PREVALENCE OF BETA-2 ADRENERGIC RECEPTOR (β_2AR) POLYMORPHISMS IN MALAY POPULATION AND RELATIONSHIP OF THESE POLYMORPHISMS TO A MODEL ASSESSING MACROVASCULAR ENDOTHELIAL FUNCTION

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by

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LIST OF ABBREVIATION

ACE	angiotensin converting enzyme
ADMA	asymmetric dimethylarginine
AIx	augmentation index
ANCOVA	analysis of covariance
ARB	angiotensin receptor blocker
Arg	arginine
BLAST	Basic Local Alignment Search Tool
BMI	body mass index
BP	blood pressure
bp	base pair
BUP	beta-upstream peptide
β_2AR	beta-2 adrenergic receptor
β_2-agonist	beta-2 agonist
CAFÉ	Conduit Artery Function Evaluation Study
CAM	cellular adhesion molecule
CI	confidence interval
c-GMP	cyclic guanosine monophosphate
CRP	C-reactive protein
CV	coefficient of variation
DBP	diastolic blood pressure
DdH_2O	double distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynuclease triphosphate
DMSO	dimethyl sulfoxide
ECG	electrocardiogram
EDTA	ethylenediaminotetra-acetic acid
EDRF	endothelium derived relaxing factor
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
FBC	full blood count
FMD	flow mediated dilatation

Gi	inhibitory G protein
Gln	glutamine
Glu	glutamate
Gly	glycine
Gs	stimulatory G protein
GTN	glyceryl trinitrate
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HR	heart rate
hs-CRP	high-sensitivity C-reactive protein
ICAM-1	intercellular adhesion molecule
Ile	isoleucine
KCl	potassium chloride
LDF	laser Doppler flowmetry
LDL	low-density lipoprotein
L-NMMA	N^G-monomethyl-L-arginine
MDI	metered dose inhaler
Met	methionine
m/s	meter per second
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
OD	optical density
ONTARGET	Ongoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial
ORF	open reading frame
PAI	peripheral augmentation index
PAI-1	plasminogen activator inhibitor
PCR	polymerase chain reaction
PWA	pulse wave analysis
PWV	pulse wave velocity
QCA	quantitative coronary angiography
REASON	Preterax in Regression of Arterial Stiffness in a controlled Double Blind Study

ROS	reactive oxygen species
rpm	rotations per minute
SD	standard deviation
SBP	systolic blood pressure
SNPs	single nucleotide polymorphisms
SOP	standard operating procedure
TBE	tris-borate EDTA
Thr	threonine
t-PA	tissue plasminogen activator
TRANSCEND	Telmisartan Randomised Assessment Risk Management Study in ACE Intolerant
UTR	untranslated region
UV	ultraviolet
Val	valine
VCAM-1	vascular adhesion molecule
vWF	von Willebrand factor
x^2	chi-square
1 X	one time
5’LC	5’ leader cistron

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KAJIAN KEKERAPAN POLIMORFISME GEN RESEPTOR BETA-2 (β_2AR) DI KALANGAN MASYARAKAT MELAYU SERTA KESAN PEWARISAN GEN TERSEBUT KE ATAS SATU MODEL MENGUKUR FUNGSI SALUR DARAH BESAR

ABSTRAK

Disfungsi endotelium mendahului proses aterosklerosis. Pengukuran fungsi endotelium akan membolehkan pengesanan awal ke atas aterosklerosis dan membantu strategi rawatan. Baru-baru ini, "pulse wave analysis" (PWA) yang digandingkan dengan provokasi farmakologi menunjukkan potensi sebagai metodologi pengukuran endotelium secara tidak invasif. Provokasi farmakologi menggunakan "sublingual Glyceryl Trinitrate" (GTN) sebagai vasodilator yang tidak bergantung kepada endotelium manakala "inhaled salbutamol" sebagai vasodilator yang bergantung kepada endotelium. Tindakbalas terhadap salbutamol, iaitu agonis beta-2 berkemungkinan dipengaruhi oleh faktor polimorfisme genetik reseptor beta-2 adrenergic (β_2AR). Polimorfisme β_2AR turut dikaitkan dengan patogenesis pelbagai penyakit, termasuk perbezaan tindakbalas pesakit terhadap rawatan yang dipengaruhi β_2AR.

Objektif tesis ini adalah untuk menentukan kekerapan polimorfisme genetik β_2AR di kalangan masyarakat dan juga untuk menentukan sama ada kesan pewarisan polimorfisme tersebut mempengaruhi model yang digunakan untuk mengukur fungsi endotelium salur darah besar menggunakan PWA.

Proses genotip dibuat untuk mengesan 5 "single nucleotide polymorphisms" (SNP) yang penting dari segi fungsi di kalangan 388 subjek Melayu yang sihat. Kesemua SNP dikesan sekaligus menggunakan metodologi "allele-specific multiplex PCR". Dari kalangan subjek tersebut, seramai 298 menjalani kajian klinikal melibatkan pengukuran fungsi endotelium salur darah besar. Pengukuran garis dasar "augmentation index" (AIx) dan "carotid-femoral pulse wave velocity" (PWV) dibuat menggunakan

alat SphygmoCor yang tidak invasif. Subjek kemudian diberi GTN diikuti dengan rakaman bacaan AIx dan PWV. Selepas tempoh penyucian, 400 µg salbutamol diberi dan diikuti rakaman bacaan AIx dan PWV. Perubahan maksima AIx dan PWV selepas setiap provokasi farmakologi diambil sebagai bacaan akhir.

