pressure is the difference between the systolic and diastolic blood pressure (Figure 5.8)
- Subendocardial viability ratio (SEVR) is an index of myocardial oxygen supply to demand ratio of the diastolic area under curve divided by the systolic area under curve (Figure 5.10) (Tsiachris et al., 2012). It is a ratio of myocardial perfusion to myocardial contraction and serves as a non-invasive measure of myocardial perfusion relative to cardiac workload (Prince et al., 2010).

The SphygmoCor software incorporates an algorithm that normalises AIx to a heart rate of 75 beats per minutes (AIx@75) since AIx varies with heart rate (Sharman et al., 2009).

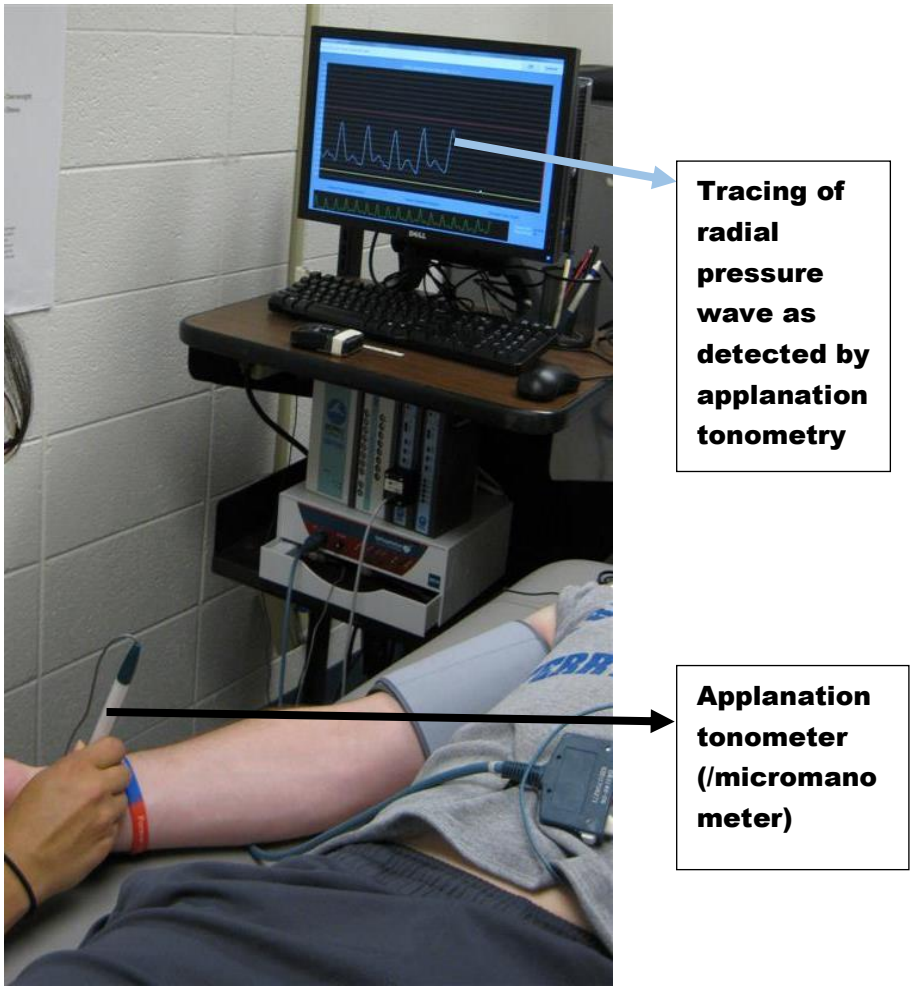


Figure 5.6 Set up of PWA in the research study setting. The applanation tonometry is performed by placing the micromanometer over the radial artery. Fifteen consecutive high quality peripheral pressure waveform recordings were taken and can be seen as the images in the computer screen.

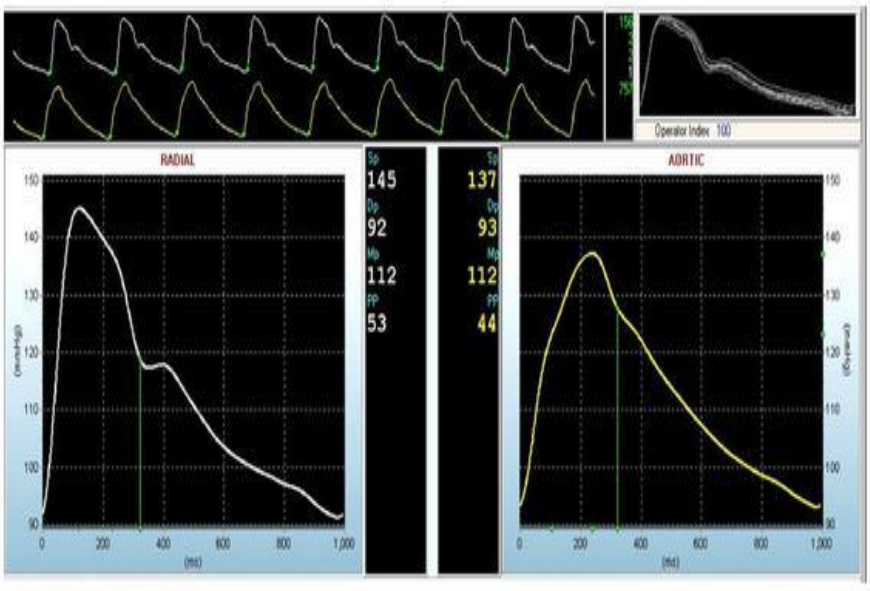


Figure 5.7 Recording of the peripheral (radial) artery applanation tonometry. The upper long panel shows the radial pressure waveform above the derived central pressure waveform. The upper right panel shows the superimposed radial waveforms, including the operator index, and the middle panel in green shows the quality control indices. The bottom right panel demonstrates a magnified derived central pressure waveform. Central pressure is 137/93 mmHg.

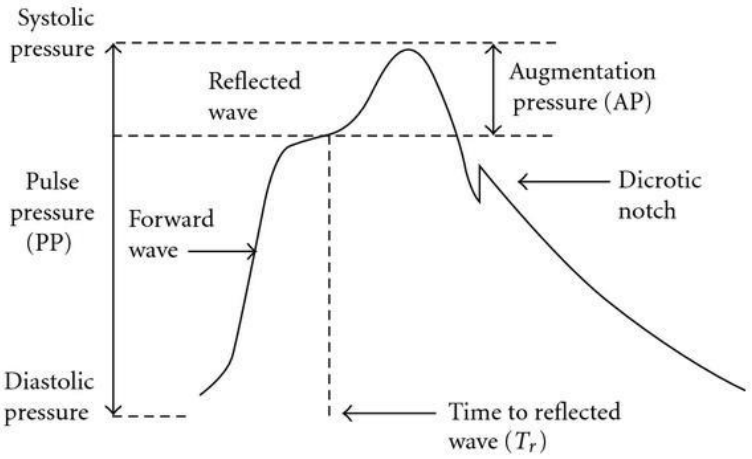


Figure 5.8 Central (aortic) pressure waveform. Systolic and diastolic pressures are the peak and trough of the waveform. Augmentation pressure is the additional pressure added to the forward wave by the reflected wave. The dicrotic notch denotes closure of the aortic valve and is utilised to calculate ejection duration. Time of return of the reflected wave (T_r) represents the time starting from the point of rise in the initial ejected pulse waveform to the onset of the reflected wave. (Adapted from Stoner et al., 2012 and Nelson et al., 2009)

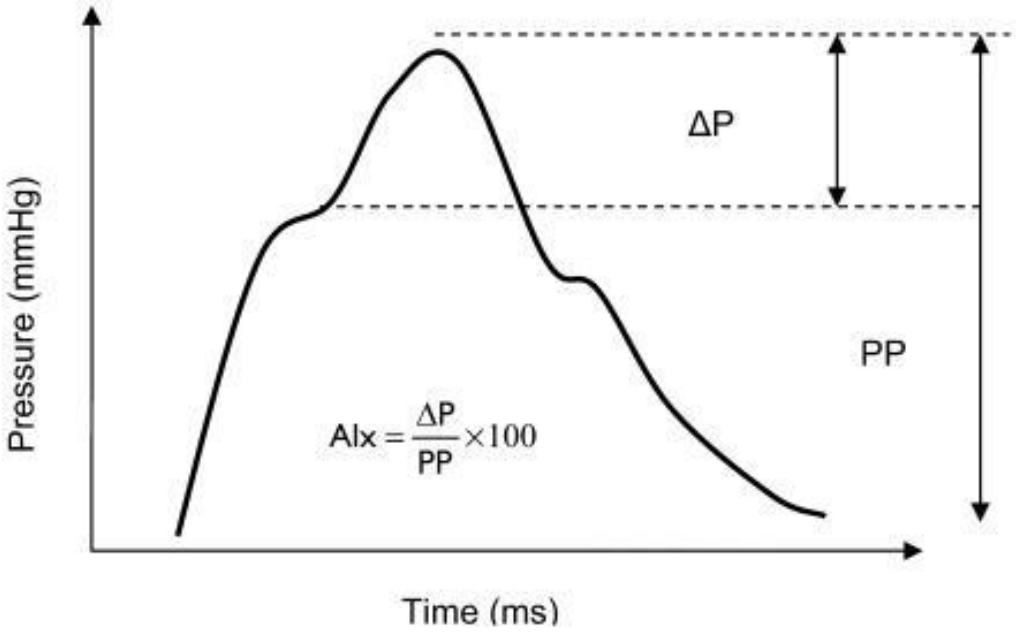


Figure 5.9 Augmentation index (AIx) (%) is calculated as augmentation pressure (ΔP) divided by pulse pressure (PP) multiply by 100 (Adapted from Lane et al., 2006)

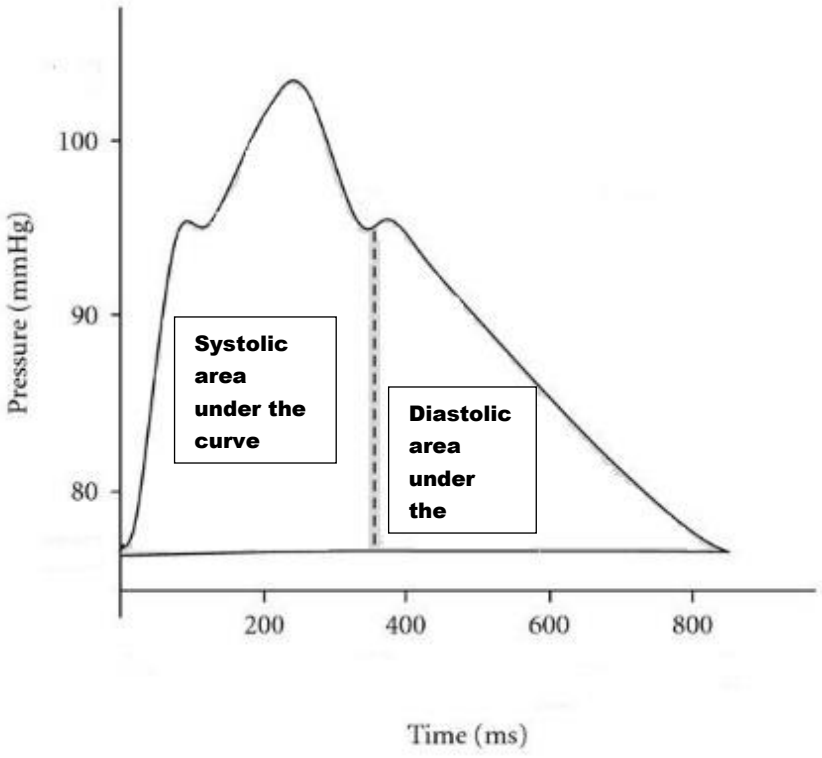


Figure 5.10 Subendocardial viability ratio (SEVR) (%) is the ratio of systolic area under the curve over diastolic area under the curve (Adapted from Mac-Way et al., 2011)

5.6 Statistical analysis

Pearson's correlation was used to assess the relationship between the levels of K_{ATP} channels in blood and vascular function tests such as LDF with iontophoresis, pulse wave analysis, PORH and brachial FMD data. Furthermore, gender differences were investigated when looking at correlation between the levels of K_{ATP} channels in blood and vascular function tests. Statistical analyses were carried out using SPSS for Windows version 18.0 (SPSS Inc.). Data are presented as mean ± SD and a probability value <0.05 was considered statistically significant.

