MICROVASCULAR ENDOTHELIAL FUNCTION IN A MUTLI-ETHNIC GROUP OF APPARENTLY HEALTHY SOUTH AFRICAN MEN AND WOMEN: IMPLICATIONS FOR TESTING AND THE RELATIONSHIP BETWEEN INSULIN RESISTANCE AND MICROVASCULAR REACTIVITY

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DECLARATION

I, Paula Roxana Pienaar, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is submitted for another degree in this or any other university.

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Pienaar, P.R., Micklesfield, L.K., Gill, J.M.R., Shore, A.C., Gooding, K.M., Levitt, N.S., Lambert. E.V. *Ethnic differences in microvascular endothelial function in apparently healthy South African men and women.* ***Journal of Experimental Physiology*** May 2014, Epub ahead of print

ABSTRACT

Introduction: Endothelial dysfunction is associated with cardiovascular disease risk factors and precedes structural changes in the blood vessels that occur prior to clinical manifestations[1]. Measuring ethnic differences in microvascular endothelial function will provide insight into the disparate cardio-metabolic disease profiles that exist within a multi-ethnic South African population [2;3] Moreover, it has been shown that insulin resistance is associated with microvascular endothelial dysfunction in obese persons[4], but it is unclear whether this relationship is mediated by body fat in apparently healthy individuals with and without insulin resistance. To our knowledge this has not been done in a South African population.

Aims: The overall aim of this thesis was to measure microvascular endothelial function using Laser Doppler Imagery (LDI) and iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) in a multi-ethnic, apparently healthy group to determine whether: 1) there are ethnic differences in microvascular endothelial function and 2) whether the association between microvascular endothelial function and insulin resistance (IR) is independent of body fat in women with and without IR.

Methods:

Study 1: 33 non–obese, apparently healthy individuals (19–29 yrs) were divided into 3 groups based on their self-reported ethnicity (Black, n=9; Mixed Ancestry, n=12; Caucasian, n=12). Body mass index (BMI), body fat (sum of 4 skinfolds and bio-electrical impedance assessment), physical activity (Global physical activity questionnaire assessment) and microvascular reactivity (LDI and iontophoresis of endothelial dependent ACh and endothelial–independent SNP) were measured.

Study 2: 37 apparently healthy women (20–45 yrs) were grouped as insulin-resistant (IR, n=16) or insulin-sensitive (IS, n=21) based on the Homeostasis Model Assessment for Insulin Resistance (HOMA-IR). Body fat (dual x-ray absorptiometry), waist circumference (WC), blood pressure (BP), fasting glucose, insulin and free fatty acid concentrations were measured. Endothelial–dependent microvascular reactivity was measured by laser Doppler imagery using iontophoresis of ACh. Maximum absolute perfusion, percentage change from baseline, and the area under the curve (AUC) were used as microvascular reactivity outcome measures in both studies.

RESULTS:

Study 1: There was no significant differences between the three ethnic groups for BMI (Black: 21.5 ± 3.2 vs mixed ancestry: 22.7 ± 2.5 vs Caucasian: 23.3 ± 2.5 kg/m^2; p = 0,4), body fat (Black: 22.4 ± 8.5 vs mixed ancestry: 22.1 ± 8.3 vs Caucasian: 20.5 ± 7.6 kg; p=0.7) or physical activity (Black: 921.8 ± 1389.7 vs mixed ancestry: 799.6 ± 756.2 vs Caucasian: 523.3 ± 367.1 mins/wk; p=0.6) measurements. Skin resistance (SR) was significantly different between the 3 groups for the ACh protocol (Black: 0.21 ± 0.04 vs mixed ancestry: 0.20 ± 0.02 vs Caucasian: 0.16 ± 0.03 Ω, p < 0.01) and between the Black and Caucasian groups for the SNP protocol (Black: 0.11 ± 0.02 vs Caucasian: 0.12 ± 0.01, 0.08 ± 0.01 Ω, p<0.001). ACh maximum absolute perfusion and the AUC was significantly higher in the Caucasian group compared to the other two groups before adjusting for SR, however after adjusting for SR these differences were no longer significant. After adjusting for SR, SNP maximum absolute perfusion and AUC remained significantly higher in the Caucasian group compared to the other two groups, and in addition percentage change from baseline was significantly higher in the Caucasian group compared to the Black group. Diastolic BP was inversely associated with AUC using the ACh protocol (r = -0.4, p = 0.02), and maximum absolute perfusion (r = -0.35, p = 0.05), percentage change from baseline (r = -0.56, p = 0.01) and AUC (r = -0.40, p = 0.02) using the SNP protocol. SR was inversely correlated with maximum absolute perfusion (r = -0.64, p<0.001) and AUC (r = -0.59, p<0.001) using the ACh protocol, and maximum absolute perfusion (r = -0.79, p<0.001), AUC (r = -0.77, p< 0.001), and percentage change from baseline (r = -0.37, p<0.001) using the SNP protocol.

Study 2: The IR group had a higher BMI (30.7 $\pm$ 6.4 vs. 22.9 $\pm$ 7.3 kg/m², p<0.01), fat mass (34.7±11.9 vs. 19.7 ± 9.6 kg, p<0.01) and WC (89.9 ± 13.6 vs 74.4 ± 9.7 cm, p < 0.01) compared to the IS group. ACh–induced response, expressed as percentage change from baseline, was significantly lower in IR subjects after adjusting for differences in WC and SR (420.9±166.5 vs 511.6±214.8%, p<0.05). There were associations between microvascular reactivity and SR (r=-0.34, P<0.05) and systolic BP (r=-0.36, P<0.05), but not BMI, body fat mass, WC, or HOMA-IR.

CONCLUSION: There are ethnic differences in microvascular endothelial function within a group of young, apparently healthy men and women. Black African men and women, and individuals who present with insulin resistance may be at a high risk for microvascular dysfunction, and future cardio-metabolic disease. LDI and iontophoresis has for the first time been used to measure microvascular endothelial function in South Africa, resulting in specific methodological implications for future studies nationally and internationally. These implications emphasize the importance of adjusting for skin resistance in multi-ethnic groups, as well as in groups consisting of overweight or obese individuals.

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LIST OF ABBREVIATIONS

ACh	-	Acetylcholine
AUC	-	Area Under the Curve
BIA	-	-Bio-electrical Impedance Analysis
BMI	-	Body mass index
BP	-	blood pressure
BZ	-	Biological Zero
cAMP	-	cyclic adenosine monophosphate
cGMP	-	cyclic guanosine monophosphate
cm	-	centimetres
CRP	-	C-reactive protein
CV	-	Coefficient of variation
CVD	-	Cardiovascular disease
DBP	-	Diastolic Blood Pressure
DXA	-	Dual-energy X-ray Absorptiometry
e-nos	-	endothelial-nitric oxide synthase
ET-1	-	Endothelin-1
FFA	-	Free Fatty Acids
FM	-	Fat Mass
FMD	-	Flow mediated dilation
g	-	gram
GLUT4	-	Glucose transporter type 4
GPAQ	-	Global Physical Activity Questionnaire
HOMA-IR	-	Homeostatic Model of Insulin Resistance

HOMA-β	-	beta cell function
Hz	-	Hertz
IL-6	-	Interleukin-6
kg	-	kilograms
LDF	-	Laser Doppler Flowmetry
LDI	-	Laser Doppler Imagery
LMIC	-	Low to Middle-Income Countries
m	-	metres
m²	-	metres squared
mA	-	milli amperes
mA	-	micro amperes
mA	-	milliAmpere
MAPK	-	mitogen activated protein kinase
Mc	-	milli coloumb
MCh	-	Metacholine
MetS	-	Metabolic Syndrome
MIC2	-	Moor Iontophoresis Controller 2
min/week	-	minutes per week
mmHg	-	millimetres Mercury
mmol/l	-	millimoles per Litre
ml	-	millilitre
mU/L	-	milliunits per Litre
MVPA	-	Moderate-to-Vigorous Physical Activity
NaCl	-	Sodium Chloride
NCD	-	Non-Communicable Diseases
NO	-	Nitric Oxide

PCOS	-	Polycystic Ovarian Syndrome
PI3K	-	phosphatidylinositol 3-kinase
PU	-	Perfusion units
ROS	-	Reactive Oxygen Species
s	-	seconds
SA	-	South Africa
SANHANES	-	South African National Health and Nutrition Examination Survey
SBP	-	Systolic Blood Pressure
SNP	-	Sodium Nitroprusside
SR	-	Skin Resistance
SSA	-	Sub-Saharan Africa
T2DM	-	Type 2 Diabetes Mellitus
TNF-α	-	Tumour Necrosis Factor alpha
VSMC	-	Vascular smooth muscle cell
WC	-	Waist Circumference
WHO	-	World Health Organisation
Ω	-	ohm

Chapter 1

METHODOLOGICAL REVIEW OF LASER DOPPLER IMAGERY AND IONTOPHORESIS FOR MEASURING MICROVASCULAR ENDOTHELIAL FUNCTION

1.1 Introduction

Endothelial dysfunction precedes cardio-metabolic disease including diabetes[5] and hypertension[6]. Assessment of endothelial function can therefore be used as a tool to detect early vascular changes before the clinical manifestation of disease. One of the earliest pathological findings related to cardio-metabolic disease is the decrease in endothelial derived vasodilators such as nitric oxide (NO), which usually accompanies early vascular remodelling[7]. The cutaneous circulation has emerged as an accessible and representative vascular bed to explore the mechanisms of microvascular function and dysfunction.

Furthermore, changes in skin blood flow can be measured by laser Doppler imagery (LDI) and the iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP)[8]. To date there has been no standardized protocol for this technique[9]. Consequently, many studies have produced conflicting findings.

The purpose of this literature review is to address some methodological issues associated with LDI and iontophoresis as a method of measuring microvascular reactivity. The limited literature that has used this technique in multi-ethnic populations has not effectively addressed possible differences in skin resistance associated with skin hue in these different populations and how this may influence microvascular reactivity measurements. This chapter therefore seeks to discuss factors that may confound the measurement of microvascular reactivity in a multi-ethnic group.

1.2 Endothelial dysfunction

The endothelium is a unicellular layer that lines the luminal surface of all blood vessels and plays a central role in maintaining cardiovascular homeostasis by regulating vascular tone, preventing the adhesion of leukocytes, and in so doing, reducing the risk of thrombotic formation. Normal endothelial function regulates vascular tone by maintaining the equilibrium between vasodilation and vasoconstriction. Vasodilators and vasoconstrictors which are released by the endothelium in response to mechanical and/or physiological stimuli act on the blood vessels to maintain vascular tone. The vasoconstrictors released by the endothelial cells are growth promoters, while the endogenous vasodilators, such as NO, inhibit vascular growth and have a protective vascular function. Besides vasodilation, studies have shown that NO regulates the proliferation of vascular smooth muscle

cells, and prevents platelet adhesion and aggregations, as well as the adhesion of leucocytes and their migration into the arterial wall[10], thus playing a significant role in the prevention of atherosclerosis. NO is therefore a crucial endothelial-derived molecule for contributing to vascular homeostasis.

Endothelial dysfunction, or the disruption of vascular homeostasis, creates the ideal environment for atherosclerosis and cardiovascular disease (CVD). Several studies have shown that patients who present with cardiovascular risk factors including smoking[11], obesity[12], family history of CVD [13]or insulin resistance[14], but no clinical evidence of atherosclerosis, have endothelial dysfunction. Endothelial dysfunction may therefore explain the mechanistic link between risk factors for CVD and the development of atherosclerosis. Evaluating endothelial function is therefore a good predictor of future cardiac events in individuals with cardiovascular risk factors as well as established CVD[15].

Endothelial – dependent vascular response to pharmacological stimuli such as ACh, thermal provocation (heating or cooling) or physical stimuli such as exercise, can be measured by evaluating changes in vessel diameter or blood flow and provides an important tool in the assessment of endothelial function. Although endothelial function can be measured in the coronary arteries, this procedure is highly invasive and requires specialised equipment and personnel, therefore making it less suitable for use in a research setting[16]. It is however well established that endothelial function in the coronary arteries and peripheral macro–and microvascular function are closely correlated[15;17], suggesting a broader regulation of endothelial function. Table 1.1(at the end of this chapter) briefly describes the various non-invasive macro and microvascular techniques. The table also presents the various vascular beds, outcome measures, advantages and disadvantages that characterise each of the techniques.

Most assessments of microvascular function involve the measurement of vasodilation in response to a vaso-active substance, with reduced vasodilation being an indicator for poor microvascular function. As well as being due to the direct effect of NO on the endothelium (endothelium-dependent), vasodilation is also dependent on the response of smooth muscle surrounding the blood vessel to the release of NO by the endothelial cells (endothelium-independent). It therefore means that reduced vasodilation may not only arise from poor endothelial function, but also from smooth muscle dysfunction (Figure 1.1). To distinguish between endothelial and

smooth muscle dysfunction, both endothelial-dependent and endothelial – independent responses are measured.

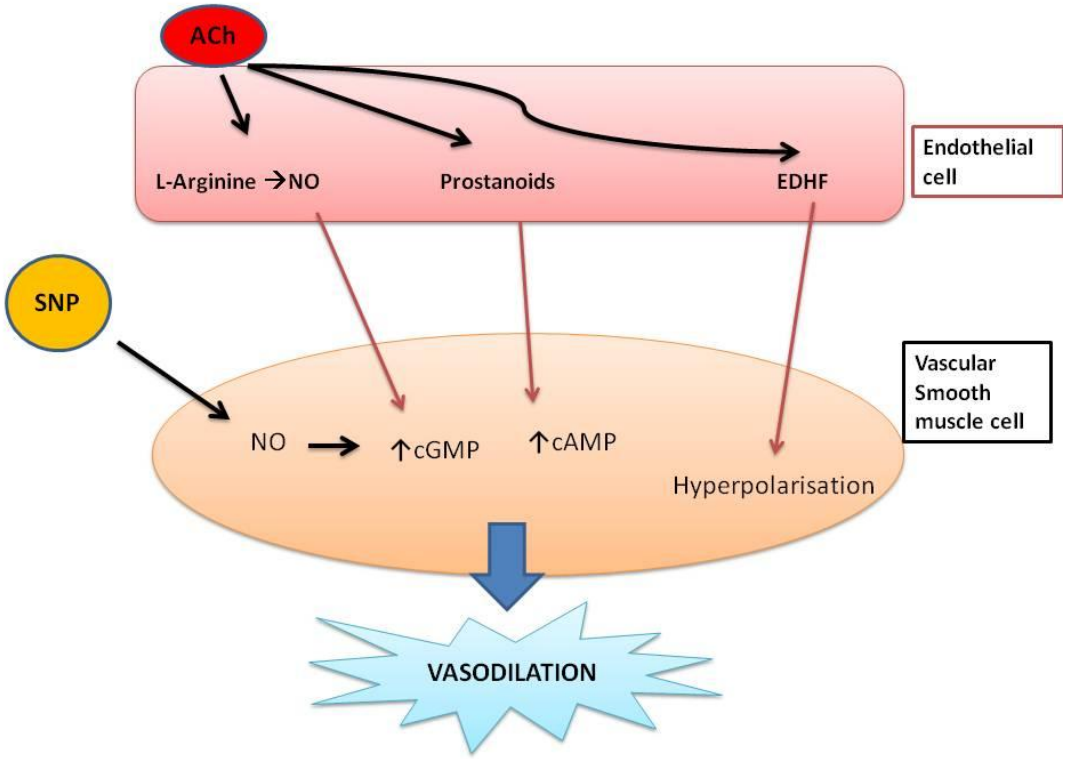

Figure 1.1 Mechanisms of action for endothelial–dependent acetylcholine (ACh) and endothelial–independent sodium nitroprusside (SNP). Binding of ACh to the endothelial cell stimulates nitric oxide (NO), prostanoid and endothelial-derived hyperpolarizing factor (EDHF) which leads to the increase in cyclic guanosine monophosphate (cGMP), cyclic adenosine monophosphate (cAMP), hyperpolarisation and subsequent vasodilation. SNP donates NO directly to the vascular smooth muscle cell and causes vasodilation by increasing cGMP production

Table 1.1 Comparison of non-invasive macrovascular and microvascular techniques for measuring endothelial function

Macrovascular Technique	Vascular bed	Stimulus	Brief description	Outcome measure	Advantage	Disadvantage
FMD	Conduit artery (brachial artery most commonly used)	Reactive Hyperemia	Endothelial dependent: Occlusion of brachial artery followed by reactive hyperemia Endothelial independent: administration of sublingual glyceryl trinitrate (GTN)	Changes in blood flow and vessel diameter AUC of reactive hyperemia Percentage change of the arterial diameter	Can be used in large epidemiological studies Inexpensive Correlation with invasive epicardial vascular function	Requires highly trained technicians Expensive to set up Lack of standardized protocol
Strain-gauge Plethysmography	Brachial artery Forearm resistance vessels	Reactive hyperemia	Forearm blood flow (FBF) measured by plethysmography. Changes in limb volume detected by strain gauge. Same endothelial-dependent and independent mechanisms as with FMD	Percentage change in flow from baseline Maximum hyperemic flow Overall time-flow curve	Easy to use Less observer dependent Does not require highly trained technicians	Requires intra-arterial infusion of vaso-active drugs to determine endothelial dependent and independent function
Applanation tonometry to measure Pulse wave analysis	Radial artery	Pressure to the radial artery causes pressure wave form	Endothelial dependent: Administration of β2-adrenergic receptor agonists Endothelial independent: sublingual administration of GTN	Augmentation index	Good reproducibility Easy to use Portable Low time commitment for subjects	Indirect information on arterial stiffness
Peripheral arterial tonometry (PAT)	Digital arteries and microcirculation	Reactive Hyperemia	Endothelial dependent: Fingertip probe used to measure changes in arterial pulsatile volume. Endothelial independent: Nitroglycerin can be used.	RH-PAT index or ENDO-SCORE Augmentation index	Easy to administer Low inter-observer and intra-observer variability Correlation with invasive microcirculatory function	Vasodilation not entirely NO-dependent Inability to take into account the impact of the ANS influences on endothelial – independent response Expensive

FMD, flow mediated dilation; AUC, area under the curve; RH-PAT, reactive hyperaemia – peripheral arterial tonometry; PORH, post-occlusive reactive hyperaemia

Microvascular Technique	Vascular bed	Stimulus	Brief description	Outcome measure	Advantage	Disadvantage
Laser Doppler flowmetry	Forearm skin microvessels	PORH; local heating and cooling Pharmacological: Ach and other vasoactive substances through iontophoresis	Iontophoresis chambers placed on skin and microcurrent applied for trandermal delivery of vaso-active agents. Laser doppler probe in direct contact.	PORH: maximal (peak) after occlusion release Time to reach peak response Half time taken to reach 50% baseline Duration of hyperemia AUC Cutaneous vascular conductance	Inexpensive Easily administered	Inability to determine absolute blood flow Great spatial and temporal variability Blood flow only measured over a single site Direct contact with skin can apply pressure and create movement artefacts
Laser Doppler imaging	Forearm skin microvessels	PORH Local heating Vaso-active agents (same as above)	Laser beam emitted at a distance above the skin Skin perfusion is therefore assessed over a wide area.	Percentage change from baseline Maximum absolute perfusion AUC	Blood flow measured over larger area No direct skin contact	Inability to determine absolute blood flow Low frequency: does not penetrate very deeply
Laser speckle contrasting imaging	Forearm skin microvessels	PORH Local heating Can be performed with or without vaso-active agents	Coupled with post occlusive reactive hyperaemia. Laser scans skin to generate a speckle pattern.	Expressed as laser speckle perfusion units Percentage of baseline Percentage of maximal vasodilation Cutaneous vascular conductance Expressed as raw values or as a function of baseline	Non contact Allows skin perfusion over a wide area but with a high frequency Deeper skin penetration Excellent reproducibility	Highly sensitive to movement Analyzing data challenging due to large amount of data
Retinal Vessel Analysis	Retinal microvessels	Diffuse luminance flicker	Measurement of arterial and venous retinal vessel diameter with DVA followed by stimulation tests: flicker light with a frequency of 12.5 Hz and bright-to-dark ratio of 25:1.	Percentage increase relative to baseline diameter	Provides excellent reproducibility and sensitivity Easily performed and quick Does not include a subjective component once optimal fundus image achieved	Good measurement quality strongly dependent on clear optical media Optimal readings require good fixation abilities

AUC, Area under the curve; PORH, post-occlusive reactive hyperaemia; DVA, Dynamic Vessel Analyzer

1.3 The skin microcirculation

Although several non – invasive techniques for measuring macrovascular function, such as flow mediated dilation (FMD), carotid intima media thickness and pulse wave velocity, are correlated with cardiovascular outcomes, these methods are only representative of conduit artery function. These macrovascular techniques focus predominantly on large arteries without providing insight into the structure and function of the microcirculation, which is the primary driver promoting target organ damage[18] and is recognised as the site where the earliest manifestations of CVD lies[19;20].