Frekuensi allel varian Gly16, Glu27, Ile164, -20C dan -47C masing-masing adalah 47%, 6.8%, 0%, 30% dan 9.3%. Dari segi frekuensi genotip, genotip paling kerap bagi kodon 16 dan 27 adalah masing-masing Arg/Gly dan Gln/Gln dengan frekuensi 81.4% dan 87.1%; bagi nucleotide -20 dan -47 adalah TT dengan frekuensi 50% dan 82.2% masing-masing; manakala 100% genotip bagi kodon 164 adalah Thr/Thr. Tiada perbezaan yang signifikan pada keputusan AIx selepas GTN dan salbutamol bagi setiap kumpulan. Begitu juga, tiada perbezaan signifikan pada keputusan PWV selepas GTN dan salbutamol bagi setiap kumpulan.

Sebagai rumusan, kekerapan polimorfisme genetik β_2AR di kalangan masyarakat Melayu menepati kekerapan di kalangan masyarakat Asia. Model yang digunakan untuk mengukur fungsi endotelium salur darah besar juga tidak dipengaruhi oleh polimorfisme β_2AR.

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ABSTRACT

Endothelial dysfunction has been shown to precede atherosclerosis. The assessment of endothelial function allows early detection of atherosclerosis and may help in preventive strategies. Recently, pulse wave analysis (PWA) combined with pharmacological challenges has been shown to be a promising non invasive method of assessing endothelium function. The pharmacological challenges involve administrations of sublingual glyceryl trinitrate (GTN) as an endothelium-independent vasodilator and inhaled salbutamol as an endothelium dependent vasodilator. The response to salbutamol, a β_2-agonist may be affected by genetic polymorphisms of Beta-2 adrenergic receptor (β_2AR). Beta-2 adrenergic receptor polymorphisms have been implicated in the pathogenesis of many diseases as well as inter individual differences in patients' response to treatment mediated by β_2AR.

The objectives of this thesis were to determine the prevalence of β_2AR polymorphisms in our population and to determine if these polymorphisms influence the model used to assess macrovascular endothelial function using PWA.

Genotyping was done to detect 5 functionally important single nucleotide polymorphisms (SNPs) of β_2AR in 388 healthy Malay subjects. All SNPs were simultaneously detected using the published method allele specific nested multiplex polymerase chain reaction (PCR). Out of this, 298 subjects proceeded with clinical measurement of macrovascular endothelial function. Augmentation index (AIx) and carotid-femoral pulse wave velocity (PWV) recordings were taken at baseline, done non-invasively using SphygmoCor. Subjects were then administered with 500 µg of

sublingual GTN followed by AIx and PWV recordings. After a wash-out period, 400 µg of inhaled salbutamol was given followed by AIx and PWV recordings. The maximum changes in AIx and PWV after each pharmacological challenge were taken as final measurements.

The frequencies of variant alleles Gly16, Glu27, Ile164, -20C and -47C were 47%, 6.8%, 0%, 30% and 9.3% respectively. In term of genotype frequencies, for codons 16 and 27 the most common genotypes were Arg/Gly and Gln/Gln with frequencies 81.4% and 87.1% respectively; for nucleotides -20 and -47 were TT with frequencies 50% and 82.2% respectively; whereas at codon 164, 100% of the genotype was Thr/Thr. No significant differences were noted in AIx after GTN and salbutamol in all groups. Similarly, no significant differences were noted in PWV after GTN and salbutamol in all groups.

In conclusion the prevalence of β_2AR polymorphisms in Malays in our population correspond to that reported in other Asian populations. Model used to assess macrovascular endothelial function using PWA was not influenced by β_2AR polymorphisms.

CHAPTER 1
INTRODUCTION

1.1 Endothelial function and cardiovascular health

The history of endothelial function started back in the days of William Harvey where it has already been accepted that blood is fluid in living and healthy vessels but coagulates in dead ones. Later, the discovery by Furchgott and Zawadzki (1980) described the role of endothelium in vasodilatation, whereby acetylcholine was shown to require an intact endothelium in order to produce vasodilatation. This landmark study lead to the current understanding that endothelium plays an essential role in cardiovascular homeostasis.

The functions of endothelium include regulating the vascular tone, maintaining the fluidity and coagulation of blood and producing mediators of inflammatory processes. The vascular tone is controlled by balancing the production of vasodilators and vasoconstrictors. Fluidity and coagulation of the blood is controlled by endothelium through the production of factors that regulate platelet activity, clotting cascade and the fibrinolytic system. Additionally, endothelium also produces cytokines and adhesion molecules which are responsible for inflammatory processes (Endemann and Schiffrin, 2004).

Endothelial dysfunction is associated with most forms of cardiovascular diseases as shall be discussed in detail below. Atherosclerosis, the earlier manifestations of cardiovascular disease has been closely linked to endothelial dysfunction. Over the last 30

years, since the discovery by Furchgott and Zawadzki (1980), it has increasingly become clear that the initiation and progression of cardiovascular disease largely depends upon the changes in the vascular homeostasis. This important implication leads to an increasing number of research geared towards understanding the functions and dysfunctions of endothelium, in the hope that cardiovascular disease may be prevented at the earlier stage of endothelial dysfunction.

In Malaysia, data from the Ministry of Health showed that cardiovascular disease had been the principal cause of death in the government hospitals, accounting for 23 – 36% of deaths from 1994 to 2001 (Zambahari, 2004). The National Health and Morbidity Survey (1996) also showed that atherosclerotic risk factors are common among Malaysians with 61% having one or two risk factors (Lim *et al.*, 2000). Taking preventive measures at an earlier stage of endothelial dysfunction would definitely help in the preventive strategies against cardiovascular disease. The tool to assess endothelial function should ideally be simple, non-invasive and largely applicable in large population studies. To date however, the consensus on the most ideal tool has not been reached.

