Exploring leptin from endothelial activation to subclinical organ damage in young black and white adults: The African-PREDICT study

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Thesis submitted for the degree Doctor of Philosophy in Science (Physiology) at the Potchefstroom Campus of the North-West University

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TABLE OF CONTENTS

PREFACE

This thesis is presented and organised in the article-format, which is an approved and recommended format according to the North-West University's guidelines for postgraduate studies. Below is an outline of this Ph.D. thesis.

- Chapter 1 comprises of a brief background, detailed literature review, problem statement, aim, objectives and hypotheses.

- Chapter 2 consists of the study design, research procedures and methodology and is in line with the African-PREDICT study protocol - the umbrella study for this present Ph.D. study, as well as the statistical analyses.

- Chapter 3 consists of the first manuscript titled, Leptin and its Relation to Autonomic Activity, Endothelial Cell Activation and Blood pressure in a Young Black and White Population: The African-PREDICT Study. This manuscript has been published in *Hormone and Metabolic Research* (2018).

- Chapter 4 consists of the second manuscript titled, Leptin and the Vasculature in Young adults: The African-PREDICT Study. This manuscript has been published in the *European Journal of Clinical Investigation (2019).*

- Chapter 5 includes the last and third manuscript titled, Leptin and the Retinal Microvasculature in Young Black and White Adults: The African-PREDICT Study. This article will be submitted to the *European Journal of Endocrinology* for publication.

- Chapter 6 is composed of the summary of main findings, recommendations and conclusions.

The references for each manuscript are at the end of each manuscript chapter division and listed according to the author's instructions for the specific journal in which the papers were either published or submitted for publication. For the rest of the thesis, references are at the end of each chapter in the Vancouver referencing style, which is the recommended referencing style

of the North-West University. References are in the order that they appeared or cited in the text. A maximum of 3 authors are listed, and in cases where there are more than three authors, the first three authors are recorded and then followed by *et al.*

AUTHOR CONTRIBUTIONS

Blessing O. Ahiante

The Ph.D. student participated in data collection of the African-PREDICT study, design and planning of research articles, data cleaning, statistical analyses, interpretation of data and writing of the draft manuscripts and the entire Ph.D. thesis.

Prof. Aletta E. Schutte

The study promoter and also the principal investigator of the African-PREDICT study was responsible for the initial research planning and design, acquisition and interpretation of data and revising critically for intellectual content, as well as provided guidance and direction for the entire thesis.

Prof. Wayne Smith and Dr. Leandi Lammertyn

As the study co-promoters of this Ph.D. study, they were actively involved in the following - initial research planning and design, collecting and interpretation of data, revised critically for intellectual content and publication, as well as provided guidance and direction for the entire thesis

Below is the statement of attestation and signed signatures of all the co-authors, attesting to their roles in the study and also giving consent that the manuscripts should form part of this thesis.

I hereby declare that I approved the above manuscripts and that my role in this thesis as mentioned above, is a true representation of my actual contribution to the study. I also give my consent that the manuscripts should be published as part of the Ph.D. thesis of Blessing Osemengbe Ahiante

Prof. AE. Schutte

Prof. W. Smith

Dr. L. Lammertyn

PUBLICATIONS, CONFERENCE PRESENTATIONS AND OTHER ACTIVITIES RELATING TO THE THESIS

Publications

- Ahiante BO, Smith W, Lammertyn L, Schutte AE. Leptin and its Relation to Autonomic Activity, Endothelial Cell Activation and Blood Pressure in a Young Black and White Population: The African-PREDICT study. *Hormone and Metabolic Research*. 2018;*50(03):257-266.* **Appendix B**

- Ahiante B), Smith W, Lammertyn L, Schutte AE. Leptin and the Vasculature in young adults: The African- PREDICT study*. European Journal of Clinical Investigation. 2018;49(1):e13039.* **Appendix C**

Conference presentations

- The first manuscript, Leptin and its Relation to Autonomic Activity, Endothelial Cell Activation and Blood Pressure in a Young Black and White Population: The African-PREDICT study was presented at the First Conference of Biomedical and Natural Science and Therapeutics (CoBNest) conference, Stellenbosch, South Africa, 07-10 October 2018*.* ***Poster presentation****.*

- The second manuscript, Leptin and the Vasculature in young adults: The African- PREDICT study was presented at the 18th International Congress of Endocrinology, Cape Town, South Africa, 01-04 December 2018. ***Poster presentation****.*

Other activities

- I won the CoBNest travel grant award to present the first manuscript of this Ph.D. study at the First Conference of Biomedical and Natural Sciences and Therapeutics conference in Stellenbosch, South Africa.

- As the study aims to contribute towards a hypertension prevention strategy in Africa, I was actively involved in science engagements and communications, where I raise blood

pressure awareness and also gave health education (dietary advice such reducing of salt intake and consumption of beets) in different provinces of South Africa, including the community of the study participants. My contribution in this regard was featured in Nature Outlook with Nobel Laureate Peter Agre**.https://www.nature.com/articles/d41586-018-06972-3**

- I was the International Society of Hypertension New Investigator Spotlight in April 2018, where the Ph.D. study and potential contributions were also featured. **http://ish-world.com/new-investigators-spotlight/i/April-2018-spotlight-of-the-month/.**

- I was selected among the top 600 scientists from 84 nations by the Council of Nobel Laureates Meetings to visit 43 Nobel Laureates in medicine and physiology in Germany (2018 June). Highlights of this Ph.D. study were featured in the book of abstracts of the Lindau Nobel Laureate meeting.

- Lastly, this Ph.D. study also attracted the NRF-DST innovation scholarship award.

SUMMARY

Title: Exploring leptin from endothelial activation to subclinical organ damage in young black and white adults: The African-PREDICT study

Motivation

Cardiovascular diseases (CVDs) are the most predominant non-communicable diseases worldwide. The most prominent risk factor that is accountable for the development of CVD, namely hypertension, is drastically increasing particularly in sub-Saharan Africa. Recently, obesity emerged as the single most essential risk factor for the development of hypertension and other related metabolic disorders. The mechanisms by which obesity may lead to hypertension development is not yet fully established. Epidemiological studies suggest leptin as a crucial factor underpinning obesity-associated hypertension and cardiovascular disorders primarily in older, obese and diseased individuals. To increase our understanding of the earlier phases of hypertension and CVD development, we, therefore, investigated what role leptin is already playing in healthy young adults who are at risk of hypertension development.

Aim

The central aim of this study was to determine the relationships that exist between measures of autonomic activity, endothelial activation, blood pressure, large arterial structure and function, as well as the retinal microvasculature with leptin in young black and white adults.

Method

This present cross-sectional study is a part of the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT study). All the available baseline data of the first 820 participants in the African-PREDICT study were used in this present Ph.D. study. The study participants comprised of both white (n= 389) and black (n= 431) young healthy men and women between the age bracket 20-30 years.

Participation in the study was voluntary, and the participants signed consent forms before conducting any measurements.

Standard procedures and methods were used to capture all data and included questionnaire data (standard general health and demographic questionnaire), body composition and accelerometry assessments (anthropometric, bioelectrical impedance and accelerometry assessments) and cardiovascular measurements (measures of autonomic function, blood pressure, micro- and macro vascular functions), as well as biochemical analyses of all relevant biomarkers used in the study.

For statistical analyses, variables that were not normally distributed were log transformed, means and proportions were compared using either independent t-tests, Chi-square tests or analysis of variance or covariance. Single, partial and multiple regression analyses were used to investigate associations between main variables of interest (independent variable: serum leptin and dependent variables: measures of autonomic function, endothelial cell activation and function, blood pressure, large artery structure and function as well as retinal microvasculature). In either the partial or multiple regression analysis, confounders were adjusted for. In all cases, $p \leq 0.05$ was used to indicate statistical significance.

Results and conclusion of each manuscript

Article 1: We investigated the associations between measures of autonomic function (24-hour heart rates and heart rate variability measures), endothelial cell activation (intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1)) and blood pressure (24-hour blood pressure measures) with leptin, in a young black and white population. Our results showed an independent and a consistent association between markers of autonomic activity (such as 24-hour heart rate, day and night-time heart rate as well as heart rate variability total power) and leptin in both white (all $p \leq 0.001$) and black men (all $p \leq 0.040$). These particular associations were either not found or less prominent in women, despite their almost ten-fold higher leptin levels than men. An independent association was observed between 24-hour diastolic blood pressure with leptin

(Std β = 0.37; p=0.006) only in white men. No independent association was observed between leptin and indicators of endothelial cell activation, irrespective of ethnicity or sex. This study, therefore, suggests the potential role that leptin might play in future blood pressure elevation especially in men.

Article 2: We explored the relationships that existed between leptin and large artery structure and function and a marker of endothelial dysfunction, namely von Willebrand (vWF) factor in young healthy men and women of the African-PREDICT study. The results from this cross-sectional association showed that in healthy young men, leptin was independently and negatively associated with carotid intima-media thickening (CIMT) (R^2=0.05; β=-0.20; p=0.036) and cross-sectional wall area (CSWA) (R^2=0.05; β=-0.20; p=0.035) in a multivariable-adjusted regression analysis. The association was confirmed in only the overweight healthy men after dividing the participants into the various body mass index categories (CIMT: R^2=0.15; β=-0.41; p=0.007; CSWA: R^2=0.21; β=-0.47; p=0.002). No association was observed in women or between pulse wave velocity and vWF with leptin in any group. This study suggests the potential vascular protective role of leptin in the macrovasculature especially in healthy young men without any overt CVD.

Article 3: This article examined whether measurements of the retinal microvasculature are associated with leptin in healthy young black and white men and women. Black men had lower body weight and lower leptin than white men, whereas black women had increased adiposity and leptin compared to white women (all p<0.001). Black individuals had narrower artery, and greater maximum arteriolar and venular dilations in response to light flicker than the white groups (p<0.001). In all groups except black women, arterio-venous ratio was inversely associated with leptin (all p≤0.044), but the association was lost upon adjustment for body mass index and other covariates. We also observed a negative relation between maximal venular dilation and leptin only in black men in single and multiple regression analyses (Std β=-0.22; R^2= 0.05; p=0.035). Lastly, no associations were found between other retinal measures with leptin in the other groups. The study suggests the potential detrimental role of leptin in

microvasculature of black men who also have been reported to be at a greater risk of CVD development.

General conclusion

Despite the higher leptin levels in women than in men, leptin showed an independent association with more measures of cardiovascular function in men than women by showing a consistent association with more markers of autonomic activity in all men, and blood pressure only in white men. Leptin also showed an independent and negative association with measures of subclinical atherosclerosis in all men. However, in the young black men, leptin associated negatively with retinal venular dilation in response to external stimulus. This study, therefore, highlights the potentially beneficial and detrimental associations of leptin with cardiovascular estimates in healthy young adults, particularly men. Our findings suggest that leptin-induced cardiovascular action in young adults are influenced by sex, ethnicity and the different vascular bed.

Keyword: Adipokine, heart rate variability, ethnicity, sex, vascular function, atherosclerosis, healthy, overweight, carotid intima-media thickness, endothelium, microcirculation.

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LIST OF ABBREVIATIONS

African-PREDICT	African Prospective study on the Early Detection and Identification of Cardiovascular disease and HyperTension
ATP	Adenosine triphosphate
AMS	Artery Measurement Systems software
Akt-eNOS	AKT-endothelial nitric oxide synthase
AEE	Active energy expenditure
ABPM	Ambulatory blood pressure monitoring
AVR	Arterio-venous ratio
ANCOVA	Analysis of covariance
Adj	Adjusted
BMI	Body Mass Index
BHS	British Hypertension Society
BP	Blood pressure
Bpm	Beats per minute
BW	Body weight
β	Standardised beta regression coefficients
CVDs	Cardiovascular diseases
CD4	Cluster of differentiation 4
CIMT	Carotid intima-media thickness
CSWA	Carotid cross-sectional wall area
CRAE	Central retinal artery equivalent
CRVE	Central retinal vein equivalent

CSCR	Central serious chorioretinopathy
CRP	C - reactive protein
CM	Centimetre
CI	Confidence interval
cfPWV	Carotid-femoral pulse wave velocity
DBP	Systolic blood pressure
DVA	Dynamic vessel analysis
D	Diameter
Et al.	*Et alia* ‘and others’
EO	Energy output
EI	Energy input
ERK1/2	Extracellular signal-regulated
ECG	Electrocardiography
EJCI	European Journal of Clinical Investigation
eNOS	endothelial nitric oxide synthase
FMD	Flow-mediated dilation
FLIP	Flicker light-induced provocation
GPAQ	Global physical activity questionnaire
GGT	Gamma-glutamyltransferase
HART	Hypertension in Africa Research Team
HIV	Human immunodeficiency virus
HR	Heart rate
HRV	Heart rate variability

HF	High frequency
HDL-C	High-density lipoprotein-cholesterol
IRS/ PI3K	Insulin receptor substrate / phosphatidylinositol 3-kinase
JAK2/ STAT3	Janus kinase 2 / activator of transcription 3
Kb	kilobase
kCal	kilocalorie
LEP/OB gene	Lep for leptin/ Ob for obese
LEPR (OBR)	Leptin receptor (OB for obese) receptor
(LEPRb)	long LEPR isoform of leptin
LF	Low frequency
LDL-C	Low-density-lipoprotein cholesterol
L	Litre
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
MVPA	Moderate-Vigorous Physical activity
MAP	Mean arterial blood pressure
MCP-1	Monocyte chemoattractant protein-1
MVD	Maximum venular dilation
M	Metre(s)
mmHg	Millimetres of mercury
m/s	Metres per second
mmol/l	Millimole per litre
mm	Millimetre(s)

min	Minute
MU	Measuring units
MMM	May Measurement Month
NWU	North-West University
NF	Nuclear factor
n. u.	Normalised unit
ng/ml	nanogram/ millilitre
N	Number of
NN	Normal to normal
%	Percentage
Ph.D.	Doctor of Philosophy
PI3k	Phosphatidylionositol-3-kinase
PK	Protein kinase
PWV	Pulse wave velocity
P	Probability
pg/ml	Picogram/ millilitre
RhoA	Ras homolog gene family, member A
ROS	Reactive oxygen species
RSNA	Renal sympathetic nervous system hyperactivity
RVA	Retinal vessel analysis
R^2	Relative predictive power of a model
RAS	Renin-angiotensin system
r	Correlation coefficient

SES	Socio-economic status
sICAM-1	Soluble intercellular adhesion molecule-1
sVCAM-1	Soluble vascular cell adhesion molecule-1
SDNN	Standard deviation of normal to normal interval
SBP	Systolic blood pressure
SNA	Sympathetic nervous system activity
SOCS3	Suppressor of cytokine signalling 3
SD	Standard deviation
SE	Standard error
TP	Total power
U/L	Units per litre
VSD	Vein segment diameter
vWF	von Willebrand factor,
WHtR	Waist-to-height ratio
χ^2	Chi-square tests
zBMI	Body mass index z-score

CHAPTER 1

Background, Literature Overview, Aim, Objectives and Hypotheses

1 Background

Deaths from cardiovascular disease (CVD) are increasing globally, and in sub-Saharan Africa, it is the primary cause of death from age 45 [1, 2]. The combinations of effects from an epidemiological change in CVD, population growth and ageing are the primary driver of death resulting from CVD [2, 3]. Several factors contribute to the global burden of CVD and include - hypertension [4, 5], obesity [6], diabetes, dyslipidaemia [7], genetic [8], stroke, heart diseases [9] and lifestyle factors. The lifestyle factors involved are wide-ranging and include excessive alcohol consumption [10], smoking [11], physical inactivity and unhealthy diets [6]. In South Africa (an upper-middle income country), over 22% of all deaths are due to stroke and heart diseases [5].

Obesity is associated with increased systemic inflammation [12] and has a marked impact on aspects related to CVD, such as increased sympathetic activity [13, 14], blood pressure [15], endothelial cell activation [16] and alterations to vascular structure and function [17]. It contributes to the development of hypertension [18], atherosclerosis [19], renal pathology [20, 21] resulting in an increased burden of CVD. In the long run, especially when ignored, obesity can result in cardiovascular morbidity and mortality [22]. Currently, the mechanisms responsible for obesity-related cardiovascular pathologies are not yet fully understood [23, 24]. In this regard, the adipocytokine, leptin, has been suggested as a possible link between obesity and its related cardiovascular complexities [25-31].

Leptin was initially known to regulate appetite, energy balance and weight gain [32, 33], but research over recent years has shown its involvement in several cardiovascular functions that may be either beneficial [34] or detrimental [28]. Leptin has been shown to maintain vascular homeostasis by inducing nitric oxide synthesis [35] which then results in vasodilation in both humans [36] and experimental animal studies [34]. Regarding the detrimental effect, leptin in its pathophysiological state can result in the following - enhanced sympathetic activity [37], hypertension development [38, 39], vascular dysfunction [40, 41], cardiovascular-related target organ damage [42] and increased risk of mortality [43-45]. The leptin-induced complexities are

particularly noted within the context of older, obese and diseased populations [31, 46, 47]. Although beyond the scope of this study, most studies evaluating leptin levels with mortality in the elderly found a U-shape relationship with greater risk of death at both low and high levels of leptin [31, 47].