In recent years, it has become well established that the skin is an acceptable vascular bed for measuring peripheral microvascular reactivity[9;21], as the skin is easily accessible for testing, and perfusion in the skin relates to the blood flow in the microcirculation. However, the skin microcirculation must be representative of the microcirculation in other organs before it may be regarded as a model for investigating the underlying mechanisms of vascular disease. Khan et al.[21] used the combination of laser Doppler flowmetry (LDF) and trans-thoracic Doppler echocardiography to examine the direct relationship between peripheral and coronary resistance vessel function in healthy volunteers (n=28). Coronary velocity reserve, as measured by trans-thoracic Doppler echocardiography, was significantly associated with ACh ($r=0.399$, $P<0.039$) and SNP response ($r=0.466$, $P<0.020$) measured by LDF. These results support the idea that peripheral measurements of skin blood flow are representative of the generalized microvascular function.

The presence of diabetes and hypertension demonstrate the link between microvascular and macrovascular dysfunction[4;22]. Work by Pessina et al.[22] describes this relationship as a “vicious cycle” whereby arterial stiffness and accompanying elevated pulse pressure causes microvascular damage, resulting in increased capillary rarefraction, wave reflection and ultimately completing the cycle by elevating systolic and diastolic blood pressure. Evidence that microvascular damage precedes hypertension is provided by de Jongh[4] who wanted to investigate whether obesity is the cause for microvascular dysfunction which may contribute to microangiopathy, hypertension and insulin resistance. Their study used nailfold capillaroscopy and LDI and iontophoresis of ACh and SNP to compare microvascular function between 16 lean and 12 obese women at baseline state and during physiological systemic hyperinsulinemia. Results of the study showed that

obese, compared with lean women, are characterized by impaired skin microvascular function. More specifically, postocclusive capillary recruitment and endothelial – dependent vasodilation at baseline state and only capillary recruitment during physiological systemic hyperinsulinemia is impaired.

It has been established that skin microvascular reactivity, more specifically endothelial dependent microvascular function, is reduced in hypertension[6;23;24], diabetes[22;25-27], insulin resistance[25;28-30], obesity and other chronic conditions [29;31;32] and will be reviewed in more detail in Chapter 3.The human cutaneous microcirculation can therefore be used as a model for generalized systemic microcirculation, as altered skin microvascular function can act as a surrogate marker in cardio-metabolic disease and its risk factors [33-35].

Pathology-induced macrovascular dysfunction is evident in the cutaneous circulation[7], thus making it a translational model for providing pre-clinical information in individuals at high risk for CVD[36]. To investigate whether microvascular function in the skin is also a valid model, the relationship between CVD risk factors and microvascular reactivity was examined in a study by Izjerman et al.[33] who used the iontophoresis of ACh and SNP in 46 healthy Caucasian volunteers between the ages of 30–70 years. Results of the study showed that increased CVD risk (according to the Framingham heart study) is associated with impaired endothelial–dependent vasodilation in men and women, independent of BMI.

Microvascular function is predominantly measured by detecting changes in skin blood flow. These dynamic changes are in response to provocation tests such as the transdermal delivery of vasoactive substances, local heating, occlusion or local cooling. For the purpose of this thesis, I shall briefly discuss the non–invasive laser Doppler techniques, but will focus more on laser Doppler imagery and iontophoresis.

1.4 Laser Doppler techniques

1.4.1 Method

Laser Doppler assessment is a standard technique used to measure changes in the microcirculation and is based on the backscattering of a laser beam[37;38]. Once the laser beam penetrates the skin and hits the moving blood cells, it undergoes changes in wavelength (Doppler shift) which are photodetected by an optical detector. The magnitude and changes in frequency of these wavelengths are related to the number and velocity of red blood cells. "Flux" is the term used to describe the product of the red blood cell velocity and concentration (blood volume), and is expressed as arbitrary perfusion units (PU)[38]. Therefore an increase in microvascular reactivity translates to an increase in perfusion and flux. The wavelength of the laser beam determines the depth at which it penetrates the skin and since the microcirculation refers to the microvessels 1–1.5mm below the skin surface, most laser Doppler techniques apply wavelengths from 632.8 nm to 780 nm[8;37] . LDI equipment includes a neon laser, a scanner head, an optical detector and computer software for data analysis

1.4.2 Laser Doppler Flowmetry (LDF) and Laser Doppler imagery (LDI)

The first laser Doppler technique that was developed was single point Laser Doppler flowmetry (LDF) which measured blood flow over a small volume (∽1mm^3). This results in spatial heterogeneity of skin blood flow, which together with movement artefacts, leads to poor reproducibility[8;37]. This was demonstrated by Roustit et al. [17] who evaluated the reproducibility of post-occlusive reactive hyperaemia and local thermal hyperaemia assessed by LDF on the finger pads and the forearms of 14 Caucasian, healthy volunteers for one week. In this study a CV% < 35% was considered acceptable. Results showed that LDF was reproducible on the finger pad, but not on the forearm (CV% ranged from 38–89% for post-occlusive reactive hyperaemia and 87–92% for local thermal hyperaemia).

In contrast, laser Doppler imaging (LDI) measures perfusion over a larger area which in turn leads to a lower spatial heterogeneity, thus being more reproducible and reliable. Morris et al.[39] showed that the CV% for the maximum response to ACh

in 8 healthy subjects (26.8 ± 4.2 years) was 12.1 ± 15.4%. In the same study, when the outcome variable was measured by LDF in 7 healthy subjects (36.6 ± 9.1 years), CV% was 42.0 ± 26.1%. In a separate study, the seven day reproducibility CV% for peak SNP iontophoresis in 10 healthy subjects was of 22%[40]. In contrast, Roustit et al. showed that LDF provided a CV% ranging from 63% to 95%[37].

Given that both LDF and LDI produce arbitrary units (PU) as an indicator of changes in blood flow, the microvessels require a provocation test prior to laser Doppler assessment. Table 1.2 presents the various laser Doppler provocation tests that have been used to measure microvascular function and include mechanical stimulation, thermal provocation, electrical stimulation, and the local administration of pharmacological agents including iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP).

Table 1.2 Provocation tests[41]

Reactivity tests			
Mechanical stimulation	**Thermal provocation**	**Electrical stimuli**	**Local administration of pharmacological agents**
Arterial occlusion	Local heating	Current induced vasodilation	Iontophoresis of Ach
Pressure induced vasodilation:	Local cooling		Iontophoresis of SNP

1.5 Iontophoresis

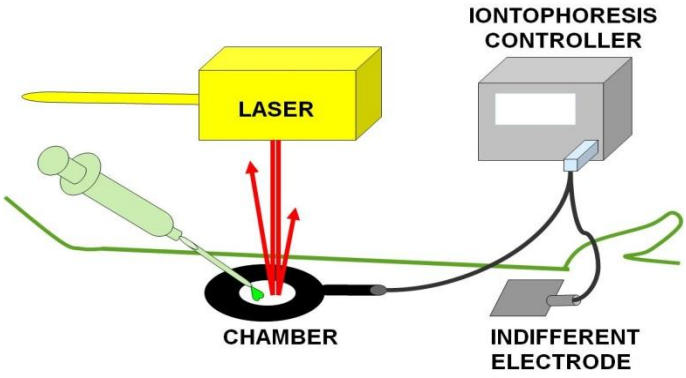

Figure 1.2 Experimental set–up showing drug application, back-scattering of laser light and iontophoresis controller *(illustration from Dr. Kim Gooding, University of Exeter Medical School and NIHR Exeter Clinical Research Facility)*

Iontophoresis is a non–invasive method of transporting charged micro-particles through the skin using a low density electrical micro-current. The equipment used for iontophoresis includes a circular electrode chamber (anode or cathode) and an indifferent (reference) electrode connected to an iontophoresis controller (Figure 1.2). An electrical circuit is created by attaching the indifferent electrode to the wrist and a chamber electrode to the forearm; both these positions are on the volar aspect of the arm. The polarity of the induced current is adapted to the charge of the vaso-active molecules involved with a positively charged solution applied with an anodal chamber while a negatively charged solution will use a cathodal chamber. The quantity of the drug delivered depends on the magnitude and duration of the induced current.

Several protocols exist to perform iontophoresis, including changing the duration of the current, length of rest interval between scans, and current magnitude. For example, in a study comparing microvascular reactivity between subjects with and without essential hypertension, Farkas et al.[42] used the following iontophoresis protocol: 60 s baseline flow, followed by 2 doses of ACh (0.1mA for 30s and 0.16mA for 30s) with 120s intervals and for SNP the 2 doses were at 0.1mA for 20s and 0.1mA for 30s, with 120s intervals. In another study assessing microvascular reactivity in a group of women with polycystic ovarian syndrome, Lakhani et al.[32] measured a longer baseline flow (100s) followed by drug delivery at 10, 15 and 20 mA, sequentially, each for 100s, followed by 800s at zero current for both ACh and SNP. In yet another study, Shantsila et al.[24] assessed ethnic differences in

microvascular reactivity using the same protocol for ACh and SNP, but in this study no mention of interval time were given, instead authors only disclosed that the current strength and duration was 0.1mA for 60s followed by one minute of baseline scanning. Despite these differences, it appears that in most [32;42-44], but not all [24;45] studies, the concentration of ACh and SNP were consistent at 1%. To date however, there does not seem to be any internationally accepted standard protocol for the iontophoresis of vaso-active drugs during microvascular reactivity evaluations.

The vaso-active substance most commonly delivered with iontophoresis is ACh (positively charged solution) at the anode, often complemented by SNP (negatively charged solution) at the cathode, and the mechanism by which they produce vasodilation is what distinguishes them apart and determines whether they are endothelium–dependent or endothelium-independent. The transport pathways for charged molecules are via the hair follicles and sweat ducts, whereas neutral molecules are transported through intercellular spaces on the stratum corneum[46;47] (Figure 1.3).

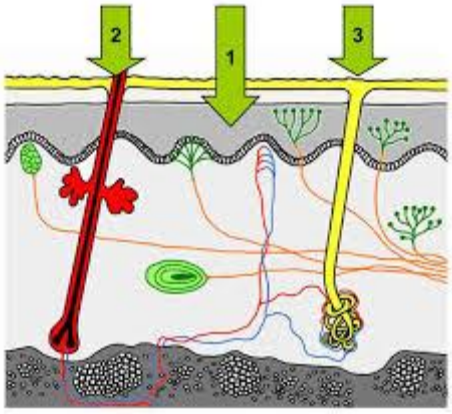

Figure 1.3 Pathway through skin during iontophoresis. Neutral molecules may travel through the skin across the intact stratum corneum (1). Charged molecules travel through the hair follicles (2) and via the sweat gland ducts (3).[48]

1.5.1 Mechanisms of ACh action

ACh is a muscarinic receptor antagonist, dependent on an intact endothelium[49]. Furthermore, it is endothelial-dependent because it binds directly to the endothelial cells and catalyzes the conversion of L-Arginine to NO (Figure 1.1). It is this production and release of NO that leads to the relaxation of the surrounding smooth muscle resulting in vasodilation. However, in addition to NO, prostanoids and endothelium–derived hyperpolarizing factors are also released, resulting in an increase in cAMP and hyperpolarization in the smooth muscle cells. Therefore there is more than one pathway in which ACh could possibly induce vasodilation.

Conflicting studies show that prostanoids may[50;51], or may not [39], be involved in endothelial–dependent vasodilation[52]. Noon et al.[50] have demonstrated that aspirin inhibits dermal iontophoretic response to ACh, and therefore suggested that vasodilation is mediated, at least in part, by the synthesis of prostanoids. Prostanoids may cause vasodilation via the C-fiber-mediated axon reflex and its action with C-nociceptor fibers[53] resulting in the release of histamine from the mast cells which indirectly leads to vasodilation and increased vascular permeability[7;54].

In contrast, a study by Morris et al.[39] showed that the ACh response following aspirin did not differ from the response after placebo administration. Both studies tested these mechanisms in a young healthy group of men and women. A possible explanation for these contrasting findings is the different protocols used during LDI and iontophoresis.

More specifically, Noon et al.[50] made use of the same iontophoresis protocol for both ACh and SNP. The protocol consisted of an increasing current duration and intensity (100μA for 10s; 200μA for 10s; 200μA for 20s; 200μA for 40s; 200μA for 80s) with increasing response periods of 60s, 90s and 180s. In contrast, Morris et al.[39] used different current protocols for ACh (seven pulses of 100μA for 20s followed by one pulse of 200μA for 20s with 60s between each dose) and SNP (two pulses of 100μA for 20s followed by one pulse of 200μA for 20s with 180s between each dose). Furthermore, Morris et al. used LDI and Noon et al. used LDF, the latter being the less reproducible of the two. These examples further reinforce the demand for standardized iontophoresis protocols when measuring microvascular reactivity.

1.5.2 Mechanisms of SNP action

Often when microvascular function is measured, a complementary control drug is used in addition to ACh. SNP is an endothelial-independent vasodilator, because it acts as a NO donor that reacts with tissue sulhydryl groups to produce NO directly and thereby stimulate smooth muscle cell relaxation through an increase in cGMP formation, independent of the endothelium (Figure 1.1). A reduction in SNP microvascular reactivity can be interpreted as a structural change within the blood vessel resulting in a reduction in vasodilatory capacity[8]. Alternatively, oxidative stress can reduce NO availability to the smooth muscle. More specifically, free radicals inactivate NO released by NO donor activity[55]. Taken together, reduced NO availability and activity prevent the smooth muscle from causing vasodilation.

Studies in which both SNP and ACh are reduced, suggest that the resulting decrease in microvascular reactivity may be as a result of the inability of NO to be released from endothelial cells, thus preventing smooth muscle cell relaxation[8], and is therefore a combination of compromised endothelial-dependent and independent function. In most studies where the iontophoresis of ACh and SNP are used, either ACh alone, or both drugs show changes, but changes in SNP alone are rare. Table 1.3 describes 12 studies over the past 10 years in which the iontophoresis of ACh and SNP were used to evaluate microvascular reactivity in individuals at risk for CVD. Within this table, only four of the twelve studies show reduced ACh and SNP responses, however, no studies show a diminished response to SNP alone.

Table 1.3 Studies using LDI or LDF and iontophoresis to assess forearm microvascular reactivity over the past 10 years

Reference	Subjects (n); Age (mean or range in years)	ACh	SNP
Fronek et al. 2013[56]	Healthy control (n=208); CAD(n=42); PAD(n=14); HT(n=28); Hypercholesterolemia(n=16); DM (n=16) All subjects divided into older (>50 years) and younger (<50 years) age category	Lower in older (>50 yrs) than younger (<50 yrs) subjects Lower in those with a CVD risk factor Lower in young women vs young men and the opposite in the older group.	No difference
Monotsori et al. 2010[57]	Healthy Controls (n=19); Lean HT (n=10); Overweight HT (n=13); Obese HT (n=10); Age category (6 – 19 years)	No ACh difference between NT and HT irrespective of BMI. Lower ACh in lean and obese HT vs control.	No difference
Shantsila et al. 2011[24]	Healthy controls (n=40); High risk HT (n=40); Malignant HT or blood pressure > 160/120 mmHg (n=15) Mean Age (50 ± 11 years)	Lower in Malignant HT vs control Lower in Malignant HT vs high risk HT Lower in HT vs control	Lower in Malignant HT vs control Lower in HT vs control
Farkas et al. 2003[42]	Essential HT (n=25); NT (n=25) Mean Age (51 ± 2 years)	Lower in subjects with HT	No difference
Rossi et al. 2007[44]	Healthy control (n=15); Hypercholesterolemia (n=15) Mean Age (58 ± 11 years)	No difference between hypercholesterolemic patients and healthy controls.	
Agarwal et al. 2012[58]	Healthy control (n=25); CAD (n=48) Mean Age (56 ± 8 years)	Lower in CAD after correction for CAD risk factors	No difference

Beer et al. 2008[59]	Healthy controls (n=38); NIDDM (n=54) Mean Age (55 ± 9 years)	Lower in NIDDM group	Lower in NIDDM group
De jongh et al. 2004[4]	Lean (n=16); Obese (n=12) Mean Age (40 ±7 years)	Lower in obese.	No difference
Izjerman et al. 2003[60]	Healthy smokers (n=12) Mean Age (26 ± 6 years)	Acute effect of smoking lowers ACh in smokers.	No difference
Vecchia et al. 2004[61]	Healthy smokers (n=9); Non-smokers (n=11) Mean Age (28 ± 2 years)	Lower in non-smokers before and after smoking a cigarette. Similar in smokers and non smokers before acute smoking, but decreased in smokers after smoking.	Lower in non-smokers before and after smoking a cigarette. Not different before or after smoking
Edvinsson et al. 2008[11]	Non-smokers (n=17); Current smokers (n=17) Mean Age (64 ± 2 years)	Lower in smokers compared to non smokers.	Lower in smokers compared to non smokers.
Tao et al. 2004[62]	Young healthy (n=12) Age range (20 – 30 years) Elderly healthy (n=12) Age range (60 – 70 years)	Lower in elderly group compared to the young healthy group.	No difference

ACh, acetylcholine; SNP, sodium nitroprusside; CAD, coronary artery disease; NIDDM, non-insulin-dependent diabetes mellitus; PAD, peripheral arterial disease; HT, hypertensive; DM, diabetes mellitus; CVD, cardiovascular disease; NT, normotensive

1.6 Interpretation of data/outcome measures

1.6.1 Measurement of blood flux rather than blood flow

A limitation of LDI is that it does not measure the volume of tissue that is being tested (i.e the cutaneous blood flow in ml/min relative to the volume of weight of the tissue). Therefore, rather than being expressed quantitatively, it is expressed as units of flux, where flux is the blood cell velocity multiplied by blood cell concentration, and is represented as arbitrary perfusion units.

1.6.2 Expression and representation of results

Flux can be presented as the area under the curve (AUC); percentage change from baseline; and maximum absolute perfusion (Figure 1.4), however there does not appear to be any consensus in the literature on the best way to express the data. It has been reported that the maximum response observed in an experiment may not reflect the true physiological maximum, because the response to a drug over a period of current application contains more information than the maximum response alone[52;63]. For example, Henricson et al.[64] found that in healthy volunteers, the maximum response to ACh was not different to that of SNP. However, the dynamics of the response was different between the groups. To quantify the differences between drug responses, they added a sigmoidal curve and calculated the differences in terms of Hill slope parameters, however this model is not based on physical processes underlying microvascular reactivity.

Accordingly, many researchers have measured the response over time, or AUC, following multiple current pulses.

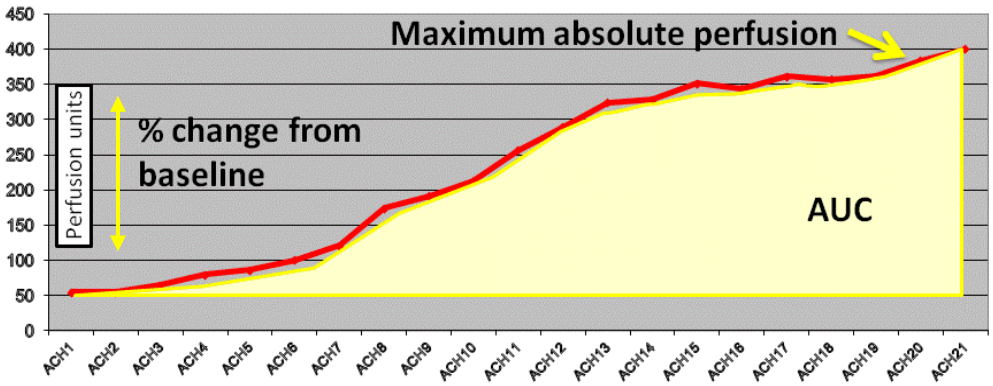

Figure 1.4 A graph showing the increase in flux (perfusion units) over time. Percentage change from baseline, maximum absolute perfusion and area under the curve (AUC) are three outcome measures that are used in this thesis. The y-axis represents flux in perfusion units and the x-axis shows the number of scans measured.