1.2 Anatomy and physiology of endothelium

Endothelium is a single cell layer in the tunica intima, the innermost lining of the blood vessel wall. It comes from the words *endo* meaning within and *–thelium* meaning covering. Other than the arteries and veins, it also lines other cavities including the heart and lymphatic vessels (Tortora and Grabowski, 2003).

Physiologically, other than acting as a barrier between the circulating blood and vessel wall, endothelium plays a crucial role in maintaining vascular homeostasis. It senses either mechanical stimuli such as pressure and shear stress, or biochemical stimuli. In response to these stimuli, it releases a number of active substances in the form of vasodilators and vasoconstrictors. These also act as antiproliferative and proliferative substances, antithrombotic and prothombotic agents, as well as angiogenesis, permeability and inflammatory markers (Verma and Anderson, 2002).

Vasodilators released by the endothelium include nitric oxide (NO), originally termed endothelium derived relaxing factor (EDRF). Other vasodilators are prostacyclin, bradykinin and endothelium-derived hyperpolarising factor. They act synergistically to overcome the effect of vasoconstrictors, proliferative and prothrombic agents. For instance, both prostacyclin and NO inhibit platelet aggregation. Bradykinin stimulates the release of these vasodilators to contribute more in inhibiting platelet aggregation. In addition, it also stimulates the production of tissue plasminogen activator (t-PA), resulting in fibrinolysis (Drexler, 1998, Luscher and Barton, 1997). Among the vasoconstrictors released by the endothelium are endothelin-1, angiotensin II, thromboxane A_2 and reactive O_2 species (ROS). These vasoconstrictors are also responsible for proliferation of smooth muscle cells and thus contribute to plaque formation. In order to maintain the vascular homeostasis, these endothelium-derived vasodilators and vasoconstrictors are kept in balance. Any injurious stimulus that disrupts the balance will lead to a condition known as “endothelial dysfunction”.

1.3 Endothelial dysfunction and its pathophysiology

Endothelial dysfunction is characterized by a reduction in the bioavailability of vasodilators, particularly NO, and a reciprocal increase in the vasoconstrictors. This leads to a state of reduced vasodilatation and an increase in vasoconstriction, as well as inflammatory, proliferative and procoagulatory state.

The association of endothelial dysfunction and the risk factors for atherosclerosis and cardiovascular disease is well-established. However, the mechanism leading to the lesion is complex and not well-defined. A few mechanisms leading to the dysfunction of the endothelium have been proposed, as discussed below. It involves the interplay of many factors, the most important being NO.

1.3.1 Nitic oxide (NO)

Nitric oxide is one of the most potent vasodilators released by the endothelium. As well as acting as a vasodilating agent, it also inhibits inflammation and platelet aggregation. In the endothelial cells, NO is formed from its precursor L-Arginine by the action of the enzyme endothelial NO synthase (eNOS), with the presence of cofactors such as tetrahydrobiopterin (Werner *et al.*, 2003). Nitric oxide then diffuses to the vascular smooth muscle cell and activates guanylate-cyclase, leading to c-GMP mediated vasodilatation. This is the result of normal physiological response by eNOS to stimuli such as shear stress.

In endothelial dysfunction, NO has been reported to be reduced due to reduced activity of eNOS. One proposed mechanism is 'uncoupling of eNOS', whereby there is a switch in the normal function of eNOS from generating NO to instead generating reactive oxygen species (ROS) (Endemann and Schiffrin, 2004). The resulting ROS production leads to the generation of hydrogen peroxide or superoxide formation (Estevez and Jordan, 2002). Moreover, ROS further reduces NO bioavailability and thus promotes more cellular damage (Bonetti *et al.*, 2003b).

1.3.2 Asymmetric Dimethylarginine (ADMA)

N^G,N^G-dimethylarginine or asymmetric dimethylarginine (ADMA) is the product of protein turnover in the cytoplasm and eliminated via the kidney. It is also the endogenous competitive inhibitor of eNOS. In a group of patients with chronic renal diseases, inhibition of eNOS was shown to be correlated with plasma ADMA levels (Xiao *et al.*, 2001). In subjects with hypercholesterolaemia, high ADMA levels were shown to be inversely correlated with endothelial-dependent vasodilatation, which is a form of assessing endothelial function (Boger *et al.*, 1998). Moreover, in response to shear stress, ADMA generation was also shown to be increased (Osanai *et al.*, 2003). Thus, ADMA generation is another proposed mechanism in the pathophysiology of endothelial dysfunction.

1.3.3 Oxidative excess

Many of the risk factors of atherosclerosis including hyperlipidaemia, hypertension, smoking and diabetes are found to be associated with oxidative stress or excess. Oxidative

excess which has been shown to diminish NO, also correlates with the impairment of endothelium-dependent vasodilatation and cardiovascular events (Heitzer *et al.*, 2001). Findings from animal and human studies showed that treatment with antioxidants may improve endothelial function (Cross *et al.*, 2003, Vaziri *et al.*, 2002). The mechanism of oxidative excess includes the generation of ROS as mentioned above.

1.3.4 Others

Other factors which have also been implicated in the pathophysiology of endothelial dysfunction include excess of angiotensin II, hyperhomocysteinaemia and insulin resistance.