CHAPTER 6

Results

6.1 Kir6.1 mRNA, but not SUR2B mRNA, can been detected in human blood

Real time RT-PCR was successfully completed in 40 of 50 subjects. Only Kir6.1 mRNA was detected and the average threshold cycle (C_t) value for Kir6.1 was 26.57±2.32 (n=40; Table 6.1). No signals corresponding to SUR2B mRNA were detected by real time RT-PCR. A higher average threshold cycle C_t value for Kir6.1 mRNA means less Kir6.1 mRNA levels were expressed in the blood. In simple terms, when there is a higher quantity of gene expression, i.e. Kir6.1 mRNA, available, fewer cycles are needed (lower threshold cycle, C_t) for the same amount of time to reach the threshold line to be detected. Conversely, when there is a lower quantity of gene expression available, more cycles are needed (i.e. higher threshold cycle) for the same amount of time to reach the same threshold line. The threshold line is the point where the expressed gene is detected when it reaches a fluorescent intensity above background.

Table 6.1 K_{ATP} channels subunit levels measured (n=40; 23 males, 17 females). Data are presented in mean (±SD).

K_{ATP} channels subunit levels	**N**	**Mean (±SD)**
Kir6.1 (average)	40	26.57 (±2.32)
SUR2B	NIL	NIL

6.2 Kir6.1 mRNA levels in the blood correlate with brachial and aortic systolic blood pressure

C_t values for Kir6.1 mRNA showed a significant positive correlation with both brachial (r=0.388, p=0.013, n=40; Figure 6.1) and aortic (r=0.364, p=0.021, n=40; Figure 6.2) systolic blood pressure. As C_t values is higher, the level of Kir6.1 mRNA is lower, so a positive correlation means that higher blood pressure is associated with lower expression of Kir6.1 in the blood.

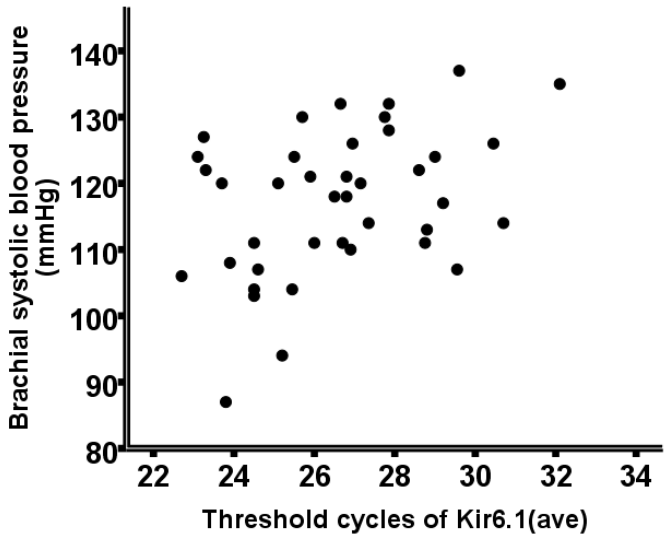


Figure 6.1 C_t values for Kir6.1 mRNA showed significant positive correlation with systolic blood pressure (r=0.388, p=0.013, n=40) for subjects.

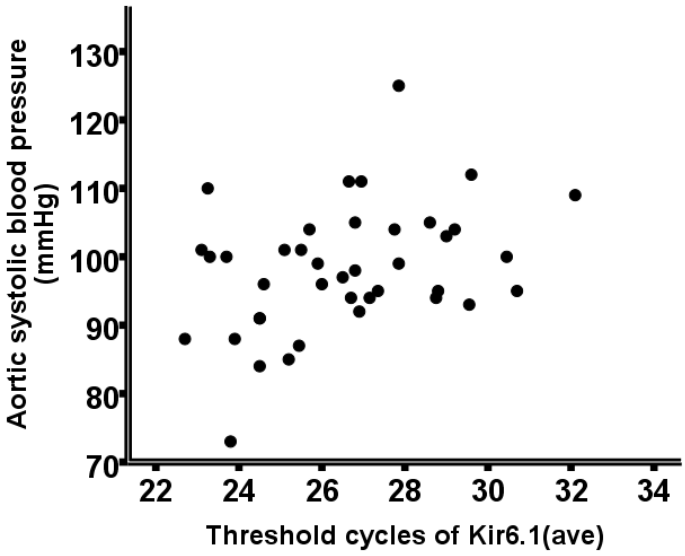


Figure 6.2 C_t values for Kir6.1 mRNA showed significant positive correlation with aortic systolic blood pressure (r=0.364, p=0.021, n=40) for subjects.

6.3 Correlations between Kir6.1 mRNA levels in blood and vascular function

Vascular function was assessed using (i) LDF with iontophoresis, (ii) PORH using FLPI, (iii) FMD and (iv) PWA. FMD was used to assess the function of macrovessels. PWA was used to evaluate arterial stiffness, whereas the other two remaining tests were utilised to measure function of microvessels.

In LDF with iontophoresis, there were incremental increase in vascular responses as the current increases (Table 6.2).

Table 6.2 Incremental increase in vascular responses as current increases in LDF with iontophoresis (n=50).

Current in LDF with iontophoresis	**Vascular responses (A.U.)**
Baseline blood perfusion, i.e. at 0μA	59.92 ± 28.94
Blood perfusion at 10μA	203.78 ± 125.79
Blood perfusion at 15μA	320.10 ± 159.02
Blood perfusion at 20μA	373.79 ± 150.83
Blood perfusion at 50μA	427.01 ± 148.15
Blood perfusion at 100μA	441.47 ± 145.04

PORH baseline skin perfusion was 65.86 ± 24.72 P.U. (n=50). Release of the cuff after 5 minutes occlusion resulted in a peak hyperaemia of 227.25 ± 64.21 P.U. (n=50). The average reactive hyperaemia response over 2 minutes of recovery (PORH 2 minutes recovery) was 128.03 ± 39.74 P.U. (n=50).

In FMD test, the baseline measurement of the brachial artery diameter was 3.55 ± 0.53 mm (n=50). Release of cuff after 5 minutes resulted in an increase of brachial artery diameter to a maximum of 3.83 ± 0.55 mm (n=50). Percentage change i.e. FMD was 7.98 ± 4.05 % (n=50).

For PWA, the data collected included time to reflected wave (T_r) (time), SEVR (%), AIx and AIx@75 and they respectively are 153.24 ± 12.86, 170.02 ± 40.06, -0.64 ± 10.16 and -6.50 ± 11.82 (n=50).

The correlation between functional vascular response and Kir6.1 mRNA levels were assessed by looking into relationship between C_t for Kir6.1 mRNA levels and functional measures of vascular response. On bivariate analysis, the C_t values for Kir6.1 mRNA levels were significantly correlated with the average response to acetylcholine (ACH (ave) response) ($r=-0.579$, $p<0.001$, $n=40$; Figure 6.4) of LDF with iontophoresis test. ACH (ave) response is the average of the blood perfusion recorded at 0µ, 10µA, 15µA, 20µA, 50µA and 100µA. As C_t values are lower, the level of Kir6.1 mRNA is higher, so a negative correlation means that lower vascular responses to iontophoresis are associated with lower expression of Kir6.1 in the blood. A positive significant correlation was noted between the C_t value for Kir6.1 mRNA and PORH 2 minutes recovery PORH response ($r=0.366$, $p=0.020$, $n=40$; Figure 6.5) (this means higher PORH is associated with lower expression of Kir6.1 which is opposite to response to ACH. In contrast, no significant correlations were demonstrated between the C_t values for Kir6.1 mRNA and PWA parameters (augmentation index: $r=0.080$, $p=0.266$, $n=40$ (Figure 6.6); augmentation index@75: $r=0.051$, $p=0.754$, $n=40$; SEVR: $r=-0.183$, $p=0.258$, $n=40$ and T_r: $r=0.243$, $p=0.130$, $n=40$) as well as FMD ($r=0.181$, $p=0.264$, $n=40$; Figure 6.7).

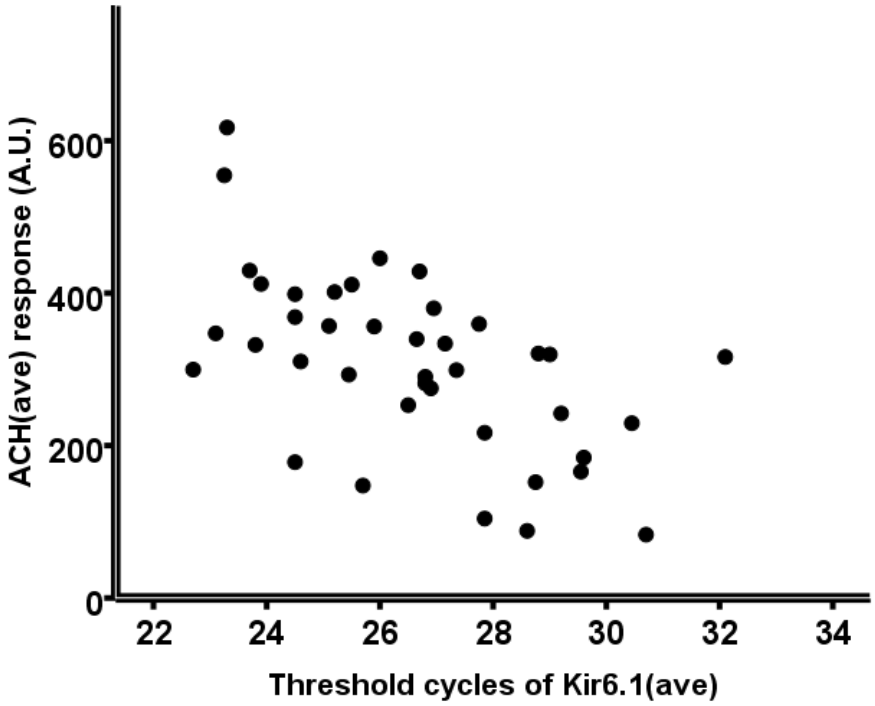


Figure 6.3 C_t values for Kir6.1 mRNA were significantly correlated with the average response of acetylcholine (ACH (ave) response) of iontophoresis with laser Doppler flowmetry (r=-0.579, p<0.001, n=40).