Leptin is not only associated with detrimental cardiovascular health in the older, obese and diseased individuals [30, 31, 47-49], but a study has shown leptin's association with reduced arterial compliance in healthy children (13-16 years) independent of adiposity [43]. Reduced arterial compliance is an important predictive factor in the development of hypertension [50, 51], increased afterload and left ventricular dysfunction [51]. Also, in a group of normotensive middle-aged (34-65 years) Japanese men, leptin associated positively with diastolic blood pressure [52]. Following the above findings, it is therefore essential to investigate what role leptin is playing in the cardiovascular profile of healthy young adults - particularly those from Africa, who may be at risk of hypertension development [53-55], as well as the epidemic of obesity [55].

A comprehensive literature overview of the relevant background of leptin and its possible relationships with cardiovascular function follows. Also, in this section, the possible mechanisms by which the adipokine, leptin, may result in the development of various cardiovascular diseases, as well as the arguments surrounding leptin's role on cardiovascular health are elaborated.

2 Literature overview

2.1 Discovery of leptin

The discovery of leptin by Zhang et al. [56] in the year 1994 through the process of positional cloning in the ob/ob mice was original due to its potential for the treatment of obesity [57]. After its discovery, leptin was named leptos - a Greek word meaning thin [56, 58] and the identification of leptin has helped to uncover the role of the adipose tissue as an endocrine organ [59]. The adipose tissue with a specific emphasis on the white fatty tissue synthesises

and releases the hormone leptin. This synthesised leptin is primarily responsible for the regulation of energy input (food intake), expenditure and weight regulation [60].

2.2 Leptin's structure, sites of production and receptors

Leptin, a pleiotropic and vasoactive peptide hormone derivative, is encoded by the LEP/OB gene [56]. Leptin's encoded gene is located on the 7 alpha 31.3 chromosome [61] and comprises of three exons and two introns, occupying about 18 kilobase pairs (kb) of the human brain [62, 63]. The crystal structure of leptin is similar to the long-chain helical structure of the cytokine family [64]. The anti-parallel elongated leptin peptide is composed of four different α-helices (A, B, C and D) and is superimposed to each other with several hydrophobic residues which gives it its high solubility features [65].

Apart from the white adipose tissue which is reported to be the primary site of leptin's production, leptin is also synthesised in small amounts in other regions of the body such as the stomach, mammary gland, placenta and heart [66-69]. In humans, leptin is released into the circulatory system in a pulsatile fashion and has a circadian rhythm [70]. Leptin's release is at its highest peak between midnight and early morning and is mostly attenuated in the mid-afternoon [71].

After leptin's production and secretion, leptin acts through its receptor called the LEPR (OBR) which belongs to the subclass called, the class I type of the cytokine receptor family [64]. Numerous LEPR isoforms exist from alternative mRNA splicing but are all products of one LEPR gene. The LEPR isoforms or subtypes fall into three categories and include - murine (LEPRe), short (LEPRa) and long (LEPRb) LEPR isoforms [72]. Although Lee et al. [73] originally proposed five LEPR isoforms namely: LEPRa, LEPRb, LEPRc, LEPRd, and LEPRe [74], it is the LEPRb isoform of leptin that is the most extended and critical for leptin's action [75]. The long leptin subtype is mostly expressed in the brain, including the hypothalamic neurons and implies that the hypothalamus is the major site for leptin's action [74, 75]. The short LEPR subtype was observed to mediate the transport of leptin across the blood-brain barrier in rat brain microvasculature [76].

Besides the hypothalamus, the presence of leptin receptors in other regions of the body such as in the vasculature, heart, stomach, pancreas and placenta [67, 74, 77, 78], depicts several other potential roles for leptin in the periphery [74, 79].

2.3 Leptin levels, sex and ethnicity

Although, plasma leptin levels are directly proportional to body fat mass [80] as well as insulin levels [81], the exact normal physiological levels of leptin may be difficult to define due to physiological inter-individual differences (gender, race and genetic variability). Regardless of this, women are reported to have higher leptin levels than men [82] and the suggested explanations for the gender differences are, (a) higher percentage of fat mass and (b) increase in the production of leptin per unit mass of the white adipose tissue in women [83, 84]. Another plausible explanation is sex differences in hormone production [85]. Estrogen is known to aggravate leptin production in both animals and humans [86].

Apart from sex, leptin levels may also differ by ethnicity [87]. A study conducted in the American population showed that black individuals have higher leptin concentration than their white counterparts (70-79 years). In this study, higher leptin concentration in black individuals was not explained by the different body fat distribution [87]. Also, in a group of the South Africa population (mean age 45 years), leptin was observed to be higher in persons from black than white ethnicity [45, 88].

2.4 The primary physiological role of leptin

Leptin reflects the status of long-term energy stores [89]. The interplay between leptin and its receptors in the hypothalamus - mainly the arcuate nucleus of the hypothalamus [90], results in satiety and energy balance and consequently, weight regulation [91]. Leptin reaches its receptor site in the hypothalamus by its saturable transport system. Its interaction with the hypothalamic leptin receptor results in the transcription and activation of many signal transduction pathways [75]. The main signalling pathways that are activated include the Janus kinase 2 (JAK2)/ activator of transcription 3 (STAT3) and insulin receptor substrate (IRS)/phosphatidylinositol 3-

kinase (PI3K) pathways. The activation of suppressor of cytokine signalling 3 (SOCS3) pathways inhibits the JAK2/STAT3 activity through a negative-feedback mechanism. The combinations of action, including the activation, expression and downstream phosphorylation of the JAK2/STAT3 signal transduction pathway is vital for the long term regulation of energy balance [75, 92].

The physiological responses following leptin's signalling and second messenger interactions that bring about satiety and energy balance are as follows: (A) The downregulation of the orexigenic neuropeptides (the hunger hormones) such as - neuropeptide Y, melanin-concentrating hormone, orexins and agouti-related peptide, as well as their hormonal activities. (B) The upregulation of the anorexigenic neuropeptides (the appetite suppressant neuropeptides) such as an α-melanocyte-stimulating hormone, cocaine and amphetamine-regulated transcription and corticotropin-releasing-hormone and, with their neuronal activities [93, 94]. In addition to this, several other studies reported that the activation of pro-opiomelanocortin neurons in the arcuate nucleus of the hypothalamus also brings about satiety [95, 96]. The physiological effects of leptin membrane receptor interactions aredescribed in Figure 1 below.

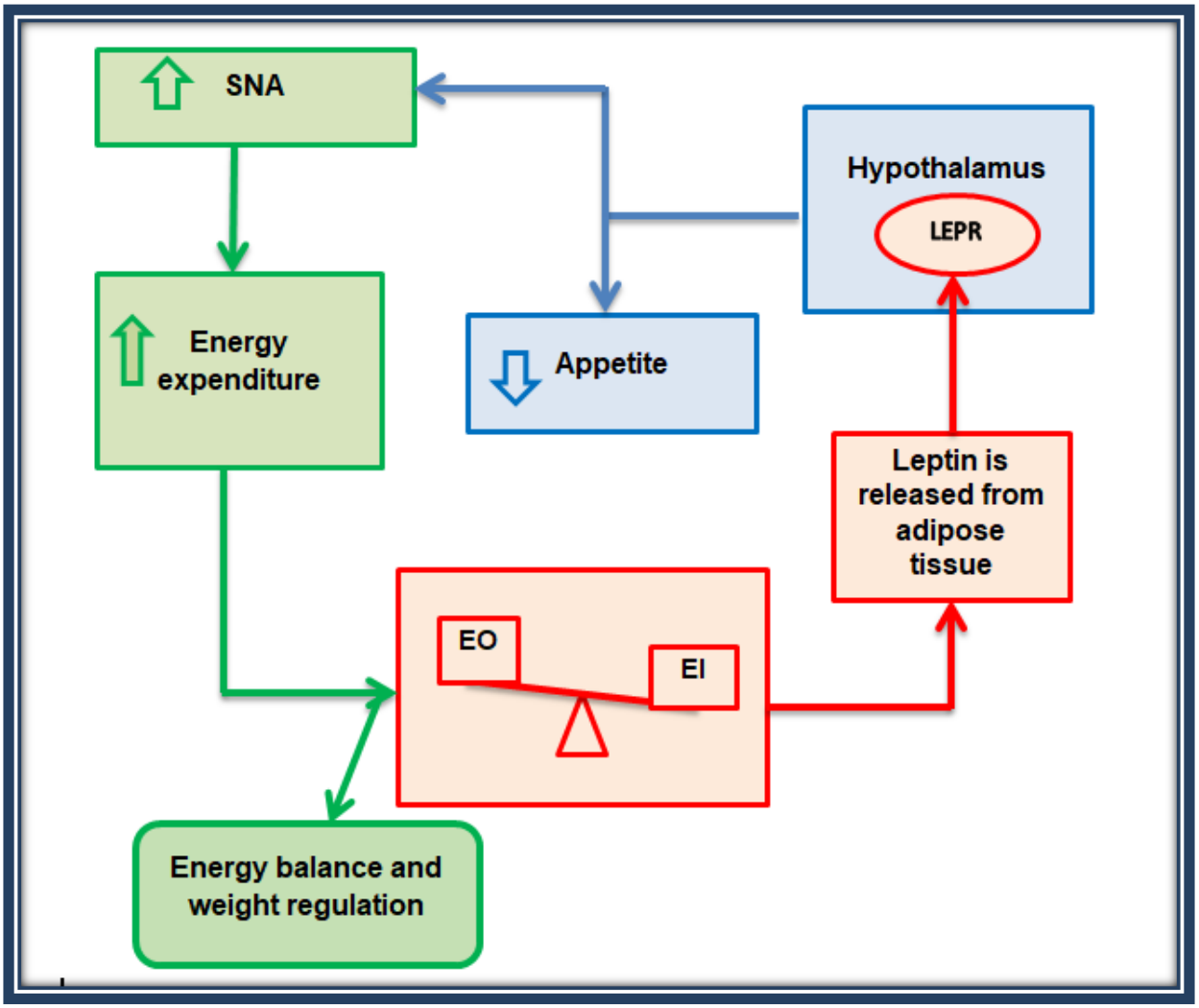

Figure 1: A feedback loop regulatory system showing leptin's role in the regulation of appetite, energy balance and weight regulation. Modified from Morris and Rui [97]. EO, Energy output; EI, Energy input; LEPR, leptin receptor and SNA, sympathetic nervous system activity.

***Legend**: The inhibition of the orexigenic and the activation of the anorexigenic neuropeptides as well as their neuronal activities involves a complex feedback loop system with three different regulatory steps as shown in Figure 1. Step A (indicated in red) shows leptin as a sensor of energy imbalance that is released when energy intake exceeds energy output. Step B (indicated in blue) shows the hypothalamus which is the site of action for leptin. The binding of leptin to its receptor in the hypothalamus stimulates a cascade of behavioural attitude events that ultimately leads to decreased appetite. Step C (indicated in green) shows the activity of the sympathetic nervous system as it helps to reinstate energy balance by increasing energy expenditure (increased metabolism) [98, 99].*

Apart from the main actions of leptin (satiety and energy balance), the pleiotropic nature of leptin also allows leptin to have a paracrine and autocrine function in the periphery [100]. This includes its participation in glucose and lipid metabolism [78, 101-103], growth development [104, 105], normal reproductive function [12, 106], immune responses [12, 107] and many cardiovascular functions like vascular tone regulation [108-113]. It is also involved in the normal regulation of myocardial metabolism and cardiac function [114, 115]. Conversely, leptin in its abnormal state termed hyperleptinaemia results in several adverse effects both centrally and peripherally, where leptin resistance may occur as noted in obese individuals [116, 117]

2.4.1 Leptin resistance

Obesity is regularly accompanied by high circulating leptin and diminished sensitivity to leptin's usual physiological role further indicating a state of leptin resistance (Figure 3) [118]. Resistance to leptin may result from the following suggested underlying factors: (a) an alteration in the propensity of leptin's uptake by the blood-brain barrier, (b) the down- regulation of the leptin receptor (LEPRb) in the hypothalamus [32, 105] and (c) an alteration of its signalling at both the central and peripheral levels [118]. Other factors that could lead to the development of this condition are high triglycerides [119] and inflammatory activities [116]. Despite this, the detailed mechanisms behind the development of leptin resistance remain unclear and form part of the characteristic features of obesity in humans [120, 121]. Leptin resistance is characterised by sympathetic hyper-reactivity, systemic vascular inflammatory responses [12], vascular smooth muscle remodelling and dysfunction [48]. Resistance to leptin is also associated with clusters of the metabolic syndrome such as abdominal obesity, hypertension and atherosclerosis [122]. These are all crucial in the pathogenesis of the CVD burden and mortality [123-127].

It is well established that in an obese individual, leptin resistance occurs in the central nervous system, where the sensitivity of leptin's effect in the hypothalamus to cause satiety is reduced [118]. Controversial findings still exist as to whether leptin resistance also extends to the peripheral system like the vasculature. Some studies suggested that in the condition of obesity,

leptin resistance might extend to the periphery thereby resulting in detrimental vascular function, progression of atherosclerosis and other related obesity-associated cardiovascular diseases [128, 129]. Also, in the microvasculature, leptin displayed the potential to cause retinal microvascular deterioration in obese children [130]. In contrast, some others studies showed that even with leptin's reduced sensitivity in the hypothalamus (state of hyperleptinemia), leptin still showed a potential vascular protective role in the macrovasculature and also in the microvasculature [131-133]. Momin et al. [132] supported the concept of tissue specificity and suggested that leptin resistance may vary depending on the different cell types with no negative impact on the vasculature in obese individuals. Another study conducted in severely obese children - where leptin is noted to be elevated withresistance occurring in the hypothalamus [118], suggested a beneficial vascular protective role of leptin [134].

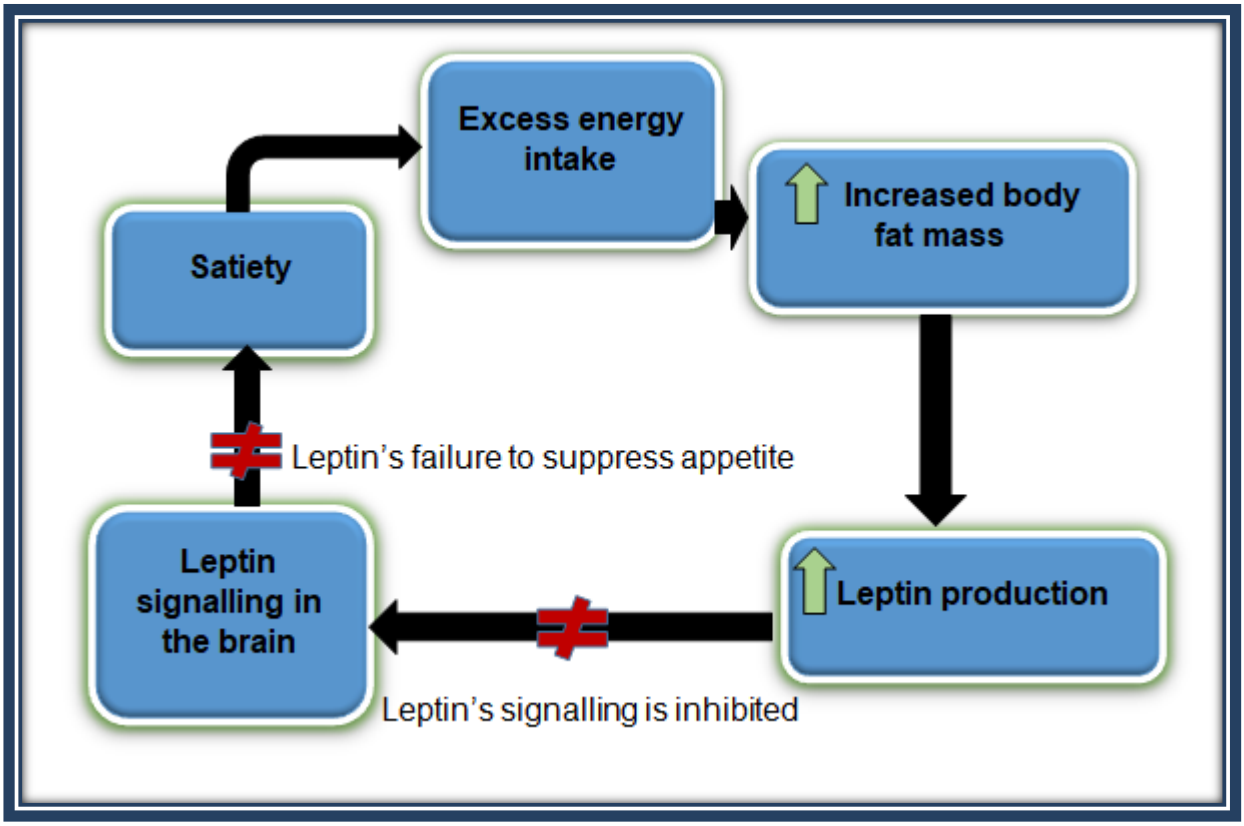

Figure 2: A model illustrating the processes involved in the condition of leptin resistance.