Angiotensin II infusion in animals has been shown to induce endothelial dysfunction and vascular inflammation (Diep *et al.*, 2002a, Diep *et al.*, 2002b). Similarly in humans, angiotensin II infusion in healthy volunteers has been shown to be associated with arterial stiffness (Rehman *et al.*, 2001). Treatment of hypertensive patients with angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers had an additional effect of improving endothelial function in contrast to treatment with β-blockers which had no similar effect on endothelial function (Schiffrin *et al.*, 2002, Schiffrin, 1996). The proposed mechanism includes the increase in nicotinamide adenine dinucleotide phosphate oxidase (NADPH) activity caused by angiotensin II. This leads to an increase in the production of ROS and further inactivation of NO (Griendling *et al.*, 2000).

As for hyperhomocysteinaemia, it has been evidenced in both animals and human studies that hyperhomocysteinaemia is linked to endothelial dysfunction (Virdis *et al.*, 2003, Virdis *et al.*, 2001). Some studies have shown that treatment with folic acid which reduces homocysteine levels improved endothelial function (Bennett-Richards *et al.*, 2002, Title *et al.*, 2000).

An emerging aspect in the study of endothelial function is the role of endothelial progenitor cells (EPC). Endothelial progenitor cells are the primitive bone marrow cells which have the ability to mature as endothelial cells as well as having the ability to repair endothelial lesions (Szmitko *et al.*, 2003). It has been shown that the levels of circulating EPC inversely correlate with endothelial dysfunction (Hill *et al.*, 2003). Thus, deficiency of EPC may be another possible mechanism for endothelial dysfunction.

1.4 Clinical implications of endothelial dysfunction

As mentioned earlier, endothelial dysfunction has been implicated in the pathogenesis and clinical courses of many cardiovascular related diseases. The study of endothelial function in humans was first described in patients with coronary artery disease, whereby impaired endothelium-dependent vasodilatation was detected in the presence of atherosclerosis (Ludmer *et al.*, 1986).

Many studies have shown that the coronary risk factors which predispose to the development of atherosclerosis are actually associated with endothelial dysfunction. It was first being associated with hypertension, when impaired vasodilatation was described in

hypertensive patients (Panza *et al.*, 1990). Thereafter, endothelial dysfunction was also being described with other coronary risk factors such as smoking, diabetes mellitus, chronic renal failure and hypercholesterolaemia (Covic *et al.*, 2004, Endemann and Schiffrin, 2004, Engler *et al.*, 2003, Vita *et al.*, 1990). More importantly, as well as in patients with established diseases, endothelial dysfunction has also been described in a group of children and adults who were at risk of atherosclerosis, but had not developed any clinical manifestations or any plaque formation in the vessel wall (Celermajer *et al.*, 1992). This leads to an important postulation that endothelial dysfunction precedes atherosclerosis. Additionally, in patients with established coronary artery disease, the presence of endothelial dysfunction has been shown to be associated with future events (Schachinger *et al.*, 2000, Suwaidi *et al.*, 2000). Thus, detection of endothelial dysfunction may serve as a preventive, monitoring as well as a prognostic tool as far as cardiovascular disease is concerned.

Based on the association between endothelial dysfunction and cardiovascular related diseases, it has been postulated that early intervention may reverse endothelial dysfunction and in turn reduce the risk of cardiovascular disease. To date, no gold standard treatment has yet been available for endothelial dysfunction. However, several pharmacological and non-pharmacological measures have been shown to improve endothelial function as well as cardiac events.

1.4.1 Non-pharmacological interventions

Exercise is a lifestyle modification that has been proven to improve cardiovascular function and reduce cardiac events. An increase in NO bioavailability especially in the coronary vessels has been associated with exercise (Gokce *et al.*, 2002, Gielen *et al.*, 2001, Taddei *et al.*, 2000). Not surprisingly, regular exercise has been shown to improve endothelium-dependent vasodilatation in young men of average fitness (Clarkson *et al.*, 1999) as well as in patients with established coronary artery disease (Bonetti *et al.*, 2003a, Hambrecht *et al.*, 2003, Hambrecht *et al.*, 2000).

Dietary modifications i.e. low in fat and high in fruits and vegetables as recommended by the American Heart Association (2000) has been proven to reduce cardiovascular risk (Krauss *et al.*, 2000). These flavonoids-rich foods may improve endothelial function (Widlansky *et al.*, 2003), as evidenced by a reversal in endothelial dysfunction after intake of tea (Duffy *et al.*, 2001), red wine (Agewall *et al.*, 2000) and dark chocolate (Vlachopoulos *et al.*, 2005). On the other hand, consumption of high-fat meals in a group of healthy volunteers was shown to worsen endothelial function (Vogel *et al.*, 1997).

Other lifestyle modifications which improve cardiovascular risk were also shown to improve endothelial function, such as weight loss, blood pressure reduction and smoking cessation (Ziccardi *et al.*, 2002, Modena *et al.*, 2002, Celermajer *et al.*, 1993).

1.4.2 Pharmacological interventions

A reduction in plasma low-density lipoprotein (LDL) has been shown to improve endothelial function. These include non-pharmacological measures like low cholesterol diet as well as treatment with bile acid resins (Widlansky *et al.*, 2003). Treatment with 3-hydroxy-3-methyl-glutaryl-Co-A (HMG Co-A) reductase inhibitor or statins has been shown to reduce cardiovascular events, partly by improving the endothelial function (Wolfrum *et al.*, 2003). Treatments with statins have consistently been shown to reverse endothelial dysfunction (Masumoto *et al.*, 2001, Perticone *et al.*, 2000, Anderson *et al.*, 1995a). While the effect of lowering LDL-cholesterol may be the predominant mechanism for the improvement in endothelial function (Tamai *et al.*, 1997), its cholesterol-independent (pleiotropic) effect has increasingly been shown to be partly responsible (Takemoto and Liao, 2001, Masumoto *et al.*, 2001, O'Driscoll *et al.*, 1997).