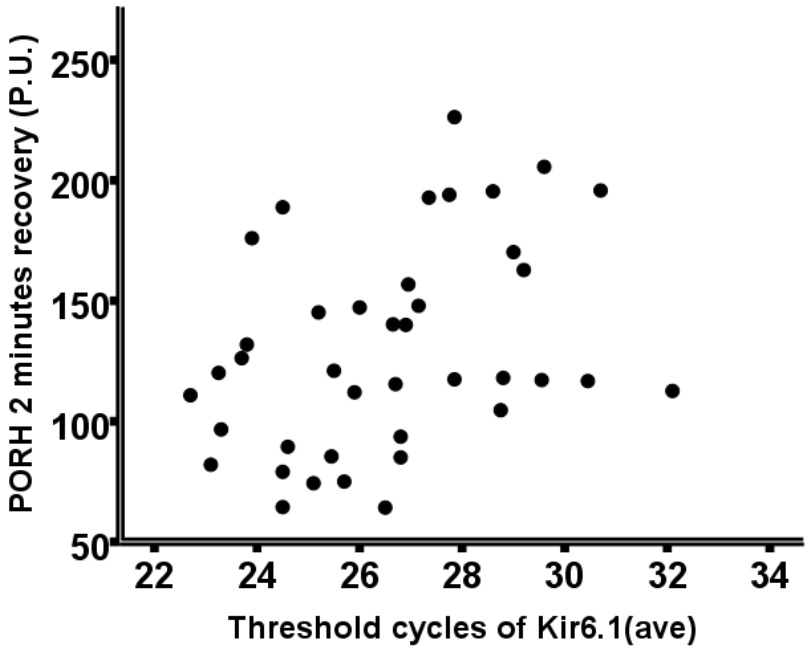


Figure 6.4 C_t value for Kir6.1 mRNA were significantly correlated with PORH 2 minutes recovery (r=0.366, p=0.020, n=40).

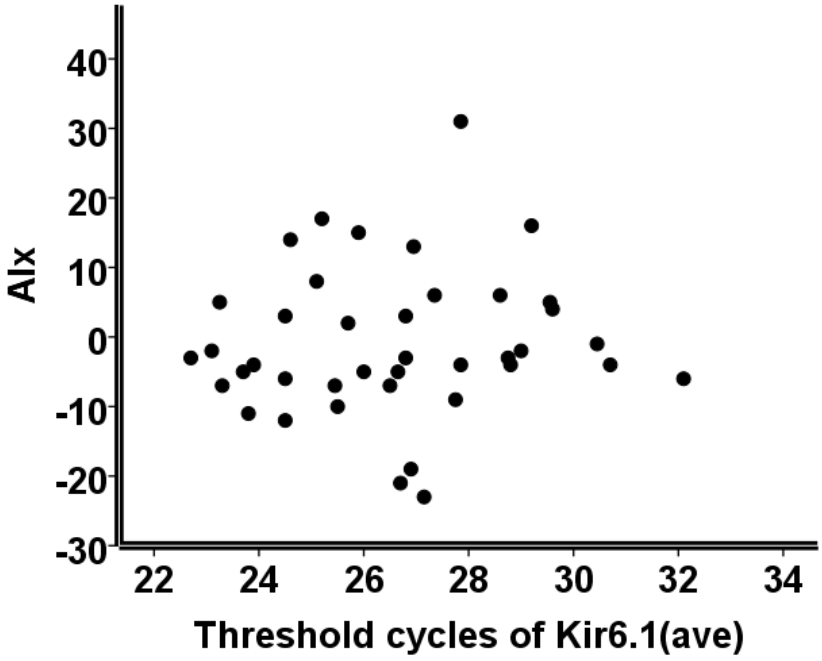


Figure 6.5 No correlation was noted between C_t values for Kir6.1 mRNA and AIx with levels (r=0.080, p=0.266, n=40).

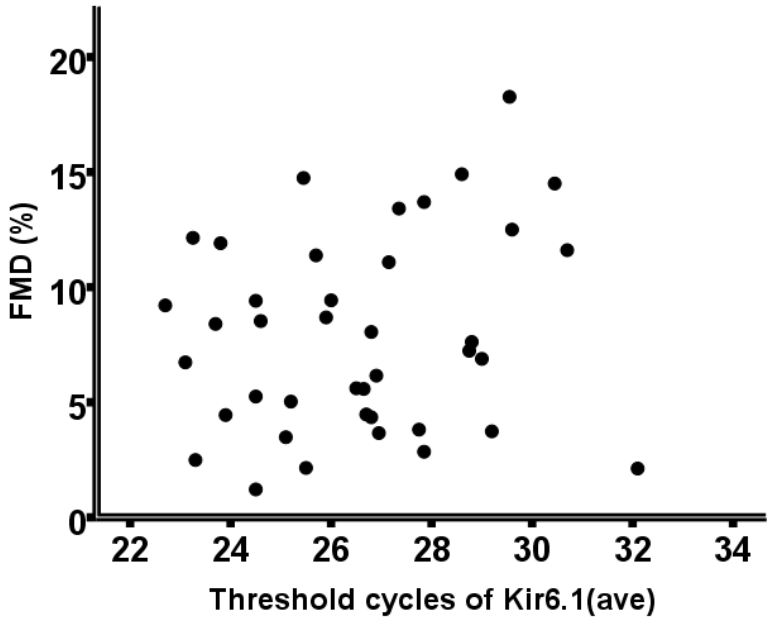


Figure 6.6 No correlation between the C_t values for Kir6.1 mRNA and FMD (r=0.181, p=0.264, n=40).

6.4 Correlation between Kir6.1 mRNA levels in the blood and lipid profile

No correlations were noted between the C_t values for Kir6.1 mRNA and all lipid profile parameters except LDL (r=0.341, p=0.039, n=46; Figure 6.8). Data are presented in mean (±SD) (Table 6.3).

Table 6.3 Lipid profile for 46 (26 males, 20 females) of 50 subjects.

Lipid profile	**N**	**Mean (±SD)**	**r**	**p value**
Total cholesterol	46	4.25 (±0.83)	0.228	0.175
HDL	46	1.48 (±0.44)	0.020	0.907
Triglycerides	46	1.17 (±0.65)	0.010	0.952
LDL	46	3.86 (±1.02)	0.341	0.039
Non-HDL	46	2.70 (±0.84)	0.249	0.137
Total cholesterol/HDL	46	3.05 (±1.15)	0.145	0.393

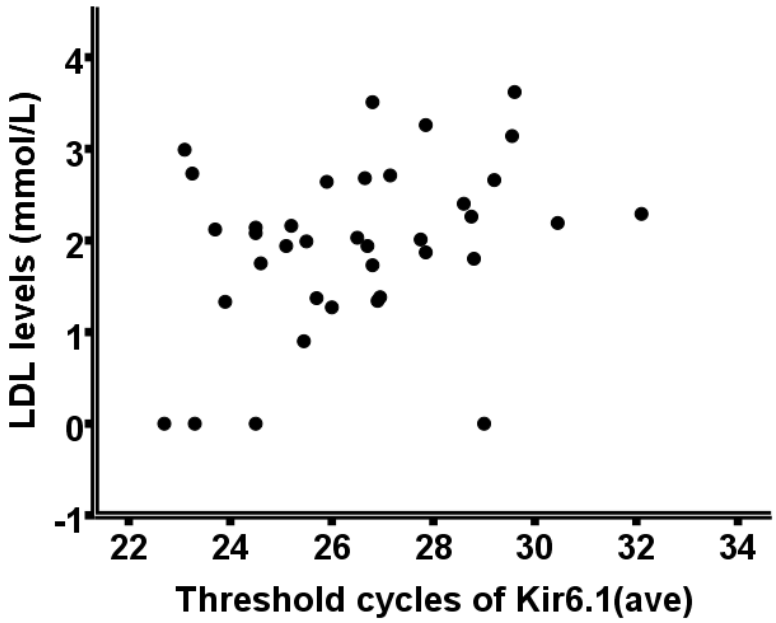


Figure 6.7 There was significant correlation between the C_t values for Kir6.1 mRNA and LDL (r=0.341, p=0.039, n=46).

6.5 Gender differences

Gender had no influence on relationships between C_t values for Kir6.1 mRNA and vascular function apart from relationship between C_t values for Kir6.1 mRNA and ACH (ave) response and PORH 2 minutes recovery. In males, we observed a significant correlation between the C_t values for Kir6.1 mRNA and ACH (ave) response (r=-0.561, p=0.005, n=23; Figure 6.9) and PORH 2 minutes recovery (r=0.418, p=0.047, n=23; Figure 6.10). In females, only significant correlation demonstrated was between the C_t values for Kir6.1 mRNA and ACH (ave) response (r=-0.628, p=0.007, n=17; Figure 6.11). In males, significant correlations were noted between the C_t values for Kir6.1 mRNA and both brachial (Figure 6.12) and aortic (Figure 6.13) systolic blood pressure (r=0.481, p=0.02, n=23 and r=0.417, p=0.048, n=23 respectively). This is not the case for females (brachial: r=0.294, p=0.253, n=17 and aortic:

r=0.339, p=0.183, n=17). No correlations were found between the C_t values for Kir6.1 mRNA and brachial as well as aortic diastolic blood pressure in either males or females. There was no statistical significant difference between males and females in the C_t values for Kir6.1 mRNA nor were there any significant differences in PORH, PWA and FMD. No significant correlations were found between the C_t values for Kir6.1 mRNA and PORH (Table 6.4), PWA (Table 6.5) and FMD (Table 6.6) in either males or females except in males, the C_t values for Kir6.1 mRNA correlates with PORH 2 minutes recovery (r=0.418, 0.047, n=23).

Table 6.4 No correlation noted between C_t values for Kir6.1 mRNA and PORH in males and females except in males, the C_t values for Kir6.1 mRNA correlates with PORH 2 minutes recovery.

PORH parameter (in males)	**r**	**p**	**n**	**PORH parameter (in females)**	**r**	**p**	**n**
Baseline perfusion	0.326	0.129	23	Baseline perfusion	0.240	0.354	17
Peak PORH	0.076	0.732	23	Peak PORH	-0.067	0.799	17
PORH 2 minutes recovery	**0.418**	**0.047**	**23**	PORH 2 minutes recovery	0.226	0.384	17

Table 6.5 No correlation noted between C_t values for Kir6.1 mRNA and PWA in both males and females.