1.7 Factors that may influence LDI and iontophoresis testing

The following section covers the factors that influence the outcome of microvascular reactivity testing using LDI and iontophoresis, and is summarized in Figure 1.5

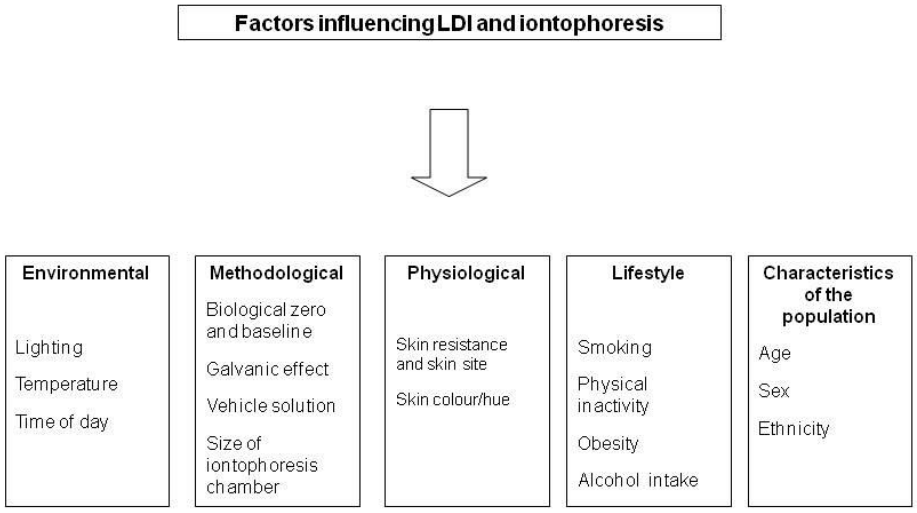

Figure 1.5 Schematic of factors that may influence iontophoresis

A report from the Standardization Group of the European Society of Contact Dermititis[65] suggests that ambient light may interfere with the laser beam as it scans the region of interest when measuring cutaneous blood flow with LDI.

Performing the test in a well-lit room may alter the magnitude of actual blood cell perfusion dependent on the intensity of the light source (fluorescent compared to light bulb). To identify whether the light source interferes with the laser measurement, the user is encouraged to test a region of interest in a dark room as well as with the lights switched on. In this way the discrepancy, if any, will determine whether lights can be used for further testing[65].

In addition, the room's temperature must remain constant at a temperature between 22 – 24°C, or as noted in the manufacturer's manual. Higher temperature may increase skin vasodilation whereas colder temperatures may impede circulation prior to testing. This was demonstrated by Abraham et al.[66] who studied 15 healthy participants at different ambient temperatures, leading to a range of skin temperatures from low (28.0 +/− 2.0 °C) to high (34.1 +/− 1.3 °C). When microvascular response to ACh and SNP was compared at the various temperatures, results showed that a lower skin temperature induced a significantly lower vasodilation for both ACh and SNP respectively. Therefore this study suggests that skin temperature interferes with skin microvascular reactivity at the forearm level.

Time of day is another factor that needs consideration when measuring microvascular reactivity. This was demonstrated in a study by Elherik et al.[67] using LDI and iontophoresis of ACh and SNP, which showed that endothelial NO and endothelin-1 (ET-1) are affected by circadian rhythms. They showed in their study of 9 men measured over a 24-hour period that endothelial–dependent and independent vasodilation was highest at 16:00 hrs and lowest at 04:00 hrs. These findings were attributed to ET-1 levels which showed a significant inverse correlation with ACh and SNP response in the morning. In contrast, the lowest measures of ET-1 were obtained in the afternoon, when microvascular response to ACh and SNP were highest.

Implications for LDI and iontophoresis testing:

- Ensure that the room's lighting does not interfere with outcome measurements.
- Testing should be performed in a temperature controlled room, after acclimatization.
- Be consistent and test at the same time of day.

1.7.1 Methodological factors

1.7.1.1 Biological zero and Baseline

Biological zero (BZ) refers to the small detectable flux signal that remains after arm occlusion. Flux never reaches a value of zero, due to the Brownian movement of macromolecules arising from the interstitial space[9]. It is for this reason, and the fact that it tends to remain constant between and within subjects, that few researchers believe one needs to subtract the BZ value from the absolute perfusion flux signal measured. A review by Turner et al.[8] concludes that subtracting "biological zero" values rarely, if ever, changes the results of a study.

Two studies by by Kernick et al.[68;69] have addressed the influence of BZ on microvascular function results. In the first study Kernick identified the origin of the BZ and provided recommendations for measuring and subtracting it from the flow signal[68]. However, these recommendations were based on measurements using LDF, as opposed to LDI. A subsequent study was therefore performed by the same group to show that these recommendations also hold true for LDI. In the second study, Kendrick compared LDI to LDF and concluded that the BZ contributes a similar proportion (17.9 vs 17.3 arbitrary units) of the output signal in both techniques[69]. However, these findings were limited to one model of LDI equipment only; therefore it cannot be generalized to other designs with different wavelengths.

In contrast, Mayrovitz et al.[70] investigated the extent to which BZ adds to the LDI perfusion signal by determining the magnitude of the LDI-BZ relationship and how it related to ACh and SNP responses to local heating of the forearm. Three scans were done in 30 post-menopausal healthy women: one baseline scan (non-heated), one heated scan and one BZ scan. Results from this study showed that BZ only increased by 3% for each unit increase during heat perfusion, suggesting that the BZ contribution is very small in relation to perfusion changes and not to a similar proportion as concluded by Kendrick et al.[69] Therefore BZ may be additive when recorded with LDF, but not to the same degree in LDI assessments.

Moreover, subtracting the baseline from the maximum response may reduce LDI reproducibility. Jadhav et al.[71] investigated the reproducibility and repeatability of LDI and iontophoresis of ACh and SNP. In this study they compared measures

between arms and 8 weeks apart. Findings from this study revealed that subtracting baseline from maximum perfusion had deleterious effects on reliability. Subtraction of the baseline value from AUC showed an inter-arm variation of 5.9 ± 6.8% for ACh and 12.2 ± 15.8% for SNP. Temporal variations showed 11.4 ± 10.7% (ACh) and 15.5 ± 14.7% (SNP). This suggests that baseline should not be subtracted from the maximal perfusion response when using iontophoresis. Taken together, it is recommended that the author always report on whether biological zero was used and/or subtracted.

Implications for LDI and iontophoresis testing:

- BZ need not be corrected for
- Baseline should not be subtracted from maximum responses when using LDI and iontophoresis.

1.7.1.2 Galvanic effect, vehicle solution and size of iontophoresis chamber

During iontophoresis, the non-specific effect of the current itself results in vasodilation. The increase in cutaneous blood flow due to the current alone, in the absence of drugs, when using a pharmacologically neutral vehicle solution, is referred to as a "galvanic effect"[72]. This galvanic response is related to the voltage required to establish an iontophoretic current[73].

Furthermore, it is well established that a range of vehicle solutions used to dilute drugs, such as de-ionized water, tap water and sodium chloride (NaCl) induce various galvanic responses[8] . Although Khan et al. found that the choice of vehicle did not influence the voltage applied, work by Ferrel et al.[74] showed that diluted water alone induces a more pronounced vehicle-response than saline.

Ferrell et al.[74] compared distilled water, 0.1, 1 and 5.8% NaCl vehicle solutions during iontophoresis of ACh and SNP in 20 healthy volunteers. More specifically, two iontophoresis protocols were used: an incremental protocol with a cumulative charge of 8mC, and a "galvanic" protocol with a high cumulative charge of 15mC. Voltage was monitored to measure the electrically induced hyperemic responses of vehicle solutions and a cut-point for hyperemic response was set at a flux greater than 100 perfusion units. The distilled water produced a hyperemic response during

the incremental protocol in 75% of the subjects. This was predominantly noted at the cathode, and corresponded with the SNP response. In contrast, none of the NaCl vehicles produced a hyperemic response; instead it was associated with voltage-time integrals below the threshold for triggering a response. Given that the presence of ions in the ACh and SNP solutions used in iontophoresis result in little effect by NaCl and deionized water, the choice of vehicle solution should be taken into account when used as a control[37].

Moreover, the size of the iontophoresis chamber appears to influence the galvanic response by changing the charge density of the solution within the chamber. This was clearly demonstrated by Ferrell et al.[74] who used iontophoresis chambers of different sizes to measure the ACh and SNP response in healthy volunteers. Using a smaller chamber (internal diameter of 1.5 mm) resulted in a higher voltage and almost tripled the perfusion integral of the galvanic response at the cathode compared to the larger chamber (internal diameter of 22 mm).

Taken together, vehicle solutions with low ionic strength result in high circuit resistance and increased voltage, leading to the development of electrically induced hyperemia, particularly at the cathode. Similarly, a small chamber size creates a higher voltage at the cathode. As a result, SNP response may be inaccurate.

Implications for LDI and iontophoresis testing:

- For a 1% drug solution, use 0.5% NaCl as a vehicle solution.
- Rather use larger (22mm internal diameter) iontophoresis chambers as it will eliminate electrically induced hyperemia that would influence SNP response.

1.7.2 Physiological factors

1.7.2.1 Skin resistance and skin sites

Skin resistance also influences the trans-dermal delivery of vaso-active substances during iontophoresis. Variability in skin resistance is determined by the density of sweat ducts and hair follicles which may also vary according to skin site, for example the volar aspect of the arm compared to the hand[8]. Inter-subject variations in calculated resistance were observed in studies by Ramsay et al.[75], who for the first time showed that an inverse relationship exists between skin resistance and blood flow response to ACh and SNP. In their study using LDI and the iontophoresis of ACh and SNP to measure cutaneous microvascular function in 20 healthy volunteers, significant inverse relationships were observed between skin resistance and measures of microvascular function. These findings occurred despite a low cumulative charge (8mC) suggesting that there are several resistance pathways in the skin and that skin resistance should be corrected for.

Despite this finding, subsequent studies using iontophoresis have failed to correct for variations in skin resistance[43;44;58;76]. Two explanations are often cited: firstly, investigators believe that a low cumulative charge negates the influence of skin resistance and prevents the galvanic response[32]; secondly, it has been mentioned that the MIC2 iontophoresis controller applies voltage as a function of skin resistance, reducing the effect on microvascular response[57]. Nevertheless, many studies with equivalent or greater cumulative charges[43;44;58;76] continue to ignore skin resistance.

Implications for LDI and iontophoresis testing:

- SR should be corrected for, particularly when the iontophoresis protocol consists of a cumulative charge greater than 8 mC.
- Current (Amperes) and voltage (volts) should be monitored during scans, and used to calculate resistance (ohm).

1.7.2.2 Skin hue/colour

The use of LDI to measure changes in flux within a multi-ethnic group can be influenced by differences in skin colour[77]. The structures of tissue that absorb photons are called chromophores and have different wavelengths of absorption. The three main chromophores of the skin are melanin, haemoglobin and water, and its distribution in the tissue determines its absorptive abilities. Darker skins contain more melanin and are more difficult for light to penetrate. Therefore longer near-infrared wavelengths (780–830nm) are required to allow for deeper skin penetration[77], however, most LDI wavelengths are set at 633–780 nm.

Furthermore, skin pigmentation has been shown to positively correlate with stratum corneum (the superficial layer of the epidermis) thickness. In a study comparing the epidermal permeability of 14 light (skin type ii/iii) and 7 dark (skin type iv/v) skin types, results showed that the darker pigmented skin required more tape strippings, a method for studying the kinetics and penetration depths of drugs [78], and was therefore more resistant than lighter skin. This finding has important implications for the transdermal delivery of topical agents.

Implications for LDI and iontophoresis testing:

- Skin preparation prior to iontophoresis and correcting for skin resistance are recommended when testing individuals with darker skin.

1.8 Lifestyle factors: confounders of microvascular reactivity testing

1.8.1 Smoking

Acute as well as chronic smoking has been shown to impair endothelial function[79]. Studies that have investigated the detrimental effects of smoking on endothelial function include those that have measured macrovascular endothelial-dependent function using FMD[79;80], microvascular endothelial-dependent and independent function using LDI and iontophoresis of ACh and SNP[61], impaired capillary recruitment measured by nailfold capillaroscopy[60] and increased brachial wall thickness as measured by high-resolution ultrasonography[79].

In the study by Dalla et al.[61], 20 healthy male volunteers (9 habitual smokers and 11 non-smokers) were asked to smoke a cigarette. Skin microvascular reactivity was measured before, and 10 minutes after, smoking. Compared to the habitual smokers, ACh and SNP response was higher in the non-smokers before and after smoking a cigarette. Furthermore, non-smokers had lower ACh response after smoking a cigarette compared to the resting period before smoking the cigarette.

Mechanisms include the increase in leukocyte adhesion to endothelial cells, the reduction in NO activity resulting from elevated angiotensin II levels, as well as the increase in oxidative stress caused by nicotine[79].

Not only is active smoking of great concern, but additional studies have found that passive smoking may also impair endothelial function[81-83]. When FMD was measured in 61 healthy, non-smokers before and after 30 minutes of exposure to a smoking room, FMD was reduced by 33%[82]. According to the authors of this study, endothelial–independent vasodilation was not measured as prior studies failed to show changes after passive smoking.

In a study by Celermajer et al.[83], FMD (endothelial–dependent) and the sublingual administration of nitroglycerin spray (endothelial–independent) was measured in n=26 control subjects who had never smoked nor had regular exposure to environmental tobacco smoke; n=26 subjects who had never smoked but had been exposed to tobacco smoke for at least one hour daily for at least 3 years; and n=26 active smokers. Endothelial–dependent vasodilation was similar in the passive smokers to the active smokers, both of which have lower measures than non-smokers. Furthermore, in passive smokers, FMD was inversely associated with the intensity of exposure to environmental tobacco smoke[82].

Implications for LDI and iontophoresis testing:

- Smoking should be avoided at least 6 hours prior to testing.

1.8.2 Physical inactivity

Cross sectional studies have reported reduced endothelial function in physically inactive individuals when compared to athletes[84-86]. Furthermore, in a number of intervention studies[87;88], moderate physical activity has been shown to improve endothelial function in older patients, and in patients with CVD or with CVD risk factors[89].

Combined resistance and cardiovascular training has been shown to increase vascular function in type 2 diabetics after three hourly sessions per week, for 8 weeks. Forearm plethysmography and intrabrachial infusions of ACh and SNP, as well as FMD accompanied by sublingual nitroglycerin, were measured in n=16 type 2 diabetics. FMD increased from 1.7 ± 0.5% to 5.0 ± 0.4% following training ($P<0.001$), forearm blood flow in response to ACh improved, but endothelial independent measures (SNP and nitroglycerin) showed no change[90].

Most recently, FMD of the popliteal artery and brachial artery were measured after 5 days of reduced physical activity (<5000 steps per day) in apparently healthy, active (>90 minutes aerobic exercise on >3 days per week and >10000 steps per day) men. Vascular function was measured on days 1, 3 and 5 of reduced daily physical activity. After 5 days, popliteal FMD was lower and the brachial artery diameter was smaller, suggesting that transition from high to low levels of physical activity leads to deleterious vascular consequences[91].

Implications for LDI and iontophoresis testing:

- Subjects should refrain from exercise 6 hours before testing.
- If physical inactivity is to be used as the intervention, investigators should control for the time from which baseline is set.

1.8.3 Body fat

Obese and overweight individuals have lower endothelial-dependent function when compared to normal weight controls[92-94]. Several studies have also investigated the relationship between microvascular function and body fat, but seldom have studies used the iontophoresis of ACh and SNP as its method.

Al-Tahami et al.[94], compared microvascular reactivity in 72 healthy subjects (mean age 26.5 years), separated into a lean group (n=26) and an obese group (n=26) according to BMI. Microvascular reactivity was measured by LDF and the iontophoresis of ACh and SNP. The main finding of this study was that ACh response was lower in the obese group even after correcting for blood pressure, HDL cholesterol and triglycerides.

Khan et al.[95] determined the relationship between percentage body fat and microvascular reactivity as measured by LDI and iontophoresis of ACh and SNP in 158 healthy children between the ages of 11–14 years. Endothelial-dependent and independent microvascular reactivity was inversely associated with percentage body fat and lower in subjects with elevated post-feeding glucose level. After correcting for sex, body fat remained inversely associated with endothelial-dependent and independent microvascular reactivity. Authors concluded that the reduced microvascular reactivity response with poor glucose handling and adiposity would predispose them to insulin resistance in adulthood.

Body weight and BMI, as measures of body fat stores, can also influence skin sensitivity[96]. Correlations between BMI and epidermal function were investigated in 63 healthy men and women, divided into 3 BMI categories (BMI<25; 25–30;BMI>30). Skin parameters that were assessed include trans-epidermal water-loss and measurement of skin blood flow with LDI. Data showed a significant correlation between BMI and trans-epidermal water-loss, suggesting a fundamentally changed epidermal skin barrier in the heavier individuals, possibly explained by greater sweat gland activity. Interestingly, this study also showed a significant correlation between BMI and skin blood flow. Possible reasons given for this finding were that obese individuals were more likely to have a higher blood pressure and increased superficial blood flow caused by their physiological temperature-regulating system.

Implications for LDI and iontophoresis testing:

- High body fat may interfere with the trans-dermal drug delivery. More specifically, it may result in an increase in voltage, resulting in greater skin resistance which needs to be corrected for.

1.8.4 Alcohol intake

Alcohol consumption may either have a protective effect on the cardiovascular system, or increase the risk of coronary artery disease. These contrasting effects may partly be attributed to the influence of light or moderate versus heavy or binge drinking on the vascular endothelium[97].

The apparent favourable effect of alcohol at low doses may be attributed to its effect on other risk factors. More specifically, by increasing high-density lipoprotein-cholesterol; lowering levels of plasma fibrinogen and decreasing platelet adhesiveness[97]. Furthermore, studies have suggested that wine, particularly red wine, improve endothelial function. However, it is unclear whether the favourable effects are due to the alcohol or the polyphenols and anti-oxidants in red wine.

Vlachopoulos et al.[98] investigated the acute effect of pure alcohol on endothelial function in 12 healthy subjects by measuring FMD before and after the consumption of grape juice with and without one ounce of pure alcohol. Results showed that pure alcohol caused significant dilation in the brachial artery at rest and at reactive hyperemia, but did not alter FMD (percentage change in diameter from baseline) or endothelial-independent response.

Similar findings by Agewall et al.[99] showed that 250 ml red wine increased resting brachial artery blood flow and diameter without affecting the percentage increase of the artery diameter during FMD, however, FMD was measured after brachial artery blood flow and diameter had already been increased at rest. De-alcoholized red wine did however increase endothelial-dependent vasodilation. Although both studies showed that ethanol increases brachial artery diameter and blood flow at rest, Agewall et al. suggests that it is the anti-oxidant properties in red wine that

increases endothelial-dependent vasodilation. These studies provide insights into the mechanisms involved in the vascular effects of alcohol.

Any beneficial effects of low dose alcohol consumption may be negated or reversed by excessive or binge drinking. Goslawski et al.[100] assessed microvascular and macrovascular function in 19 apparently healthy, alcohol abstaining and binge-drinking college students studied 1 to 4 days after their last binge, where binge drinking was defined as consuming more than 4 or 5 standard drinks (13 g/drink) in a 2 hour period. Results showed that macrovascular endothelial-dependent and independent vasodilation was reduced in the binge-drinking group compared to the abstainers, but there were no differences in microvascular endothelial function. Despite unchanged microvascular function, when the resistance arterioles were exposed to endothelin-1 in a separate experiment, vasoconstriction was enhanced in the binge-drinking group compared to the abstainers. Goslawski and colleagues concluded that their data showing alterations in macrovascular and microvascular function associated with binge drinking were similar to those seen in association with recognized cardiovascular risk factors.

Despite these findings, studies addressing the effect of alcohol on microvascular endothelial function using LDI and iontophoresis are limited. In one study, Vauzour et al.[101] assessed whether acute, moderate Champagne wine consumption was capable of modulating microvascular endothelial function in 15 healthy volunteers. The consumption of either Champagne wine or alcohol control induced a rapid increase in endothelial-dependent vasodilation which returned to basal level after 8 hours. In contrast, only the Champagne wine intervention was able to maintain increased endothelial-independent vasodilation after 8 hours. These results were attributed to the absorption of Champagne wine polyphenols which were detected as metabolites in subjects' urine following Champagne wine consumption.