Legend: *In leptin resistance, leptin signalling and effect in the brain is altered, and as a result, the brain is not able to effectively suppress appetite.*

2.5 Leptin and its potential beneficial role in cardiovascular health

Physiologically, intact endothelial cells function as the chief regulator of vascular homeostasis [135], by keeping a balance between vasoconstrictory and vasodilatory agents [136, 137]. Leptin may play a role in the regulation of vascular tone [138] through local mechanism since its receptors are also present in the endothelium [139] and vascular smooth muscle cells [140]. Studies reported that the endothelium and vascular smooth muscle cell wall express different isoforms of leptin receptor and can modulate a wide range of intracellular signalling pathways, mediating many relevant biological actions [141, 142] Importantly, leptin has been shown to increase vascular endothelium and smooth muscle cell nitric oxide synthesis, which then stimulate vasodilation [35, 142]. According to Vecchione et al. [35] leptin stimulates nitric oxide synthesis through the activation of Akt-endothelial nitric oxide synthase (eNOS) phosphorylation pathway. Another study showed leptin's role in maintaining the balance between vasodilatory (nitric oxide) and vasoconstrictory (endothelin-1) substances in individuals without associated metabolic disturbances [143].

Besides leptin's vasodilatory effects through the endothelial-derived nitric oxide-dependent mechanism [35], some other studies have demonstrated leptin's direct vasodilating effect on vascular smooth muscle cells which is not dependent on nitric oxide [36, 132]. A study conducted by Momin et al. [132] proposed that leptin may exert its direct vasodilating effects through hyperpolarisation - which involves activation of the ATP-sensitive potassium channels, similar to its impact in the pancreatic beta-cells [144]. Also, recent studies have shown leptin's role in the mobilisation of beneficial vascular endothelial progenitor cells [108, 134, 145], which are also known for the maintenance of vascular homeostasis and reduction in plaque formation [146].

Whether leptin possesses anti-hypertrophic properties on the vascular smooth muscle cells is still not clear but seems plausible. Rodríguez et al. [147] showed the anti-proliferative effect of leptin on vascular smooth muscle cells that were induced by angiotensin II, through a nitric oxide-dependent mechanism. Also, leptin induced-beneficial endothelial progenitor cells which

were recruited into the neointima cell of the blood vessel, have been observed to reduce neointima growth after vascular injury [148]. Lastly, the injection of leptin in ob/ob mice has shown a significant reduction in wall thickness and size of cardiac myocyte [115] and may be related to its anti-proliferative properties on the vascular smooth muscle cells [147].

The physiological roles of leptin in the maintenance of vascular homeostasis and cardiac smooth muscle cells are predictive for a normal systemic haemodynamic and will be of relevance in the normal functioning of the cardiovascular system.

2.6 Leptin and its potential detrimental role in cardiovascular health

Several studies implicate leptin as a risk factor involved in the development of various cardiovascular diseases including atherosclerosis, stroke, myocardial infarction and coronary events in either overweight or obese individuals [88, 149, 150]. The suggested mechanisms linking leptin to detrimental cardiovascular health are complex. According to Rahmouni et al. [141], leptin has several actions on the endothelium, and little is known about the exact mechanism by which leptin receptor regulates the different molecular pathways in the endothelium. However, Huang et al. [151] suggested the involvement of phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), and nuclear factor (NF)-kBp65 pathway in vascular smooth muscle cell proliferation and migration. Other suggested signalling pathways and mechanisms by which leptin may induce atherosclerosis disease progression include JAK/STAT, Phosphatidylinositol-3-kinase (PI3K)/Akt, p38 mitogen-activated protein kinase (MAPK), mammalian target of rapamycin (mTOR), and RhoA/ROCK second messenger/signal transduction pathways [142]. Leptin has also been suggested to play a role in vascular deterioration through the generation of reactive oxygen species (ROS) and leptin-induced activation of angiotensin and endothelin systems and activities [142, 152].

2.6.1 Leptin and endothelial cell activation

The most widely acceptable barometer for endothelial dysfunction is the loss of vascular endothelium-dependent vasodilatation, which is crucial in the development of atherosclerosis

and other related CVDs [153, 154]. Hyperleptinaemia has been suggested as a possible risk factor for endothelial dysfunction in obese individuals [113]. Herein, leptin may contribute to the development of endothelial dysfunction by subjecting the surface of the endothelial cell to structural disorder. Park et al. [155] reported that leptin could stimulate endothelial cell activation and proliferation and also stimulate matrix metalloproteinases expression. In the presence of high circulating leptin, leptin preferentially induces the production of Ang II and endothelin-1 over nitric oxide production and thus mediate endothelial cell activation [124, 142, 156, 157].

Activated endothelium increases the production of inflammatory cytokines, expression of monocyte chemoattractant-1 protein (MCP-1), soluble intercellular adhesion molecules-1 (sICAM-1) and soluble vascular adhesion molecules-1 (sVCAM-1), among others [158-160]. The study by Santos-Alvarez et al. [161] showed that leptin act as a stimulatory hormone of the human circulating monocyte, increasing the appearance of monocyte producing cytokines in a dose-dependent fashion. Another study by Yamagishi et al. [162] showed that leptin induces a dose-dependent increase in the expression of ROS and MCP-1 by increasing fatty acid activation through protein kinase (PK) A signaling pathway in bovine aortic endothelial cells. Leptin is therefore suggested to be a pro-inflammatory cytokine and pro-oxidant agent [161, 163]. However, a study showed a dose-dependent reduction in the expression of MCP-1 in aortic root atherosclerotic lesion in leptin-deficient low-density lipoprotein receptor knockout female mice. The dose-dependent reduction of the chemokine, MCP-1, occurred from within the sub-physiological (0.1 and 0.5 mg/kg BW/day leptin doses) to physiological range (3.0 mg/kg BW/day leptin dose) compared to control (normal saline) [164]. An increase in the amount of MCP-1 transcription and expression is suggested to occur in arteries with atherosclerotic lesions and not in normal arteries [165].

Another possible mechanism by which leptin may contribute to CVD risk is through the haemostatic system [166]. Previous studies showed leptin’s positive and independent association with fibrinogen (an acute phase protein) and von Willebrand factor (also a marker of

endothelial function) in diabetic men (60-79 years old) [166]. This further depicts the potential role of leptin in atherosclerotic CVD development through altered endothelial function.

Lastly, a cross-sectional study by Porreca et al. [167] reported leptin's positive association with sVCAM-1 in obese women (mean age 48 years) before and after weight loss and was independent of body mass index (BMI).

2.6.2 Leptin and autonomic function

Leptin is known to achieve its regular physiological duties (appetite regulation and energy expenditure) through the activation of the sympathetic nervous activity (SNA) [99]. However, when leptin signalling is impaired as in the case of leptin resistance – where leptin is noted to be high, it could lead to exaggerated SNA modulations with a wide-spread effect reaching to other regions of the body like the kidney [168].

Sympathetic hyperactivity, a consequence of autonomic dysfunction, has been associated with overall obesity and body fat distribution [169] and is a crucial factor in the pathogenesis of hypertension and target organ damage [170, 171]. Correia et al. [172], showed that chronic leptin-induced elevation of the SNA and arterial blood pressure, are mediated by the activity of corticotropin-releasing-hormone (supposed anorexigenic neuropeptides) possibly through the release of cortisol [173]. Other studies suggested that the established association between adiposity and enhanced sympathetic activity leading to elevated blood pressure is due to leptin-induced renal sympathetic nervous system hyperactivity (RSNA) [32, 38]. The discovery of RSNA has further led to an increased understanding of the concept of selective leptin resistance and its effects on the periphery. In this concept, hyperleptinemia is suggested to impair hypothalamic regulation, while increasing the overexpression of sympathetic activity in selected tissues or organs like the kidney [174, 175].

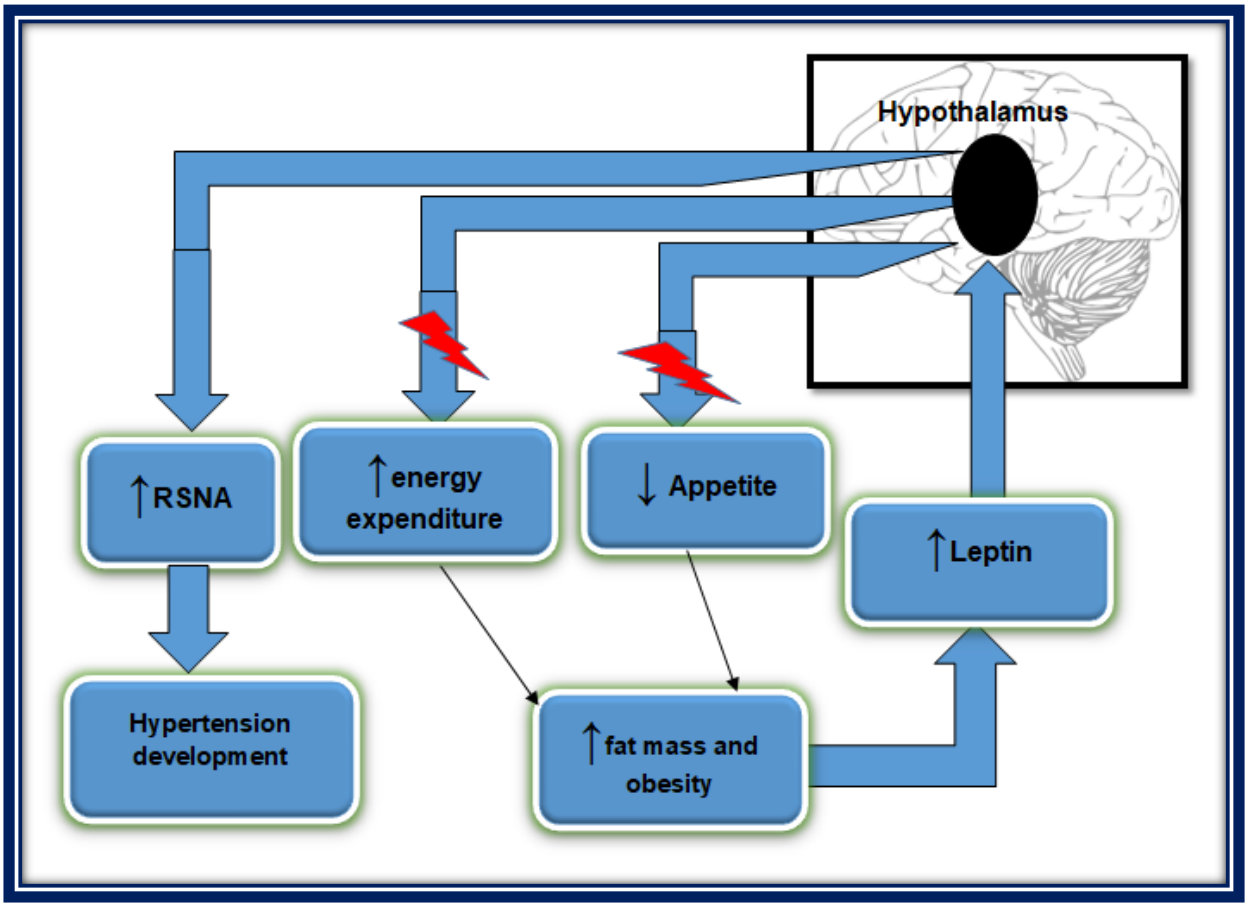

Figure 3: Leptin-induced renal sympathetic nervous system activity and its possible impact on blood pressure. Adapted from Rahmoun et al. [18].

Legend: Red colours indicate impaired leptin function due to leptin resistance. *Leptin selective resistance as a result of increased body fat mass and obesity may result to increased renal sympathetic nervous system activity (RSNA) and possibly hypertension development.*

Controversial findings exist in the association between measures of autonomic function with leptin in men and women. A study found that leptin associates with measures of sympathetic activity especially in men [45], another showed leptin's influences on the sympathetic nervous system of women and not in men [176]. The effects of leptin in the autonomic nervous system may, therefore, be different between men and women. More studies are needed to help explain the sex-specific differences that may exist between leptin and autonomic function. To support this notion, a study by Licinio et al. [177] already suggested that women may show more resistance to the physiological effects of leptin than men. According to this survey, women may

require twice as high plasma leptin levels to maintain their body weight, which includes SNA regulation of metabolism leading to energy expenditure and maintenance of weight compared to men [99, 178]. However, a study by Lambert et al. [179], showed no influence of leptin in muscle sympathetic nerve activity in men or women.

Several techniques have been proposed for the assessment of sympathetic nervous system activity such as heart rate, heart rate variability, microneurography, sympathetic adrenergic neuroimaging [180] and the norepinephrine spill over technique [181] etc. Due to limited resources and the expensive and invasive nature of some of these techniques, for this present study, we focused on heart rate and heart rate variability as indirect measures of autonomic activity and modulation [182, 183].

2.6.2.1 Leptin and heart rate

Resting heart rate is a simple indicator used in cardiovascular risk assessments [184]. Although the average value for RHR ranges from 60 to 80 beats per minute (bpm), it can exceed 100 bpm in sedentary individuals and be below 30 bpm in endurance athletes undergoing high and consistent training [185]. The elevated heart rate observed with hyperleptinaemia or a leptin-resistant state is presumed to result from the leptin-induced sympathetic over-reactivity [110]. In hypertensive subjects, leptin was reported to have a positive association with heart rate in patients with untreated essential hypertension [186]. Also, with regard to sex and ethnicity, Pieterse et al. [45] found a positive association between measures of ambulatory heart rate with leptin in both black ($\beta = 0.184$) and white men ($\beta = 0.376$) but not in black or white women. Another study confirmed leptin's positive association with heart rate in men with normal blood pressure, but not in women [187]. Leptin seems to play a significant role in the inherent rhythmic activity of the heart, especially in men and also in diseased individuals (such as hypertensive and obese individuals) [186, 187].

2.6.2.2 Leptin and heart rate variability

Heart rate variability (HRV) is a non-invasive, moderately inexpensive and simple prognostic indicator for the assessment of autonomic modulations [180]. HRV evaluates changes in heart rate by measuring the oscillations between the R-R intervals of the sinoatrial node rhythmic activity [188]. It also ascertains the central autonomic nervous system modulations as well as its input to the heart [189-191]. HRV in its depressed state is independently associated with increased sympathetic nervous system activity [192], CVD development (such as myocardial infarction, and heart failure) [193], hypertension development [189, 194], as well as cardiovascular morbidity and mortality [195].

Few studies show the role of leptin on HRV especially in older individuals compared to the study population used in this Ph.D. study (20-30 years old). In a study conducted by Paolisso et al. [196], leptin was positively associated with a low frequency to high frequency ratio in healthy non-obese men (mean age 43 years) at the 4^{th} quartile of leptin and was independent of obesity. In a group of South Africa population at risk of CVD development (mean age 45) (some of the participants had diabetes and hypertension), leptin showed an independent association with depressed HRV in black men and women as well as in white men [45]. Also, a study by Kurajoh et al. [197] suggests the involvement of high circulating leptin in cardiac autonomic dysfunction in diabetic patients (mean age 63 years) irrespective of gender. In the same study, a negative association between leptin and standard deviation of normal to normal R-R intervals (SDNN) – a time domain index of HRV and known to be reduced in individuals with untreated hypertension was reported. Furthermore, Charles et al. [198] showed an association between leptin and depressed heart rate variability in police officers whose body weights were higher than 25 kg/m^2 irrespective of BMI, gender and ethnicity (mean age 41 years). Since HRV has been associated with increased cardiovascular disease risk and mortality in older individuals, it is, therefore, necessary to investigate leptin's role in the variability of the heart of young healthy and normal weight individuals. This will be for a better understanding of leptin's physiological role in cardiac autonomic modulation.

2.6 3 Leptin and blood pressure

Several studies suggest a pathophysiological role of leptin in obesity-associated hypertension, especially in obese humans and animals [199, 200]. Others have also observed positive associations between leptin with blood pressure in obese and hypertensive individuals independent of BMI [201, 202]. Therefore, the lack of influence of BMI in the association between leptin and blood pressure suggests other mechanisms of actions of leptin on blood pressure that may not be related to obesity.

The association between leptin and blood pressure may be affected by gender [82]. To support this notion, Mallamaci et al. [203] showed leptin's positive association with blood pressure in normotensive men but not women. A plausible explanation for the lack of associations between leptin and blood pressure in women may be due to their reduced susceptibility to SNA activation - a factor that is responsible for the modulation of peripheral vasoconstrictor response [204]. However, in a multi-ethnic group of lean, overweight and obese hypertensive men and women (aged >20 years of age) from the United State of America, leptin associated with hypertension prevalence irrespective of gender after adjusting for BMI, ethnicity and other covariates [39]. Although only conducted in men, leptin showed a positive association with diastolic blood pressure in healthy non-obese Japanese men (34-65 years old) independent of obesity [52].

A study conducted among obese black South African women (28-37 years) revealed leptin's positive association with systolic blood pressure in obese hypertensive women. The above association was independent of age, BMI and insulin resistance when compared to normal weight and non-hypertensive obese black women [205]. Also, in underweight South African black men, Smith et al. [49] showed a positive association between leptin and blood pressure. Leptin's association with blood pressure both at lower (underweight) and higher (obese) plasma leptin concentration suggests the dose dependency and U-shaped relationships between leptin and adverse cardiovascular health is seen in the older and diseased individuals [108, 206, 207].