Angiotensin II has been implicated in the pathophysiology of endothelial dysfunction as mentioned before. Patients treated with angiotensin converting enzyme (ACE) inhibitors have been shown to improve endothelial function, independent of blood pressure reduction (Schiffrin *et al.*, 2002, Anderson *et al.*, 2000, Prasad *et al.*, 2000, Mancini *et al.*, 1996, Schiffrin, 1996). The benefit is further supported by similar findings in treatment with angiotensin receptor blockers (ARB) (Schiffrin *et al.*, 2002, Wassmann *et al.*, 2002, Hornig *et al.*, 2001, Schiffrin *et al.*, 2000). The currently ongoing large scale clinical trial, The Ongoing Telmisartan Alone and in combination with Ramipril Global Endpoint Trial (ONTARGET) is expected to provide the ultimate evidence that treatment with ARB and / or ACE inhibitors reverse endothelial dysfunction and whether that reduces

cardiovascular events in high risk patients (Teo *et al.*, 2004). This trial is expected to be completed in 2008.

There are a number of potential treatments undergoing studies which may be promising, some of which have been shown to improve endothelial function. The administration of L-Arginine (precursor of NO) in healthy volunteers has been shown to improve endothelial function in some studies involving healthy volunteers as well as patients with coronary artery disease (Bode-Boger *et al.*, 2003, Lerman *et al.*, 1998, Adams *et al.*, 1997). Other potential pharmacological interventions being studied include tetrahydrobiopterin (co-factor for eNOS), protein-kinase c and cyclooxygenase inhibitions (Chenevard *et al.*, 2003, Beckman *et al.*, 2002, Heitzer *et al.*, 2000).

1.5 Assessment of endothelial function

Early detection of endothelial dysfunction before any clinical manifestations develop will help in the preventive measures and reduce possible complications. It can also help in making the prognosis, predict the possible outcome and monitor the efficacy of treatments given. An ideal assessment tool will have to be safe, non-invasive, simple, reproducible, practical, reliable and cost-effective. To date, no particular test has yet to fulfill all the requirements, each having its own advantages as well as disadvantages.

Over the years, endothelial function is assessed via various methods. The main assessment methods would either be testing the endothelial vasomotor function or measuring the circulating biomarkers of endothelial dysfunction in the blood.

1.5.1 Endothelial-dependent vasomotor function assessment

The endothelial vasomotor function can readily be assessed whether centrally in the coronary artery or in the peripheral circulation. The test of endothelial vasomotor function is based on the principle that introduction of certain stimuli trigger the release of NO from the endothelium causing endothelium-dependent vasodilatation (Lerman and Zeiher, 2005). Endothelium-dependent vasodilatation involves the introduction of a stimulus, either physiological or pharmacological; resulting in an increase in the production of endothelium-derived NO and other vasoactive substances. Common stimuli used include increase shear stress induced by reactive hyperaemia or administration of receptor-dependent agonists such as acetylcholine, bradykinin, β_2-receptor agonists and substance P (Farouque and Meredith, 2001). The parameter used is endothelium-dependent vasodilatation, whereby a resultant normal dilatation indicates a healthy endothelium whereas a reduced dilatation indicates endothelial dysfunction. It is often performed in comparison with vascular responses to endothelium-independent vasodilators e.g. sodium nitroprusside and glyceryl trinitrate (GTN), which acts as control.

In different vascular beds, different types of assessment tools are used. Thus, the assessment tools and methods for micro and macrovascular are different. Macrovascular or conduit vessels are those measuring above 100 μm in internal diameters. These include all the large vessels for example coronary artery in the centre and brachial and femoral arteries in the periphery.

1.5.1.1 Assessment in coronary circulation

Earlier studies involved the assessment of endothelial function in the coronary vessels. The methods used in the coronary circulation are coronary angiography and intracoronary Doppler. These measurements provide the direct calculation of coronary blood flow (in macrovascular) and coronary vascular resistance (in microvascular) respectively (Schachinger *et al.*, 2000, Suwaidi *et al.*, 2000).

Ludmer *et al*. (1986) were the first to describe the assessment of endothelial function in humans, whereby angiography was used. They demonstrated the effect of acetylcholine on endothelium in humans by assessing the epicardial coronary vessels using the method called quantitative coronary angiography (QCA). It involves an invasive intracoronary infusion of acetylcholine and GTN assessed by coronary angiography. Acetylcholine is a muscarinic endothelium-dependent vasodilator which stimulates the endothelial cells to release vasoactive agents including NO (Newby *et al.*, 1997). A healthy endothelium is indicated by a mild vasodilatation, whereas endothelial dysfunction results in a paradoxical vasoconstriction. Glyceryl trinitrate is an endothelium-independent vasodilator and thus all patients were expected to respond with vasodilatation, indicating the integrity of smooth muscle cells.

In the coronary microvasculature, intracoronary Doppler measurement involves the placement of Doppler wire into the coronary artery (mid-left anterior descending artery). Blood flow velocity is then measured after the infusions of endothelium-dependent (e.g.

acetylcholine) and endothelium-independent (e.g. papaverine and adenosine) vasodilators respectively (Treasure *et al.*, 1993).