Implications for LDI and iontophoresis testing:

- Include alcohol intake and drinking pattern into research questionnaires
- Subjects should be fasted overnight or for longer than 10 hours before testing (no food or beverages, except water)

1.9 Characteristics of the population

1.9.1 Age and sex

In healthy adults, the capacity for the endothelium to produce NO declines with age[102], and has been associated with structural vascular alterations[103]. Specifically, aging is associated with a decline in endothelial–dependent microvascular function[104], as well as vascular function of the coronary arteries[105]. A study by Taddei et al. reported that endothelial-dependent vasodilation gradually declines with age, with up to a 50% loss in the oldest group (65.2 ± 7.4 years) tested, as measured by blood flow response to ACh. Furthermore, when response to SNP was also assessed, ACh was lower in older adults, but SNP remained unchanged[104;106;107]. In contrast, Al-Shaer et al.[108] found that both ACh and SNP forearm vasodilation were blunted with aging, independent of the presence of atherosclerosis, suggesting an overall decline in peripheral endothelial function. For the purpose of this thesis, the mechanisms associated with age-related endothelial dysfunction will not be discussed, however, collectively; these findings demonstrate that age should be accounted for when measuring microvascular function.

Differences in hormone production lead to sex differences in skin physiology and endothelial-dependent vasodilation. Androgens have been shown to lead to a greater sebum production and pH differences in men[109], which affect the transdermal delivery of vaso-active agents during iontophoresis. These differences can however be eliminated by proper skin preparation prior to testing. It has also been shown that oestrogen production in females contributes to greater endothelial-dependent vasodilation[110]. A study by Lieberman et al., showed that short term oestrogen replacement therapy increased flow mediated endothelial–dependent dilatation of the brachial artery in 13 postmenopausal women. The menstrual cycle with its hormonal variations however shows no influence on the iontophoresis of ACh and SNP[31;111].

When microvascular function was measured using skin iontophoresis of ACh and SNP, capillaroscopy, and vasomotion (LDF) in 21 healthy, normal-weight women with regular menstrual cycles, there were no differences in microvascular function during early follicular, late follicular nor mid luteal menstrual phases. Similarly, when Rossi et al.[111] investigated whether changes in female sex hormones associated

with follicular and luteal phase affect microvascular reactivity in healthy, non-smoking women with evidence of having one ovulatory cycle, no differences in ACh or SNP responses were found. More specifically, the women with regular menstrual cycle days and evidence of ovulation (n=9) were not different to those with evidence of one ovulatory cycle (n=6), suggesting that female sex hormone changes during menstruation do not affect ACh and SNP iontophoresis.

In contrast, a study by Adams et al.[112] showed that sex differences in endothelial-dependent macrovascular function were due to the smaller vessel size of the females when compared to males, rather than due to hormonal differences. In a separate study by Jensen-Urstad et al.[113], endothelial-dependent macrovascular function was not significantly different between a group from a community (57 men and 47 women) and a previous studied 35 year old population (52 men and 56 women). In their study however the younger women had higher macrovascular function compared to the older women, suggesting that age rather than sex contributed to macrovascular outcomes.

Implications for LDI and iontophoresis testing:

- Although the menstrual cycle or female sex hormones appear to have no significant effect on microvascular reactivity, researchers are encouraged to record this information for completeness, when including female participants in their study.

1.9.2 Ethnicity

It has been well-established that skin physiology differs between ethnic groups[114]. As discussed earlier, skin colour/hue influences LDI. The most obvious differences in skin of different ethnicities are skin hue. It has been well established that skin barrier function and permeability differ between Black, Caucasian and Asian skin types and is characterized by measuring transepidermal waterloss[115]. Indeed several studies have reported greater transepidermal waterloss in black skin[114], however transepidermal waterloss of the volar forearm is not different between Black and Caucasian skin[116;117].

ACh and SNP travel through the stratum corneum through hair follicles and via sweat glands (Figure 1.4). Although it has been shown that there are no differences in the stratum corneum between Black African and Caucasian individuals[118;119] there appears to be more stratum corneum layers in Black individuals, as tape strips have been shown to remove layers when compared to the Caucasian group. Furthermore, in a study by Sandy-Moller et al.[120], stratum corneum thickness was positively correlated with cutaneous pigmentation, suggesting that black skin has better skin integrity and cohesion. This may also contribute to the greater mean electrical resistance in Black compared to Caucasian skin .

In a study by Kompoare et al.[121], forearm stratum corneum between Asian, Black and Caucasian individuals were compared using transepidermal waterloss and laser Doppler velocimetry of methyl nicotinate. Transepidermal waterloss was greatest in the Asian and Black group, and vasodilation lag times to determine skin permeability, was lowest in the Black group when compared to Asians and Caucasians. In a separate study, Berardesca et al.[122] compared the vasodilation of 2 topical agents between Black and White individuals to show the weaker response in the Black group, indicating a decreased percutaneous penetration. Further support showing a more intact skin barrier in the Black skin was provided when the drug response was shown to be greatest in the Black skin after delipidisation.

Most studies comparing endothelial function in a multi-ethnic group do so by performing techniques to measure macrovascular differences. However, in a study by Elherik et al.[123] , LDI and the iontophoresis of ACh and SNP were used to compare microvascular function between Scottish and Arabic individuals, and

showed that endothelial-dependent response was greater in the Arabic group, with no differences observed in endothelial-independent results. The authors were unable to provide reasons for these differences and suggested that it is possibly linked to differences in lifestyle, diet, environmental and genetic factors.

Salt sensitivity, defined as an increase in blood pressure of 5–10 mmHg over baseline after a brief salt loading[124], may contribute to ethnic differences in microvascular function. When capillary recruitment, microvascular function and salt sensitivity was measured in a group of 27 normotensive and 26 hypertensive Caucasian individuals, de Jongh et al.[125] demonstrated that capillary recruitment and endothelial-dependent vasodilation were inversely associated with salt sensitivity after adjustment for age, sex, BMI and waist-to-hip ratio. Moreover, salt sensitivity occurs more frequently in Blacks compared to Caucasians[126], suggesting a potential reason for microvascular endothelial difference.

1.10 Summary

It is for this reason that the present study aims to compare microvascular endothelial function among Black African, Caucasian and Mixed Ancestry groups with no known cardiovascular disease or physiological factors that could influence testing. By correcting for differences in skin resistance this study can provide a clearer understanding as to whether differences observed between groups are due to skin resistance or intrinsic physiological differences.

LDI and iontophoresis of ACh and SNP provides a non-invasive measure of microvascular endothelial function. Whereas macrovascular techniques predominate the literature, it is necessary to also consider alterations in the microvasculature, as studies have shown microvascular changes to precede macrovascular endothelial dysfunction.

Given that many factors influence the use of this technique, the first part of the thesis compares ethnic differences in microvascular reactivity within an apparently healthy group. Moreover, the limited use of this technique within a multi-ethnic group provided the reasoning behind recruiting 3 groups of different ethnic background. In this way we could also investigate whether differences in microvascular reactivity

could assist in providing insight to ethnic disparities that exist in cardiovascular disease.

1.11 Aims

The aims for the next chapter are:

1. To measure microvascular reactivity between young, apparently healthy Caucasian, Mixed Ancestry and Black African South African men and women
2. To determine whether LDI and the iontophoresis of ACh and SNP is an acceptable technique to measure microvascular function in a multi-ethnic group and whether there are methodological implications for future use

Chapter 2

ETHNIC DIFFERENCES IN MICROVASCULAR ENDOTHELIAL FUNCTION IN APPARENTLY HEALTHY SOUTH AFRICAN MEN AND WOMEN

Paper in review:

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2.1 Introduction

Cardiovascular disease (CVD) is the leading cause of death globally[127], but its progression differs between ethnicities[128], particularly in Sub-Saharan Africa (SSA) where the epidemiological transition has resulted in a rise in non-communicable diseases [129]. The prevalence of hypertension (38.9%)[130] and diabetes (12.1%)[2] in Black South Africans are amongst the highest in SSA and globally, the Black population have a greater risk for microvascular disease[131]. South African people of mixed ancestral origin, have a high prevalence of diabetes (15.6%)[132], while the cholesterol–attributable mortality is highest in the Indian (22.2%) and Caucasian (20.5%) populations[133] .However, it remains unclear whether physiological differences between ethnic groups, including differences in endothelial function, exist prior to the clinical manifestation of disease.

Endothelial dysfunction, an early marker of atherosclerosis, precedes cardiovascular complications such as hypertension[134], diabetes[135] and coronary artery disease[136]. In a healthy state, the release of endothelial nitric oxide (NO) enables cardiovascular homeostasis and vascular tone. However, in the presence of CVD or its risk factors, reduced NO bioavailability promotes structural and functional alterations, resulting in endothelial dysfunction [134;135].

Previous studies comparing ethnic differences in endothelial function have shown reduced macrovascular function in non-Caucasian groups, however, these studies predominantly used invasive or macrovascular techniques such as the infusion of vasodilators[137], flow mediated dilation (FMD)[138;139] or measures of carotid-intima media thickness[140;141]. Results in most[139-141], but not all[142], of these studies have shown that Black ethnic groups have attenuated endothelial function and increased arterial stiffness when compared to Caucasian groups. Moreover, multi–ethnic studies usually compare vascular function in healthy controls, to those with known disease such as hypertension[143], heart failure[144] or diabetes[145]. In South Africa, only macrovascular function have been compared between Black African and Caucasian subjects with and without disease [143;146-148], but to date, no studies have been published assessing microvascular endothelial function. Given that disturbances in microvascular function have been shown to precede macrovascular disease [149],

assessment of endothelial microvascular function may provide valuable insight into the etiology of CVD risk disparities between ethnic groups.

Microvascular endothelial function, measured non-invasively with laser Doppler imagery (LDI) and iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP), have predominantly been performed in Caucasian and Asian populations[150]. Furthermore, skin resistance, a factor known to differ amongst ethnicities[151], is rarely corrected for with this technique and may therefore influence interpretation of findings.

To our knowledge there are no studies using LDI and iontophoresis comparing microvascular function in a multi-ethnic group of healthy, young, individuals with very different disease profiles. For this reason, young individuals with no CVD risk factors and with no known disease were purposively selected for the present study. The aim was to examine ethnic differences in microvascular function between young, apparently healthy Black Africans, Caucasians and individuals of Mixed Ancestry using LDI and iontophoresis.

2.2 Methods and Procedures

2.2.1 Subjects

The study population consisted of 33 South African men and women who were assigned to three groups based on self-reported ethnicity (9 Black African, 12 Mixed Ancestry, and 12 Caucasian).The “Mixed Ancestry” group refers to South African people of mixed ancestral origin, referred to as ‘coloured’, who descended from slaves brought from East and central Africa, the indigenous Khoisan who lived in the Cape at that time, indigenous Africans, white settlers, and an admixture of Malay, Indian and Asian groups. Subjects were recruited from the surrounding areas through posters and an e-mail service. They were included in the study if they were between 18-30 years of age, normotensive, free from known metabolic disease or CVD, non-smokers, not pregnant or lactating, and did not use any medication except oral contraceptives. Ethics approval was obtained from the Faculty of Health Sciences Research Ethics Committee of the University of Cape Town. Prior to testing, written informed consent was obtained from each study participant.

2.2.2 Testing Procedures

A questionnaire was administered to document family history of diabetes, cardiovascular disease amongst first-degree relatives and alcohol intake. The Global Physical Activity Questionnaire (GPAQ) was used to collect self-reported physical activity data in minutes per week of moderate-vigorous physical activity (MVPA)[152].

Body weight and standard height (to the nearest 0.1 cm) were measured, and BMI (kg/m^2) was calculated. Body fat (kg and %) was measured using 2 methods. Firstly, the sum of 4 skinfolds, namely bicep, tricep, subscapular and supra-iliac sites were measured and body fat was calculated using the Womersely and Durnin method [153]. Secondly, subjects were asked to remove their shoes, socks and any jewellery before supine bio-electrical impedence (Quantum II body composition analyser, RJL system, Michigan, USA) was recorded, and body fat was measured using the equation by Sun et al.[154].

Blood pressure was measured twice after 5 minutes seated rest using an aneroid sphygmomanometer (Flaemnuova, Brescia, Italy). These measurements were then averaged to give a final reading of systolic and diastolic blood pressure.

2.2.3 Experimental protocol for measuring microvascular reactivity

All subjects fasted overnight and were allowed to acclimatize for 15–20 minutes before testing microvascular reactivity in a temperature-controlled (22°C ± 2°C) room. An indifferent electrode was placed on the wrist and iontophoresis chambers were applied to the volar aspect of the left arm with double-sided adhesive tape, avoiding any area with hair, freckles or broken skin. The scanner head of the laser Doppler imager (Moor LDI2, Moor Instruments Ltd., Axminster UK) was placed 30 cm above the arm to allow the non-invasive measurement of erythrocyte flux directly over the site of drug application. A direct current for drug iontophoresis was provided by a battery-controlled iontophoresis controller (MIC 2, Moor Instruments, Axminster, UK).

2.2.4 Iontophoretic drug delivery

To assess endothelial–dependent microvascular reactivity, acetylcholine (ACh) vehicle (0.45% saline) was measured at one site and a 1% ACh solution (Sigma Chemical Co., St. Louis, MO, U.S.A) at two adjacent sites on the forearm. The chamber containing the ACh vehicle was connected to the anodal lead and attached to the volar aspect of the forearm. After completing the vehicle, a similar chamber was placed on a new untouched neighbouring area for the iontophoresis of ACh. The protocol for ACh involved the application of five 20-s pulses of 100μA current, with a 60-s rest period between each pulse: total charge of 10 mC. Skin microvascular response was quantified by a laser Doppler imager at baseline and then every 20 seconds for 7 minutes after the start of the charge protocol. The mean of the two drug sites represented the drug response.

To assess endothelial independent vasodilation, a chamber filled with the vehicle for sodium nitroprusside (SNP) (0.45% saline) was connected to the cathodal lead and placed on a new part of the arm. The 0.25% SNP (Sigma) containing chamber was then placed on a clear area of the volar aspect of the forearm. Iontophoresis of SNP was performed in 14 scans with the delivery of 2 consecutive 30-s pulses of 200μ: total charge of 12mC.

Microvascular response was measured in perfusion units (PU) and expressed as flux in three ways: maximum absolute perfusion; the area under the curve (AUC); and percentage change from baseline. The average coefficient of variation (CV%) of the maximum absolute response was 14.7 ± 8.9% for ACh and 37.5 ± 28.7% for SNP, Microvascular testing was completed by one technician to minimise the inter-tester reliability.

Skin resistance (SR) was calculated using Ohm's law by monitoring voltage. Voltage readings were obtained at the beginning and end of each scan during the application of charge, resulting in 10 readings for ACh and 4 for SNP. Voltage produced for these scans were divided by the current to obtain the resistance which was averaged for each research subject.

2.2.5 Statistical analysis

The data were analyzed using STATISTICA (version 10, StatSoft Inc., Tulsa, OK, USA) and expressed as unadjusted means and standard deviations. Normality of data distribution was assessed by Kolmogorov-Smirnof and Shapiro-Wilk tests. If data were not normally distributed, values log transformed for parametric statistical analysis. One-way ANCOVA, with and without adjustment for SR was used to compare microvascular reactivity outcomes between the three groups. In attempting to explain the ethnic differences encountered, we performed multiple regression modeling, taking into account putative confounders or modifiers of the relationship. To determine whether baselines measures had a confounding effect when comparing microvascular reactivity between groups, a multiple regression including SR and the respective baseline measurement was completed. Pearson correlation coefficients were used to examine the relationships between microvascular reactivity outcomes, blood pressure and SR.

Sample size estimates were calculated based on the study by Tew et al. in which the iontophoresis of ACh and SNP was used to investigate the effects of aging on skin microvascular function[155]. Based on the differences in microvascular function between older and younger groups in the study by Tew et al.[155], with 80% statistical power and an alpha level of 0.05, the sample size required ranged between 9-15 subjects per group, depending on which parameter was used for the estimate.

2.3 Results

2.3.1 Subject's basic characteristics

Descriptive statistics of all participants combined and within each self-identified ethnic group are presented in Table 2.1. The mean age for the whole group was 24.4 ± 2.7 yrs and mean BMI was 22.6 ± 2.7 kg/m². The three groups were similar for age, BMI, body fat, physical activity (MVPA mins/wk), systolic and diastolic blood pressure and alcohol intake (data not shown). Skin resistance (SR) when using the ACh and SNP iontophoresis protocols was significantly lower in the Caucasian group compared to the other ethnic groups (all $P < 0.01$).

2.3.2 Endothelial-dependent microvascular function (ACh)

Baseline, maximum absolute perfusion and AUC flux measurements were significantly higher in the Caucasian compared to the other two ethnic groups (Table 2.2 and Figure 2.1). However, after adjusting for differences in SR, these differences were no longer significant. The percentage change from baseline (relative change) was not significantly different between the groups before or after adjusting for SR.

2.3.3 Endothelial–independent microvascular function (SNP)

Baseline flux was significantly higher in the Caucasian group when compared to the other 2 groups (both P<0.01), and this remained significant after adjusting for SR (Table 2.2). Maximum absolute perfusion was significantly different between all three groups, and AUC outcome measurements were different between Caucasian and Black Africans, before and after adjusting for SR (all P<0.01) (Figure 2.2). Percentage change from baseline was significantly higher in the Caucasian compared to the Black African group (P<0.01), however this difference was no longer significant after adjusting for SR.

Given that baseline measurements for SNP and ACh were significantly different between groups, the respective baseline measurements were included with SR into a multiple regression model (Table 2.3), but did not change the findings (data not shown). This was to exclude the confounding effect that baseline may have had when comparing microvascular reactivity between groups. Furthermore, AUC calculations are normalised for baseline, thus controlling for these differences in the data.

2.3.4 Correlations between microvascular function and blood pressure

Diastolic BP was inversely correlated with ACh derived AUC (r = -0.40, P = 0.02), as well as SNP maximum absolute perfusion (r = -0.35, P = 0.05), percentage change from baseline (r = -0.56, P = 0.01) and AUC (r = -0.40, P = 0.02). Systolic BP was not correlated with any of the measures of microvascular function.

2.3.5 Correlations between microvascular function and SR

SR was inversely correlated with ACh maximum absolute perfusion (r = -0.64, P<0.001) and AUC (r = -0.59, P<0.001), and SNP maximum absolute perfusion (r =

-0.79, P<0.001), AUC (r = -0.77, P< 0.001), and percentage change from baseline (r = -0.37, P<0.001). These correlations are presented in Figure 2.3.

2.4 Discussion

The present study demonstrates that ethnic differences in microvascular endothelial function exist between young, apparently-healthy Black Africans, Caucasians and individuals of Mixed Ancestry. Microvascular reactivity is the highest in Caucasians when compared to those individuals of Black African and mixed ancestral descent however some of these differences may not be independent of SR. Given that endothelial-independent response remained attenuated after correcting for SR in the Black African and Mixed Ancestry groups, differences in the sensitivity of smooth muscle to the vasodilatory effect of NO may be an important factor contributing to ethnic differences in disease risk.

Ethnic differences in skin physiology[116;151;156] have previously been reported, but whether iontophoresis is affected remains unclear. By measuring trans-epidermal waterloss (TEWL) and stratum corneum integrity, Muizzuddin et al.[157] confirmed that African American skin displayed the strongest barrier function and thickest stratum corneum when compared to Asian and Caucasian skin. This may explain the present ethnic differences in SR, thus asserting the need to take SR into account during iontophoresis. Although microvascular techniques such as cutaneous microdialysis may eliminate the effect of SR, these techniques are invasive[158;159].

In the present study, a higher SR was associated with a smaller vasodilatory response for both ACh and SNP. These findings support that of Ramsay et al.[75] who showed significant inter-subject variability in SR when using the same technique, but at a lower cumulative charge compared to the present study (8mC vs 10mC). Indeed Ramsay's 20 subjects were all healthy, but their ages ranged from 22–50 years and no mention of their ethnic background was disclosed. To our knowledge, there have been no studies investigating the specific relationship between SR and ethnicity during iontophoresis. Monitoring the voltage during iontophoresis and calculating SR in all ethnic groups is therefore critical, especially when the cumulative charge is greater than 8mC. Indeed Ramsay was the first to highlight the importance of SR, but to our knowledge, our findings are the first to show these

differences in a young, multi-ethnic group of normal–weight, apparently healthy subjects with no cardiovascular risk factors.