2.6.4 Leptin and the macrovasculature

Large artery stiffness (often measured using pulse wave velocity (PWV)), and carotid intima-media thickness (CIMT) are both important cardiovascular risk markers for the prediction of vascular damage and cardiovascular events [208, 209]. In addition to predicting cardiovascular events, CIMT is also a useful tool for the assessment of subclinical atherosclerosis [210] and PWV to predict cardiovascular mortality [209].

Hyperleptinaemia has been postulated to increase the risk of developing obesity-associated cardiovascular diseases such as atherosclerosis [128]. To further support the atherogenic potential of leptin, leptin independently associated with carotid cross-sectional wall area (CSWA) in obese and overweight black and white school teachers (mean age 45 years) [88]. In middle-aged hypertensive women (51 years), leptin associated with PWV independently of obesity. In this same group of women, leptin associated with CIMT, but the association was dependent on BMI [211]. Another study by Ciccone et al. 2002, also found leptin's association with CIMT in obese men and women (18-45 years) to be dependent on obesity [19], and implies that obesity is a determinant in the development of atherosclerosis

In young, healthy children with a moderate degree of obesity, high plasma leptin level was associated with reduced arterial distensibility independent of BMI [43]. Also, in obese Romanian children (4-20 years), leptin weakly correlated with CIMT in an unadjusted regression analysis [212]. Leptin's association with either reduced elasticity or CIMT in adolescent and children gives an indication for adult-onset of CVD development in young individuals.

2.6.5 Leptin and the microvasculature

The study of the body's microvasculature is important because microvascular changes are thought to precede macrovascular pathologies [213, 214]. The retinal microvascular photographic imaging technique is now recognised as a reliable new tool for microvascular disease research [215] (such as those relating to hypertension), as well as for CVD risk prediction [215, 216]. As the direct visualisation and investigation of the microcirculation is

difficult especially in human studies and also for routine clinical follow-up, the retinal microvasculature can easily be used non-invasively for this investigation. Essential information can be obtained from the non-invasive assessment of the retinal microvasculature since it bears a close resemblance with the coronary and cerebral vasculature [216-218].

Retinal imaging devices such as the Retinal Vessel Analyzer coupled to specific software, make it possible for retinal vessel calibres to be measured [219, 220]. In this regard, alterations in the retinal vessel calibers (central retinal artery equivalent (CRAE), central retinal vein equivalent (CRVE) and arterio-venous ratio (AVR; the ratio between CRAE and CRVE), could indicate independent risk for early diagnosis and prognosis of incident cardiovascular and other related diseases [220-222]. Reduced central retinal artery equivalent is linked to increased blood pressure [216] and has been shown to associate with incident hypertension [223]. Widening of the CRVE is associated with an atherosclerotic profile - including markers of inflammation, greater BMI, dyslipidaemia [224] and incident stroke [225].

Only a few studies have shown leptin's relationships with measures of retinal vessel calibres. One study found leptin to be positively associated with CRVE and negatively with AVR in obese German children, and was dependent on BMI [130]. In contrast, another study observed a positive association between continuous leptin (before stratifying by quartiles) with CRAE in children and adolescents and was also dependent on BMI. After dividing leptin into quartiles, leptin also associated positively with CRAE at highest leptin quartile (>90th percentile), but the association was independent of obesity despite their higher cardio-metabolic risk [133]. However, there is limited information on the relationship between leptin and retinal vessel calibres - particularly in Africa. This warrants further investigation.

Additionally, the Retinal Vessel Analyzer which helps to assess microvascular responses to flicker light-induced provocation (FLIP, acute light stimulation) is also informative providing an indication of retinal vessel function, including vascular reactivity, functional hyperemia, neurovascular coupling and retinal blood flow autoregulatory mechanism [226-228]. Photic

stimulation of the retinal induces retinal vessel dilation in a process mediated by several factors, including nitric oxide synthase and nitric oxide [214]. Reduced vessel dilation to acute stimuli may imply endothelial dysfunction [229, 230] and is also related to several systemic diseases such as: [231], obesity [232], hypercholesterolemia [229], untreated hypertension [233], coronary artery disease [234], and ocular pathologies [235, 236].

There are no studies that have investigated the relationship between leptin with retinal vessel dilatory responses to FLIP, but some studies have shown the microvascular action of leptin using other methods and within different microvascular beds.

A study demonstrated with a laser Doppler flow-meter showed that leptin increases perfusion rate during a microvascular stimulation with increase temperature [237]. Another study showed that leptin improves forearm blood flow in normotensive young Japanese men [238]. Studies that address the role of leptin – a known cardiovascular risk factor, in the retinal microvasculature are therefore recommended and maybe a valuable tool in early detection of hypertension and CVD risk [239]

3 Problem statement and motivation

In the literature, relationships were shown between circulating leptin and several measurements of cardiovascular function, namely sympathetic activity [45, 197, 240], blood pressure [52, 205], endothelial cell activation [30, 167] and large artery structure [88, 241] either in the older overweight or obese individuals, those with the metabolic syndrome (hypertension and diabetes), diseased (CVDs; myocardial infarction, coronary heart disease and stroke) or in Western populations [43, 88, 143, 197, 242-244], as well as in South Africans with a high prevalence of CV risk factors [30, 45, 88]. It seems plausible that leptin may be involved within obesity-related hypertension recorded so far among aged obese hypertensive adults [45, 205, 245, 246]. To the best of our knowledge, the role of leptin on early vascular deterioration has not yet been studied thoroughly. In order to increase our understanding on the earlier phases of CVD development, it is necessary to investigate the potential role of leptin on cardiovascular function, by determining whether leptin already associates with measures of autonomic and

vascular endothelial activation and function, blood pressure, macrovasculature and the microvasculature, particularly in healthy young adults without any overt CVD.

This Ph.D. thesis is written in the article-format, and therefore the main motivation for each of the three manuscripts was based on limited information available regarding a potential role for leptin on: (A) autonomic activity, endothelial activation and blood pressure (manuscript 1), (B) large artery structure and function (manuscript 2) and the retinal microvasculature (manuscript 3), in healthy young adults at risk of CVD development [54]. Detailed and other motivations for these manuscripts are expressed in each manuscript (chapter 3, 4 and 5), and the final chapter (chapter 6) of this Ph.D. thesis.

4 Research aim, objectives and hypotheses

4.1 Aim

The central aim of this study was to determine the relationships that exist between measures of autonomic activity, endothelial activation, blood pressure, large arterial structure and function and, retinal microvasculature with leptin in young black and white adults.

4.2 Objectives

In a group of young black and white men and women from South Africa, the objectives and hypotheses of the study are as follows:

4.2.1 Manuscript 1:

Objective

To determine whether leptin relates to measures of autonomic activity (ambulatory heart rate and heart rate variability (HRV)), endothelial activation (MCP-1, sVCAM-1, sICAM-1), and 24-hour blood pressure.

Hypotheses

- Leptin will associate negatively with heart rate variability (HRV) high frequency (HF), 24-hours total power, triangular index, standard deviation of normal to normal R-R intervals (SDNN), and positively with, low frequency (LF), LF/HF ratio and ambulatory heart rates,
- Leptin will associate positively with markers of endothelial activation (monocyte chemo-attractant protein-1 (MCP-1), soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular adhesion molecule-1 (sVCAM-1)),
- Leptin will associate positively with 24-hour blood pressure, and the above relationships will be observed in all the groups.

4.2.2 Manuscript 2:

Objective

To determine whether leptin relates to measures of large artery structure and function (PWV, CIMT and CSWA) and a marker of endothelial dysfunction (von Willebrand factor, vWF) in young healthy black and white men and women.

Hypotheses

- Leptin will associate positively with large artery structure and function (pulse wave velocity (PWV), carotid intima-media thickness (CIMT) and cross-sectional wall area (CSWA) and a marker of endothelial dysfunction (vWF), and the above relationships will be observed in all the groups.

4.2.3 Manuscript 3:

Objective

To establish if associations exist between leptin and retinal vessel calibres (CRAE, CRVE, AVR) and functional responses to light flicker provocation (maximum arteriolar and venular dilations).

Hypotheses

- Leptin will relate positively to central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE) and negatively to arterio-venous ratio (AVR).
- Leptin will relate positively to maximum arteriolar and venular dilations following a light flicker provocation, and the above relationships will be observed in all the groups.

5 Organisation of the study

This literature review chapter is followed by a chapter describing the study design and methods (Chapter 2), followed by the presentation of the three individual manuscripts in the following order:

Chapter 3; Manuscript 1:

Title: Leptin and its Relation to Autonomic Activity, Endothelial Cell Activation and Blood Pressure in a Young Black and White Population: The African-PREDICT study.

Chapter 4; Manuscript 2:

Title: Leptin and the Vasculature in Young Adults: The African- PREDICT study.

Chapter 5; Manuscript 3:

Title: Leptin and the Retinal Microvasculature in Young Black and White Adults: The African-PREDICT Study

After the manuscript presentations, a summary of the study's main findings and concluding remarks are presented in chapter 6.

6 Relevance of the study

This thesis should provide a better physiological understanding of the relationship between leptin and specific markers of autonomic modulation, endothelial activation, blood pressure, and

micro and macrovascular function in a young normotensive bi-ethnic population. This may be of public health relevance as a better understanding of how obesity and leptin associate with cardiovascular function, may aid in initiatives towards primary prevention strategies for the reduction of the CVD burden – the emergent source of death resulting from non-communicable diseases [9].

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CHAPTER 2

Study Design and Methodology

1 Study design

This study forms part of the African Prospective study on the Early Detection and Identification of Cardiovascular disease and HyperTension (African-PREDICT study) [1].

The African-PREDICT study employs a prospective study design to longitudinally characterise and monitor the early stages of hypertension development in 1202 healthy young black and white individuals (aged 20-30 years) over a follow-up period of 10 years. Its goal is to identify and understand early pathophysiological changes in cardiovascular function and specific predictors contributing to the development of hypertension and hypertension-mediated organ damage. This Ph.D. study included cross-sectional data of the first consecutive 820 participants (Figure 1) with a completed data-set that were relevant to this study. The study participants included apparently healthy black and white men and women.

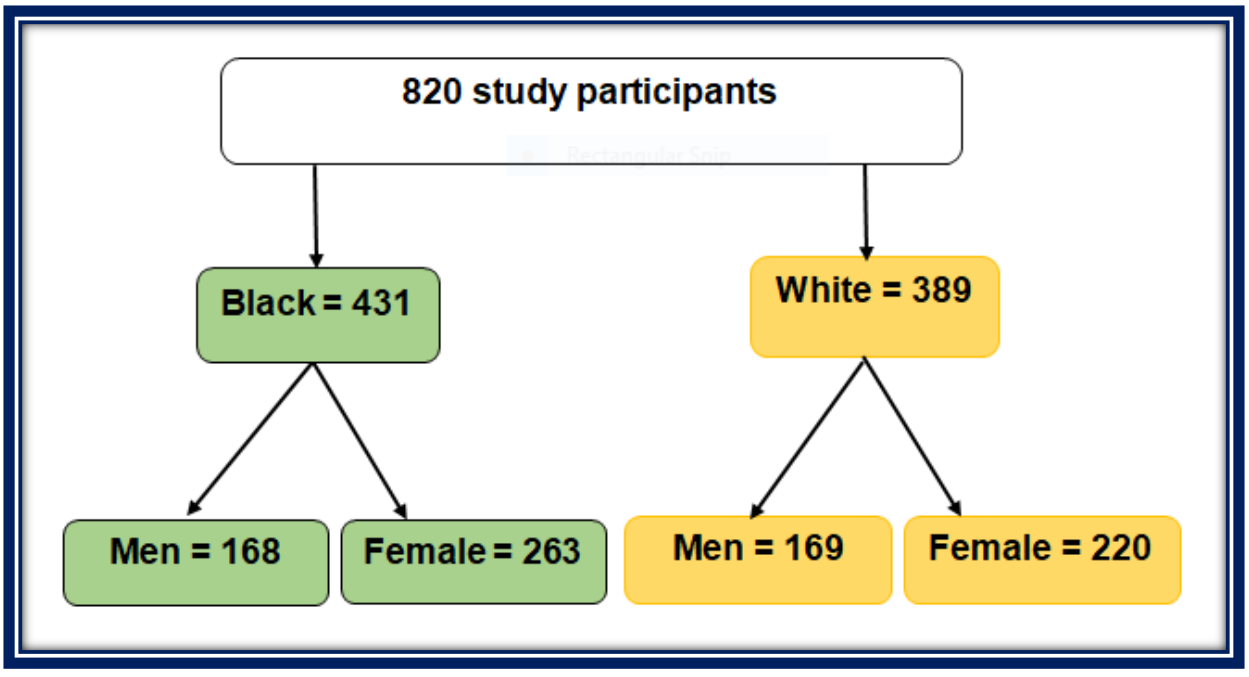

Figure 1. A flow chart depicting the participant distribution in terms of ethnicity and sex for this Ph.D. study.

This Ph.D. study was prepared in the article format and from the total 820 participants that were included in the study, manuscript 1 and 2 (chapter 3 and 4 respectively) made use of all the available 820 participants (white N=389 and black N=431), while manuscript 3 (chapter 4),

included only the participants with available data for retinal vessel analyses (RVA) amounting to a total of 572 participants (black N = 283 and white N = 289).

2 Methodology

2.1 Organisational procedures

The African-PREDICT study is conducted at the Hypertension Research and Training Clinic on the Potchefstroom campus of the North-West University (NWU) and is currently registered on ClinicalTrials.gov (NCT03292094). Permission for the execution of the African-PREDICT study was obtained from the following authorities (a) Health Research Ethics Committee of the North-West University with approval number: NWU-00001-12-A1. (b) The National Department of Health, Section Non-Communicable Diseases and (c) North West Province Department of Health. Similarly, this Ph.D. study was also approved by the Health Research Ethics Committee of the North-West University with approval number: NWU-00066-16-S1 (Appendix A). The study complied with all applicable requirements of the US and International Regulations, in particular, the Helsinki Declaration of 1975 and subsequent revisions. All participants voluntarily participated in the study and gave written informed consent.

2.2 Participant recruitment and screening for eligibility

Participants were invited to participate in the study through various strategies such as advertisements in newspapers, on notice boards, by health screening in public places and also by direct recruitment at workplaces. Participants were recruited on a voluntary basis and therefore constituted a convenience or availability sample, stratified into different ethnic (black and white), sex and socio-economic class groups (low, mid, high). The young age, sample size and groupings are ideal for addressing the specific objectives of this study as it constitutes a young population, with roughly equal distribution of black and white men and women.

Volunteers were screened for eligibility and to be eligible participants were required to have the following: clinic brachial systolic blood pressure (BP) less than140 mmHg and diastolic BP less than 90 mmHg [2], be HIV free, have no previous diagnosis or medication use for chronic

disease (self-reported), not be pregnant or breastfeeding if women. The HIV status of the participants was determined with the ABON HIV 1/2/O Tri-Line Rapid Test (ABON Biopharm, Hangzhou, China) using whole blood. If positive, the HIV test was repeated with a First Response (PMC Medical, Nani Daman, India) rapid HIV test. Test results were communicated to individuals by trained counsellors in a private room, and those who tested positive for HIV were referred to their physician, local clinic or hospital to measure their CD4 count.

2.2.1 Inclusion and exclusion criteria of the African-PREDICT study.

Table 1. The inclusion and exclusion criteria, as well as the justification used in the screening phase are shown below

Inclusion criteria:	**Justification**:
1. Black or white ethnicity	The African-PREDICT study is a longitudinal study, aimed to include and track young healthy individuals over 10-20 years in order to monitor especially the early phases of cardiovascular disease development. In South Africa, our research group and others have shown that black individuals, which constitute about 80 to 90% of the South African population, present with very high blood pressure and were therefore included in the study. Furthermore, it is necessary to study specifically this group to get a better understanding of biomarkers and processes involved during the early

	stages of cardiovascular disease development. Black participants were compared with white participants (used as a comparison group and also constitute about 5% of the South African population), as per the aims of the study.
2. Aged 20-30 years	As the aim of the study is to understand the early phases of hypertension and cardiovascular disease development, a population of 20-30 years is required, as they are adults, but generally considered to be at the peak of health and at a stage prior to cardiovascular deterioration.
3. Men and women (equally distributed)	Both men and women are used to determine whether differences occur according to gender.
4. Apparently healthy	As per the aim of the study, the focus is on early disease development, hence healthy participants were required as part of the baseline phase.
5. Normotensive or pre-hypertensive (SBP<140 and DBP<90mmHg) based on four clinic BP measures in one day	International and South African Guideline for hypertension cut off points are used to determine if a participant is hypertensive or normotensive [3]
Exclusion criteria	**Justification**
1. Indian, Asian or mixed origin ethnicity	From all known populations black individuals present with the highest blood

	pressure and are therefore used in the study, whereas the white population is used as a comparison group. Our focus has always been on ethnic differences between black and white populations.
2. Not a permanent resident of Potchefstroom surrounding area (i.e. intend to move to another area)	Due to the longitudinal nature of the study, researchers ensure that participants can be followed over the required time period.
3. Diagnosed type 1 or 2 diabetes mellitus 4. Elevated glucose >5.6 mmol/L and confirmed glycated haemoglobin (HbA1c) ≥ 6.5% 5. HIV infected 6. Fever (internal ear temperature > 37.5ºC on the research day) 7. Known liver disease, cancer, tuberculosis or renal disease 8. Microalbuminuria > 30 mg/ml in spot morning urine or proteinuria 9. Medication use for chronic disease, i.e. antihypertensive, anti-diabetic, antiretroviral or anti-inflammatory medication	Individuals with any known diseases or risk factors that may influence cardiovascular health are excluded**.**
10. Pregnant or lactating women	Due to the known influences of hormones on cardiovascular health pregnant and lactating women were excluded.