PWA parameter (in males)	**r**	**p**	**n**	**PWA parameter (in females)**	**r**	**p**	**n**
Time to reflected wave (T_r) (time)	0.302	0.161	23	Time to reflected wave (T_r) (time)	0.124	0.634	17
Subendocardial viability ratio (SEVR) (%)	0.010	0.964	23	Subendocardial viability ratio (SEVR) (%)	-0.365	0.150	17
Aix	0.075	0.734	23	Aix	0.074	0.504	17
AIx@75	-0.038	0.863	23	AIx@75	0.209	0.420	17

Table 6.6 No correlation noted between C_t values for Kir6.1 mRNA and FMD in both males and females.

FMD parameter (in males)	**R**	**p**	**n**	**FMD parameter (in females)**	**R**	**p**	**n**
Baseline measurement of the brachial artery (mm)	0.131	0.552	23	Baseline measurement of the brachial artery (mm)	-0.118	0.653	17
Measurement of brachial artery after 5 minutes cuff release (mm)	0.197	0.367	23	Measurement of brachial artery after 5 minutes cuff release (mm)	-0.014	0.956	17
Percentage change i.e. FMD (%)	0.229	0.293	23	Percentage change i.e. FMD (%)	0.190	0.465	17

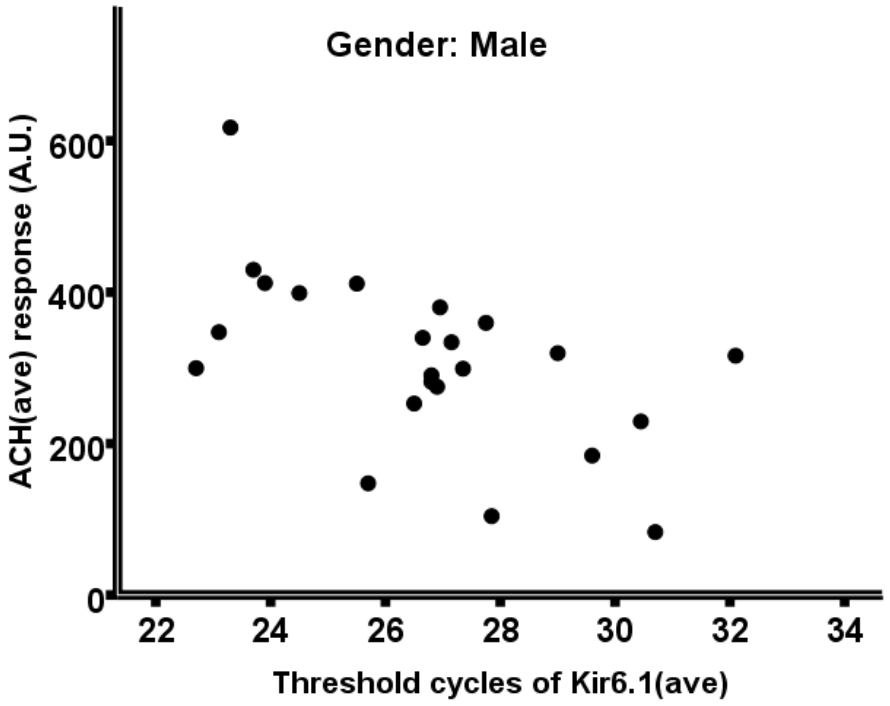


Figure 6.8 A significant correlation was noted between the C_t values for Kir6.1 mRNA and ACH (ave) response in males (r=-0.561, p=0.005, n=23).

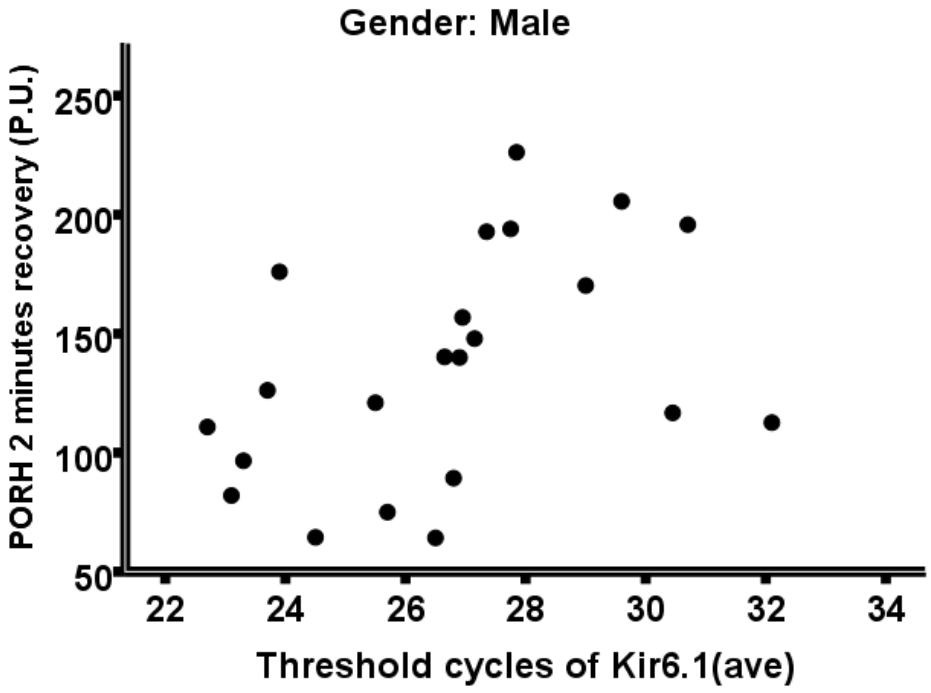


Figure 6.9 The C_t values for Kir6.1 mRNA were correlated with PORH 2 minutes recovery in males (r=0.418, 0.047, n=23).

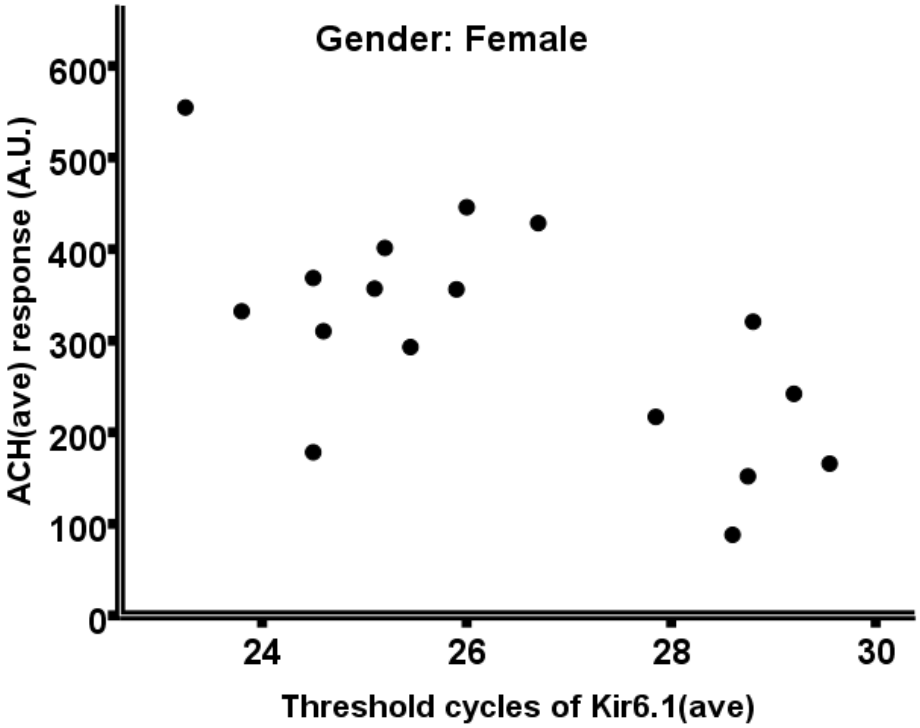


Figure 6.10 The C_t values for Kir6.1 mRNA were correlated with ACH (ave) response in females (r=-0.628, p=0.007, n=17).

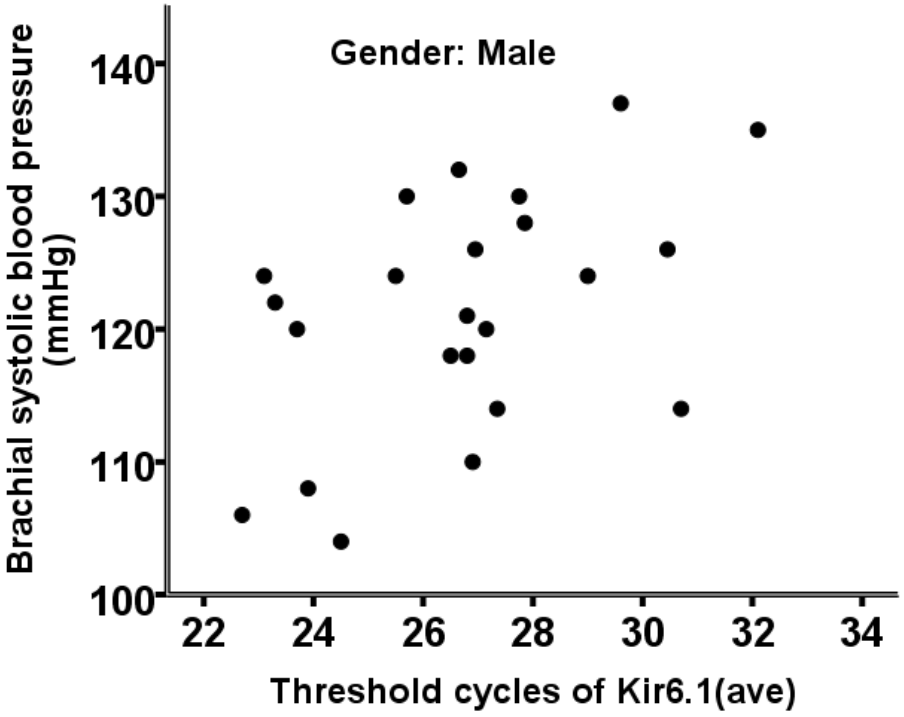


Figure 6.11 The C_t values for Kir6.1 mRNA were correlated with brachial systolic blood pressure in males (r=0.481, p=0.02, n=23).