The present findings have critical implications for the interpretation of ACh-induced iontophoresis in multi-ethnic studies. For example, Shantsila et al.[144] reported significantly reduced endothelial-dependent microvascular function in South Asians with heart failure compared to other ethnic groups. In addition, microvascular function was significantly associated with ethnicity after adjustment of age,, however, despite a similar cumulative charge to the present study, they did not adjust for SR. In addition, Shantsila et al., used laser Doppler flowmetry (LDF) which has shown to be less reproducible than LDI[37], as it only measures flux over a single site and involves direct contact with the skin which may also influence blood flow via pressure and movement artefacts.

Furthermore, comparison of ethnic differences among apparently-healthy subjects show higher endothelial function in Caucasians when compared to Asian[138;150], Black[160;161] and Mexican–American[162] groups. In most studies, endothelial function was measured using macrovascular techniques[138;161;162] , and in the studies using iontophoresis, neither ethnicity, nor SR was taken into account[163-165].

For example, in multi-ethnic studies where iontophoresis was used to measure the microvascular response to ACh and SNP in groups with diabetic neuropathy[76] or hypertension [24] compared to healthy controls, both endothelial-dependent and -independent function were reduced in the groups presenting with disease. When FMD was included in these studies to measure macrovascular function, only endothelial-independent function was reduced. Vascular smooth muscle dysfunction is associated with hypertension[166] and diabetes[167], and the prevalence of these cardiovascular risk factors are highest in Black Africans[130] and individuals of mixed Ancestry[132], therefore the present SNP findings may well be an early predictor of disease risk in a healthy group.

Previous work by Schutte et al. reported differences in arterial stiffness and carotid-intima media thickness between Black African and Caucasian South Africans. Schutte et al. concluded that muscular arterial stiffness is elevated in young normotensive Black South Africans when compared to age-matched Caucasians[146]. In line with Schutte's work, the Black SA group in the present study showed lower

microvascular function when compared to the Caucasian group, and for the first time provides microvascular research to support SA macrovascular studies. Given that changes in smooth muscle function precede arterial stiffness[168], the present findings suggest that lower endothelial-independent microvascular reactivity may precede pathophysiology in apparently healthy Black Africans. The present work may therefore provide further insight into the mechanisms underlying ethnic disparities in disease profiles in South Africa.

Various mechanisms may explain the ethnic differences in endothelial-independent vasodilation observed in this study. Firstly, the difference in responsiveness to SNP may be through both CGMP–and CAMP–dependent pathways. This was demonstrated by Cardillo et al.[137], who showed that the response to brachial infusion of SNP in normotensive Black individuals was blunted when compared to matched Caucasians, and attributed these differences to the attenuation of cyclic nucleotide–mediated smooth muscle relaxation in people of Black ethnicity. Another possible mechanism may be via β-adrenergic–mediated vasodilation, which is blunted in patients with hypertension [169]. The infusion of isoprotenerol, a β–adrenergic agonist that induces direct smooth muscle relaxation, has been shown to be blunted in young, normotensive Black compared to Caucasian groups[137;169], suggesting that ethnic differences exist at the level of the vascular smooth muscle. Anatomical differences such as the number of microvessels perfusing skeletal muscle may be decreased in Black individuals, resulting in a reduced response to vaso-active agents. Ama et al.[170] reported an increased percentage of fast-twitch muscle fibres in Black compared to Caucasians individuals. Considering that there is low vascularisation in fast twitch fibres, these findings suggest that differences in fibre type and vascularisation contribute to higher peripheral resistance in Black individuals[171]. Lastly, increases in superoxide production in smooth muscle cells have been demonstrated to inhibit intravascular signalling[172]. It has previously been shown that endothelial cells in Black populations produce more superoxide than Caucasians and may explain differences in endothelial dysfunction[173].

These endothelial–independent differences may contribute to the increase in total peripheral vascular resistance associated with essential hypertension[169]. It is therefore reasonable to speculate that physiological differences may be operative in the microcirculation of Black African and Caucasian subjects. Given that changes in endothelial–independent response were observed in both invasive[137;138] and now,

non-invasive techniques, our findings may well reflect physiological differences between the ethnic groups. The differences in endothelial–independent microvascular response in the present study may therefore be an early marker of future essential hypertension in groups with lower SNP-induced microvascular reactivity.

2.4.1 Strengths

Several risk factors such as obesity, physical inactivity and hypertension are associated with reduced endothelial function. By excluding these risk factors, it was possible to explore whether differences observed in the different ethnic groups were due to the specific methodological protocol, or whether there are indeed physiological differences within the microvasculature. The differences in endothelial-independent response between Black African and Caucasian subjects suggest real physiological differences in microvascular smooth muscle dysfunction. To our knowledge, this is the first study to show the importance of correcting for SR in a young, normal–weight, healthy multi-ethnic group, also essential when the iontophoresis protocol cumulative charge is greater than 8 mC. Moreover, this is the first study to show microvascular endothelial–independent differences between young, apparently healthy ethnic groups, and in a South African context, the first to address endothelial microvascular function in a South African multi-ethnic group. Consequently the present findings provide a foundation from which future studies can assist to understand the ethnic disparities in CVD within South Africa.

2.4.2 Limitations

This study is limited by its cross-sectional design and small sample size. Future studies should include larger groups and could include alternate methods for measuring microvascular function to enhance and to confirm these findings. Another limitation is that this study did not account for phase of the women's menstrual cycle or contraceptive use. However, it has been previously reported that that there are no significant differences in endothelial dependent and independent response during the different phases of the menstrual cycle[31], and Virdis et al.[174] have shown that the effect of oral contraceptives on vascular function remained unchanged in young women after 6 months of contraceptive use, suggesting that it is unlikely to have influenced the present results.

In conclusion, variability in SR appears to account for ethnic differences in ACh-induced microvascular response, suggesting that SR should always be corrected for when using LDI and iontophoresis in a multi-ethnic group or when cumulative charge is greater than 8mC. In contrast, ethnic differences in SNP-induced microvascular response were independent of SR suggesting physiological differences in microvascular smooth muscle between ethnic groups.

Table 2.1 Subject characteristics for Black, Mixed Ancestry and Caucasian apparently healthy men and women

	Whole group	Black	Mixed Ancestry	Caucasian
n	33	9	12	12
Age (yrs)	24.4 ± 2.7	25.2 ± 1.9	23.3 ± 2.3	24.9 ± 3.42
Gender (M/F)	17/16	5/4	6/6	6/6
Weight (kg)	69.3 ± 14.9	65.2 ± 17.8	66.1 ± 12.0	75.6 ± 14.3
Height (m)	1.74 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.1
Body mass index (kg/m^2)	22.6 ± 2.7	21.5 ± 3.2	22.7 ± 2.5	23.3 ± 2.5
%Body fat (bio-electrical impedance)	21.6 ± 7.9	22.4 ± 8.5	22.1 ± 8.3	20.5 ± 7.6
%Body fat (sum of skinfolds)	19.1 ± 7.6	19.3 ± 8.8	20.8 ± 8.4	17.6 ± 5.8
Body fat (kg)	14.8 ± 6.1	14.8 ± 7.8	14.5 ± 6.2	15.0 ± 5.2
Systolic blood pressure (mmHg)	112.1 ± 9.5	113.2 ± 13.4	111.3 ± 7.1	112.1 ± 9.0
Diastolic blood pressure (mmHg)	74.1 ± 9.4	78.7 ± 12.2	74.7 ± 5.2	70 ± 9.42
Family history of hypertension (n, %)	19, 57.6	5, 55.6	8, 66.7	6, 50.0
Family history of diabetes mellitus (n, %)	12, 36.4	2, 22.2	6, 50.0	4, 33.3
MVPA (mins/week)	423.0 ± 395.5	421.7 ± 601.5	485.4 ± 396.6	361.7 ± 162.3
ACh Skin Resistance (Ω)	0.19 ± 0.04	0.21 ± 0.04^{A}	0.20 ± 0.02^{B}	0.16 ± 0.03^{AB}
SNP Skin Resistance (Ω)	0.10 ± 0.02	0.11 ± 0.02^{A}	0.12 ± 0.01^{B}	0.08 ± 0.01^{AB}

MVPA, moderate-vigorous physical activity; ACh, acetylcholine; SNP, sodium nitroprusside. Values are expressed as unadjusted means ± standard deviation (SD). Family history is expressed as number of subjects within group and percentage. $^{a}p<0.05$ and $^{A}p<0.01$ for Black vs Caucasian; $^{b}p<0.05$ and $^{B}p<0.01$ for Mixed Ancestry vs Caucasian.

Table 2.2 Microvascular reactivity outcomes

	Unadjusted Values				Adjusted for skin resistance		
	Overall	Black	Mixed Ancestry	Caucasian	Black	Mixed Ancestry	Caucasian
n	33	9	12	12	9	12	12
Acetylcholine (ACh)							
Baseline	74.9±0.9	57.3 ± 6.0^{A}	67.9 ± 33.9^{b}	95.2 ± 9.5^{Ab}	60.4±31.0	69.2±28.3	91.6±33.4
Maximum absolute response (PU)	461.1±188.2	317.5 ± 130.2^{A}	421.3 ± 174.7^{B}	608.7 ± 133.5^{AB}	370.5±158.5	442.8±144.7	547.5±171.2
%change from baseline	583.0±266.7	574.8±419.8	600.4±187.1	571.8±207.8	589.0±314.8	606.2±287.5	555.3±339.9
AUC	$8.7\times10^{4}\pm5.0\times 10^{4}$	$4.8\times10^{4}\pm3.2\times10^{4A}$	$7.8\times10^{4}\pm4.1\times 10^{4B}$	$1.3\times10^{5}\pm4.4x10^{4AB}$	$5.9\times10^{4}\pm4.4\times10^{4}$	$8.2\times10^{4}\pm4.0\times10^{4}$	$1.1\times10^{5}\pm4.7\times10^{4}$
Sodium Nitroprusside (SNP)							
Baseline	93.8±56.5	64.8 ± 28.7^{A}	72.7 ± 27.8^{B}	136.9 ± 68.9^{AB}	67.7 ± 31.3^{A}	73.8 ± 49.5^{B}	133.4 ± 58.5^{AB}
Maximum absolute response (PU)	343.2±205.6	152.2 ± 66.2^{AC}	276.7 ± 168.8^{BC}	552.9 ± 92.9^{AB}	209.1 ± 110.1^{AC}	316.3 ± 106.8^{BC}	470.7 ± 125.1^{AB}
%change from baseline	413.2±233.5	256.7 ± 178.3^{A}	403.5± 32.1	540.3 ± 209.3^{A}	274.3±232.7	415.8±225.9	514.8±264.4
AUC	$1.0\times10^{5}\pm9.2\times10^{4}$	$3.2\times10^{4}\pm3.9\times10^{4A}$	$6.8\times10^{4}\pm7.0\times10^{4B}$	$1.9\times10^{5}\pm6.7\times10^{4AB}$	$5.9\times10^{4}\pm5.8\times10^{4A}$	$8.6\times10^{4}\pm5.7\times10^{4B}$	$1.5\times10^{5}\pm6.6\times10^{4AB}$

PU, perfusion units; AUC, area under the curve. Unadjusted and adjusted values are means of microvascular reactivity measures ± SD. $^{a}p<0.05$ and $^{A}p<0.01$ for Black vs Caucasian; $^{b}p<0.05$ and $^{B}p<0.01$ for Mixed Ancestry vs Caucasian; $^{c}p<0.05$ and $^{C}p<0.01$ for Black and Mixed Ancestry

Table 2.3 Multiple regression analysis for Acetylcholine (ACh) and Sodium Nitroprusside (SNP) microvascular reactivity outcome measurements

	ß	B	SEE	P value
ACh				
Maximum Absolute perfusion				
Ethnicity	0.24	56.51	168.63	0.16
Skin Resistance	0.33	2.04	0.83	0.03
Baseline	-0.34	-1770.23	704.90	0.04
	R=0.76	R^2=0.57	*Adj* R^2= 0.53	0.00
SNP				
Maximum Absolute perfusion				
Ethnicity	0.40	101.47	28.24	0.00
Skin Resistance	0.23	0.83	0.34	0.02
Baseline	-0.45	-5107.92	1158.58	0.00
	R=0.90	R^2=0.82	*Adj* R^2= 0.80	0.00
AUC				
Ethnicity	0.49	55588	15748.8	0.00
Skin Resistance	-0.21	-334	191.8	0.09
Baseline	-0.55	-2764214	646156.5	0.00
	R=0.84	R^2=0.71	*Adj* R^2=0.68	0.00

ß, partial correlation coefficient; B, parameter estimate; SEE, standard error of the estimate; AUC, area under the curve

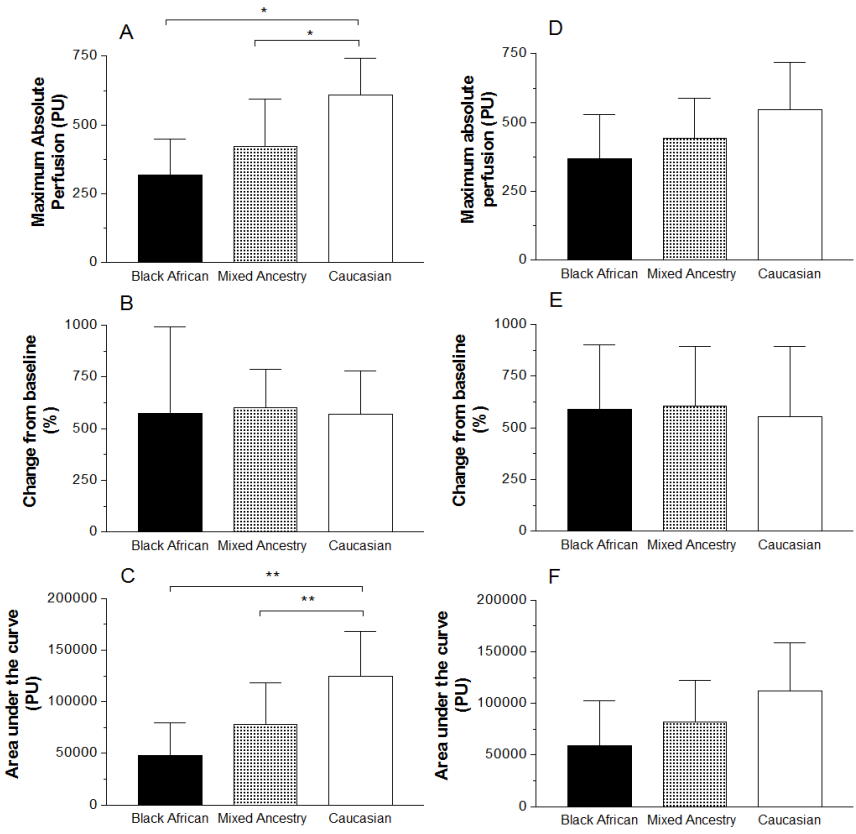

Figure 2.1 ACh Outcome measures of microvascular reactivity for Black African (solid bar), Mixed Ancestry (dotted bar) and Caucasian (open bar) groups. Graphs A-C represents uncorrected outcome measurements for microvascular reactivity and graphs D-F are corrected for skin resistance. *P<0.01; **P<0.001. Values represent means ± standard deviation

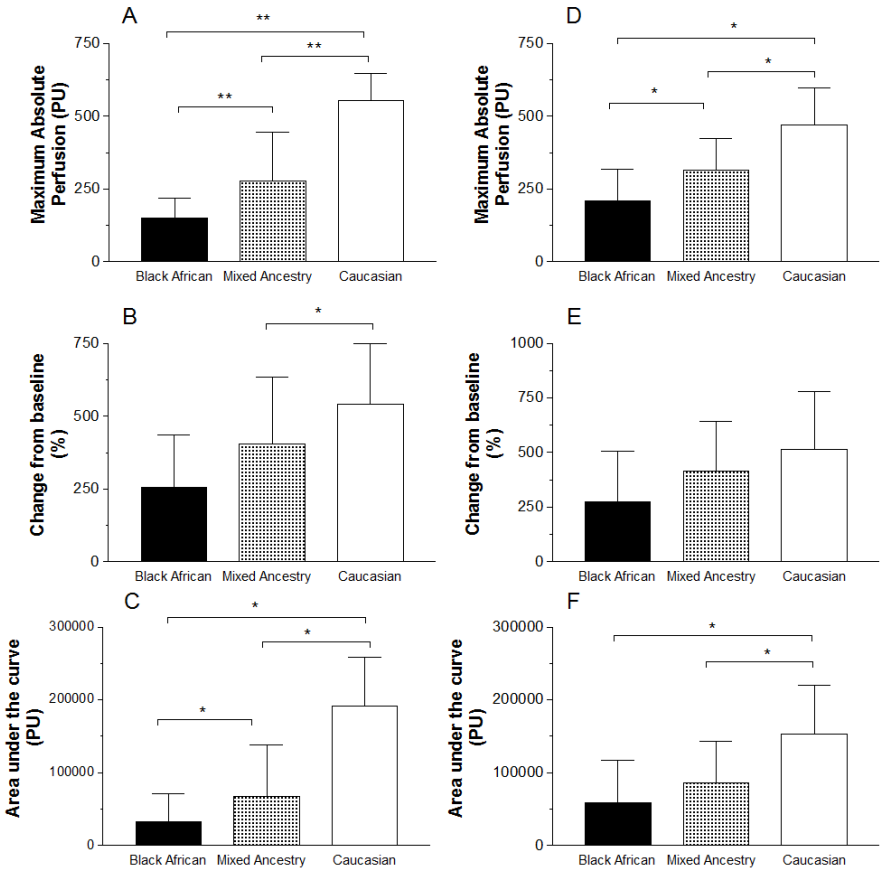

Figure 2.2 SNP Outcome measures of microvascular reactivity for Black African (solid bar), Mixed Ancestry (dotted bar) and Caucasian (open bar) groups. Graphs A–C represent uncorrected outcome measurements for microvascular reactivity and graphs D–F are corrected for skin resistance. For A, C, D *P<0.01; B *P=0.02; E *P=0.14; F *P=0.01. Values represent means ± standard deviation.

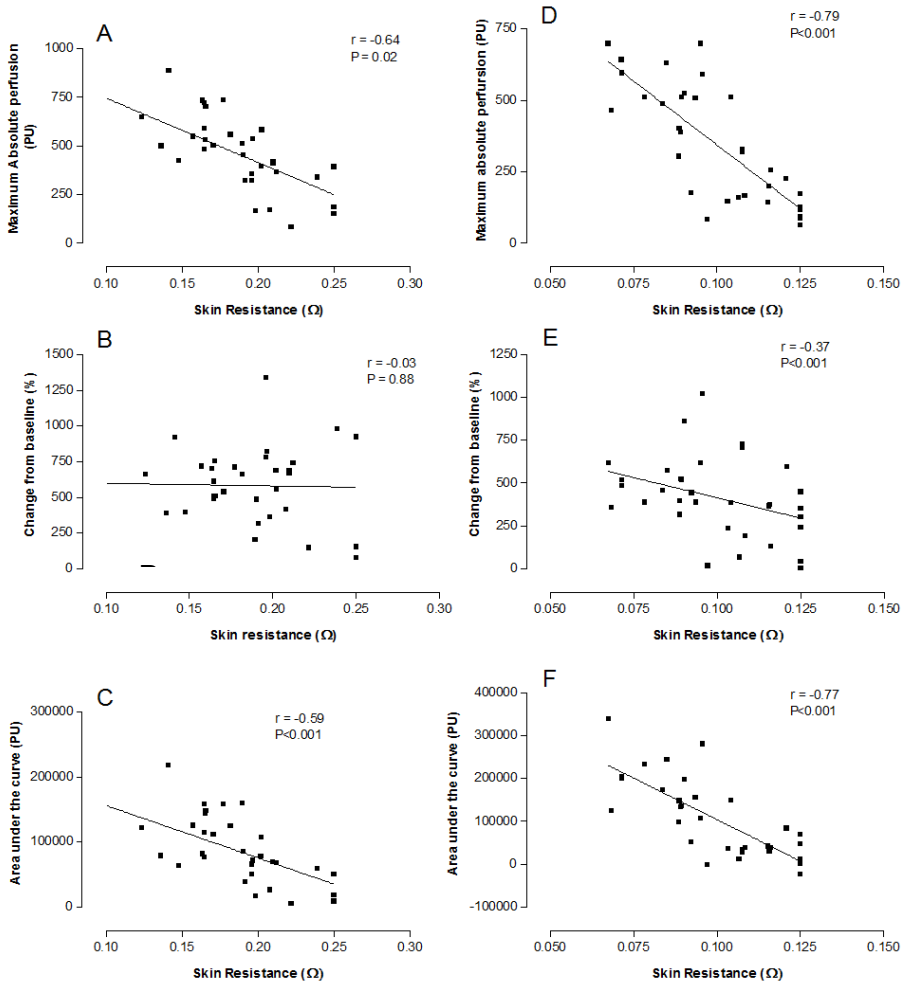

Figure 2.3 Relationships between microvascular reactivity and skin resistance. Graphs A–C show the relationship between the outcome measures of microvascular reactivity and skin resistance for the Acetylcholine (ACh) protocol. Graphs D–F show the relationship between the outcome measurements of microvascular reactivity and skin resistance for the sodium nitroprusside (SNP) protocol**.**

Chapter 3

LITERATURE REVIEW: ENDOTHELIAL DYSFUNCTION AND INSULIN RESISTANCE

3.1 Introduction

In 2008 the World Health Organization (WHO) estimated that 17.3 million people died of cardiovascular disease (CVD) worldwide and over 80% of these deaths occurred in low to middle income countries. Of greater concern is the fact that by 2030 it is predicted that the annual death rate as a result of cardiovascular disease will rise to 23 million (http://www.who.int/cardiovascular_diseases/en/). Equally alarming is that in the same year, the number of individuals with diabetes is predicted to rise to an estimated 552 million, and of those, only half will be aware of their diagnosis. A 2011 report by the International Diabetes Federation reported that 300 million individuals were at risk of developing type 2 diabetes (T2DM) in the future. These risk factors included fasting hyperglycemia, impaired glucose tolerance and insulin resistance (International Diabetes Federation 2011. Global Burden: Prevalence and Projections, 2011 and 2030. http://www.diabetesatlas.org/content/diabetes-and-impairedglucose-tolerance.).