11. Recent surgery or trauma (within the past three months) 12. Previous history of stroke, angina pectoris or myocardial infarction	Same as the justification for 3-9

2.3 African-PREDICT study research procedures

The eligible participants that were identified through the screening phase were invited to another clinic visit appointment for baseline data collection. The median time between screening and research data collection was 15 days (interquartile range 7–40 days). Baseline exam cycle and data collection took place within the Hypertension Research and Training Clinic on the Potchefstroom campus of the North-West University. The participants' voluntarily participated in the study and also consented prior to data collection. All measurements were performed in a private room. The participants were from Potchefstroom, South Africa and surrounding towns (Figure 2).

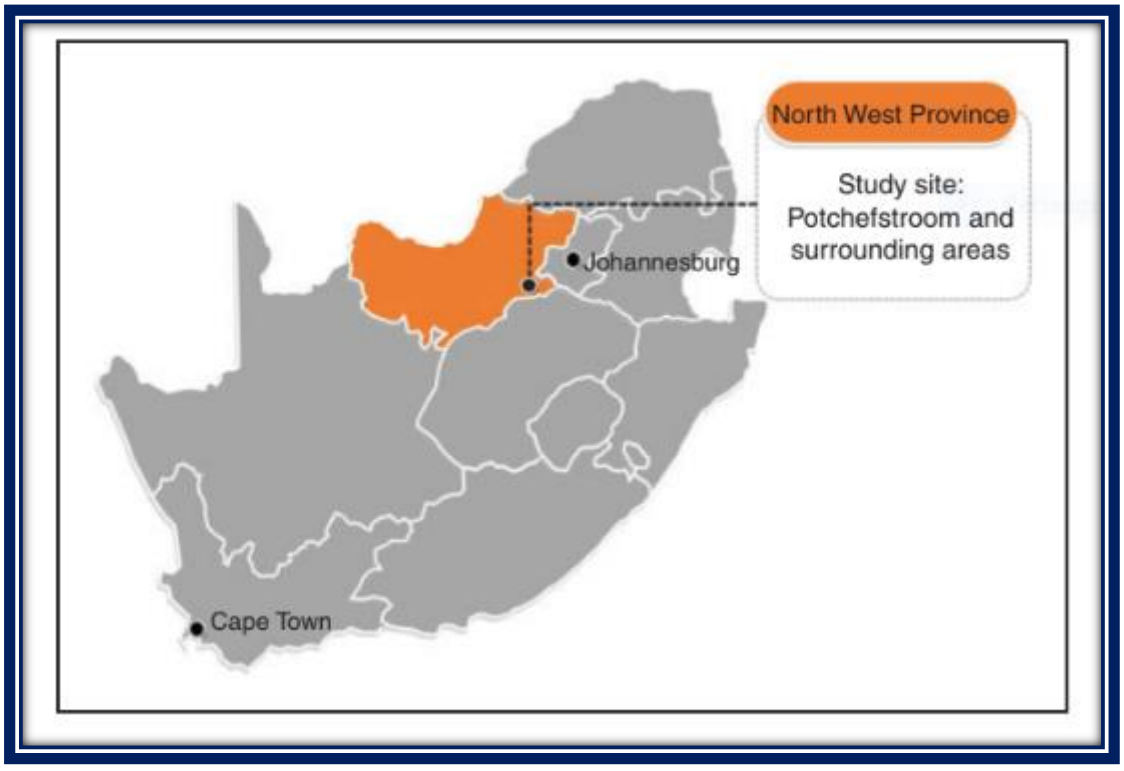

Figure 2. A map showing the location where the study was conducted in Potchefstroom of the North West province of South Africa [1].

2.4 Questionnaires

We used a standard general health and demographic questionnaire to gather information such as medical history, socio-economic status and traditional risk factors (including sex, age, as well as smoking and alcohol consumption) from the participants. Self-reported alcohol and smoking included a no/yes response on previous and current use, as well as questions to quantify alcohol and tobacco. For the present study only the no/yes response was used. Demographic factors such as age and gender have been proposed as a secondary regulators of circulating leptin levels [4]. Both alcohol consumption and cigarette smoking (self reported) are regarded as independent predictors of plasma leptin in young normotensive men [5]. Socio-economic status was calculated by a point system adapted from Kuppuswamy's Socioeconomic Status Scale 2010, in alignment with the South African environment [6]. The participants were then scored based on three categories namely: skill level, education and household income. Skill level was classified according to the South African Standard Classification of Occupation. These three factors were scored and used to group the participants into low, middle and high socio-economic groups both as a categorical and continuous variable.

2.5 Body composition and accelerometry assessments

2.5.1 Anthropometric measurements

We conducted the following anthropometric measurements - weight in kg, which was taken to the nearest 0.01 kg (SECA electronic scales, SECA, Birmingham, UK). Height in m, measured to the nearest 0.1 cm (SECA stadiometer, SECA) and waist circumference in cm measured in triplicate using a validated non-flexible tape measure (Holtain, Crymych, UK), and the measurement was made to the nearest 0.1 cm. In all determined anthropometric parameters, the median of the three measurements was determined and recorded. Body mass index (BMI)

which is an indirect assessor of overall adiposity [7, 8], was calculated from the standard weight kg/height^2 (m^2) and waist-to-height ratio (WHtR), an index of abdominal obesity [9] was calculated using waist circumference (cm)/height (cm). Previous studies have shown leptin's positive associations with overall adiposity and fat distribution [8, 10]

The anthropometric assessments were done according to the measurement guidelines recommended by the International Society for the Advancement of Kinanthropometry and National Institutes of Health sponsored Arlie Conference [11].

2.5.2 Bioelectrical impedance measurement

We measured bioelectrical impedance to estimate lean body mass and body fat percentage with a Bodystat 1500MDD dual-frequency analyser (Bodystat, Ltd, Ballakaap, British Isles). During this assessment, electrodes were placed on the individual's right arm and leg while in a supine position. The bio-impedance analyser works by passing a safe battery-generated signal through the body and measuring the impedance (a function of resistance and reactance) at two frequencies of 5 kHz and 50 kHz. These non-invasive arm-to-leg measurements were then used to indirectly estimate body fat percentage that is calculated by the Bodystat software and equation formula [12].

2.5.3 Accelerometry assessment

The participants were fitted with a combined heart rate and accelerometry device (ActiHeart, CamNtech Ltd., Cambridge, UK). The device was worn on the chest and gives recording of physical activity, total energy expenditure and activity energy expenditure for a maximum of 7 consecutive days. During the measurement, acceleration was captured mechanically and then converted into an electrical signal through an accelerator sensor and data collected at 60-s epochs length [13]. Activity energy expenditure was furthered divided by body weight to compensate for increased energy expenditure accompanied with an increase in body mass.

2.6 Cardiovascular measurements

2.6.1 Ambulatory blood pressure and heart rate variability measurements

Participants were fitted with a 24-hour ambulatory blood pressure monitoring (ABPM) and electrocardiography (ECG) apparatus (Card(X)plore, MediTech, Hungary, British Hypertension Society (BHS) validated). An appropriately sized cuff was fitted to the participant's non-dominant arm, with programmed measurements taken at half an hour intervals during the day (6 am to 10 pm) and one-hour intervals during the night (10pm to 6am). In order to achieve successful ABPM measurements, the participants were taught on the process of monitoring across the 24-hour measurement period. Instructions were given on how to ensure successful inflations. An ambulatory diary card was provided and was completed by the participants during the measurements. The successful mean inflation rate over the 24-hour ambulatory blood pressure measurement for this Ph.D. study was 88.5%. Banegas et al. [14] showed 24-hour ambulatory blood pressure measurement as a superior predictor of all-cause and cardiovascular mortality than clinic blood pressure measurement.

We used Cardio Visions 1.15.2 Personal Edition (Meditech, Budapest, Hungary) software to obtain 24 hours, day and night heart rate, and 24 hour blood pressure and heart rate variability (HRV) measures The 24 hour blood pressure included systolic and diastolic blood pressure (SBP and DBP respectively), while the mean arterial blood pressure (MAP) was calculated with the formula as follows: (SBP-DBP)1/3 + DBP. The measurements of the 24-h HRV indices were automatically calculated by the software, and readings taken from the time, frequency and geometric domains. The time domain analysis consists of the SDNN (Standard Deviation of Normal to Normal interval), which is a representative of overall HRV activity in the time domain. The frequency domain analysis (determined by the fast Fourier transformation) involves low frequency, (a major index of sympathetic cardiac tone but also having a parasympathetic component, LF=0.04–0.15 Hz) and high frequency (a major reflector of the parasympathetic activity, HF=0.15–0.4 Hz). The LF and HF analyses were measured at the normalised unit. The low frequency-to-high frequency ratio (LF/HF, reflect sympatho-vagal autonomic balance) was

also determined, as well as total power (TP), which is considered a global determinant of overall autonomic modulation [15], being an estimation of the variance of HRV over a certain period of time. We assessed the geometric domain using the HRV triangular index which also represents the entire HRV and is the total number of NN intervals divided by the number of NN intervals in the modal bin of the NN interval histogram [15-18].

2.6.2 Measures of subclinical target organ damage

Based on leptin's reported contributions towards vascular pathophysiology, including its atherogenic potential [19, 20], we thus considered the following cardiovascular variables which also stand as an index of subclinical target organ damage [21-24] in healthy young adults:

2.6.2.1 Pulse wave velocity (PWV)

Pulse wave velocity, an index of stiffness [25], was measured along the descending thoraco-abdominal aorta using the foot-to-foot velocity method, while the participant was in the supine position. The participant's right carotid artery pulse wave was recorded with a tonometer while the femoral pulse was measured with a femoral cuff placed around the upper thigh (positioned as high as possible) of the participant (Figure 3). During this assessment, 80% of the direct carotid-femoral distance measure (infantometer (SECA)) and the femoral-to-cuff distance measurements (tape measure) was calculated [25] and then entered onto the SphygmoCor software on a laptop that is linked to a SphygmoCor. The result of the PWV calculated as distance/pulse transit time was then automatically generated. Prior to this test and for accurate measurements, participants were not allowed to eat at least 3 hours before the test procedure. Two consecutive measurements were done and the last one used for analysis, but in cases where the PWV differed by more than 0.5 m/s between the two measurements, a third measurement was conducted.

Figure 3. Measurement of cfPWV as part of the African-PREDICT study. (A) Illustrates the placement of the tonometer on the carotid artery. (B) Shows the position of the cuff on the thigh during the measurement.

2.6.2.2 Carotid intima-media thickness (CIMT) and cross-sectional wall area (CSWA)

B-mode ultrasonography, a high-resolution and non-invasive technique (General Electric Vivid E9, GE Vingmed Ultrasound A/S, Horten, Norway), was used to measure the carotid intima media thickness (CIMT) of the study participants by a sonographer. CIMT has been shown to predict cardiovascular risk [26, 27] and has also been associated with cardiovascular events [28]. The measurement was done on the left and right common carotid artery by a sonographer. Images from at least two optimal angles of the left and right common carotid artery were obtained. A single reader conducted measurements using a semi-automated program, namely the Artery Measurement Systems software v1.139 (Chalmers University of Technology, Gothenburg, Sweden). A maximum of 10mm segment length of the artery segment was used for the analysis and the intraobserver variability for the far wall was 0.04 mm between measurements. The arterial wall segment was assessed in a lateral view and with measurement made on the far wall. Measurement was preferably done on the far wall because measurement made on the near wall requires in part gain setting (operating an additional setting in order to increase performance) and is also less reliable [26, 27].

Figure 4. Measurement of CIMT as part of the African-PREDICT study.

The cross-sectional wall area (CSWA) was calculated to confirm structural and not functional changes in luminal diameter: CSWA = (d/2+ CIMT) 2-d/2)2, where d denotes luminal diameter.

2.6.2.3 Retinal microvascular calibres and dilation in response to a light flicker provocation

Retinal fundus photography aids the non-invasive assessment of the retinal vessel structure and responses to light stimuli and is a reliable tool for cardiovascular risk prediction [29, 30]. In this Ph.D. study, the status of the retinal vessel calibres, and dynamic changes after flicker light-induced stimulation was assessed with the Retinal Vessel Analyzer (IMEDOS Systems, Jena, Germany) fitted with a Zeiss Fundus camera FF-450. This assessment was conducted in a half-lit and temperature controlled room.

Before the commencement of this measurement, participants were asked not to eat food or drink fluid at least 1 hour prior to the measurement. The participants also refrained from smoking and exercise during this time. Participants with a history of epilepsy were not included in the study. Thirty minutes before the measurement, a drop of Tropicamid (1% Alcon) was administered in the right eye (used the majority of the time) of the participants to induce mydriasis. After the induction of the mydriatic condition in the participant's eye, dynamic retinal

vessel responses to light flicker provocation (dynamic vessel analysis) was assessed, followed by fundus photography for the determination of retinal vessel calibres (static vessel analysis).

Assessments of the retinal vessel calibres (static analysis)

For the static retinal vessel analysis, coloured and monochrome images of the retinal were taken using the Visualis 2.81 software (Imedos Systems, Jena, Germany), with the camera position at an angle of 50°. The monochrome image was predominantly analyzed with the VesselMap2 software version 3.02 (Imedos Systems, Jena, Germany) for vessel calibre determination. Arteriolar and venular trunk segments located within 0.5-1.0 optic disc diameters from the margin of the optic disc were selected (Figure 5).

Using the six largest arteriolar and venular segments, the central retinal artery and vein equivalents (CRAE and CRVE), were then calculated using revised formulas described by Knudtson et al. [31]. These revised formulas for quantifying retinal vessel calibres were chosen for the following reasons: it offers the advantages of being more robust against variability in the number of vessels under investigation, it does not rely on image scale, and much easier to apply or implement [31]. Both CRAE and CRVE were measured in measuring units (MU). The MU correlates with a micrometre (1:1) if the measuring extent of the eye is equivalent to the normal Gullstrand eye. The arteriolar to venular ratio (AVR) was calculated as the ratio of CRAE/CRVE.

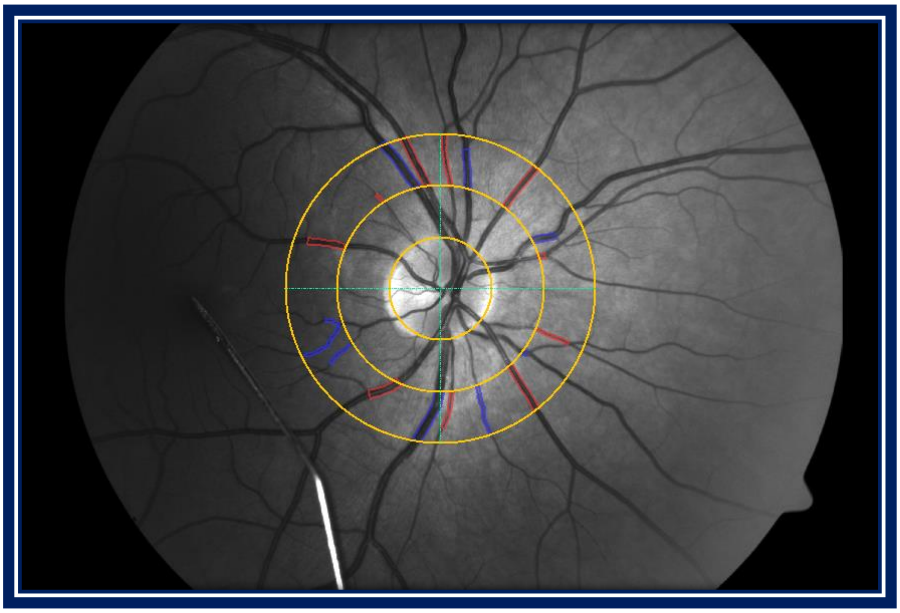

Figure 5. Selected vessel segments within 0.5 – 1.0 optic disc diameters from the margin on the optic disc: blue colour represents the venular trunk and red arteriolar trunk.

Assessments of retinal vessel responses to a flicker light stimulation (Dynamic responses).