Other than the pharmacological stimuli above, the physiological stimuli are also used such as cold pressor test and dynamic exercise. Cold pressor test is based on sympathetic stimulation which is achieved after immersing a patient's hand in ice-cold water for 90 seconds. This is followed by measurements of blood flow velocity and angiography (Nabel *et al.*, 1988). Dynamic exercise test is performed using ergometer, with continuous haemodynamic monitoring and repeated angiography before, during and after exercise, as well as after GTN infusion (Deanfield *et al.*, 2005).

Although the assessment in the coronary vascular bed may provide the direct measurement of coronary endothelial function which is also the target of atherosclerosis, its invasive nature is the main limitation. Moreover, coronary angiography and intracoronary Doppler are operator dependent. Thus, all these factors confine the assessment to only patients scheduled for certain interventional procedures and are not suitable to be used in large scale studies.

1.5.1.2 Assessment in peripheral circulation

The assessment of endothelial function in the peripheral circulation is based on the principle that endothelial function is a systemic disorder and thus can be measured in any vascular beds. This is evidenced by findings that impaired vascular responses in the

coronary circulation have also been confirmed with the same impaired responses in other vascular beds in the peripheral circulations (Takase *et al.*, 1998, Anderson *et al.*, 1995b).

Using the same endothelium-dependent vasodilatation principle, the stimuli in the peripheral circulation include intra-arterial local infusion of vasodilators (e.g. acetylcholine) or the less invasive approach of shear stress induced by reactive hyperaemia. Vasodilators are also administered systemically in a non-invasive manner; e.g sublingual GTN or inhaled salbutamol.

Flow mediated dilatation (FMD) is a popular and widely used assessment method of macrovascular function in the peripheral circulation. It involves physiological stimulus of shear stress for the release of NO and other vasoactive substances by the endothelium. Shear stress is induced by the introduction of reactive hyperaemia which represents an ischaemia in the distal tissue. In response to the short ischaemia, there is a transient increase in the blood flow with a resultant shear stress. The change in the diameter in the conduit artery (e.g. brachial artery) is measured using a high resolution 2D ultrasound system. Flow mediated dilatation is defined as the change in the artery diameter following the stimulus, expressed as percentage of baseline diameter. In most studies the endothelium-independent vasodilator given is sublingual GTN (Deanfield *et al.*, 2005, Corretti *et al.*, 2002, Celermajer *et al.*, 1992). Given the non-invasive nature of the measurement together with its relative simplicity, this method has been widely used in relatively large scale trials. It is however highly operator dependent as well as involving some technical variances between centres which may limit the interpretation of the study results.

Another method for studying macrovasculature is the forearm perfusion technique by measuring the forearm blood flow (FBF). It involves an invasive placement of a catheter in the brachial artery for direct infusion of pharmacological agents. The measurement of blood flow is then performed non-invasively using strain gauge plethysmography or venous occlusion plethysmography, whereby the variations in the limb volume placed in the mercury strain gauge are reflected in the change of the circumference of the strain gauge and recorded by the plethysmograph. This technique is based on the principle that obstruction in the venous outflow is reflected in the increase in the limb volume, and it is directly proportional to the rate of arterial inflow (Deanfield *et al.*, 2005). The downside of this technique is its invasive nature which may also limit the application.

Recently, a non-invasive method based on the technique of pulse wave analysis (PWA) has been implemented in the assessment of macrovascular endothelial function. Pulse wave analysis is a non-invasive technique to assess arterial stiffness via the measurement of augmentation index (AIx). The method of applanation tonometry is used, involving the placement of a tonometer (pen-like probe) over the maximal and superficial arterial pulsation under study (e.g. radial artery), from which a corresponding central aortic waveform is generated by using a validated transfer function. Augmentation index is the difference between the first and second systolic peaks of the central waveform, expressed as a percentage of pulse pressure. In the study of endothelial function, pharmacological challenge in the form of β_2-receptor agonist (e.g. salbutamol) is administered systemically via inhalation technique (Hayward *et al.*, 2002, Wilkinson *et al.*, 2002a). The changes in AIx elicited after the administration of this endothelium dependent vasodilator can be used as a tool to measure endothelial function. The endothelium-independent vasodilator

administered which acts as a control is sublingual GTN. It has been shown that β_2-receptor agonist induced, but not GTN induced changes could be inhibited by the N^G-monomethyl-L-arginine (L-NMMA), a specific inhibitor of eNOS. This indicates that the effect of β_2-receptor agonist is in part, mediated through the endothelial NO release, and thus pulse waveform analysis can be applied as a non-invasive and simple method for assessing endothelial function (Wilkinson *et al.*, 2002a). A recent study compared FMD, PWA and pulse contour analysis as methods for assessing endothelial function in adults and children (Donald *et al.*, 2006). While FMD was still concluded as the non-invasive technique of choice followed by PWA, the role of PWA is promising. There have been increasingly more studies on endothelial function using this method, and further studies are needed to look into the reproducibility and possible factors that may affect the measurement.

In the microvasculature, a relatively new and non-invasive method of assessment is laser Doppler flowmetry (LDF). It has been implemented to evaluate the endothelial function in the skin microvasculature using the technique of postocclusive hyperaemia, local thermal hyperaemia or acetylcholine iontophoresis (Cracowski *et al.*, 2006). However, recent findings suggested that the mechanism of postocclusive hyperaemia in this microvasculature is not primarily NO mediated and thus does not represent a specific marker for endothelial function but rather a form of assessing overall microvascular function (Wong *et al.*, 2003).