Furthermore, individuals with type 2 diabetes mellitus (T2DM) have a markedly higher risk for microvascular complications and macrovascular disease[175]. In many individuals, atherosclerosis precedes the development of T2DM, and cardiovascular complications such as hypertension and dyslipidemia, are often present in newly diagnosed diabetic patients. A survey[176], involving 4196 patients, found that 70% of patients diagnosed with CVD have some form of glucose intolerance, indicating that CVD and diabetes mellitus may share underlying causes which could include endothelial dysfunction.

Insulin resistance represents one of the earliest detectable abnormalities of T2DM and hyperinsulinemia, a key feature of insulin resistance, contributes to the development of endothelial dysfunction, and therefore plays a key role in the progression of atherosclerosis. In addition, insulin resistance is central to both the progression of normal glucose tolerance to T2DM, and to a constellation of cardiovascular risk factors known as the metabolic syndrome (MetS). Moreover, visceral adiposity, a metabolic risk factor present in the MetS, plays a crucial role in the pathogenesis of insulin resistance through metabolic abnormalities which will be discussed in the literature review.

The purpose of this literature review is therefore to explore the link between insulin resistance and microvascular endothelial dysfunction in apparently healthy obese and non-obese individuals.

3.2 Insulin resistance: mechanisms

Insulin is an anabolic hormone that exerts its effect on skeletal muscle, adipose tissue and the liver. Insulin regulates glucose homeostasis by promoting the glucose uptake into the skeletal muscle and adipose tissue, and by inhibiting hepatic glucose production (gluconeogenesis). Moreover, it also regulates nutrient delivery to target tissues by actions on the microvasculature[177] .

The binding of insulin to its receptor on target tissues such as skeletal muscle and the endothelium of blood vessels, leads to the activation of complex insulin-signalling pathways. The phosphatidylinositol 3-kinase (PI3K) and the mitogen activated protein kinase (MAPK) signalling pathways contribute to cardiovascular and metabolic homeostasis (Figure 3.1). The PI3K pathway mediates the metabolic actions of insulin, including regulation of glucose metabolism in the muscle, as well as the adipose and hepatic tissues; and the regulation of nitric oxide (NO) from the endothelium and vascular smooth muscle cells (VSMC)[178-180]. The MAPK signalling pathway regulates the non-metabolic actions of insulin, including VSMC proliferation, secretion of endothelin-1 (ET-1) from endothelial cells, and increased expression of adhesion molecules on the endothelium. Taken together, the PI3K-pathway, which is NO-dependent, results in vasodilation, an increase in blood flow, stimulation of capillary recruitment and enhanced glucose uptake in the muscle. In contrast, the MAPK pathway, causes vasoconstriction, VSMC proliferation, and contributes to the development of atherosclerosis[181].

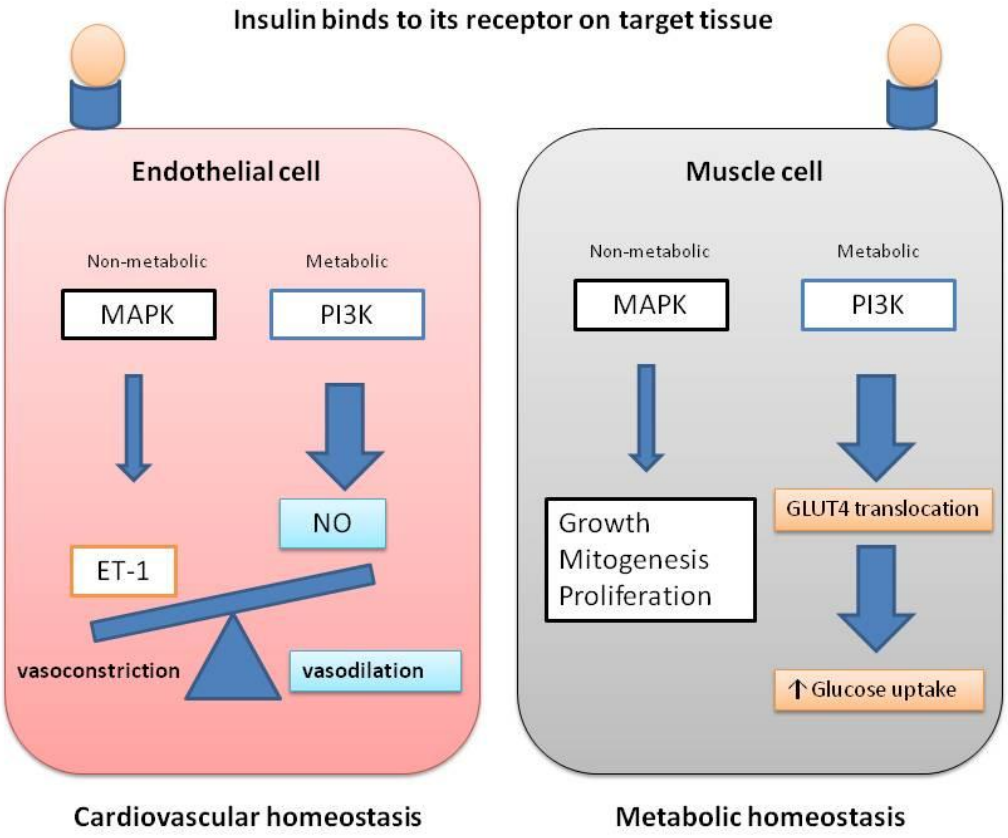

Figure 3.1 Insulin helps maintain cardiovascular and metabolic homeostasis by stimulating the PI3K-dependent pathways in endothelial and muscle cells. The MAPK pathway regulates the non-metabolic actions of insulin. The PI3K pathway regulates its metabolic actions. MAPK, mitogen activated protein kinase; PI3K, phosphatidylinositol 3-kinase; ET-1, endothelin-1; GLUT4, glucose transporter 4

Under normal conditions, insulin stimulates NO production via the PI3K pathway, but in insulin resistant conditions, this pathway is impaired while the MAPK pathway remains unaffected. Compensatory hyperinsulinemia, which accompanies insulin resistance in order to maintain euglycemia, overstimulates the unaffected MAPK pathway resulting in an imbalance between these two pathways[182]. As a consequence, the reduced bioavailability of NO and increase in ET-1, pro-inflammatory and pro-atherogenic factors result in endothelial dysfunction. Furthermore, the overproduction of ET-1 as a result of insulin resistance and hyperinsulinemia, contributes to the activation of the sympathetic nervous system leading to the development of systemic hypertension[183]. In this way, the reciprocal

relationship between insulin resistance and endothelial dysfunction contributes to the link between cardiovascular and metabolic (cardio-metabolic) disease. Moreover, endothelial dysfunction also contributes to impaired insulin action. For example, a decrease in NO production leads to both endothelial dysfunction as well as reduced glucose uptake in the muscle, resulting in insulin resistance.

3.3 Obesity and endothelial dysfunction

Obesity is associated with insulin resistance and impaired insulin-mediated vasodilation[93]. It is well-established that insulin is an endothelial-dependent vasodilator and that insulin-mediated NO production is reduced in obesity[184;185]. In order to determine whether obesity and insulin resistance were associated with both impaired endothelial-dependent vasodilation and insulin-mediated augmentation of endothelial-dependent vasodilation, Steinberg et al. measured leg blood flow after the intra-femoral infusion of metacholine chloride (MCh), an endothelial-dependent vasodilator, and sodium nitroprusside (SNP), an endothelial-independent vasodilator, in a group of obese (classified as BMI $\geq$ 28kg/m² in this study) and non-obese (BMI < 28 kg/m²) apparently healthy women (n=44). When compared to the lean controls, the obese group showed a 40-50% reduction in endothelial-dependent vasodilation; no increase in endothelial-dependent vasodilation during euglycemic hyperinsulinemia, and no difference in endothelial-independent response[93]. Interestingly and of importance, the differences in endothelial-dependent vasodilation between insulin-sensitive and insulin-resistance subjects were accentuated by euglycemic hyperinsulinemia, which increased endothelial-dependent vasodilation in the insulin-sensitive, but not insulin-resistant group.

According to these findings and others[12;186;187], obese, insulin resistant individuals are characterized by endothelial dysfunction and endothelial resistance to insulin-mediated vasodilation. Furthermore, there is increasing evidence that insulin also increases endothelial-dependent vasodilation in the microcirculation, leading to enhanced blood flow and glucose uptake in muscle cells[5]. Failure of this microvascular action that occurs with hyperinsulinemia is also associated with obesity[184].

3.3.1 Mechanisms by which obesity leads to microvascular dysfunction

Obesity associated microvascular dysfunction may be caused by cellular defects that disrupt the balance between the MAPK and PI3K pathways. Obesity is associated with elevated free fatty acid (FFA) concentration which increases the production of reactive oxygen species (ROS)[188;189]. ROS limits the bioavailability of NO via reduced NO production as well as the direct inactivation of NO by superoxide anions[187]. Furthermore, the increase in ROS leads to the production of pro-inflammatory cytokines such as IL-6 and TNF-α. In the muscle, endothelial-nitric oxide synthase (eNOS) expression is diminished leading to a decreased in NO production[190;191]. Moreover, visceral adipose tissue secretes a variety of pro-inflammatory adipocytokines such as angiotensinogen, angiotensin II, IL-6 and TNF-α. TNF-α downregulates eNOS and increases ET-1 secretion via the MAPK pathway in endothelial cells[192;193]. As a consequence of these cellular defects, the PI3K pathway is disrupted, while the MAPK pathway remains unaffected. The imbalance between the two pathways results in an increased production of ET-1 and a shift from vasodilation to vasoconstriction. Similarly, angiotensin II disrupts intracellular signaling pathways contributing to elevated systemic blood pressure in obese individuals[194].

3.3.2 Obesity and microvascular endothelial function

Several studies have indicated that obesity impairs microvascular function in a number of ways[4;93;195;196]. Obese subjects show diminished vasodilation in response to the endothelial-dependent vasodilators in the skin and capillaries. For example, de Jongh et al.[4], measured microvascular function in 16 lean and 12 obese healthy women during basal state and during physiological systemic hyperinsulinemia. Results showed that both skin capillary recruitment and ACh-mediated vasodilation were reduced in the obese group, but SNP-mediated vasodilation remained similar although the significant differences in waist-to-hip ratio between the groups were not adjusted for in this study. In addition, free fatty acids were higher in the obese group, but did not affect microvascular function once adjusted for. This study concluded that compared to the lean group, endothelial-dependent microvascular function was impaired in obese women during basal and hyperinsulinemic states, and that reduced microvascular function contributes to the development of obesity-related insulin resistance.

In another study, macrovascular and microvascular endothelial function was compared between non-diabetic, non-obese (BMI<30; n=21) and obese (BMI≥30; n=20) men and women. Interestingly, there were no differences in FMD measures between non-obese and obese groups, but ACh-induced vasodilation, as measured by video microscopy, was lower in the obese group, after adjusting for differences in sex. Furthermore, only macrovascular function (FMD) was inversely correlated with waist circumference, but both macro- and microvascular function were inversely correlated with BMI[197].

Contradictory to these studies, Czernichow et al.[198] found no differences in skin capillary recruitment or LDI with iontophoresis of ACh and SNP between two groups of apparently healthy men and women, categorized by their BMI (BMI<25 and BMI≥25). Despite these results, there was a positive association between BMI and capillary recruitment; therefore it is insulin resistance and its classical association with inflammatory markers that impair vascular reactivity. This study concluded that insulin-sensitive overweight and obese individuals do not exhibit structural capillary rarefaction or endothelial-dependent vasodilation in comparison with lean individuals. They further suggested that alterations in the microcirculation of insulin-sensitive obese individuals, independent of blood pressure, T2DM and endothelial dysfunction, may be considered the earliest marker of CVD.

Although most[199-201] but not all[198] of these studies have shown that obesity is characterized by microvascular dysfunction, they have not investigated the role of body fat distribution. Various studies have shown that body fat distribution rather than the obesity attenuates endothelial function[187;196;202].

3.3.3 Body fat distribution and microvascular dysfunction

The study by de Jongh et al.[203] investigated how body fat distribution relates to microvascular dysfunction in 52 healthy adults (ages 44.0 ± 4.9 years). Microvascular function was measured by post-occulsive skin capillary recruitment and LDF with the iontophoresis of ACh and SNP. Concentrations of adipocytokines were analyzed to determine their possible role in the relationship between visceral obesity and microvascular function. The results of their study showed that visceral adiposity as measured by magnetic resonance imaging, was inversely associated with capillary recruitment, and that this association was partly explained by C-reactive protein (CRP), IL-6 and TNF-α.

Weight loss has been shown to decrease pro-inflammatory cytokines and improve endothelial function in a study by Ziccardi et al.[204] where they measured circulating levels of pro-inflammatory cytokines, endothelial function and body fat distribution before and after a one year sustained weight reduction programme. Endothelial function was assessed by the haemodynamic response to intra-arterial infusion of L-Arginine. This study comprised 56 healthy obese and 40 age-matched normal weight women. Before the intervention, all circulating pro-inflammatory cytokines, including TNF-α and IL-6, and endothelial function, were higher in the obese group compared to the normal-weight group. After the intervention, all obese women had lost at least 10% of their initial total body weight, and their weight loss was associated with significant decreases in waist-to-hip ratio, TNF-α, IL-6 and various adhesion molecules. Endothelial function significantly improved and was no longer different to the normal-weight group at the end of the intervention, and changes in cytokine concentration and endothelial function were related to the decrease in BMI and waist-to-hip ratio.

Taken together, endothelial dysfunction in apparently healthy, insulin sensitive, obese individuals may be specific to their increased visceral adiposity, and mediated by pro-inflammatory cytokines. Furthermore, attenuation of endothelial function may precede obesity-related insulin resistance.

3.4 Insulin resistance in non-obese individuals

Not all obese individuals are insulin resistant[205-207], and insulin resistance may also occur in normal weight individuals for the following reasons: first degree relatives of diabetics, and women with polycystic ovarian syndrome. Several studies have investigated whether these conditions are associated with impaired endothelial function, dependent or independent of insulin resistance.

3.4.1 Family history of diabetes

Microvascular endothelial dysfunction may be both the cause and a consequence of T2DM. On the one hand T2DM leads to microvascular complications such as nephropathy and retinopathy, while on the other hand, microvascular endothelial dysfunction has been shown to precede T2DM[208]. There is consensus in the literature that there is a strong genetic component associated with type 2 diabetes (T2DM)[209]. Individuals with at least one parent with T2DM have a 50% increased

chance of developing T2DM, independent of other risk factors such as obesity and physical inactivity[210]. Given that insulin resistance also precedes and predicts T2DM, it may impart a greater risk for individuals with a genetic risk for T2DM compared to those with no family history of the disease[211].

Early abnormalities in vascular function have been reported in individuals with a family history of T2DM. A study by Olive et al.[212] found that FMD was lower in 42 young (20-30 years), healthy, insulin-sensitive women with a family history of T2DM compared to those with no family history of the disease. Another study by Goldfine et al.[211] also found similar results using FMD in 19 non-diabetic individuals (38 ± 2 years) with a family history, compared to 19 individuals without a family history (37 ± 2 years) of T2DM. In addition to lower macrovascular function as measured by brachial FMD, Caballero et al.[25] reported that microvascular reactivity was lower in first degree relatives of type 2 diabetics when compared to a healthy control group with no family history of T2DM. Further, they used iontophoresis of ACh and SNP to assess endothelial dependent and independent vasodilation and when compared to the control group, both measures of microvascular reactivity were lower in the relatives of diabetics.

It therefore remains unclear as to whether these vascular abnormalities are related to insulin resistance or genetically determined vascular defects. Abnormalities in the endothelium may therefore be present early in individuals at risk of developing type 2 diabetes, even at a stage where normal glucose tolerance exists.

3.4.2 Polycystic ovarian syndrome (PCOS)

Polycystic ovarian syndrome is a condition associated with insulin resistance and visceral adiposity both of which may contribute to endothelial dysfunction[213]. Most studies that have compared endothelial function have included overweight or obese women with PCOS[214], with few studies being done in normal-weight women. The study by Orio et al.[215] compared FMD (% change in arterial diameter), carotid-intima media thickness and endothelin-1 (ET-1) level, between 30 young, normal weight women with PCOS but without dyslipidemia or hypertension, and 30 age- and BMI-matched controls. FMD was lower, and both carotid-intima media thickness and ET-1 levels were increased, in the PCOS group, suggesting early functional, structural and biochemical vascular impairment in women with PCOS, independent of body weight.

Moreover, microvascular function measured using LDI and the iontophoresis of ACh and SNP, was compared between 12 women with PCOS (BMI: 31.1 ± 7.1 kg/m^2) and 12 healthy controls (BMI: 24.5 ± 3.5 kg/m^2). Final results showed that maximal ACh-induced microvascular response was lower in the PCOS group, independent of differences in BMI[32].

Taken together, these findings suggest that insulin resistance may contribute to the early development of endothelial function, independent of obesity.

3.5 Conclusion

The presence of microvascular endothelial dysfunction in individuals with insulin resistance plays a key role in the development and progression of cardio-metabolic disease. Conditions that are associated with insulin resistance including obesity, and in particular visceral obesity, have also been shown to be associated with reduced endothelial-dependent microvascular function[203] .

As microvascular endothelial dysfunction usually precedes the clinical manifestations of cardio-metabolic disease, the study of microvascular function in individuals with insulin resistance, and the role of visceral adiposity, is important to contribute to our understanding of the underlying mechanisms behind the association of these two factors prior to the development of diabetes.

3.6 Aims

The aims of the following chapter are to:

1. Compare ACh–induced microvascular reactivity between insulin resistant and insulin sensitive South African women without known disease
2. Determine whether differences are independent of body fat

Chapter 4

INSULIN RESISTANCE IS ASSOCIATED WITH LOWER ACETYLCHOLINE-INDUCED MICROVASCULAR REACTIVITY IN NON-DIABETIC WOMEN

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4.1 Introduction

Cardiovascular disease (CVD) is the leading cause of death in individuals with type 2 diabetes (T2DM), with more than 50% of the deaths due to coronary artery disease and a further 15% from stroke[216]. T2DM and CVD are closely linked, at least in part, via concomitant risk factors such as dyslipidemia, hyperglycemia, hypertension, altered fibrinolysis and obesity, that often manifest prior to the diagnosis of T2DM[217;218]. Individuals with hyperinsulinemia, characteristic of insulin resistance, are at a high risk of developing microvascular and macrovascular complications[219-221].

The mechanisms linking vascular disease and insulin resistance are multiple and complex[217]. The vascular response to insulin is mediated by the balance between phosphatidylinositol 3-kinase-(PI3K)-dependent insulin-signalling pathways that regulate nitric oxide (NO) production, and mitogen-activated protein kinase (MAPK)-dependent insulin-signalling pathways regulating the secretion of the vasoconstrictor endothelin-1[5]. Insulin resistance is accompanied by reduced NO production, creating disequilibrium in vascular homeostasis which may contribute to endothelial dysfunction[222;223], so although insulin itself is a weak vasodilator, other factors associated with insulin resistance appear to markedly potentiate endothelial-dependent vasodilation[28].