For dynamic retinal vessel analysis, a standard flicker protocol by IMEDOS Systems was used. In the flicker assessment, the baseline phase lasted for 50 seconds followed by a 20 seconds flicker period and a 80 seconds recovery/2nd baseline period. In total there were three flicker cycles, and the measurement lasted a total of 350 seconds. With the camera set at a 30°degree angle and the participant focusing on the tip of a fixation rod, arteriolar and venular segments (Arteriolar and venular median segment lengths were 1125 and 1255 MU, respectively in this study) were primarily selected in the upper or lower temporal quadrant of the fundus image (Figure 7). As indicated by the retinal vessel analysis (RVA) software manual, vessels were selected if they were at least between 0.5-2.0 optic disc diameters away from the margin of the optic disc. A subjective scoring methods by Dr W Smith and Prof KE Kotliar, was used to assessed the quality of the measurement, and the criteria were as follows: (1) The possibility of raw data to be analysed, (2) the availability of at least one good flicker recording (baseline/flicker pattern), (3) the availability of consistent measuring points during the flicker, (4) low noise and (5) almost no gaps. For each category, a score was given (0, 0.5 or 1) which

carried the same weight for a total of 5 points (0 = poor quality and 5 = excellent quality; in extreme cases a negative score could be allocated). For the African PREDICT study, only participants with a vessel segment score of 2.5 or above were considered.

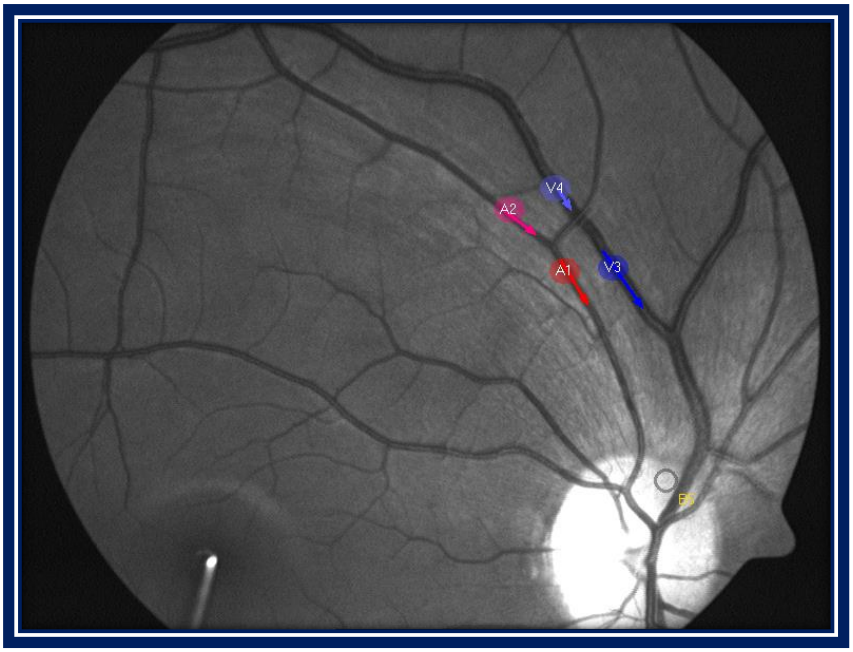

Figure 6. Represents the vessel segments that were selected during the dynamic measurements.

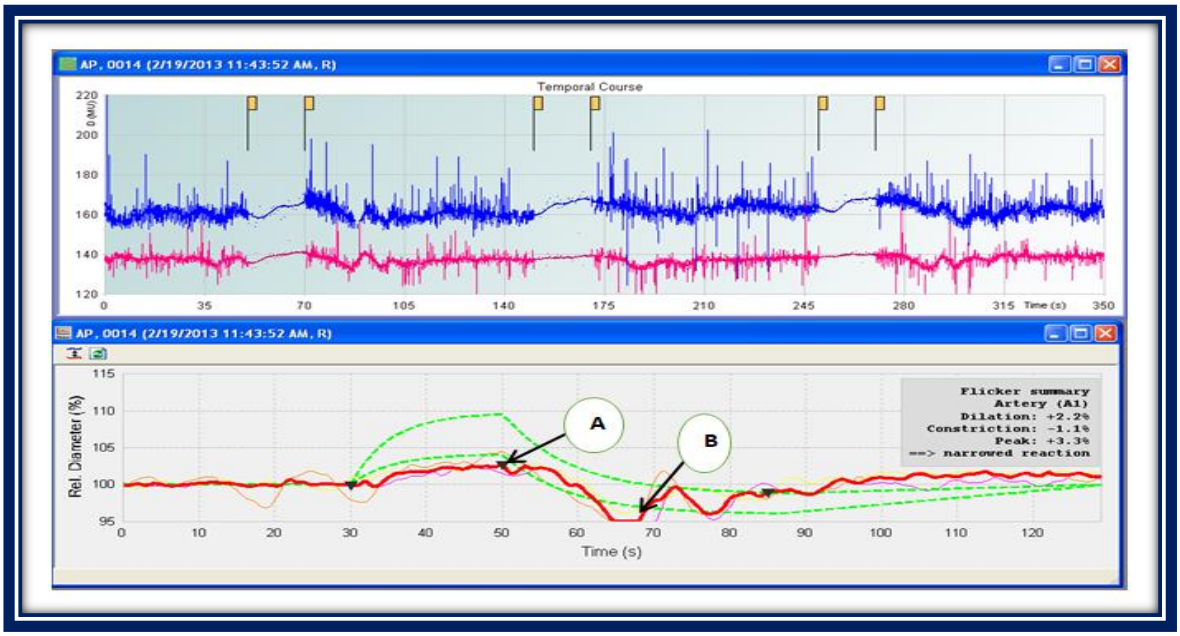

Figure 7. Represents retinal vessel responses to flicker light-induced stimulation (A= maximum dilation and B=maximum constriction).

After the measurement, the raw data from the DVA was exported into an Excel Microsoft template for further sorting, processing and analysis. The following variables were measured: (A) Vessel diameters prior to FLIP and are measured in measuring units (MU). This represents the median values calculated from over the last 30 seconds of the initial baseline phase. (B) Maximum arteriolar and venular dilations and was derived from the smoothed maximum median curve between the initial and 30 seconds after FLIP, and are expressed in percentages.

2.7 Blood sample handling and biochemical measurements

2.7.1 Blood sample handling

Prior to the blood sample collection in the morning, participants were asked to fast overnight for a period of eight to ten hours. A research nurse extracted blood from the brachial ante-cubital vein using a syringe and dispensed the blood sample into different vacutainer tubes. A sterilised needle was used to draw the blood, and all necessary precautions were considered according to Good Clinical Practice.

A research assistant, trained in the handling of biological samples, collected the blood tubes in the Research Clinic and placed the tubes in a closed container according to a pre-specified protocol. The blood samples were then prepared according to standard procedures and aliquoted into cryovials for short- and long-term storage in the bio freezer at –80^{o}C until analysis was carried out. The validity of the stored samples was ensured by a temperature alarm system linked to a researcher's cell phone and the University campus security.

2.7.2 Biochemical measurements and methods

The biochemical variables, as well as the analysis used in this Ph.D. study are described as follows: The intra- and inter-assay variability of all the biochemical variables were calculated to ensure the specificity, sensitivity, reliability and reproducibility. Intra- and inter-assay variability for all biochemical variables are <10%, while the inter-assay variability for all biochemical variables are <12%.

- Leptin, an adipocyte-derived hormone [32], is the main independent variable in this Ph.D. study and has been implicated in obesity-associated hypertension [32], as well as other related cardiovascular diseases [20, 33, 34]. The biomarker leptin was determined from serum in duplicate using an enzyme-linked immunosorbent assay kit (Quantikine, R&D Systems, MN, USA). Intra- and inter-assay variability were 3.1% and 2.9%, respectively. All biochemical analyses of leptin were performed by a single trained biochemist.
- Serum intercellular adhesion molecule-1 (ICAM-1; intra- and inter-assay variability were 5.2% and 6.7%, respectively), vascular cell adhesion molecule-1 (VCAM-1; intra- and inter-assay variability were 3.6% and 7.8%, respectively), and plasma monocyte chemo-attractant protein-1 (MCP-1; intra- and inter-assay variability were 5.9% and 5.9%, respectively),) were assayed using Quantikine ELISA kits (R&D systems, Minneapolis, MN, USA). We determined vWF from citrate plasma using a sandwich ELISA. Polyclonal rabbit anti-vWF antibody and rabbit anti-vWF-horseradish peroxidase antibody (DAKO, Glostrup, Denmark) were used to perform the assay. Vascular endothelial dysfunction is associated with serum levels of elevated biomarkers such as - cellular adhesion molecules and chemokine, and haemostatic factors in patients with essential hypertension [35-37].

Other biochemical variables that were used to describe the general characteristics of the participants and those that may also be potential cofounders in the relationship between leptin (independent) and our dependent variables are described below:

- Serum adiponectin levels were determined with the Human Adiponectin ELISA kit (BioCat GmbH, Heidelberg, Germany) and, the intra- and inter assay variability for adiponectin were 3.7% and 8.6% respectively. We performed analyses of serum high-sensitivity C-reactive protein (intra- and inter-assay variability were 1.3% and 3.5%, respectively), total cholesterol (intra- and inter-assay variability were 0.51% and 1.9%, respectively), low-density lipoprotein cholesterol (LDL-C; intra- and inter-assay variability were 1.5% and 1.9%, respectively), high-density lipoprotein-cholesterol (HDL-C; intra- and inter-assay variability were 1.13% and 1.0%, respectively), triglycerides, (intra- and inter-assay variability were 1.6% and 1.9%,

respectively) and gamma-glutamyltransferase (Intra- and inter-assay variability were 1.8% and 1.8%, respectively), were determined in serum, as well as glucose in sodium fluoride plasma (intra- and inter-assay variability were 1.8% and 2.1%, respectively) (Cobas Integra 400plus, Roche, Basel, Switzerland). Cotinine was determined using the chemiluminescence method on the Immulite (Siemens, Erlangen, Germany). The intra- and inter-assay variability for cotinine were 10.7% and 5.50%, respectively.

3 Statistical considerations

The detailed statistics that were used in this study are described within each manuscript (chapter 2, 3 and 4).

4 Student contributions

The Ph.D. student participated diligently within a larger research team (Hypertension in Africa Research Team) contributing to data collection. The Ph.D. student participated in the advanced research measurements of the African-PREDICT study, where I was responsible for the following:

- Preparing urine and blood samples for storage in the bio-freezers at -80^{o}C until analyses were ready to be carried out: I prepared serum, plasma, EDTA and citrated samples and also aliquoted the prepared samples into individual cryovials for storage.
- Tonometry assessments, where I measured the pulse wave velocity of the subjects- an index of large artery stiffness, using the sphygmoCor XCEL device. The right common carotid artery pulse amplitude was recorded with a tonometer (probe) while the femoral pulse wave velocity was measured with a femoral cuff placed around the upper thigh of the participants.
- The Ph.D. student also participated in the measurement of flow-mediated dilation (FMD) within the HART research team. Although FMD measurements were not included in this study, an association has been observed between FMD and Dynamic Retinal Vessel (DVA) responses to light flicker stimulation. Most importantly, both FMD and DVA have shown

similarities in their application and have shown reduced responses in patients with known endothelial dysfunction and underlying pathologies [38].

- Although not directly relevant to this Ph.D. thesis, the National Research Foundation that funds this Ph.D. thesis, has a policy for every research to be involved in community engagement as part of their research. The Ph.D. student was actively involved in the May Measurement Month (MMM17 and 18) blood pressure global awareness campaign. The MMM is an initiative of the International Society of Hypertension as a goal towards the reduction of blood pressure. HART contributed maximally in this initiative, and as a student under HART, the Ph.D. student voluntarily measured blood pressure of individuals above 18 years, gave health talk and made referrals of individuals with extremely high blood pressure that were unaware of their condition. The outcome of the nationwide MMM17 blood pressure awareness campaign and the student's contributions in South Africa - Sub-Saharan Africa MMM17 is now published in The Lancet Global Health [39] and European Heart Journal (Supplement) [40].
- Apart from this, the Ph.D. student designed the original proposal for this particular study, did the literature search, wrote the manuscripts and conducted all statistical analysis, including cleaning of the data sets, checking for normality and all other advanced statistics which are already described in the three manuscripts). The thesis was written under the guidance of both my promoter (Prof. AE Schutte) and co-promoters (Prof. W Smith and Dr. L Lammertyn).

Although trained personnel took the measurements for the retinal vessel analysis and carotid intima-media thickness, the Ph.D. student observed the measurement procedures and also understood the principle on which the measures were based on.

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CHAPTER 3; Manuscript 1

Leptin and its Relation to Autonomic Activity, Endothelial Cell Activation and Blood Pressure in a Young Black and White Population: The African-PREDICT Study

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Thieme

Leptin and its Relation to Autonomic Activity, Endothelial Cell Activation and Blood Pressure in a Young Black and White Population: The African-PREDICT Study

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Key words
adipokine, heart rate variability, ethnicity, sex, vascular function

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ABSTRACT

An increasing prevalence of obesity-related hypertension is observed in the youth and may have severe consequences for future cardiovascular disease development. Previous studies portrayed leptin as a potential factor involved in obesity-related hypertension development. In order to understand leptin's contributions to early cardiovascular deterioration, we investigated leptin and its associations with measures of autonomic activity, endothelial activation, and blood pressure in young healthy black and white men and women. We included 820 participants (aged 20–30 years) and determined serum leptin and endothelial cellular adhesion molecules. We measured

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Summary of Instruction to authors used for the preparation of this manuscript

Journal Details	
Title:	Hormone and Metabolic Research
Impact factor	2.560
Aims & scope	Hormone and Metabolic Research publishes original articles and short communications on cutting edge topics of clinical and basic science research, covering the field of endocrinology and metabolism.
Publisher	Thieme
Author guidelines	
Original paper	It should not exceed 6 printed pages, including references, tables, figures and legends. A maximum of 4 figures and 3 tables is allowed.
Preparation of manuscripts	The following outlines were followed: **Page 1**: a) title, b) short running title (limit: 40 characters), c) name of the author and address of the institute(s), d) complete mailing address of corresponding author **Page 2**: a) an abstract containing maximum of 250 words, b) keywords between 3–6 words. **Page 3 and onwards:** a) introduction also indicating the aim of the study, b) materials and methods, c) results, d) discussion and conclusions, e) list of references, f) acknowledgements, legends and tables and authors declaration
	Complete details of the instruction to author can be viewed using the following web-address: *https://www.thieme.com/media/ita/pubid-388413336.pdf*

(Please note that some of the formats were changed to ensure uniformity throughout the thesis)

Leptin and its Relation to Autonomic Activity, Endothelial Cell Activation and Blood Pressure in a Young Black and White Population: The African-PREDICT Study

Short running title: Leptin in Young South Africans

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Abstract:	246
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1 Abstract

Objectives: An increasing prevalence of obesity-related hypertension is observed in the youth and may have severe consequences for future cardiovascular disease development. Previous studies portrayed leptin as a potential factor involved in obesity-related hypertension development. In order to understand leptin's contributions to early cardiovascular deterioration, we investigated leptin and its associations with measures of autonomic activity, endothelial activation and blood pressure in young healthy black and white men and women.

Methods: We included 820 participants (aged 20-30 years) and determined serum leptin and endothelial cellular adhesion molecules. We measured 24-hour blood pressure, heart rate and heart rate variability components.

Results: In multivariate-adjusted regression analyses, we found consistent associations between markers of autonomic activity (such as 24-hour heart rate, day and night-time heart rate as well as heart rate variability total power) and leptin in both white (all $p \leq 0.001$) and black men (all $p \leq 0.040$). These findings were absent or less prominent in women, despite their almost ten-fold higher leptin levels than men. Only in white men, 24-hour diastolic blood pressure was associated with leptin (Std $\beta = 0.37$; $p=0.006$). This association was found to be partly mediated by autonomic activity (24-h heart rate variability total power). No independent associations were observed between leptin and markers of endothelial cell activation, irrespective of race or gender.

Conclusion: Leptin's independent association with autonomic neural activity in a young apparently healthy population suggests an early influence of leptin on autonomic function and future blood pressure elevation especially in men.

Keywords: Adipokine, heart rate variability, ethnicity, sex and vascular function.

2 Introduction

Obesity is a growing and significant health threat not only globally, but also in sub-Saharan Africa [1]. Excess adiposity is associated with a myriad of metabolic and cardiovascular disorders, including hypertension- a key mediator of obesity-induced cardiovascular disease (CVD) [2]. Of note, increasing prevalence of obesity-related hypertension development is currently observed in the youth and may predict future CVD development in sub-Saharan Africa [3]. The exact link in obesity-associated hypertension development remains poorly understood. In this regard, findings from animal and human studies implicate an important role for the adipocytokine, leptin [4, 5]. Besides leptin's satiety regulatory role [6], it has beneficial and deleterious effects on the vasculature. Leptin contributes to vasodilation in its physiological state [7], and when elevated, it increases the sympathetic outflow to blood vessels leading to vasoconstriction and ultimately results in endothelial dysfunction [8].

Elevated circulating leptin is known to be associated with an increased risk for CVD development in older, obese and hypertensive individuals, likely through sympathetic nervous system (SNS) activation [2, 9, 10]. Studies within South Africa reported leptin's possible contribution to CVD development, to be either dependent or independent of obesity [11, 12]. In non-obese healthy adolescents outside Africa, leptin also showed a potential role for CVD development [13, 14]. Despite this, little is known regarding the potential link between leptin, early endothelial activation and cardiovascular function in young healthy black adults known to be prone to hypertension development [15, 16].