Pulse wave amplitude in the microvasculature is assessed by measuring the digital pulse volume in the finger using pulse contour analysis. The mechanism is similar to the application of PWA in assessing the macrovascular endothelial function as explained

above. Beta 2-receptor stimulation in the microvasculature was also shown to result in characteristic changes in digital pulse volume, and thus the agonist is also used to assess endothelial function (Chowienczyk *et al.*, 1999). Another form of stimulation used to record the digital volume changes is reactive hyperaemia, which has been shown to correlate with the brachial artery FMD measurements (Kuvin *et al.*, 2003).

1.5.2 Circulating biomarkers of endothelial function

A number of circulating biomarkers have also been studied, as they were shown to be associated with endothelial dysfunction in human. These include the direct products of the endothelium as well as markers of endothelial damage and repair. Their levels in the circulation may serve as "surrogate markers" of endothelial function. At present, however, many of these circulating biomarker levels are still expensive and difficult to measure.

Nitric oxide is a labile substance and thus not a good candidate to measure. Its generation by the endothelium on the other hand, may be reflected by the circulating levels of its metabolites, nitrite and nitrosylated proteins (Rassaf *et al.*, 2004). However, these are technically difficult to measure and furthermore may be confounded by other sources of NO including dietary sources (Wang *et al.*, 1997). Asymmetric dimethylarginine which has been implicated in the pathophysiology of endothelial dysfunction is also a good candidate as a surrogate marker. As mentioned above, high levels of ADMA were found in patients with risk factors such as hypercholesterolaemia. At present, the assay is still expensive and not widely used. C-reactive protein (CRP), which is synthesised by the liver has been a known marker of inflammatory response. In human plasma, it can be measured by high-

sensitivity (hs) assays. Serum CRP levels has been shown to be inversely correlated with forearm blood flow response in patients with coronary artery disease (Fichtlscherer *et al.*, 2000). Endothelial vasodilator responses also improved when hs-CRP levels normalised. Thus, hs-CRP may serve as an indirect marker of endothelial dysfunction. Von Willebrand factor (vWF) is a high molecular weight glycoprotein synthesized mainly by the endothelium. In the coagulation cascade, it acts as a cofactor in platelet aggregation and adhesion. Elevated levels of vWF have been associated with risk factors of atherosclerosis. Furthermore, the levels were also shown to be reduced with treatment (Seljeflot *et al.*, 1999). Tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI-1) are also endothelium-derived regulators of fibrinolysis. Their levels have been associated with increased cardiovascular risk and may be predictive of adverse events (Juhan-Vague *et al.*, 1996). However, they may not be specific for endothelial dysfunction, as these factors are not exclusively produced by the endothelium. Moreover, their levels may also be influenced by other pathological states (Farouque and Meredith, 2001).

Cellular adhesion molecules (CAMs) mediate migration and adhesion of leukocytes to the endothelial wall. These include E-selectin, P-selectin, vascular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). The soluble forms of these molecules can be quantified by the enzyme immunological assays (Gearing and Newman, 1993). Elevated levels of soluble CAMs have been shown to be associated with cardiovascular risk in healthy subjects as well as predictors of adverse outcome in patients with established coronary disease (Blankenberg *et al.,* 2001, Hwang *et al.,* 1997). Circulating endothelial cells that detach into the circulation following endothelial activation can be measured by flow cytometry or fluorescent microscopy (Goon *et al.,* 2005).

Elevated levels of endothelial cells in the peripheral circulation have been detected in patients with atherosclerosis and vascular inflammation (Diamant *et al.*, 2004). Likewise, circulating EPC may also be detected by flow cytometry. This is in support of the hypothesis that EPC deficiency may play a role in the pathogenesis of endothelial function as mentioned above. However, the measurements of both circulating cells are still under extensive studies and have not been applied clinically.

Even though the measurements of circulating biomarkers may seem to be good surrogate markers for endothelial dysfunction, in reality many further studies are needed before it can be fully and widely applied. The measurements of circulating biomarkers are currently only used in a research setting and have not been applied clinically. This is owing to the fact that they are non-specific, technically difficult to perform as well as expensive.

1.6 Pulse wave analysis

Pulse wave analysis (PWA) is a non-invasive method to assess central pressure and arterial stiffness (Siebenhofer *et al.*, 1999, Liang *et al.*, 1998, Wilkinson *et al.*, 1998). Using the SphygmoCor system developed by O'Rourke *et a*l. (1993), it involves the principle of applanation tonometry whereby a maximal pulsation of superficial artery (e.g. radial, carotid or femoral) is minimally flattened or applanated by a tonometer. Minimal flattening of the artery allows accurate recording of the pressure waveform. The piezoelectrical crystal within the tonometer records the changes in the electrical resistance of the pressure waveform obtained. An approximately 10-second recording of the peripheral artery waveform generates a corresponding central aortic waveform by using a validated transfer

function (Pauca *et al.*, 2001, Chen *et al.*, 1997). SphygmoCor system generates 2 indices of arterial stiffness namely augmentation index (AIx) and pulse wave velocity (PWV) (O'Rourke *et al.*, 2001, Wilkinson *et al.*, 1998).

The measurement of AIx is defined as the difference between the second and first systolic peaks, expressed as percentage of pulse pressure (%). This is based on the principle that large arteries are compliant structures, thus absorb some of the pressure which result from the ventricular ejection of blood into the aorta. These pressure waves which are reflected back from the periphery form the second peak, whereas the forward waves from the ventricular ejection form the fist peak. Normally the reflected waves arrive in the central arteries after aortic valve closure. However, in a stiff vessel, the velocity and amplitude of the reflected waves increase, resulting in an earlier arrival which then augments the central systolic pressure. This augmentation is then calculated as the index of arterial stiffness (O'Rourke *et al.*, 2001, Wilkinson *et al.*, 1998). The utilisation of AIx as an index to measure arterial stiffness has been well established, for instance in the Conduit Artery Function Evaluation Study (CAFÉ) (Williams *et al.*, 2006) and Preterax in Regression of Arterial Stiffness in a controlled Double Blind Study (REASON) (Asmar *et al.*, 2001).