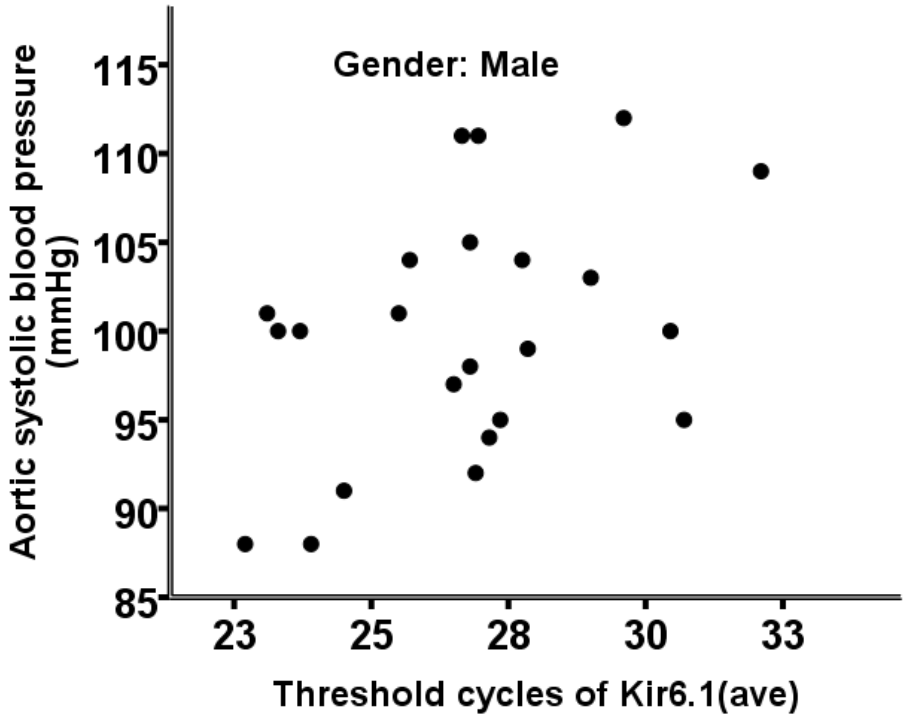


Figure 6.12 The C_t values for Kir6.1 mRNA correlate with aortic systolic blood pressure in males (r=0.417, p=0.048, n=23).

CHAPTER 7

7.1 Discussion

In the current study, the relationship between the levels of Kir6.1 mRNA subunits in blood with microvascular and macrovascular function of healthy normal subjects without cardiovascular disease was evaluated. It was demonstrated that Kir6.1, but not SUR2B, mRNA subunits can be detected in human blood. This finding is in keeping with previous animal studies which demonstrated expression of Kir6.1 mRNA subunits but not SUR subunits (Yasu et al., 2002). In addition, Yasu et. al (2002) have demonstrated fully functioning K_{ATP} channels in polymorphonuclear neutrophils (PMNLs) despite only detecting the pore-forming subunit, Kir6.1 or Kir6.2 mRNA subunit, using real time RT-PCR. Since it is understood that expression of Kir alone does not result in fully functional K_{ATP} channels (Shyng et al., 1997), it is likely that an unknown SUR isoform is expressed in PMNLs. Another possibility, as suggested by Yasu et al (2002), is that there exist novel channels which mimic the activity of K_{ATP} channels in blood cells (Yasu et al., 2002). Since the primer used to detect human SUR2 subunit in this study is similar to those designed by Jovanovic and Du (unpublished) to detect SUR2 subunits in animal model, it could possibly mean that a different primer needs to be designed to be compatibility with human SUR2 subunit. However, up to date, no studies have designed compatible primer and experimented on human samples. Currently, the possible SUR variant has not yet been identified, however, based on the findings of this study, it is unlikely to be SUR2B, which is the known regulatory

subunit of K_{ATP} channel in VSMCs in the literature (Shi et al., 2007, Morrissey et al., 2005).

A key issue raised in this study was whether a link could be established between the level of K_{ATP} channel subunits and vascular function. It is recognised that K_{ATP} channels play a central role in the regulation of vascular tone via various vasodilators and vasoconstrictors and hence in the regulation of blood pressure and blood flow. Therefore, it is possible that the degree of vasoreactivity of the vasculature, is dependent on the number of K_{ATP} channels in VSMCs i.e. increased number of activated K_{ATP} channels permit higher degree of blood vessel vasodilation, therefore, associated with increased blood flow i.e. better vascular function. Since only Kir6.1 mRNA subunits are detected, the question remains as to whether the level of Kir6.1 subunits are representative of the level of fully functional K_{ATP} channels in blood. It has been shown that the level of the least expressed K_{ATP} channel subunit can be representative of the level of K_{ATP} channels. Ranki et al (2001) and Jovanovic (2008) demonstrated that the level of the least expressed K_{ATP} channels subunit can be used as a proxy measure for the number of fully assembled-sarcolemmal K_{ATP} channels in the heart and skeletal muscle (Ranki et al., 2001, Jovanović et al., 2008). Therefore, Kir6.1 mRNA subunit could be the rate limiting subunit which forms VSMC K_{ATP} channels and be a proxy measure for these channels. Another key issue raised in this study is can the level of K_{ATP} channels in blood be a direct reflection of those in the VSMCs? Definite proof is still lacking, however, it has been reported that since the expression of Kir6.2, another Kir6 isoform, is regulated by similar control elements in different tissues i.e cardiac, skeletal muscle and pancreas, the levels of Kir6.1 mRNA subunit

between blood and VSMCs could potentially be similar (Ashfield and Ashcroft, 1998, Moritz et al., 2001).

In this study, the levels of Kir6.1 mRNA subunit were measured using real time RT-PCR. Real time RT-PCR is a relatively new technique used to detect and quantify mRNA targets and has been shown to yield accurate and reliable results with significantly less quantities of blood required compared to any other techniques used to measure mRNA levels. During each cycle of PCR, this technique monitors the production of amplification products using fluorescent indicators. Since both amplification of DNA and detection of DNA levels occur simultaneously within one homogenous assay, this omits the need for post-PCR processing, hence reducing intra-operative error, thus, allowing higher reproducibility of results (Kennedy and Oswald, 2011).

In this study, microvascular function was assessed by measuring LDF with iontophoresis and PORH while macrovessels was assessed using FMD. PWA was carried out to assess arterial stiffness. LDF with iontophoresis of ACH measures real-time microvascular flow and NO-dependent vasodilator reactivity in each subjects using chemical stimulus. On the other hand, PORH, which is dependent on shear stress (mechanical stimulus), measures microvascular flow in downstream resistance vessels. Hojs et. al. (2009) demonstrated that during LDF with ACH iontophoresis, ACH mediated vasodilatation was attenuated following introduction of glibenclamide, suggesting the important role of K_{ATP} channels in human cutaneous vasodilatation (Hojs et al., 2009). This is consistent with the findings of this study whereby a correlation was found between levels of Kir6.1 and ACH (ave) response. Cankar and Štrucl (2008)

and Banitt et al (1996) both shown that K_{ATP} channels contribute to vasodilation of human blood vessels during reactive hyperaemia particularly PORH 2 minutes recovery but not peak PORH flow (Cankar and Štrucl, 2008, Banitt et al., 1996). These findings are in keeping with this study whereby a significant correlation was seen between the levels of Kir6.1 mRNA subunit and PORH 2 minutes recovery but this was not seen with peak PORH blood flow.

Based on the study's hypothesis, the higher the levels of Kir6.1 mRNA subunit i.e. lower threshold cycles of Kir6.1, the better the vascular function. Although there was significant correlation between the threshold cycles of Kir6.1 levels and PORH 2 minutes recovery, unlike ACH (ave) response, the correlation between the former was a positive one i.e. the lower the Kir6.1 subunit levels, the better the vascular function. The reason for this observation is currently unknown but two possible conclusions can be drawn to explain the discrepancy between both tests: (i) this observation could be due to the involvement of different physiological mechanisms in these different vasodilator stimuli, (ii) paradoxical up-regulation of K_{ATP} channel expression which was only detected with PORH test but not with LDF with ACH iontophoresis. LDF with ACH iontophoresis depends on transdermal iontophoretic delivery of a chemical stimulus, i.e. ACH through the interstitium surrounding blood vessels, to elicit a blood flow response. A gradual increment in electric current allows blood flow to increase from baseline to reach a maximum plateau in response to ACH delivery transdermally. Unlike LDF with ACH iontophoresis, the stimulus for PORH is quite different compared with the former. PORH is elicited using mechanical stimulus, i.e. blood pressure cuff for occlusion, and is known to be partially dependent on myogenic-mediated vasodilation in downstream

resistance vessels. As described previously, reactive hyperaemia occurs after cuff released as a result of decreased vascular resistance due to a build-up of vasodilator metabolites intra-arterially which dilate arterioles. Hence, reactive hyperaemia does not occur in an incremental fashion as seen in LDF with ACH iontophoresis. Instead, PORH involves a rapid increase in blood flow from zero blood flow after blood pressure cuff release to reach peak flow in less than one minute before a gradual decrease in blood flow occurs until normal vascular tone is regained downstream. Although previous studies have shown that K_{ATP} channels contribute towards blood vessel vasodilation in both microvascular function tests, it is unclear whether the difference in the nature of stimulus could influence the level of expression of K_{ATP} channels in VSMCs. As for the second explanation, there is a possibility that VSMC K_{ATP} channels are up-regulated in conditions of reduced perfusion therefore acting as a compensatory mechanism to reduce vascular damage. This compensatory mechanism was demonstrated in cardiac muscle under metabolic stress by Bao et al. (2010) whereby an increased in surface density of K_{ATP} channels was seen leading to increase cellular resistance to hypoxia thus minimising cardiac damage (Bao et al., 2010).

In macrovessels, no correlation was found between levels of Kir6.1 subunits and FMD. Previous studies have shown that K_{ATP} channels play a more apparent role in resistance arteries and less so in conduit arteries. Wu et. al. demonstrated the involvement of K_{ATP} channels in resistance arteries in endotoxin-induced smooth muscle hyperpolarisation while Hodnett et. al. showed that in obese Zucker rats, arteriolar vasodilation was mediated by K_{ATP} channels (Wu et al., 2004, Hodnett et al., 2008). Therefore, it is possible that

the vasoregulatory mechanism in conduit arteries are not mediated by K_{ATP} channels. It is, therefore, not unreasonable to conclude that Kir6.1 could be an indicator for microvascular function but not macrovascular function since no correlation was seen between Kir6.1 levels and FMD. In addition, no correlation was found between the levels of Kir6.1 subunit and arterial stiffness indicating that Kir6.1 level is not a good marker of arterial stiffness. Currently, no other studies have examined the relationship between arterial stiffness and VSMC K_{ATP} channel levels.

This study also showed a strong correlation between the threshold cycles of Kir6.1 levels with brachial and aortic systolic blood pressure i.e. the lower the expression of Kir6.1, the higher the systolic blood pressure. Since lower expression of Kir6.1 means poorer vascular function and that higher blood pressure is recognised to be proportionately related to increased CV events risk i.e. poorer vascular function, this finding was not unexpected (Prospective Studies Collaboration, 2003). Nonetheless, similar relationship was not seen between Kir6.1 levels and diastolic blood pressure. This observation could be explained by evidence which showed that systolic blood pressure increases steadily with age, whereas diastolic blood pressure increases until about age 50 and then declines (Rockwood & Howlett, 2011).

Although real-time RT-PCR represent a fast and effective method enabling quantification of mRNA levels with high sensitivity and precision, there are several challenges associated with this technique. These include careful isolation of RNA to ensure the integrity of the RNA itself and that the RNA template is free from contaminant. Contaminant such as DNA could contribute

to the final amplification signal thereby affecting the final product. Further studies are required to test reproducibility of Kir6.1 levels.