A recent global analysis in 19 million adults showed that raised blood pressure recently transitioned from high income to low-income countries particularly sub-Saharan Africa [17]. Together with this transition, is the growing prevalence of overweight/obesity in South Africa [18, 19]. According to the 2016 South African Demographic and Health Survey [19], 68% of women and 31% of men are overweight or obese. Given the above relevant findings, it is important to understand leptin's contributions to early CVD development in younger individuals, and also to understand the underlying pathophysiological processes. We, therefore,

investigated leptin and its associations with measures of autonomic activity, endothelial activation and blood pressure in young black and white men and women.

3 Subject and methods

Study design

This study forms part of the African Prospective study on the Early Detection and Identification of Cardiovascular disease and HyperTension (African-PREDICT study). Young black and white individuals were invited to participate in the study. Participants were recruited on a voluntary basis and were all from Potchefstroom and surrounding areas in South Africa. We included black and white men and women, aged 20 to 30 years, with systolic BP (SBP) <140 mm Hg/ diastolic BP (DBP) <90 mm Hg and no known CVD, not taking any BP medication, no chronic disease, not HIV positive and not pregnant or breastfeeding. Participants gave written informed consent after the procedures of the study were explained to them. The Health Research Ethics Committee of the North-West University approved the study, and the study complied with the Helsinki Declaration of 1975. The data of the first 820 participants (white N=389 and black N=431) was available for this cross sectional sub-study.

Questionnaire data

Demographic and lifestyle questionnaires, as well as the global physical activity questionnaire (GPAQ) [20] were used to assess medical history, lifestyle, physical activity, social status and traditional risk factors.

Body composition measurements

We measured weight (SECA electronic scales, SECA, Birmingham, UK), height (SECA stadiometer, SECA) and waist circumference (taken in triplicate with a non-flexible tape measure, Holtain, Crymych, UK) [21]. Body mass index (BMI) was calculated from the standard formula: weight (kg)/ height ^{2} (m^2). Participants with a BMI >30 kg/m^2 were considered obese.

Bioelectrical impedance was measured to assess lean body mass and body fat percentage (Bodystat 1500MDD dual-frequency analyser, Bodystat, Ltd, Ballakaap, British Isles).

Cardiovascular measurements

Participants were fitted with a 24-hour (h) ambulatory blood pressure and ECG apparatus (CardioXplore®, and Meditech CE120 Cardiotens; Meditech, Budapest, Hungary). An appropriately sized cuff was fitted to the participant's non-dominant arm with programmed measurements taken at half an hour intervals during the day (6 am to 10 pm) and one-hour intervals during the night (10 pm to 6 am). If less than 70% of the ambulatory blood pressure monitoring recordings for a particular participant were successful, the measurement was repeated the next day.

We used Cardio Visions 1.15.2 Personal Edition (Meditech, Budapest, Hungary) software to obtain 24-hour, day and night heart rate, and 24-hour blood pressure and heart rate variability (HRV) measures as previously described [12]. The measurements of the 24-hour HRV indices were automatically calculated by the software, and readings taken from the time, frequency and geometric domains. The time domain analysis included SDNN (Standard Deviation of Normal to Normal interval) which is a representative of overall HRV activity in the time domain. The frequency domain analysis (determined by the fast Fourier transformation) involve low frequency, (a major index of sympathetic cardiac tone but also having a parasympathetic component, LF=0.04–0.15 Hz) and high frequency (a major reflector of the parasympathetic activity, HF=0.15–0.4 Hz) which were measured at the normalized unit. The low frequency-to-high frequency ratio (LF/HF, reflect sympatho-vagal autonomic balance) was also determined, as well as total power (TP), which is considered a global determinant of overall autonomic modulation, being an estimation of the variance of HRV over a certain period of time. Given that LF/HF [22] is a controversial measure, it is reported for descriptive purposes and also to support our main findings [23]. In this study, we also assessed the geometric domain using the HRV triangular index. It equally represents the entire HRV and is the total number of NN intervals divided by the number of NN intervals in the modal bin of the NN interval histogram [23-25].

Biological sampling and biochemical measurements

A research nurse took blood samples from participants that had fasted (8-10 h overnight) and the different fractions of blood were prepared and stored at -80 degrees Celsius. We performed analyses of serum high-sensitivity C-reactive protein, total cholesterol, low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides, gamma glutamyltransferase, as well as glucose in sodium fluoride plasma (Cobas Integra 400plus, Roche, Basel, Switzerland). Serum cotinine was determined with a chemiluminescence method on the Immulite (Siemens, Erlangen, Germany). Serum intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and plasma monocyte chemo-attractant protein-1 (MCP-1) were assayed using Quantikine ELISA kits (R&D systems, Minneapolis, MN, USA). Serum leptin levels were determined using an enzyme-linked immunosorbent assay kit (Quantikine, R&D Systems, MN, USA) and adiponectin levels were determined with the Human Adiponectin ELISA kit (BioCat GmbH, Heidelberg, Germany).

Statistical analysis

We performed statistical analysis using Statistica Software Version 13.2 (Dell Software, Round Rock, Texas, 2016). Variables that were not normally distributed were log transformed (including BMI, waist circumference, night-time heart rate, 24-hour HRV LF/HF, 24-hour HRV total power, 24-hour HRV triangular index, leptin, adiponectin, MCP-1, total cholesterol, LDL-C, HDL-C, C-reactive protein, active energy expenditure, moderate-vigorous physical activity, cotinine and gamma-glutamyltransferase) and presented as the geometric mean with 5^{th} and 95^{th} percentiles. Logarithmically transformed variables were also employed in all statistical analyses. We tested the interaction effects between leptin and either ethnicity or sex in their association with autonomic activity markers, endothelial cell activation and blood pressure. Independent t-tests were done to compare means between groups and the Chi-square test (χ^2) to compare proportions. Single and partial correlations were used to assess the associations of markers of interest with leptin. For the partial correlations, we adjusted for age and BMI. We performed multiple regression analyses to test for associations of markers of autonomic activity,

endothelial cell activation and blood pressure with leptin. The following covariates were included in this model: age, BMI, socio-economic status, active energy expenditure, total cholesterol, triglycerides, glucose, C-reactive protein, gamma- glutamyltransferase, alcohol, smoking and cotinine. These covariates were selected among many others using appropriate selection criteria and were based on their relationship with our main variables. We conducted both mediation and sensitivity analyses by substituting a measure of an overall index of HRV (24-hour HRV total power) and sympatho-vagal balance (24-hour HRV LF/HF ration) with leptin in the multiple regression model. The sensitivity analysis was performed to determine the robustness of the results from mediation analysis.

4 Results

Characteristics of the study population

Our study participants were stratified according to ethnicity and sex because of the presence of significant interactions between leptin and each of ethnicity and sex in their relationships with measures of ambulatory heart rate, HRV, 24-hour SBP and MCP-1 with leptin (Supplementary Table 1S). The group stratification was also based on the already established differences in leptin levels between older black and white adults [26]. **Table 1** represents the characteristics of black and white men and women, aged 20-30 years. White men displayed higher leptin ($p=0.003$) and BMI ($p<0.001$) than black men and a total of 49 (14.5%) men (black: 11 (6.55%) and white: 38 (22.5%)) were obese. For the cardiovascular variables, we found that black men had lower 24-hour SBP, pulse pressure ($p<0.001$) and 24-hour heart rate (HR) ($p=0.026$) as well as day time HR ($p=0.023$) than their white counterparts. White men displayed higher LF/HF and LF as well as a lower index of HF ($p<0.001$) than black men. Regarding the markers of endothelial cell activation, ICAM-1 was higher in white men ($p<0.001$) while MCP-1 was higher in the black men ($p<0.001$). In women, we observed higher values of leptin ($p<0.001$) and BMI

(p<0.001) in black women and a total of 105 (21.7%) women were obese (black: 70 (26.6%) and white: 35 (15.9%). Black women also showed higher 24-h, day and night-time HR (all p<0.001), higher 24-hour HRV HF, lower 24-hour HRV LF/HF and 24-hour HRV LF (all p<0.001) than white women. The white women displayed higher SDNN, HRV total power and triangular index (p<0.001, p=0.001, and p=0.005 respectively) than the black women. Lastly, the ICAM-1 (p<0.001) of black women were found to be lower than those of white women, but MCP-1 was higher in black than white women (p<0.001)

Table 1. Characteristics of participants, stratified by ethnicity and sex (N = 820)

	Black men (N = 168)	**White men (N = 169)**	**p value**	**Black women (N = 263)**	**White women (N = 220)**	**p value**
Age, (years)	24.4±3.03	25.5±2.86	0.001	24.6±3.26	25.2±2.83	0.045
Socioeconomic status						
Low	101 (60.1)	26 (15.4)	<0.001	146 (55.5)	41 (18.6)	<0.001
Middle	40 (23.8)	41 (24.3)		76 (28.9)	53 (24.1)	
High	27 (16.1)	102 (60.4)		41 (15.6)	126 (57.3)	
Anthropometric variables						
Body mass index (kg/m^2)	21.9 [17.5;30.3]	26.7 [20.3;36.1]	<0.001	26.1 [18.3;37.7]	24.3 [18.4;37.9]	<0.001
Waist circumference (cm)	75.1 [63.7;94.6]	89.4 [74.0;114]	<0.001	78.3 [62.0;103]	76.4 [63.4;105]	<0.001
Body fat (%)	16.1±6.12	19.0±7.02	<0.001	33.9±8.00	29.6±8.77	<0.001
Lean body mass (kg)	53.6±8.72	69.5±8.37	<0.001	43.9±6.06	48.0±6.57	<0.001
Cardiovascular variables						
24-h SBP (mmHg)	120±8.27	124±7.29	<0.001	113±8.42	113±8.68	0.65
24-h DBP (mmHg)	69.7±6.08	70.4±5.76	0.29	68.2±5.65	68.0±5.62	0.60
24-h pulse pressure (mmHg)	50.1±6.67	53.2±6.83	<0.001	44.5±5.60	45.2±5.91	0.23
24-h MAP (mmHg)	89.8±6.24	91.7±5.48	0.003	86.1±6.33	86.1±6.38	0.10
24-h heart rate (bpm)	68.2±8.36	70.4±9.83	0.026	81.2±9.15	76.7±10.27	<0.001
Day-time heart rate (bpm)	72.4±9.22	75.0±10.8	0.023	85.0±9.70	81.3±10.71	<0.001

Night-time heart rate (bpm)	59.3 [46.0;76.0]	61.1 [46.0;81.0]	0.087	72.3 [57.0;90.0]	65.6 [52.0;86.0]	<0.001
24-h HRV LF (n.u.)	61.6±11.5	69.1±11.1	<0.001	54.5±12.6	65.0±11.0	<0.001
24-h HRV HF (n.u.)	35.5±10.7	28.8±10.1	<0.001	42.4±11.9	32.8±10.5	<0.001
24-h HRV LF/HF	1.81 [0.80;4.10]	2.46 [1.10;5.70]	<0.001	1.30 [0.60;3.20]	2.04 [10.8;0.90]	<0.001
24-h HRV total power (ms^2)	629 [288;1344]	618 [209;1540]	0.77	366 [129;793]	435 [172;1240]	0.001
24-h HRV triangular index	50.2 [32.0;77.0]	45.9 [26.0;78.0]	0.087	36.5 [24.0;57.0]	39.6 [79.0;24.0]	0.005
24-h HRV SDNN (ms)	177±45.8	173±47.9	0.39	128±33.1	145±41.6	<0.001
Biochemical variables						
Leptin (ng/ml)	2.70 [0.34;25.1]	6.72 [1.02;33.10]	0.003	35.49 [8.31;103]	22.7 [6.11;92.5]	<0.001
Adiponectin (µg/mL)	3.74 [0.90;12.6]	2.54 [0.57;8.17]	<0.001	4.16 [1.02;13.15]	5.10 [1.39;15.3]	0.003
ICAM-1 (ng/ml)	156±85.7	206±66.5	<0.001	162±85.5	192±59.6	<0.001
VCAM-1 (ng/ml)	654±200	673±188	0.36	659±188	689±207	0.10
MCP-1 (pg/ml)	170 [122;261]	138 [100;200]	<0.001	150 [102;230]	121 [80.4;204]	<0.001
Total cholesterol (mmol/l)	3.70 [2.71;5.48]	4.76 [3.29;6.64]	<0.001	3.76 [2.67;5.30]	4.52 [3.19;6.40]	<0.001
LDL-C (mmol/l)	2.27 [138;3.86]	3.23 [1.97;5.14]	<0.001	2.37 [1.26;3.90]	2.81 [1.77;4.43]	<0.001
Triglycerides (mmol/l)	0.79 [0.41;1.58]	1.14 [0.55;2.85]	<0.001	0.67 [0.36;1.30]	0.87 [0.41;2.08]	<0.001
HDL-C (mmol/l)	1.23 [0.80;1.82]	1.10 [0.69;1.67]	<0.001	1.22 [0.79;1.90]	1.48 [0.94;2.36]	<0.001
Glucose (mmol/l)	4.34±0.58	5.12±0.53	<0.001	4.45±0.75	4.78±0.52	<0.001
C-reactive protein (mg/l)	0.65 [0.10;5.94]	0.89 [0.10;8.08]	0.57	1.88 [0.21;13.6]	1.06 [0.10;10.9]	<0.001
Lifestyle						

Active energy expenditure (k/Cal)	2.52 [2.13; 2.83]	2.47 (2.13; 2.83)	0.50	2.55 [2.17; 2.96]	2.57 [2.17; 2.97]	0.77
MVPA, (mins/day)	59.2 [5.00; 446]	49.1 (6.23; 309)	0.25	58.7 [8.6; 386]	42.4 [6.43;314]	0.011
Self-reported tobacco use (N/total **%**)	62/158 (39.2)	46/161 (28.6)	0.044	38/247 (15.4)	33/206 (16.0)	0.85
Cotinine (ng/ml)	3.45 [1.00;342]	4.36 [1.00;276]	0.67	3.34 [1.00;327]	3.39 [1.00;308]	0.94
Gamma glutamyltransferase (U/l)	28.5 [13.4;87.5]	25.6 [11.0;64.5]	0.11	23.1 [10.1;67.1]	14.5 [7.40;39.6]	<0.001
Self-reported alcohol use (N/total **%**)	101/156 (64.7)	102/160 (63.8)	0.85	131/243 (53.9)	118/206 (57.3)	0.47

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial blood pressure; HRV, heart rate variability; LF, Low frequency; HF, high frequency; SDNN, standard deviation of normal RR intervals; h, hour; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemo-attractant protein-1; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; MVPA, Moderate-Vigorous Physical activity**.** Data are expressed as arithmetic mean ± standard deviation or geometric mean (5th to 95th percentile intervals) for logarithmically transformed variables, or number of participants and percentages (%) and $p<0.05$ were considered significant.

Single and partial correlation analyses

We explored the associations of markers of autonomic activity, endothelial cell activation, and blood pressure with leptin before and after adjusting for age and BMI (Supplementary Table 2S and 3S). As part of our main findings, we plotted leptin against 24-hour HRV total power and 24-hour DBP (Figure 1 and 2). In all groups, 24-hour HRV total power correlated negatively ($p \leq 0.012$) and 24-hour DBP positively ($p \leq 0.001$) with leptin. Upon adjustment for age and BMI (Supplementary Table 3S), 24-hour, day time and night time heart rate correlated positively with leptin in all groups ($p<0.05$). Only in white men, all of the measures of HRV (except 24-hour HRV HF (n.u)) as well as 24-hour DBP correlated significantly with leptin ($p<0.05$).