Central obesity, which is often associated with insulin resistance, may also adversely affect endothelial function either directly or indirectly by the release of free fatty acids, tumour necrosis factor-α (TNF-α), and the adipocytokines interleukin-1 (IL-1) and interleukin-6 (IL-6) from adipose tissue[224;225]. These metabolites, hormones and inflammatory cytokines may interfere with the insulin-signalling pathways resulting in the reduction of insulin secretion and consequent reduction in nitric oxide production[178]. Moreover, insulin resistance is postulated to be the common underlying pathogenic link between obesity and T2DM, which have been associated with reduced endothelium-dependent vasodilation[30;93;224]. However it is unclear whether this is due to the direct influence of insulin resistance on endothelial-dependent function or whether it is the indirect effect of total and visceral fat accumulation, as the primary mediator.

Metabolic abnormalities such as insulin resistance do not however affect all obese individuals[226]. Ach-mediated vasodilation has been shown to be similar in

overweight individuals without insulin resistance, when compared to a lean control group[198]. Conversely, separate studies in non-obese apparently healthy women have shown inverse associations between vascular function and insulin concentration[227], and reduced endothelial function with increase in waist circumference[228]. It is therefore of interest to this study to examine whether the direct vasodilatory effects of ACh-induced iontophoresis are reduced with insulin resistance, independent of visceral body fat.

In addition to this, there is evidence that normoglycemic individuals, with a family history of T2DM in one or both parents, have lower endothelial-dependent vascular reactivity, lower plasma markers for endothelial function, and higher markers for inflammation, compared to individuals without a similar family history[13-17]. This raises the question as to whether the endothelial impairment precedes insulin resistance or whether it is a consequence of genetic vascular defects.

There is limited research examining the relationship between endothelial-dependent microvascular reactivity measured using iontophoresis and laser Doppler imagery (LDI) and insulin resistance, in apparently healthy, non-diabetic persons. Although flow mediated dilation (FMD) is the most common technique for measuring endothelial-dependent vasodilation, LDI records the continuous graded measures of endothelial response without evoking systemic vasoregulatory reflexes[229], and measurement at the microvascular, rather than the macrovascular level, may identify changes prior to the onset of disease itself[33].The current study therefore primarily aims to test the hypothesis that insulin resistance is associated with lower ACh-induced microvascular reactivity in apparently healthy individuals and secondly, to examine whether this relationship is independent of differences in body fat and visceral fat accumulation.

4.2 Methods and Procedures

4.2.1 Subjects

The study population consisted of 37 women recruited from the surrounding areas through advertisements placed in local newspapers and an e-mail service. They were included in the study if they were between the ages of 18-45 years, pre-menopausal, normotensive and free from known metabolic or cardiovascular disease, non-smokers, not pregnant or lactating, and did not use any medication. Pre-menopausal women were selected as a convenience sample because of their CVD risk and higher body fat when compared to men. Ethics approval was obtained from the Faculty of Health Sciences Research Ethics Committee of the University of Cape Town. Prior to testing, written informed consent was obtained from each study participant.

4.2.2 Testing Procedures

A questionnaire was administered to document family history of diabetes and cardiovascular disease amongst first-degree relatives. Demographic data were also collected, including self-identified ethnicity. The Global Physical Activity Questionnaire (GPAQ) was used to collect self-reported moderate to vigorous physical activity data (MVPA, min/wk) [152].

Body mass and height (to the nearest 0.1 cm) were measured. Waist circumference (WC) was measured at the level halfway between the base of the sternum and the umbilicus, and hip circumference was measured at the largest gluteal region. Whole body fat and body fat percentage were measured using dual energy x-ray absorptiometry (DXA; Hologic QDR Discovery-W; software version 12.6:7), according to standard procedures.

After 5 minutes of seated rest, resting blood pressure (BP) was measured twice, using an anaeroid sphygmomanometer (Flaemnuova, Brescia, Italy). These measurements were averaged to give a final BP reading.

4.2.3 Blood samples and biochemical analyses

Blood samples were drawn from the antecubital vein, after a 10-12 hour overnight fast for the subsequent measurement of plasma glucose and serum free fatty acids

(FFA), and insulin concentrations. The collected plasma was stored at -20°C for glucose and -80°C for insulin and FFA.

Plasma glucose concentrations were determined using the glucose oxidase method (YSI 2300 STAT PLUS, Ohio, USA). Intra- and inter-assay coefficients of variation for plasma glucose were 1.4 (5.30 ± 0.079) and 2.5% (5.38 ± 0.134) respectively. Serum FFA concentrations were analyzed using a commercial kit (FFA Half-micro test; Boehringer). The intra-assay and inter-assay coefficients of variation for serum FFA concentrations were 2.1 (0.230 ± 0.0049) and 9.2% (0.243 ± 0.0224), respectively. Serum insulin concentrations were determined by a Micro particle Enzyme Immunoassay (MEIA) (AxSym Insulin Kit, Abbot, IL, USA).

4.2.4 Homeostasis model for insulin resistance (HOMA-IR) and HOMA-β

The homeostasis model assessment (HOMA) was used to calculate insulin resistance (HOMA-IR) and beta cell function (HOMA-β) from fasting insulin and glucose concentrations[230-232]. Based on previous studies[185;233], a cut point of 1.69 was used to classify a subject as either insulin-sensitive (IS, n = 21) or insulin-resistant (IR, n = 16)[185;233]. Within the IR group, 6 subjects were Black African, 4 were of Mixed Ancestry, and 6 were Caucasian. Within the IS group, 6 were Black African, 4 were of Mixed ancestry, and 11 were Caucasian. Therefore, different ethnic groups were proportionally similarly represented in the IR and IS groups.

4.2.5 Experimental protocol for measuring endothelial-dependent microvascular reactivity

The ACh iontophoresis protocol and calculation for skin resistance was followed as described in Chapter 2's methodology section (pg 39-40).

Microvascular reactivity was measured in perfusion units (PU) and expressed in three ways: maximum absolute perfusion; the area under the curve (AUC); and % change from baseline. The average coefficient of variation (CV%) for maximum absolute perfusion was 10.6 ± 9.9%; for AUC 16.6 ± 11.1% and % change from baseline 21.5 ± 15.5%.

4.2.6 Statistical analysis

The data were analyzed using STATISTICA version 10 (StatSoft Inc., Tulsa, OK, USA) and expressed as unadjusted means and standard deviations. One-way analysis of variance (ANOVA), with and without adjustment for WC and BMI, were used to compare metabolic variables between the IR and IS groups. One-way ANOVA, with and without adjustment for WC and SR, were used to compare microvascular reactivity measures between the IR and IS groups. To account for possible ethnic heterogeneity within the sample, all variables were adjusted for ethnicity, but resulted in no difference between the IR and IS groups (data are not shown). Statistical significance was accepted at the $P < 0.05$ level. Pearson correlation coefficients were used to examine the relationships between microvascular reactivity measures and BP, SR, metabolic outcomes, WC, BMI and body fat mass (kg).

4.3 Results

4.3.1 Subject characteristics and metabolic outcomes

Table 4.1 describes the subject characteristics. The IR group was heavier ($P < 0.01$), had a greater WC ($P<0.01$), waist-to-hip ratio ($P <0.05$), BMI, % body fat, and total fat mass ($P<0.01$), and lower SR ($P<0.05$) than the IS group. Groups were not different for age, height, ethnicity, family history of diabetes and CVD, and MVPA (mins/wk).

The metabolic parameters for the two groups are presented in Table 4.2. By design, HOMA-IR, fasting insulin, and HOMA-β were significantly higher in the IR group. These differences persisted after adjustment for BMI and WC separately and together. There were no significant differences in plasma glucose and FFA between the two groups, before and after adjustment for BMI and WC. Systolic and diastolic BP were not significantly different between the groups.

4.3.1 Microvascular reactivity outcomes

None of the microvascular reactivity outcome measures were different between the groups (Table 4.3). However, percentage change from baseline was significantly lower in the IR group after adjusting for differences in WC and SR, separately and together (P = 0.05 and P = 0.04 respectively). Maximum absolute perfusion was lower in the IR group after adjusting for SR only (P = 0.01).

There were no significant correlations between the measures of microvascular reactivity and body composition, measures of glucose tolerance or insulin resistance. However, maximum absolute perfusion and AUC were inversely correlated with systolic BP (Maximum absolute perfusion: r = -0.44, P = 0.04; AUC: r = -0.36, P = 0.03). In addition, SR was inversely correlated with maximum absolute perfusion (r = -0.34, P = 0.04), BMI (r = -0.81; P < 0.001), WC (r=-0.77, P<0.001); body fat mass (r=-0.81; P < 0.001), HOMA-IR (r=-0.45; P = 0.006); HOMA-β (r=-0.59, P < 0.001) and positively correlated with systolic BP (r = 0.34, P = 0.04).

4.4 Discussion

The major finding in the present study was that ACh- induced microvascular reactivity (% change from baseline) was significantly lower in non-diabetic, apparently healthy women who were insulin resistant compared to those who were not, after adjusting for differences in WC and SR. ACh, an endothelial-dependent vasodilator, directly stimulates NO production in endothelial cells. Without the inclusion of an endothelial-independent vasodilator, the present study cannot determine whether abnormal responses are due to a decreased production of NO, inactivation of NO, or a decreased vascular smooth muscle cell response to NO. However, despite the absence of endothelial-independent results, the present findings may suggest early microvascular changes before the presentation of disease.

Although previous studies have shown an impaired response to ACh in an insulin-resistant state[234], these studies were largely conducted in persons with known diabetes or a family history of diabetes[25;235], hypertension[42] and/ or polycystic ovarian syndrome[32]. These cardio-metabolic conditions, as well as certain lifestyle behaviours such as smoking, were excluded in the present study. Despite including

individuals with a family history of diabetes, there was no difference in the percentage of these individuals between the IR and IS groups. Thus reduced microvascular reactivity may be an early marker of microvascular dysfunction that precedes clinically diagnosed cardio-metabolic conditions in apparently healthy individuals.

In agreement with a study by Irving et al.[165], ACh-mediated vasodilation was not associated with blood glucose or insulin sensitivity. Although different measures of body fat distribution were measured, both waist to hip ratio in Irving's group and WC measured in the present study were associated with insulin sensitivity, but Irving did not correct for differences in body fat distribution. In the present study, microvascular reactivity was significantly lower in the IR group only after correcting for WC, suggesting that hormones, adipokines and/or inflammatory cytokines associated with visceral adiposity may alter ACh-mediated microvascular function in apparently healthy individuals prior to metabolic aberration.

Further, while the mechanistic links between insulin resistance and alterations in microvascular function are complex, visceral adiposity has been shown to affect endothelial function[236]. In support of previous work showing that reduced endothelial-dependent occurs in obese women at basal state and hyperinsulinemia, the present study found that those who were insulin-resistant were also heavier, had a greater WC and had reduced microvascular response to ACh[4]. Conversely, the IR group in the present study did not demonstrate significantly elevated FFA[236;237], hypertension[238] or impaired fasting glucose[27;237], which have been implicated in endothelial dysfunction, suggesting that they were otherwise healthy. Therefore, it is possible that the reduced ACh-induced microvascular reactivity observed in the IR group may suggest that early microvascular alterations precede hypertension, elevated FFA or hyperglycemia despite differences in body weight or WC.

Furthermore, BP in the present study was inversely associated with ACh-induced microvascular response, but were all within normal physiological ranges. Indeed the role of microvascular dysfunction in the development of hypertension has been reported in obesity, essential hypertension and the family history of hypertension, but the present study found that neither body fat nor family history were associated with systolic BP. These results provide further support to show that microvascular changes may occur in normotensive apparently healthy individuals prior to hypertension.

The results of the present study are in agreement with that of Ardigo et al.[14], who demonstrated similar outcomes between insulin resistance, compensatory hyperinsulinemia and macrovascular endothelial-dependent vasodilation using flow-mediated dilation (FMD), in a population free of metabolic abnormalities or CVD. Given these findings, Ardigo et al. concluded that those who were more insulin-resistant or hyperinsulinemic exhibited the lowest endothelial-dependent vasodilation[14]. On the one hand, the insulin concentration in our IR group was comparable to Ardigo's group with the lowest FMD values, on the otherhand, Ardigo's findings were in a markedly older group (age ranging between 45 and 65 years) in which age was not covaried for, suggesting that it may have confounded FMD outcomes. The present study was conducted in a younger group, preventing the possible age effect and therefore our data suggests that microvascular function may also be altered in a young apparently healthy but insulin-resistant group.

The reduced microvascular response to ACh in women with insulin resistance could be related to impaired insulin signalling, characteristic of hyperinsulinemia. Hyperinsulinemia catalyzes the overdrive of endothelin-1, a powerful vasoconstrictor, while NO production is inhibited[5;183]. This resulting imbalance attributed to insulin resistance and concomitant hyperinsulinemia may help to explain the lower ACh-induced microvascular reactivity observed in the IR group.

The significant difference in SR between the IR and IS groups, as well as the inverse correlation between SR and ACh response, suggests that there may be inter-subject variability in the skin of different subjects. This methodological finding supports Ramsay et al.[75] who showed that even at a lower cumulative charge to that used in the present study (8mC vs. 10mC). SR is an important variable that influences iontophoresis[74;75]. Furthermore, the inverse association between SR and all measures of adiposity suggests that a greater availability of resistance pathways, which could be attributed to richly vascularised sweat glands, may be more active in obesity[96]. Given these findings, it appears that correcting for SR has important implications for interpreting iontophoresis results in obese subjects suggesting the likelihood that in previous studies, variations in SR and adiposity might have influenced effective drug delivery, leading to spurious results.

The limitations to the present study include the relatively small sample size, and the absence of endothelial-independent microvascular reactivity outcome measures, which would have added to the understanding of the mechanisms associated with

insulin resistance and microvascular function. From this study it is not possible to specifically determine whether this is due to impaired endothelial function or changes in the vascular smooth muscle. Further research with the use of an endothelial-independent vasodilator such as sodium nitroprusside, is needed to clarify this.

In conclusion, the present study demonstrates that the differences in ACh-induced microvascular reactivity, in non-diabetic, insulin-resistant women, utilizing LDI and iontophoresis, were only apparent when the differences in WC and SR were controlled for. Furthermore, it is anticipated that the correction for SR, particularly in obese subjects, will lead to significant improvements of the interpretation of results of the iontophoresis technique, thereby further increasing the robustness for assessing microvascular function in clinical studies. There is a need for longitudinal studies to determine if altered microvascular function is an early sequelae of insulin-resistance and associated cardio-metabolic disease.

Table 4.1 Basic characteristics of a sample of insulin sensitive and insulin-resistant South African women

	Insulin resistant (n=16)	Insulin sensitive (n=21)	P value
Age (years)	30.9 ± 7.4	31.5 ± 8.1	0.08
Height (cm)	164.8 ± 5.8	164.7 ± 6.3	0.96
Weight (kg)	84.1 ± 20.9	62.5 ± 15.0	<0.01
Waist (cm)	89.9 ± 13.6	74.4 ± 9.7	<0.01
W-H ratio	0.8 ± 0.1	0.7 ± 0.1	0.006
BMI (kg/m^2)	30.7 ± 6.4	22.9 ± 7.3	<0.01
Body fat mass (kg)	34.7 ± 11.9	19.7 ± 9.6	<0.01
Body fat (%)	40.7 ± 5.7	30.8 ± 7.3	<0.01
Ethnicity % (B, M, C)	38; 25; 38	29; 19; 52	0.43
Skin resistance (Ω)	0.24 ± 0.08	0.32 ± 0.08	0.004
Family history of diabetes (n)	5 (31.3%)	7 (33.3%)	0.29
Family history of CVD (n)	5 (31.3%)	6 (28.6%)	0.86
MVPA (minutes/wk)	287.8 ± 287.1	416.4 ± 316.7	0.21

BMI, body mass index; W-H, waist to hip ratio; B, Black African; M, Mixed Ancestry; C, Caucasian; CVD, cardiovascular disease; MVPA, moderate-vigorous physical activity. Values are expressed as unadjusted means ± standard deviation (SD). Family history is expressed as number of subjects within each group and percentage of total sample in brackets.

Table 4.2 Metabolic characteristics of insulin-resistant and insulin sensitive groups

	Insulin resistant (n=16)	Insulin sensitive (n=21)	P values			
			Unadjusted	Adjusted for:		
				Waist	BMI	Waist and BMI
HOMA – IR	3.1 ± 1.8	1.0 ± 0.4	< 0.001	0.002	0.002	< 0.001
HOMA2%B	155.5 ± 87.8	52.2 ± 23.6	<0.001	0.005	0.005	0.005
Insulin (mU/L)	13.3 ± 7.4	4.2 ± 1.7	< 0.001	0.002	0.002	< 0.001
Glucose (mmol/L)	5.3 ± 0.4	5.2 ± 0.3	0.20	0.14	0.11	0.11
FFA (mmol/L)	0.4 ± 0.1	0.3 ± 0.1	0.06	0.07	0.12	0.11
Systolic BP (mmHg)	109.6 ± 10.3	114.2 ± 10.2	0.18	0.13	0.23	0.21
Diastolic BP (mmHg)	70.4 ± 9.3	72.2 ± 8.5	0.54	0.09	0.07	0.07

HOMA-IR, homeostasis model insulin resistance; HOMA2%B, % insulin secretion; FFA, free fatty acids. Values are expressed as unadjusted means ± SD.

Table 4.3 Endothelial-dependent microvascular response of ACh in insulin-resistant and insulin sensitive groups

	Insulin Resistant (n = 16)	Insulin Sensitive (n = 21)	P values			
			Unadjusted	Adjusted for		
				WC	SR	WC and SR
Area under the curve (PU)	78238.0 ± 43132.9	89504.5 ± 50663.7	0.48	0.16	0.07	0.12
Maximum absolute perfusion (PU)	421.0 ± 176.4	463.5 ± 173.4	0.47	0.11	0.05	0.08
Change from baseline (%)	420.9 ± 166.5	511.6 ± 214.8	0.17	0.05	0.01	0.04

PU, perfusion units; WC, waist circumference; SR, skin resistance. Values are expressed as unadjusted means of microvascular reactivity measures ± SD

Chapter 5

SUMMARY AND CONCLUSIONS

In developing countries such as South Africa, the epidemiological transition is associated with an increase in non communicable diseases (NCD) of lifestyle including obesity and diabetes[239]. The latest South African National Health and Nutrition Examination Survey (SANHANES-1) report[240] has revealed that obesity amongst South Africans has increased by 22% from 2002–2013, and is currently 40.1% in adult women and 11.6% in adult men. Furthermore, the same report showed that over a third of individuals (38.2%) between the ages of 15–24 years had blood pressure readings in the pre-hypertensive range and recent data from Peer et al.[2], has reported that 13.1% of South African adults are diabetic. It is well established that endothelial dysfunction characterises the initial stages of cardio-metabolic disease, and more specifically, microvascular endothelial function appears to precede macrovascular disease and the clinical manifestation of cardio-metabolic disease. Detecting these early changes in the microcirculation may present promising future therapeutic and preventative targets in apparently healthy individuals at risk for cardio-metabolic disease.

Microvascular endothelial function can be assessed in the clinical research setting with LDI and iontophoresis. The main objective of this thesis was to measure microvascular endothelial function with LDI and iontophoresis in a South African group of apparently healthy men and women of different ethnicities, and also to compare microvascular function in non-diabetic women with and without insulin resistance, and determine the role of visceral adiposity.

The main findings of the two studies included in this thesis are:

In study 1:

1. Microvascular reactivity was higher in a Caucasian group of healthy men and women compared to a Black African and Mixed Ancestry group
2. Endothelial-independent microvascular function remained attenuated in the Black African group after correcting for differences in skin resistance

Skin resistance was identified as a significant methodological factor that must be considered in future studies using this technique, particularly when comparing individuals from different ethnic groups. Moreover, the ethnic difference in

microvascular function in this young, apparently-healthy group may provide insight into the etiology of the disproportionate disease risk profiles amongst South Africans of different ethnicities. Attenuation of endothelial–independent microvascular function, even when adjusting for differences in skin resistance, appears to indicate alterations within the smooth muscle within different ethnic groups, but further studies are required in order to confirm this.