In addition, this study showed that in males, significant correlations were found between Kir6.1 levels and ACH (ave) response as well as PORH 2 minutes recovery and both brachial and aortic systolic blood pressure. In females, only a correlation between Kir6.1 levels and ACH (ave) response was noted. This lack of correlation in females could be a result of hormonal differences or to a lesser extent due to a smaller sample size (23 males versus 17 females) compared to their male counterpart. It is recognised that hormones such as oestrogen are cardioprotective as they exert an antiatherogenic effect, mainly through the enhancement of nitric oxide by increasing endothelial nitric oxide synthase activity, on the vasculature (Ranki et al., 2001, Chambliss & Shaul, 2002). In premenopausal females, therefore, vasodilatory changes in blood vessels may be influenced by their menstrual phases. Since the different stages of menstrual cycle of female subjects were not taken into account, this could partially explained the differences observed.

It is important to remember that this study is solely based on correlations and the above relationships do not necessarily prove causality. Further in depth statistical analysis such as explorative analyses, testing for normal distribution of the variables and multivariate analysis adjusting for any relevant confounders could have been performed. It is, therefore, difficult to robustly establish whether Kir6.1 level could potentially be a good indicator for vascular function.

7.2 Limitations

Several limitations have been identified in the present study. It is understood that RNAses are present in all domains of life including atmospheric air. Therefore, whole blood RNA degradation could have occurred in heparinised blood tube prior to carrying out real-time RT PCR, possibly yielding inaccurate threshold cycles of mRNA subunits. This problem can be overcome by using special blood tubes such as Tempus or PAXgene Blood RNA Tube. These special blood tubes are costly but they collect, store and transport blood and stabilise intracellular RNA in a closed tube and allowing subsequent isolation and purification of intracellular RNA from whole blood for RT-PCR. It was suggested that since the promoter for Kir6.1 were similar for different tissues, there is a possibility that Kir6.1 levels were the same in both tissues. Nonetheless, it would be helpful if a direct relationship between the levels of Kir6.1 subunit in different tissues can be establish with specific test, yielding concrete data. This can be overcome by testing this relationship in animal models. Theoretically, rodent VSMCs can be harvested in vivo and VSMCs Kir6.1 subunit levels could be measured using laboratory techniques for comparison with the levels of Kir6.1 in blood of similar subject detected using real-time RT-PCR. Another limitation was this study was unable to identify the unknown SUR variance. It is recognised that both the pore-forming subunits and regulatory subunits are required to form fully-functioning K_{ATP} channels. Using real-time RT-PCR, this study has only identified Kir6.1 regulatory subunit from blood cells. In addition, subject sample size should be larger to investigate the relationship between Kir6.1 levels and gender differences. As this study has

only concentrated on normal healthy subjects, it would have benefited from the inclusion of older individuals or individuals with CVD, as it would have generated a wider spread of vascular function results, allowing the detection of meaningful correlations easier.

7.3 Conclusion

This present study has investigated the relationship between K_{ATP} channels in blood and vascular function. It was primarily aimed to investigate, in normal healthy subjects, whether the levels of Kir6.1 subunit expressed in blood samples are related to the function of both microvessels and macrovessles. In this study, it was demonstrated that Kir6.1 levels correlate with microvascular but not macrovascular function. Gender differences were also shown when correlating with the levels of Kir6.1 mRNA. In addition, this study found no correlation between Kir6.1 subunit levels and lipid profile as well as arterial stiffness. Despite the above findings, it appears unlikely that the levels of K_{ATP} channel has the potential to be a suitable and accurate surrogate of vascular function due to various factors as mentioned above in the 'Discussion' section. However, one would have to agree that it provides important pathophysiological insight which could act as the basis of future research.

7.4 Recommendations for future research

This research has raised some questions which require further investigations. It is recommended that further work needs to be undertaken to identify the unknown SUR subunit variance in VSMCs K_{ATP} channel. In addition, further

studies in animal models could allow the determination of the levels of Kir6.1 subunit between different tissues i.e. VSMCs and blood cells directly. This can be carried out as suggested in the section '7.2 Limitations'. More studies should also be carried out to investigate gender differences with larger sample size. Female subjects should be controlled for their menstrual cycle. Finally, further studies are required to investigate the reproducibility of Kir6.1 subunit levels with real-time RT PCR. Future studies could adapt the methods of this research project to yield data for comparison with the findings of this project. Once more answers are shed, further research could investigate the relationship between Kir6.1 levels and vascular function in patient population with vascular disease. Depending on these findings, there is scope for translational medicine to occur.

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Appendices

Appendix 1 Data collected for laser Doppler flowmetry with ACH iontophoresis

These values are the average of four readings carried out at the same electrical current. ACH (ave) response is calculated by adding the flux reading for the baseline and flux reading recorded for the different electric current and divided by six.

Subject	ACH@0μA	ACH@10μA	ACH@15μA	ACH@20μA	ACH@50μA	ACH@100μA
1	94	361.5	500.5	553	538	530.5
2	62.5	291	420	536.5	588.5	573.5
3	56	302	395	395	384	365
4	79	256.5	380	540.5	556.5	579.5
5	58	248	535	581	426	560
6	154.5	540.5	682.5	682.5	797.5	846.5
7	59	366	505	519	559	536.5

8	**58.5**	**158.5**	**360**	**451**	**580.5**	**602.5**
9	**80**	**224.5**	**566.5**	**622.5**	**662**	**519**
10	**54**	**208.5**	**424**	**451**	**480**	**466**
11	**41.5**	**50**	**113.5**	**138**	**337.5**	**388.5**
12	**60**	**186**	**286.5**	**293**	**281**	**320.5**
13	**51**	**107**	**187**	**343**	**486.5**	**475.5**
14	**53**	**158.5**	**273.5**	**366**	**493.5**	**517.5**
15	**83**	**81.5**	**117.5**	**237.5**	**332**	**370.5**
16	**47**	**79.5**	**193**	**260**	**383**	**372**
17	**77.5**	**353.5**	**465.5**	**477**	**447.5**	**468**

18	**76.5**	**236.5**	**409.5**	**408.5**	**428**	**406**
19	**49.5**	**255.5**	**347.5**	**278**	**353**	**406**
20	**50**	**229.5**	**351.5**	**405.5**	**457.5**	**498.5**
21	**53**	**82.5**	**141.5**	**175**	**192.5**	**241**
22	**58**	**183**	**298.5**	**323**	**327.5**	**327.5**
23	**56.5**	**122**	**324.5**	**399**	**413**	**426.5**
24	**59.5**	**163.5**	**282**	**377**	**364.5**	**343**
25	**46.5**	**266.5**	**375.5**	**379**	**366.5**	**363.5**
26	**49**	**189**	**321**	**357.5**	**423**	**473.5**
27	**55.5**	**117**	**324**	**400**	**465**	**511**

28	**52.5**	**271**	**495.5**	**556.5**	**583**	**612.5**
29	**55.5**	**116**	**226.5**	**240.5**	**307.5**	**365**
30	**67**	**218.5**	**450**	**498**	**541.5**	**505.5**
31	**93**	**389.5**	**424**	**438.5**	**556**	**566.5**
32	**95.5**	**102.5**	**130.5**	**241.5**	**356.5**	**374.5**
33	**97**	**66.5**	**71.5**	**426**	**605**	**651**
34	**103**	**70**	**70.5**	**67.5**	**101.5**	**116**
35	**77**	**399**	**395.5**	**414**	**438.5**	**433.5**
36	**70**	**72.5**	**80.5**	**90**	**148**	**164.5**
37	**146**	**91**	**77**	**65**	**58**	**61.5**

38	**62**	**96.5**	**212**	**360.5**	**505.5**	**557**
39	**53.5**	**198.5**	**199.5**	**190**	**234.5**	**229.5**
40	**48**	**297**	**279**	**284**	**271**	**273.5**
41	**11.4**	**627.5**	**745**	**706**	**665**	**573**
42	**14**	**195.5**	**419**	**480.5**	**529.5**	**498.5**
43	**5**	**88**	**183.5**	**185.5**	**208.5**	**241.5**
44	**19.5**	**51**	**109**	**283.5**	**425**	**487.5**
45	**20.5**	**126**	**279.5**	**360.5**	**549**	**667.5**
46	**37.5**	**21.5**	**66**	**150.5**	**325**	**394**
47	**32.5**	**149**	**327.5**	**390**	**427.5**	**431.5**

48	**51.5**	**321**	**402**	**404.5**	**422.5**	**436.5**
49	**17.5**	**157.5**	**384.5**	**471.5**	**566.5**	**544**
50	**44**	**245.5**	**396.5**	**436**	**402**	**401**

ACH@0μA means ACH flux reading at baseline

ACH@10μA means ACH flux reading at 10μA etc

All readings' units in arbitrary units (A.U.)

Appendix 2 Data collected for post-occlusive reactive hyperaemia

The five readings obtained from different sites in the forearm were averaged up to give the final reading which is used in data interpretation. Base 1 means baseline reading taken at rest in region one; base 2 means baseline reading taken at rest in region two etc. Peak 1 means reading that is taken at maximal blood perfusion post blood pressure cuff released in region one etc. Ave 1 means reading that is taken from the area under the curve 2 minutes post cuff released in region 1

Subject	Base 1	Base 2	Base 3	Base 4	Base 5	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Ave 1	Ave 2	Ave 3	Ave 4	Ave 5
1	76	72	79	78	76	233	223	234	261	246	125	107	130	141	128
2	46	44	53	46	45	236	264	293	238	259	168	177	204	166	165
3	51	49	48	49	52	197	202	172	183	222	112	105	115	116	115
4	50	49	55	56	50	217	231	218	209	196	67	58	71	64	62
5	67	60	71	70	65	298	288	326	306	298	142	131	156	156	141

6	**50**	**47**	**55**	**54**	**50**	**356**	**317**	**351**	**318**	**336**	**101**	**88**	**105**	**102**	**87**
7	**84**	**85**	**87**	**87**	**86**	**231**	**215**	**205**	**216**	**233**	**128**	**132**	**125**	**129**	**123**
8	**139**	**118**	**146**	**107**	**136**	**347**	**329**	**327**	**283**	**298**	**210**	**190**	**193**	**170**	**181**
9	**78**	**83**	**70**	**81**	**73**	**301**	**384**	**259**	**338**	**282**	**161**	**171**	**136**	**138**	**130**
10	**41**	**36**	**38**	**34**	**37**	**174**	**169**	**167**	**154**	**166**	**87**	**82**	**83**	**77**	**81**
11	**42**	**39**	**41**	**42**	**40**	**167**	**145**	**135**	**143**	**139**	**86**	**78**	**75**	**80**	**76**
12	**52**	**51**	**47**	**50**	**57**	**240**	**254**	**251**	**237**	**251**	**119**	**119**	**125**	**119**	**131**
13	**72**	**73**	**68**	**70**	**79**	**224**	**179**	**237**	**220**	**227**	**138**	**126**	**144**	**146**	**146**
14	**49**	**44**	**48**	**44**	**42**	**229**	**187**	**209**	**186**	**191**	**96**	**86**	**97**	**86**	**82**
15	**54**	**64**	**62**	**63**	**65**	**231**	**254**	**269**	**288**	**269**	**140**	**162**	**196**	**209**	**184**