The changes in AIx after the administration of an endothelium dependent vasodilator have been tested as a tool to measure endothelial function (Hayward *et al.*, 2002, Wilkinson *et al.*, 2002a). The details of this method have been explained above. It was based on the fact that systemic administration of β_2-agonist changed the arterial pressure waveform. More importantly, the changes induced by β_2-agonist could be blunted

by L-NMMA, and thus this pulse wave methodology could be applied as a method to assess endothelial function. In this thesis, assessment of endothelial function was based on this methodology whereby systemic administration of β_2-receptor agonist was used as an endothelium dependent vasodilator and sublingual GTN, an endothelium-independent vasodilator acts as a control.

Another index of arterial stiffness generated by the SphygmoCor system is pulse wave velocity (PWV). It involves a measurement of the time taken for the arterial pulse to propagate between 2 points in the arterial tree (Stewart *et al.*, 2003, Wilkinson *et al.*, 1998). Wave transit time between the 2 points is recorded using the R wave of a simultaneously recorded echocardiogram (ECG) as the referenced time. When recorded between carotid and femoral pulsations sequentially, as most commonly done, it then acts as a marker for aortic stiffness. The velocity is expressed as meter per second (m/s). Carotid-femoral PWV is also a well established method to assess arterial stiffness. In the currently ongoing clinical trial "Ongoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial" and "Telmisartan Randomised AssessmeNT Risk Management Study in aCE iNtolerant" (ONTARGET/TRANSCEND), this parameter was used to measure arterial stiffness (Teo *et al.*, 2004). In fact, compared to other parameters provided by PWA, it has been argued that PWV is a stronger predictor of cardiovascular and all cause mortality especially in high risk patients (Baulmann *et al.*, 2006, London and Cohn, 2002, Laurent *et al.*, 2001). Even though PWV is not routinely used as a tool to assess endothelial function, this simple measurement was incorporated into this thesis to observe the changes in PWV in response to GTN and salbutamol. Thus it would be interesting to test if the changes in PWV after the pharmacological challenges could also be used as a tool to assess endothelial

function. To date, there are still no published work where both entities; AIx and PWV are studied together in the assessment of endothelial function. This thesis presents the first study looking at the changes with both GTN and salbutamol using both parameters of arterial stiffness.

1.7 Beta-2 adrenergic receptors

Beta-2 adrenergic receptors or adrenoceptors (β_2AR) are the target receptors for endogenous catecholamines i.e. adrenaline and noradrenaline and thus play an important role in the regulation of the sympathetic nervous system in the body. They are expressed mostly in the bronchial and vascular smooth muscle cells, being responsible to mediate bronchodilation and vasodilatation respectively (Guimaraes and Moura, 2001). They are also found in the heart and mediate positive inotropic and chronotropic effect, but less so than that of β_1AR. To a lesser extent, they are also found on glands, adipocytes, leukocytes, hepatocytes, gastrointestinal smooth muscle and myometrial membrane of uterus (Hoffman and Taylor, 2001).

Beta-2 adrenergic receptors are 7-membrane spanning G-protein-coupled receptors, and were first cloned in 1987 (Kobilka *et al.*, 1987). In the vascular smooth muscle cells, agonist stimulation causes it to couple with stimulatory G protein (Gs). This stimulates adenylyl cyclase which results in an increase in the second messenger cyclic AMP (cAMP). The increase in cAMP levels activates protein kinase A. This in turn phosphorylates myosin light-chain kinase within the smooth muscle resulting in relaxation and vasodilatation (Lefkowitz *et al.*, 2002, Murray, 1990). Other than this classical pathway, β_2AR stimulation

also leads to NO generation by the vascular endothelium. Several possible pathways have been proposed to be responsible for the β_2AR-mediated NO generation. *In vitro* studies have shown that the elevation of cAMP and protein kinase A activation also leads to stimulation of endothelium nitric oxide synthase (eNOS) (Yao *et al.*, 2003, Ferro *et al.*, 1999). As has been explained previously, this enzyme leads to an increase in the production of nitric oxide (NO) by the endothelium. Nitric oxide, being a potent vasodilator acts by stimulating guanylyl cyclase; resulting in an increase in cGMP. Cyclic GMP induces smooth muscle relaxation and hence vasodilatation. In addition to the main pathways above, another possible pathway proposed for the β_2AR-mediated NO generation is via inhibitory G protein (Gi) coupling. It has been shown *in vitro* that following agonist stimulation, β_2AR also couples with Gi protein. This in turn stimulates phosphatidylinositol 3' kinase which then leads to stimulation of eNOS (Dimmeler *et al.*, 1999, Fulton *et al.*, 1999).

Based on its function in mediating the effects of catecholamines, β_2AR become the target receptors for adrenergic drug treatments like β_2 agonists and antagonists. Beta-2 agonist for example salbutamol, is widely used as a bronchodilator in the treatment of asthma whereas non-selective β antagonists or β blockers are used in the treatment of hypertension and several other conditions.

1.8 Beta-2 adrenergic receptor polymorphisms

Polymorphism is defined as variability in deoxyribonucleic acid (DNA) sequence that occurs with an allele frequency of more than one percent (> 1%) in the population.