Taking the findings from Study 1 and the important role of skin resistance when measuring microvascular function, all comparisons in study 2 of this thesis were corrected for skin resistance. Obesity is associated with impaired microvascular endothelial function which has been demonstrated to increase peripheral vascular resistance and decrease insulin–mediated glucose uptake. It is therefore well established that obesity and insulin resistance often co-exist, however, understanding the role of visceral adiposity in the association between microvascular function and insulin resistance, in apparently healthy individuals, remains unclear.

The main findings in study 2 were:

1. ACh-induced iontophoresis was lower in non-diabetic, insulin resistant women compared to insulin sensitive women.
2. The relationship between ACh-induced microvascular reactivity and insulin resistance was dependent on differences in waist circumference and SR.

The second study demonstrated that differences in waist circumference, a proxy for visceral adiposity, explain the lower ACh-mediated vasodilation in insulin resistant women compared to insulin sensitive women. Equally important as in study 1, skin resistance played an important role in the final interpretation of results. Skin resistance is therefore also to be considered when using LDI and iontophoresis in overweight and obese individuals.

This thesis has shown that there are ethnic differences in microvascular endothelial function within a group of young, apparently healthy men and women, and that Black African men and women and individuals who present with insulin resistance may be at a high risk for microvascular dysfunction, and future cardio-metabolic disease. Laser Doppler imagery and iontophoresis has for the first time been used to measure microvascular endothelial function in South Africa, resulting in specific

methodological implications for future studies nationally and internationally. These implications emphasize the importance of adjusting for skin resistance in multi–ethnic groups, as well as in groups consisting of overweight or obese individuals.

The studies in this thesis were not designed to understand the mechanisms for these differences in microvascular endothelial function between these groups of apparently healthy South African men and women. Accordingly, further research will be required to investigate further smooth muscle function within the Black African population and how it may be associated with future disease risk, as well as differences in endothelial dependent microvascular function between insulin resistant and insulin sensitive individuals.

Future directions using this technique include increasing the sample size and comparing both ACh and SNP iontophoresis in a multi-ethnic, normal-weight, apparently healthy group of individuals with and without insulin resistance. In this way we can further unravel the complex underlying mechanisms linking microvascular endothelial dysfunction, insulin resistance and ethnicity between individuals without disease.

Furthermore, having found that visceral adiposity contributes to decreased endothelial-dependent microvascular function in insulin resistant women, evidence-based interventions would be a meaningful area to explore. More specifically, dietary[241-243] and/or physical activity[87;88;244] interventions have shown to improve endothelial function, therefore including such a strategy in future studies may yield significant public health implications that ultimately could lend itself to the reduction of insulin resistance and consequent prevention of type 2 diabetes mellitus.

Together, these studies have contributed to the understanding of LDI and iontophoresis methodology in different ethnic groups, and the association between endothelial-dependent microvascular function and visceral adiposity in non-diabetic, insulin resistant women.

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APPENDICES

STUDY 1

1.1 Informed Consent and study outline

Subject information sheet and consent

Endothelial function in normal weight, healthy individuals: is there a relationship between vascular health and body fat?

Aims of study

The aims of this study are therefore to measure vascular reactivity or the ability of the blood vessels on the surface of the arm to change diameter. This does not involve giving a blood sample. Instead, we simply apply solutions to the skin, that may change blood flow in the arm. We measure the change in skin blood flow with a laser. This technique is not unpleasant or painful. From these measures we hope to gain more knowledge on how body fat is associated with measures of endothelial function (blood vessel health).

Procedure:

This procedure involves coming to our laboratory (you will be reimbursed for your travel), and resting as you wait in our examination room. The skin on your forearm will be prepared with some distilled water and a small amount of sandpaper, to ensure that the equipment can "read" a signal from your arm. This may be mildly uncomfortable. You will have 6 different measurements, each lasting 7 minutes on your arm. Two different solutions, both which should increase skin blood flow, will be placed in a little disc which is situated on 3-4 locations on your arm. This solution will move through your skin painlessly via a small electrical current, which transferred to the disc. You should not feel anything painful, and may only occasionally feel a light tingling sensation. A small laser beam will scan your arm and allow the tester to monitor the vascular reactivity in your bloodvessels.

The entire series of tests may take up to 1.5 – 2 hours in length, and you will need to rest quietly on your back for the entire period of time.

Risks and benefits:

There are no known risks or side effects; however you may experience a tingling sensation. If this sensation becomes painful, you may just let the tester know that you are uncomfortable. There is also a slight risk of a local skin reaction to the solutions (Sodium Nitroprusside and Acetylcholine) used on the arm.

Information, confidentiality and statement of consent:

The participation in the trial is absolutely voluntary. You may refuse to participate or withdraw from the trial, at any time, without declaration of the reasons and without prejudice. New information that might be relevant to your willingness to continue participating in the trial will be communicated immediately to you as and when it becomes available. In addition, I am able to withdraw you from the study at any time if you are unable to complete any aspect of the trial protocol. All data from the trial will be stored in a computer database, and in a manner that maintains confidentiality–your identity will not be made available to the public at any time. Regulatory authorities and members of the Research Ethics Committee will be allowed access to the data for data verification and quality control purposes. However, these will only be under strict conditions of confidentiality. When the results are published, your identity will remain unknown and you will be informed of the results from the study.

Consent:

I, _____, have read or have had explained to me the procedures described above. I have had an opportunity to ask questions and these questions have been answered satisfactorily. I understand that I am free to withdraw from this study without prejudice at any time. I give my consent to participate in this study.

Name: _____ Signature: _____Date: _____

Witness: _____ Signature: _____

1.2 Information sent with reminder 2 days before testing

BLOOD VESSEL (VASCULAR) REACTIVITY TESTING

Important points to remember:

- Time of testing is in the morning
- Arrive having fasted overnight (You will get a snack and drink after the testing)
- Don't rush to the testing room. Once you've arrived you will lie down and relax for 10–15 minutes before the testing begins
- The testing room will be kept at a constant temperature of approximately 22 ° C, but there will be a blanket for you in case you get cold
- To assure that the results are accurate, please lie still during testing

Procedure:

After the resting period (acclimatisation)

- You will remain lying down, with your left arm facing palm-upward
- Your left arm will be wiped with an alcohol swap followed by a tissue dabbed in de-ionised water
    - This ensures that your arm is disinfected to prevent inaccurate results
- An electrode (small square pad) will be placed over your wrist area
    - This allows the small current to pass to your skin and may sometimes cause a slight tingle
- A little circular chamber (looks like a ring) will be placed over another area of your arm
    - This chamber (ring) will be filled with a solution which will result in blood vessel response and increase the vessel's blood flow
    - I'll be able to see the response on the computer screen (i'll be sitting with you in the room), and will record the data for interpretation
- 3 solutions will be used
    - Solution 1 will serve as a baseline measurement (control = no drug in solution)
    - Solution 2 will be the first drug (not harmful at all) that will elicit a change in your blood vessel diameter to increase blood flow
        - This is where I see how healthy your blood vessels are (the more they respond to the drug by increasing in diameter, the greater the blood flow, and the healthier the vessels)
    - Solution 3 will be the second drug which allows increases the blood flow

- Each time I add a solution to the chamber, I'll ask you when you are ready, and then a laser beam will scan the small area of the chamber
  - This is when you should be lying as still as you can (i.e no coughing, talking or moving around)
- The scanning takes a few minutes, after which I take the chamber off your arm and place it on another spot on your arm
  - During this time you can cough, talk or just continue relaxing
- This same procedure will follow until I have scanned 6 areas on your arm
  - 1 area (control) followed by area 2 and 3 (drug 1 = Acetycholine)
  - 4 (control) followed by 5 and 6 (drug 2 = sodium nitroprusside)
- Once I have scanned all 6 areas, I'll wipe your arm and blood vessel testing is complete

Body fat measurements

- Body fat will be measured in 2 ways:
  - Skin fold measurements
  - Bioelectrical impedance analysis (see below for more details)

Bio electrical impedance testing

Bioelectrical Impedance is measured when a very small electrical signal carried by water and fluids is passed through the body. Impedance is greatest in fat tissue, which contains only 10-20% water, while fat-free mass, which contains 70-75% water, allows the signal to pass much more easily. By using the impedance measurements along with a person's height and weight, and body type (gender, age, fitness level), it is possible to calculate the percentage of body fat, fat-free mass, hydration level, and other body composition values.

Using BIA to estimate person's body fat assumes that the body is within normal hydration ranges. When a person is dehydrated, the amount of fat tissue can be overestimated. Factors that can affect hydration include not drinking enough fluids, drinking too much caffeine or alcohol, exercising or eating just before measuring, certain prescription drugs or diuretics, illness, or a woman's menstrual cycle. Measuring under consistent conditions (proper hydration and same time of day) will yield best results with this method.

How to prepare for testing:

- All metal jewelry should be removed. Although you won't feel anything, metal removal improves accuracy.
- Avoid exercise or other activity that would make you sweat at least 8 hours before your test. This is important to get accurate body fluid results.
- Avoid caffeine or alcohol in large quantities 12 hours before the test.
- Go to the bathroom before the test to get rid of any waste products.
- Your height and weight will be measured. Since an accurate weight is important, remember to empty out your pockets, remove your shoes and any other heavy clothing.
- You will be asked to remove shoes and socks and lie down on a table.
- Two electrodes will be placed on your right hand and two more on your right foot.
- Once the leads are hooked up to the electrodes, the test only takes a few seconds.
- **No eating 4 hours before**
- **No eating or drinking 2 hours before**

1.3 Test sheet for principal investigator

SN:

Subject Personal Details

Name: _____

Surname: _____

Age: _____

Contact number: _____

Email address: _____

Resting blood pressure (mmHg): _____

Weight: (Kg) _____

Height: (cm) _____

Body fat percentage

BIA	RESISTANCE		REACTANCE		Body fat (%)
Skinfolds (mm) ∑ =	Bicep	Tricep	Subscap	Suprailiac	

STUDY 2

2.1 Informed consent and study outline

Subject information sheet and consent

The effect of insulin resistance on endothelial function in South African women

Introduction:

Currently, in South Africa, cardiovascular conditions such as diabetes, insulin resistance and hypertension are estimated as a high burden of disease (Norman et al.). Obesity and overweight, in addition to being associated with high blood pressure, high blood fats and high blood sugar is better known as metabolic syndrome and is linked to disease of the large and small blood vessels, leading to cardiovascular diseases. This has several implications, and by investigating this association, medical scientists can begin to study the "reactivity" or response of the blood vessels to various chemicals, which mimic reactions in the body, and learn new information on what factors may be controlling these relationships. Results of these and other studies may lead to important new findings which improve the manner in which we medically treat, manage or prevent these conditions. This study has important implications for the South African population, where Black and White women are presenting with different disease profiles.

Aims of study

The aims of this study are therefore to measure vascular reactivity or the ability of the blood vessels on the surface of the arm to increase blood flow. This does not involve giving a blood sample. Instead, we simply apply solutions to the skin,that may change blood flow in the arm. We measure the change in blood flow with a laser. This technique is not unpleasant or painful. From these measures we hope to gain more knowledge concerning insulin resistance, and its effect on microvascular reactivity.

Who is involved?

We are recruiting women who are not pregnant (under contraception), and who are not on any medication for any known illnesses (high blood pressure, diabetes, HIV, etc.). One group will have insulin resistance (HOMA ≥ 1.69), while the other group will not have insulin resistance (HOMA < 1.69). These women must refrain from smoking, eating or drinking anything except for water at least two hours before volunteering.

Many of you will have participated in studies with us before, however, all subjects will be asked to complete a questionnaire, to indicate that they have no known metabolic diseases including diabetes mellitus or disorders of thyroid metabolism; also remember that you should not be pregnant or breastfeeding, and your blood pressure must be normal to participate (BP < 140/90 mm Hg).

Procedure:

This procedure involves coming to our laboratory (you will be reimbursed for your travel), and resting as you wait in our examination room. The skin on your forearm will be prepared with some distilled water and a small amount of sandpaper, to ensure that the equipment can “read” a signal from your arm. This may be mildly uncomfortable. You will have 6 different measurements, each lasting 7 minutes on your arm. A solution which should increase your skin blood flow, will be placed in a little disc which is situated on 3 locations on your arm. This solution will move through your skin painlessly via a small electrical current, which passes through the small discs. You should not feel anything painful, and may only occasionally feel a light tingling sensation. A small laser beam will scan your arm and allow the tester to monitor the microvascular reactivity in your bloodvessels.

The entire series of tests may take up to 1 hour in length, and you will need to rest quietly on your back for the entire period of time.

Risks and benefits:

There are no known risks or side effects; however you may experience a tingling sensation. If this sensation becomes painful, you may just let the testor know that you are uncomfortable. There is also a slight risk of a local skin reaction to the solution used on the arm.

Information, confidentiality and statement of consent:

The participation in the trial is absolutely voluntary. You may refuse to participate or withdraw from the trial, at any time, without declaration of the reasons and without prejudice. New information that might be relevant to your willingness to continue participating in the trial will be communicated immediately to you as and when it becomes available. In addition, I am able to withdraw you from the study at any time if you are unable to complete any aspect of the trial protocol. All data from the trial will be stored in a computer database, and in a manner that maintains confidentiality – your identity will not be made available to the public at any time. Regulatory authorities and members of the Research Ethics Committee will be allowed access to the data for data verification and quality control purposes. However, these will only be under strict conditions of confidentiality. When the results are published, your identity will remain unknown and you will be informed of the results from the study.

Consent:

I, _____, have read or have had explained to me the procedures described above. I have had an opportunity to ask questions and these questions have been answered satisfactorily. I understand that I am free to withdraw from this study without prejudice at any time. I give my consent to participate in this study.

Name: _____ Signature: _____Date: _____

Witness: _____ Signature: _____

2.2 Information sent with reminder 2 days before testing

Effect of impaired glucose tolerance on endothelial function in South African women

The University of Cape Town is undertaking a study at the Sport Science Institute of South Africa (SSISA) in which two groups of will be compared. The one group will have insulin resistance, while the other group will have a normal insulin response. Each group will be compared according to microvascular reactivity using the iontophoresis and laser Doppler technique. This non-invasive technique allows the investigating team to observe changes in blood vessel distension (vasodilation) in response to acetylcholine (endothelium dependent) and sodium nitroprusside (endothelium independent). The study hopes to find a difference in vascular reactivity between the two groups.

We are trying to recruit women that are pre-menopausal and not pregnant. If you have a metabolic disease such as diabetes mellitus or HIV, or if you have a cardiovascular disease, you cannot be included in the study.

You, as a subject will be required to visit the Sport Science Institute of South Africa (SSISA) laboratory on two separate occasions. The first visit will comprise of screening tests and will rely on the subject having completed an overnight fast. A blood sample will be drawn to determine the fasting serum glucose concentration. This will enable the impaired glucose tolerant group to be distinguished, and will confirm that the other group portrays a “normal” glucose concentration. The blood sample will also determine each subject’s lipid profile (triglycerides, total cholesterol, high density lipoproteins and low density lipoprotein concentrations). This information will provide a greater understanding of each subject’s metabolic status.

The second visit requires that you fast and not ingest any caffeine or alcohol, nor do any exercise for at least two hours prior to testing. Vascular reactivity will be measured using the iontophoresis and laser Doppler imaging technique. For this technique, you will be asked to lie still in a quiet dark, temperature controlled (22°C) room for approximately one hour, while a small laser beam scans an area of your arm and a minute electrical pulse enters the skin at specific time intervals. Regional body fat distribution will be measured by using dual-energy x-ray absorptiometry (DEXA). This technique will determine how your fat is distributed throughout your

body.

3. Questionnaires for study 1 and study 2

FAMILY MEDICAL HISTORY

IDENTIFICATION AND CONTACT DETAILS
Name _____
ID number: _____
Date of Birth _____ Age: _____ Ethnicity: _____
Physical Address: _____
_____
Postal Address: _____
_____
Tel No's: _____ (h) _____ (w) _____ (Cell)
Alternative contact Person: _____ Tel No:_____

NO.	QUESTIONS AND FILTERS	CODING CATEGORIES
9	Now I would like to ask you about your family. Do you have a **close blood relative** (grandparents, father, mother, brother, sister or child) who has ever had any of the following conditions:	
9A	High Blood Pressure?	YES..... 1 NO ..... 2 DON'T KNOW ..... 8 If yes, who? ……
9B	Heart attack or angina or chest pain when exerting himself/herself?	YES..... 1 NO ..... 2 DON'T KNOW ..... 8 If yes,who……
9C	Was this relative younger or older than 50 years old when they first had a heart attack, angina or chest pain?	YOUNGER THAN 50 YEARS..... OLDER THAN 50 YEARS ..... DON'T KNOW .....
9D	Stroke?	YES..... 1 NO ..... 2 DON'T KNOW ..... 8 If yes, who? ……..
9E	Diabetes?	YES..... 1 NO ..... 2 DON'T KNOW ..... 8 If yes, who? …… Adult/ child onset? …….
9F	Obesity? (Were they abnormally large? Or have difficulty moving?)	YES..... 1 NO 2

NO.	QUESTIONS AND FILTERS	CODING CATEGORIES
		DON'T KNOW ..... 8 If yes, who? ……

Physical Activity

Next I am going to ask you about the time you spend doing different types of physical activity in a typical week. Please answer these questions even if you do not consider yourself to be a physically active person.

Think first about the time you spend doing work. Think of work as the things that you have to do such as paid or unpaid work, study/training, household chores, harvesting food/crops, fishing or hunting for food, seeking employment. *[Insert other examples if needed].* In answering the following questions 'vigorous-intensity activities' are activities that require hard physical effort and cause large increases in breathing or heart rate, 'moderate-intensity activities' are activities that require moderate physical effort and cause small increases in breathing or heart rate.

	Questions	**Response**	**Code**
Activity at work			
1	Does your work involve vigorous-intensity activity that causes large increases in breathing or heart rate like *[carrying or lifting heavy loads, digging or construction work*] for at least 10 minutes continuously? *[INSERT EXAMPLES] (USE SHOWCARD)*	Yes 1 No 2 *If No, go to P 4*	P1
2	In a typical week, on how many days do you do vigorous-intensity activities as part of your work?	Number of days ⌊__⌋	P2
3	How much time do you spend doing vigorous-intensity activities at work on a typical day?	Hours : minutes ⌊__⌋⌊__⌋ : ⌊__⌋⌊__⌋ hrs mins	P3 (a-b)
4	Does your work involve moderate-intensity activity that causes small increases in breathing or heart rate such as brisk walking *[or carrying light loads*] for at least 10 minutes continuously? *[INSERT EXAMPLES] (USE SHOWCARD)*	Yes 1 No 2 *If No, go to P 7*	P4
5	In a typical week, on how many days do you do moderate-intensity activities as part of your work?	Number of days ⌊__⌋	P5

6	How much time do you spend doing moderate-intensity activities at work on a typical day?	Hours : minutes ⌊_⌊_⌋ : ⌊_⌊_⌋ hrs mins	P6 (a-b)
Travel to and from places			
The next questions exclude the physical activities at work that you have already mentioned. Now I would like to ask you about the usual way you travel to and from places. For example to work, for shopping, to market, to place of worship. [insert other examples if needed]			
7	Do you walk or use a bicycle (*pedal cycle*) for at least 10 minutes continuously to get to and from places?	Yes 1 No 2 *If No, go to P 10*	P7
8	In a typical week, on how many days do you walk or bicycle for at least 10 minutes continuously to get to and from places?	Number of days ⌊_⌋	P8
9	How much time do you spend walking or bicycling for travel on a typical day?	Hours : minutes ⌊_⌊_⌋ : ⌊_⌊_⌋ hrs mins	P9 (a-b)
Recreational activities			
The next questions exclude the work and transport activities that you have already mentioned. Now I would like to ask you about sports, fitness and recreational activities (leisure), [insert relevant terms].			
10	Do you do any vigorous-intensity sports, fitness or recreational (*leisure*) activities that cause large increases in breathing or heart rate like [*running or football,]* for at least 10 minutes continuously? *[INSERT EXAMPLES] (USE SHOWCARD)*	Yes 1 No 2 *If No, go to P 13*	P10
11	In a typical week, on how many days do you do vigorous-intensity sports, fitness or recreational (*leisure*) activities?	Number of days ⌊_⌋	P11
12	How much time do you spend doing vigorous-intensity sports, fitness or recreational activities on a typical day?	Hours : minutes ⌊_⌊_⌋ : ⌊_⌊_⌋ hrs mins	P12 (a-b)