16	**53**	**56**	**52**	**49**	**63**	**199**	**200**	**205**	**209**	**229**	**109**	**115**	**110**	**102**	**127**
17	**61**	**64**	**62**	**60**	**57**	**257**	**281**	**260**	**283**	**292**	**95**	**100**	**98**	**96**	**88**
18	**61**	**59**	**64**	**64**	**60**	**250**	**247**	**262**	**228**	**183**	**135**	**136**	**119**	**118**	**113**
19	**44**	**45**	**49**	**47**	**43**	**130**	**153**	**112**	**126**	**132**	**83**	**85**	**88**	**92**	**77**
20	**46**	**49**	**49**	**47**	**48**	**208**	**216**	**219**	**228**	**244**	**126**	**125**	**138**	**137**	**133**
21	**54**	**56**	**56**	**56**	**52**	**140**	**161**	**153**	**149**	**151**	**70**	**82**	**69**	**77**	**77**
22	**31**	**37**	**35**	**37**	**37**	**3**	**221**	**4**	**220**	**254**	**24**	**88**	**22**	**91**	**96**
23	**46**	**45**	**41**	**44**	**43**	**132**	**137**	**114**	**119**	**147**	**92**	**97**	**90**	**92**	**97**
24	**49**	**48**	**47**	**41**	**42**	**222**	**220**	**233**	**208**	**202**	**117**	**120**	**106**	**104**	**100**
25	**32**	**38**	**37**	**33**	**38**	**110**	**185**	**166**	**178**	**200**	**93**	**117**	**107**	**111**	**126**

26	**44**	**45**	**44**	**49**	**47**	**220**	**263**	**211**	**234**	**227**	**114**	**121**	**120**	**125**	**111**
27	**68**	**64**	**68**	**65**	**57**	**152**	**166**	**129**	**125**	**121**	**116**	**115**	**108**	**107**	**99**
28	**52**	**61**	**59**	**65**	**60**	**308**	**306**	**290**	**289**	**325**	**112**	**108**	**119**	**112**	**126**
29	**72**	**78**	**81**	**86**	**87**	**229**	**212**	**252**	**217**	**206**	**119**	**112**	**105**	**101**	**97**
30	**82**	**84**	**82**	**84**	**85**	**230**	**213**	**235**	**199**	**239**	**156**	**150**	**167**	**149**	**162**
31	**68**	**65**	**84**	**85**	**62**	**176**	**157**	**197**	**191**	**186**	**115**	**108**	**133**	**135**	**114**
32	**77**	**72**	**89**	**86**	**76**	**242**	**242**	**283**	**290**	**262**	**122**	**119**	**110**	**123**	**113**
33	**69**	**59**	**68**	**69**	**57**	**231**	**312**	**239**	**293**	**351**	**168**	**169**	**159**	**175**	**180**
34	**105**	**89**	**110**	**109**	**96**	**368**	**332**	**349**	**325**	**329**	**203**	**184**	**203**	**196**	**191**
35	**125**	**125**	**120**	**125**	**128**	**364**	**348**	**366**	**382**	**359**	**196**	**193**	**188**	**194**	**199**

36	**149**	**140**	**180**	**165**	**156**	**359**	**340**	**331**	**324**	**321**	**226**	**216**	**234**	**225**	**230**
37	**88**	**95**	**89**	**82**	**90**	**282**	**277**	**282**	**242**	**247**	**209**	**204**	**194**	**179**	**193**
38	**82**	**83**	**75**	**76**	**77**	**356**	**309**	**332**	**311**	**279**	**214**	**197**	**188**	**176**	**189**
39	**96**	**84**	**87**	**86**	**85**	**303**	**279**	**313**	**293**	**280**	**228**	**212**	**206**	**184**	**198**
40	**98**	**95**	**106**	**102**	**103**	**281**	**271**	**251**	**258**	**264**	**165**	**155**	**165**	**163**	**166**
41	**71.8**	**67.7**	**63.4**	**66.7**	**60.5**	**247.5**	**274.7**	**219.3**	**251.5**	**221.9**	**124.1**	**125.4**	**122.5**	**122.5**	**105.9**
42	**49**	**56.9**	**55.2**	**56.1**	**50.3**	**170**	**178.3**	**174**	**163.2**	**158.1**	**110.7**	**124.9**	**108.2**	**108**	**108.4**
43	**64.3**	**66.8**	**64.3**	**66.4**	**63.3**	**138.9**	**150.8**	**138.1**	**145.9**	**134.9**	**101.3**	**110.3**	**103.3**	**105.3**	**102.7**
44	**55.9**	**51.3**	**54.4**	**52.3**	**44.9**	**164.2**	**145**	**141.6**	**141.8**	**96.2**	**119.1**	**108.8**	**132.9**	**123.7**	**99**
45	**61.7**	**65.2**	**71.3**	**77.6**	**73.2**	**201**	**226.7**	**222**	**262**	**275.3**	**126.1**	**149.8**	**138.8**	**161**	**164**

46	**50.8**	**53.6**	**57.5**	**52.4**	**48.3**	**138**	**168.4**	**164.8**	**176**	**155.8**	**99.3**	**119.6**	**122.4**	**129.6**	**114.7**
47	**51.2**	**47.8**	**61.8**	**63.5**	**52.8**	**172.5**	**170.3**	**189.9**	**207**	**193.6**	**78.6**	**77.6**	**82.2**	**95.9**	**92.7**
48	**57.6**	**60.3**	**65**	**66.9**	**63.8**	**165.4**	**160.8**	**208.2**	**205.8**	**190.4**	**131.4**	**126.5**	**143**	**150.8**	**149.5**
49	**31.9**	**31**	**38.2**	**46.3**	**40.3**	**218.1**	**224.1**	**214.5**	**207.9**	**211.7**	**60.4**	**65.9**	**81.1**	**85.5**	**78.7**
50	**54.5**	**54.1**	**59.5**	**66.8**	**57.3**	**172.8**	**181.9**	**168.3**	**188**	**168.5**	**113.2**	**115.2**	**117.7**	**129.9**	**113.9**

All units in perfusion units (P.U.)

Appendix 3 Data collected for flow-mediated dilatation

Subjects	Baseline diameter of brachial artery (mm)	Postocclusion diameter of brachial artery (mm)	FMD percentage change (%)
1	3.69	4.00	8.40
2	3.60	3.76	4.44
3	3.78	3.86	2.12
4	3.43	3.61	5.25
5	3.18	3.34	5.03
6	3.61	3.70	2.49
7	2.76	2.97	7.60
8	2.87	3.14	9.41

9	**2.97**	**3.25**	**9.43**
10	**3.86**	**4.12**	**6.74**
11	**3.30**	**3.34**	**1.21**
12	**3.20**	**3.42**	**6.88**
13	**3.25**	**3.45**	**6.15**
14	**3.40**	**3.69**	**8.53**
15	**5.28**	**5.59**	**5.87**
16	**3.26**	**3.61**	**10.74**
17	**3.49**	**3.79**	**8.60**
18	**3.18**	**3.42**	**7.55**

19	**3.45**	**3.60**	**4.35**
20	**3.61**	**4.04**	**11.91**
21	**4.13**	**4.60**	**11.38**
22	**4.28**	**4.52**	**5.61**
23	**3.23**	**3.49**	**8.05**
24	**4.15**	**4.23**	**1.93**
25	**3.91**	**4.27**	**9.21**
26	**3.45**	**3.82**	**10.72**
27	**2.63**	**2.98**	**13.31**
28	**3.58**	**3.74**	**4.47**

29	**3.17**	**3.45**	**8.83**
30	**3.28**	**3.40**	**3.66**
31	**4.66**	**4.76**	**2.15**
32	**2.92**	**3.32**	**13.70**
33	**3.63**	**3.88**	**6.89**
34	**3.22**	**3.70**	**14.91**
35	**4.46**	**4.63**	**3.81**
36	**3.86**	**3.97**	**2.85**
37	**4.05**	**4.52**	**11.60**
38	**3.28**	**3.72**	**13.41**

39	**4.40**	**4.95**	**12.50**
40	**3.48**	**3.61**	**3.74**
41	**3.13**	**3.51**	**12.14**
42	**3.57**	**3.88**	**8.68**
43	**3.04**	**3.26**	**7.24**
44	**3.93**	**4.50**	**14.50**
45	**4.06**	**4.51**	**11.08**
46	**3.12**	**3.69**	**18.27**
47	**3.12**	**3.58**	**14.74**
48	**4.48**	**4.73**	**5.58**

49	**3.16**	**3.27**	**3.48**
50	**3.15**	**3.39**	**7.62**

Appendix 4 Data collected for pulse wave analysis

Subject	Brachial SBP (mmHg)	Brachial DBP (mmHg)	Brachial pulse pressure (mmHg)	Aortic SBP (mmHg)	Aortic DBP (mmHg)	Aortic pulse pressure (mmHg)	T_r (time)	AIx	SEVR (%)	Aix@75
1	120.00	72.00	48.00	100.00	73.00	27.00	154.00	-5.00	148.00	-8.00
2	108.00	61.00	41.00	88.00	62.00	26.00	139.00	-4.00	172.00	-12.00
3	135.00	74.00	61.00	109.00	75.00	34.00	141.00	-6.00	186.00	-17.00
4	104.00	72.00	32.00	91.00	73.00	18.00	154.00	-6.00	190.00	-12.00
5	94.00	64.00	30.00	85.00	64.00	20.00	137.00	17.00	232.00	5.00
6	122.00	68.00	54.00	100.00	68.00	32.00	157.00	-7.00	252.00	-19.00

7	**127.00**	**84.00**	**43.00**	**111.00**	**85.00**	**26.00**	**142.00**	**9.00**	**123.00**	**11.00**
8	**103.00**	**57.00**	**46.00**	**84.00**	**58.00**	**26.00**	**143.00**	**-12.00**	**151.00**	**-15.00**
9	**111.00**	**72.00**	**39.00**	**96.00**	**73.00**	**23.00**	**165.00**	**-5.00**	**154.00**	**-9.00**
10	**124.00**	**71.00**	**53.00**	**101.00**	**71.00**	**30.00**	**140.00**	**-2.00**	**139.00**	**-2.00**
11	**111.00**	**63.00**	**48.00**	**91.00**	**64.00**	**27.00**	**141.00**	**3.00**	**171.00**	**-4.00**
12	**109.00**	**63.00**	**46.00**	**89.00**	**64.00**	**25.00**	**138.00**	**-2.00**	**165.00**	**-6.00**
13	**110.00**	**68.00**	**42.00**	**92.00**	**68.00**	**24.00**	**151.00**	**-19.00**	**181.00**	**-28.00**
14	**107.00**	**73.00**	**34.00**	**96.00**	**74.00**	**22.00**	**141.00**	**14.00**	**145.00**	**10.00**
15	**127.00**	**58.00**	**69.00**	**99.00**	**59.00**	**40.00**	**157.00**	**-9.00**	**233.00**	**-21.00**
16	**119.00**	**75.00**	**44.00**	**101.00**	**76.00**	**25.00**	**157.00**	**2.00**	**169.00**	**-5.00**

17	**103.00**	**63.00**	**40.00**	**87.00**	**64.00**	**23.00**	**163.00**	**-10.00**	**179.00**	**-17.00**
18	**106.00**	**72.00**	**34.00**	**92.00**	**73.00**	**19.00**	**156.00**	**-3.00**	**223.00**	**-14.00**
19	**118.00**	**71.00**	**47.00**	**98.00**	**72.00**	**26.00**	**136.00**	**-3.00**	**154.00**	**-5.00**
20	**87.00**	**52.00**	**35.00**	**73.00**	**52.00**	**21.00**	**175.00**	**-11.00**	**357.00**	**-26.00**
21	**130.00**	**66.00**	**64.00**	**104.00**	**67.00**	**37.00**	**160.00**	**2.00**	**160.00**	**-5.00**
22	**118.00**	**68.00**	**50.00**	**97.00**	**69.00**	**28.00**	**152.00**	**-7.00**	**179.00**	**-12.00**
23	**121.00**	**80.00**	**41.00**	**105.00**	**81.00**	**24.00**	**144.00**	**3.00**	**139.00**	**3.00**
24	**128.00**	**60.00**	**68.00**	**99.00**	**59.00**	**40.00**	**146.00**	**-9.00**	**138.00**	**-15.00**
25	**106.00**	**62.00**	**44.00**	**88.00**	**63.00**	**25.00**	**159.00**	**-3.00**	**164.00**	**-7.00**
26	**105.00**	**70.00**	**35.00**	**92.00**	**71.00**	**21.00**	**157.00**	**5.00**	**178.00**	**-2.00**

27	**99.00**	**59.00**	**40.00**	**85.00**	**60.00**	**25.00**	**195.00**	**10.00**	**163.00**	**4.00**
28	**111.00**	**70.00**	**41.00**	**94.00**	**70.00**	**24.00**	**152.00**	**-21.00**	**189.00**	**-30.00**
29	**92.00**	**60.00**	**32.00**	**81.00**	**61.00**	**20.00**	**133.00**	**10.00**	**121.00**	**14.00**
30	**126.00**	**79.00**	**47.00**	**111.00**	**80.00**	**31.00**	**145.00**	**13.00**	**144.00**	**12.00**
31	**124.00**	**69.00**	**55.00**	**101.00**	**70.00**	**31.00**	**152.00**	**-10.00**	**155.00**	**-15.00**
32	**132.00**	**89.00**	**43.00**	**125.00**	**90.00**	**35.00**	**138.00**	**31.00**	**144.00**	**28.00**
33	**124.00**	**72.00**	**52.00**	**103.00**	**73.00**	**30.00**	**157.00**	**-2.00**	**130.00**	**-2.00**
34	**122.00**	**77.00**	**45.00**	**105.00**	**78.00**	**27.00**	**153.00**	**6.00**	**132.00**	**5.00**
35	**130.00**	**67.00**	**63.00**	**104.00**	**68.00**	**36.00**	**158.00**	**-9.00**	**132.00**	**-12.00**
36	**128.00**	**58.00**	**70.00**	**99.00**	**59.00**	**40.00**	**151.00**	**-4.00**	**177.00**	**-15.00**

37	**114.00**	**68.00**	**46.00**	**95.00**	**68.00**	**27.00**	**161.00**	**-4.00**	**175.00**	**-8.00**
38	**114.00**	**59.00**	**55.00**	**95.00**	**60.00**	**35.00**	**164.00**	**6.00**	**128.00**	**7.00**
39	**137.00**	**67.00**	**70.00**	**112.00**	**68.00**	**44.00**	**162.00**	**4.00**	**189.00**	**-8.00**
40	**117.00**	**76.00**	**41.00**	**104.00**	**77.00**	**27.00**	**143.00**	**16.00**	**154.00**	**10.00**
41	**127.00**	**83.00**	**44.00**	**110.00**	**84.00**	**26.00**	**145.00**	**5.00**	**143.00**	**3.00**
42	**121.00**	**50.00**	**71.00**	**99.00**	**50.00**	**49.00**	**149.00**	**15.00**	**191.00**	**1.00**
43	**111.00**	**70.00**	**41.00**	**94.00**	**71.00**	**23.00**	**156.00**	**-3.00**	**202.00**	**-15.00**
44	**126.00**	**62.00**	**64.00**	**100.00**	**63.00**	**37.00**	**195.00**	**-1.00**	**200.00**	**-12.00**
45	**120.00**	**58.00**	**62.00**	**94.00**	**59.00**	**35.00**	**152.00**	**-23.00**	**163.00**	**-29.00**
46	**107.00**	**71.00**	**36.00**	**93.00**	**71.00**	**22.00**	**156.00**	**5.00**	**138.00**	**1.00**

47	**104.00**	**63.00**	**41.00**	**87.00**	**63.00**	**24.00**	**145.00**	**-7.00**	**152.00**	**-11.00**
48	**132.00**	**80.00**	**52.00**	**111.00**	**81.00**	**30.00**	**159.00**	**-5.00**	**143.00**	**-6.00**
49	**120.00**	**65.00**	**55.00**	**101.00**	**66.00**	**35.00**	**165.00**	**8.00**	**203.00**	**-4.00**
50	**113.00**	**67.00**	**46.00**	**95.00**	**67.00**	**28.00**	**171.00**	**-4.00**	**151.00**	**-11.00**

T_r- time to reflected wave

AIx- augmentation index

SEVR- subendocardial viability ratio

AIx@75- augmentation index at 75 beats per minutes

Appendix 5 Data collected for levels of Kir6.1

Subjects	Threshold cyles of Kir6.1	Subjects	Threshold cyles of Kir6.1
1	23.70	26	23.90
2	32.10	27	24.50
3	25.20	28	23.30
4	-	29	24.50
5	26.00	30	23.10
6	24.50	31	-
7	26.90	32	24.60
8	-	33	-

9	**-**	**34**	**-**
10	**26.80**	**35**	**23.80**
11	**25.70**	**36**	**26.50**
12	**26.80**	**37**	**-**
13	**22.70**	**38**	**-**
14	**-**	**39**	**26.70**
15	**-**	**40**	**26.95**
16	**25.50**	**41**	**27.85**
17	**29.00**	**42**	**28.60**
18	**27.75**	**43**	**27.85**

19	**30.70**	**44**	**27.35**
20	**29.60**	**45**	**29.20**
21	**23.25**	**46**	**25.90**
22	**28.75**	**47**	**30.45**
23	**27.15**	**48**	**29.55**
24	**25.45**	**49**	**26.65**
25	**25.10**	**50**	**28.80**

Appendix 6 Data collected for lipid profile

Subject	Total cholesterol	HDL	Triglycerides	LDL	Non-HDL	Total cholesterol/HDL
1	3.98	1.50	0.81	2.12	2.49	2.70
2	3.02	1.45	0.51	1.33	1.57	2.10
3	3.91	1.25	0.81	2.29	2.67	3.10
4	4.55	1.91	1.22	2.08	2.63	2.40
5	3.85	1.38	0.69	2.16	2.47	2.80
6	2.87	1.46	0.51	0.00	1.41	2.00
7	4.19	1.54	0.75	2.31	2.65	2.70
8	2.97	0.60	0.51	0.00	2.37	5.00

9	**3.28**	**1.40**	**1.32**	**1.27**	**1.88**	**2.30**
10	**5.47**	**1.58**	**1.97**	**2.99**	**3.89**	**3.50**
11	**3.84**	**1.28**	**0.93**	**2.14**	**2.57**	**3.00**
12	**5.31**	**0.69**	**1.65**	**3.86**	**4.61**	**7.70**
13	**2.86**	**0.97**	**1.20**	**1.34**	**1.89**	**3.00**
14	**4.21**	**0.94**	**3.31**	**1.75**	**3.26**	**4.50**
15	**5.68**	**2.59**	**1.06**	**0.00**	**0.00**	**0.00**
16	**5.14**	**2.08**	**1.41**	**2.41**	**3.06**	**2.50**
17	**4.92**	**1.54**	**1.51**	**2.69**	**3.38**	**3.20**
18	**-**	**-**	**-**	**-**	**-**	**-**

19	**3.18**	**0.97**	**1.06**	**1.73**	**2.21**	**3.30**
20	**-**	**-**	**-**	**-**	**-**	**-**
21	**3.70**	**1.99**	**0.74**	**1.37**	**1.71**	**1.90**
22	**3.66**	**1.31**	**0.68**	**2.03**	**2.35**	**2.80**
23	**5.95**	**1.58**	**1.88**	**3.51**	**4.37**	**3.80**
24	**4.33**	**1.74**	**0.66**	**2.29**	**2.60**	**2.50**
25	**3.96**	**1.50**	**0.51**	**0.00**	**2.46**	**2.46**
26	**3.68**	**0.93**	**0.51**	**0.00**	**2.76**	**4.00**
27	**4.55**	**2.14**	**0.51**	**0.00**	**2.41**	**2.10**
28	**3.89**	**1.56**	**0.84**	**1.94**	**2.33**	**2.50**

29	**4.28**	**0.87**	**3.00**	**2.03**	**3.41**	**4.90**
30	**3.44**	**1.51**	**1.21**	**1.38**	**1.93**	**2.30**
31	**4.05**	**1.73**	**0.73**	**1.99**	**2.32**	**2.30**
32	**5.71**	**1.40**	**2.29**	**3.26**	**4.31**	**4.10**
33	**4.69**	**2.25**	**0.51**	**0.00**	**2.44**	**2.10**
34	**4.11**	**0.96**	**1.64**	**2.40**	**3.15**	**4.30**
35	**3.39**	**0.83**	**1.21**	**2.01**	**2.57**	**4.10**
36	**3.93**	**1.38**	**1.47**	**1.87**	**2.55**	**2.80**
37	**-**	**-**	**-**	**-**	**-**	**-**
38	**-**	**-**	**-**	**-**	**-**	**-**

39	**6.06**	**1.83**	**1.33**	**3.62**	**4.23**	**3.30**
40	**5.02**	**2.03**	**0.72**	**2.66**	**2.99**	**2.50**
41	**4.62**	**1.36**	**1.14**	**2.73**	**3.25**	**3.40**
42	**5.42**	**2.02**	**1.67**	**2.64**	**3.40**	**2.70**
43	**3.92**	**1.28**	**0.83**	**2.26**	**2.64**	**3.10**
44	**4.03**	**1.35**	**1.05**	**2.19**	**2.68**	**3.00**
45	**4.94**	**1.81**	**0.92**	**2.71**	**3.14**	**2.70**
46	**5.02**	**1.21**	**1.47**	**3.14**	**3.81**	**4.20**
47	**4.07**	**2.01**	**2.54**	**0.90**	**2.06**	**2.00**
48	**4.09**	**0.99**	**0.90**	**2.68**	**3.10**	**4.10**

49	**3.73**	**1.54**	**0.54**	**1.94**	**2.18**	**2.40**
50	**3.95**	**1.72**	**0.95**	**1.80**	**2.23**	**2.30**