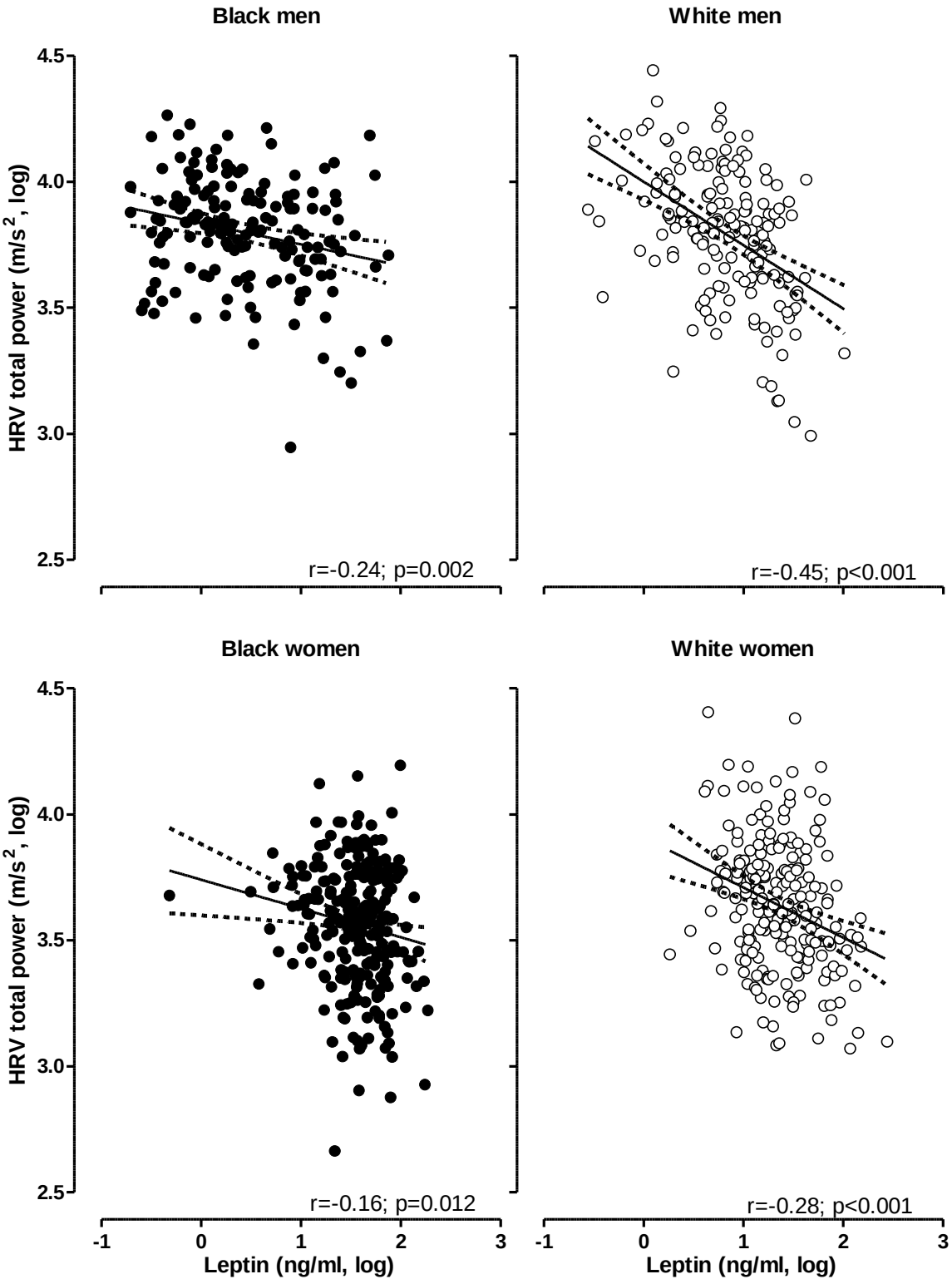

Figure 1: Leptin plotted against HRV total power in black and white men and women. Solid and dashed lines represent the regression line and the 95 % boundaries

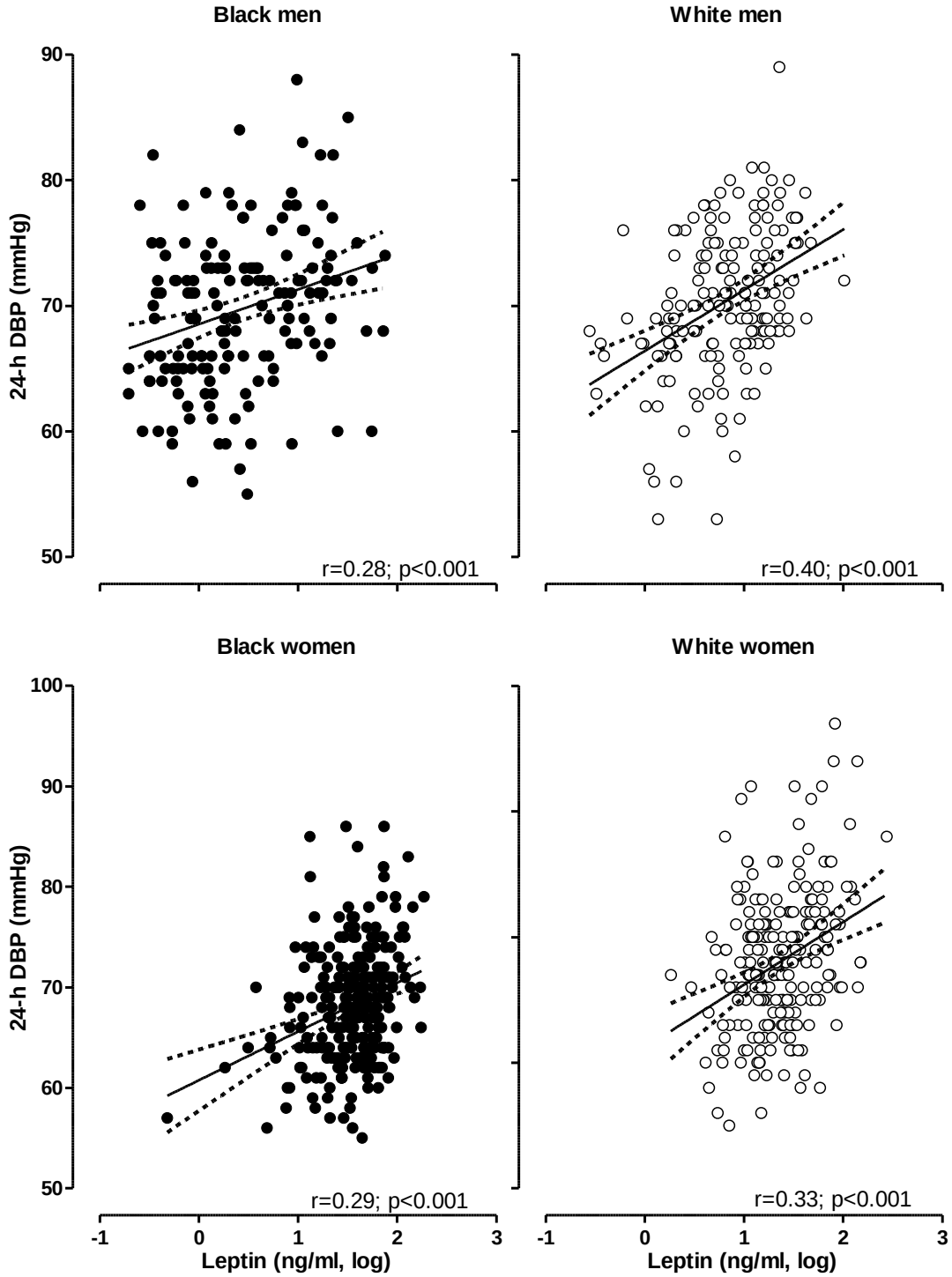

Figure 2: Leptin plotted against 24-hour diastolic blood pressure in black and white men and women. Solid and dashed lines represent the regression line and the 95 % boundaries

Analysis of covariance

We compared black and white men, and black and white women regarding 24-hour HRV total power, 24-hour DBP and leptin, after adjusting for age and socio-economic status (Supplementary Table 4S). Leptin remained higher in white men than black men ($p=0.001$) and also higher in black women than in white women ($p<0.001$).

Multi-variable adjusted regression analyses

In Table 2, we presented the results of the multiple regression analysis with markers of autonomic activity, endothelial cell activation and blood pressure employed as the dependent variable in men and women of each ethnicity. We found consistent associations between markers of autonomic activity (such as 24-hour HR, day and night-time HR as well as 24-hour HRV total power) and leptin in both white and black men. In the white men only, leptin independently and positively associated with 24-hour HRV LF/HF (Std $\beta = 0.36$; $p_2<0.015$) and 24-hour DBP (Std $\beta = 0.37$; $p_2=0.006$). In women, the associations of leptin with markers of autonomic activity were not as prominent as in men. Leptin associated positively with day and night time HR in black women and only day time HR in white women. In black women, leptin negatively associated with 24-hour HRV SDNN (Std $\beta = -0.24$; $p_2=0.035$). No associations were observed between leptin and any marker of endothelial cell activation irrespective of race or gender.

Table 2. Independent associations of markers of autonomic activity, endothelial cell activation and blood pressure with leptin

	Black men (N = 168)				White men (N = 169)			
	Model		**Leptin (ng/ml)**		**Model**		**Leptin (ng/ml)**	
Dependent variables:	**Adjusted R^2**	**p_1**	**Std β (95 % CI)**	**p_2**	**Adjusted R^2**	**p_1**	**Std β (95 % CI)**	**p_2**
Autonomic activity								
24-h heart rate (bpm)	0.11	**0.019**	**0.48 (0.18; 0.78)**	**<0.001**	0.36	**<0.001**	**0.43 (0.20; 0.67)**	**<0.001**
Day time heart rate (bpm)	0.08	0.055	**0.46 (0.16; 0.77)**	**0.004**	0.32	**<0.001**	**0.40 (0.16; 0.63)**	**0.001**
Night time heart rate (bpm)	0.06	0.12	**0.35 (0.04; 0.66)**	**0.029**	0.30	**<0.001**	**0.45 (0.21; 0.69)**	**<0.001**
24-h HRV LF (n.u.)	0.07	0.077	0.19 (-0.12; 0.49)	0.24	0.01	0.33	0.28 (-0.01; 0.57)	0.056
24-h HRV HF (n.u.)	0.05	0.16	-0.18 (-0.49; 0.13)	0.25	----	0.74	-0.21 (-0.50; 0.08)	0.16
24-h HRV LF/HF	0.05	0.13	0.11 (-0.20; 0.42)	0.49	0.03	0,23	**0.36 (0.07; 0.64)**	**0.015**
24-h HRV total power (ms^2)	0.10	**0.032**	**-0.32 (-0.63; 0.02)**	**0.040**	0.31	**<0.001**	**-0.47 (-0.71; 0.23)**	**<0.001**
24-h HRV triangular index	0.01	0.38	-0.07 (-0.39; 0.25)	0.65	0.13	**0.004**	-0.25 (-0.52; 0.01)	0.067
24-h HRV SDNN (ms)	0.06	0.10	-0.16 (-0.47;0.15)	0.31	0.16	**0.001**	-0.20 (-0.47; 0.06)	0.14
Endothelial cell activation								
ICAM-1 (ng/ml)	0.01	0.41	-0.15 (-0.47; 0.17)	0.37	0.23	**<0.001**	-0.06 (-0.31; 0.20)	0.67
VCAM-1 (ng/ml)	0.15	**0.003**	-0.29 (-0.59; 0.01)	0.053	0.05	0.11	-0.03 (-0.31; 0.25)	0.83
MCP-1 (pg/ml)	0.01	0.36	-0.03 (-0.35; 0.28)	0.83	0.10	**0.016**	0.11 (-0.16; 0.38)	0.44
Ambulatory blood pressure								
24-h SBP (mmHg)	0.09	**0.040**	-0.05 (-0.35; 0.26)	0.76	0.19	**<0.001**	-0.23 (-0.49; 0.03)	0.091

24-h DBP (mmHg)	0.22	**<0.001**	0.09 (-0.19; 0.38)	0.52	0.19	**<0.001**	**0.37 (0.11; 0.63)**	**0.006**
24-h pulse pressure (mmHg)	0.07	0.072	-0.14 (-0.45; 0.17)	0.37	0.15	**0.002**	**-0.55 (-0.82; -0.29)**	**<0.001**
	Black women (N = 263)				**White women (N = 220)**			
	Model		**Leptin (ng/ml)**		**Model**		**Leptin (ng/ml)**	
Dependent variables:	**Adjusted R^2**	**p_1**	**Std β (95% CI)**	**p_2**	**Adjusted R^2**	**p_1**	**Std β (95% CI)**	**p_2**
Autonomic activity								
24-h heart rate (bpm)	0.05	0.066	0.21 (-0.01; 0.43)	0.068	0.17	**<0.001**	0.23 (-0.01; 0.46)	0.053
Day time heart rate (bpm)	0.03	0.16	**0.17 (-0.06; 0.39)**	**0.028**	0.17	**<0.001**	**0.26 (0.03; 0.49)**	**0.028**
Night time heart rate (bpm)	0.09	**0.007**	**0.23 (0.01; 0.45)**	**0.041**	0.13	**<0.001**	0.21 (-0.03; 0.44)	0.085
24-h HRV LF (n.u.)	----	0.82	0.03 (-0.20; 0.26)	0.80	0.01	0.44	-0.05 (-0.31; 0.20)	0.67
24-h HRV HF (n.u.)	----	0.83	-0.03 (-0.26; 0.20)	0.82	0.01	0.43	0.07 (-0.18; 0.32)	0.58
24-h HRV LF/HF	----	0.86	0.03 (-0.20; 0.26)	0.77	-----	0.48	-0.04 (-0.30; 0.21)	0.73
24-h HRV total power ($logms^2$)	----	0.52	-0.13 (-0.35; 0.10)	0.28	0.19	**<0.001**	-0.03 (-0.25; 0.20)	0.81
24-h HRV triangular index	0.02	0.18	-0.11 (-0.33; 0.21)	0.34	0.09	**0.009**	-0.19 (-0.43; 0.05)	0.12
24-h HRV SDNN (ms)	0.07	**0.017**	**-0.24 (-0.45; -0.02)**	**0.035**	0.06	**0.032**	-0.16 (-0.40; 0.09)	0.21
Endothelial cell activation								
ICAM-1 (ng/ml)	0.01	0.37	-0.06 (-0.28; 0.17)	0.62	0.19	**<0.001**	-0.05 (-0.27; 0.18)	0.69
VCAM-1 (ng/ml)	0.16	**<0.001**	-0.09 (-0.30; 0.12)	0.40	0.14	**<0.001**	-0.06 (-0.29; 0.18)	0.63
MCP-1 (pg/ml)	0.03	0.14	-0.03 (-0.25; 0.20)	0.82	0.05	0.054	-0.02 (-0.27; 0.22)	0.84
Ambulatory blood pressure								

24-h SBP (mmHg)	0.26	**<0.001**	0.10 (-0.10; 0.29)	0.33	0.41	**<0.001**	0.01 (-0.18; 0.21)	0.90
24-h DBP (mmHg)	0.08	**0.012**	0.14 (-0.08; 0.56)	0.22	0.15	**<0.001**	0.04 (-0.19; 0.27)	0.74
24-h pulse pressure (mmHg)	0.21	**<0.001**	0.01 (-0.19;0.21)	0.94	0.38	**<0.001**	-0.02 (-0.22; 0.18)	0.86

Standardized β (Std β) represents the change in the dependent variable for every 1 SD change in the independent variable. β, partial regression coefficients; 95 % CI, 95 % confidence interval; Adjusted R^2, coefficient of determination of each total regression model; p_1, p value for Adjusted R^2; p_2, p value for Std β (95 % CI); All models include the following covariates: age, body mass index, socio-economic status, active energy expenditure**,** total cholesterol, triglycerides, glucose, C - reactive protein, gamma-glutamyltransferase, alcohol and smoking. HRV, heart rate variability; HF (n.u.), high frequency measured at normalized unit; LF (n.u.), low frequency measured at normalized unit of the frequency domain; SDNN, standard deviation of normal RR intervals; h, hour; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemo-attractant protein-1; SBP, systolic blood pressure; DBP, diastolic blood pressure and $p<0.05$ were considered significant

Mediation and sensitivity analyses

To better understand leptin's positive relationship with 24-hour DBP in the white men, we performed a mediation analysis (Supplementary Table 5S) by adding a marker of autonomic activity (24-hour HRV total power) to the multiple regression model with 24-hour DBP as dependent variable. The inclusion of 24-hour HRV total power in the model resulted in the change of the association between leptin and 24-hour DBP from significantly positive (Adjusted R^2=0.19; p_1<0.001 and Std β=0.37; p_2=0.006) to a non-significant (Adjusted R^2 =0.24; p_1<0.001 and Std β=0.23; p_2=0.090) one. However, a negative association was observed between 24-hour DBP and the newly introduced 24-hour HRV total power (Adjusted R^2= 0.24; p_1<0.001 and Std β=-0.30; p_2=0.003). We then extended the mediation analysis to further confirm and increase the robustness of our results in the observed association between leptin and DBP, by replacing HRV total power in this model with 24-hour HRV LF/HF (sensitivity analysis, Supplementary Table 6S). Our result from the sensitivity analysis also showed a change of association between leptin and 24-hour DBP from a significantly positive (Adjusted R^2=0.19; p_1<0.001 and Std β=0.37; p_2=0.006) to a borderline significant (Adjusted R^2 =0.23; p_1<0.001 and Std β=0.19; p_2=0.052) one. Concurrently, a positive association was observed between 24-hour DBP and the newly introduced 24-hour HRV LF/HF (Adjusted R^2= 0.23; p_1<0.001 and Std β=0.16; p_2=0.037). The observed association between 24-hour HRV total power and DBP (Std β=-0.30; p_2=0.003) (Supplementary Table 5S) was observed to be much stronger than that of the association between 24-hour HRV LF/HF and DBP (Std β=0.16; p_2=0.037) (Supplementary Table 6S). The associations with DBP were in opposite directions, where 24-hour HRV total power indicated a negative, and 24-hour HRV LF/HF a positive association with DBP.

5 Discussion

Despite an almost ten-fold higher concentration of leptin in young healthy women compared to men, leptin was shown to play a more active role in men by associating with numerous markers of autonomic function. Particularly in white men, leptin associated positively with ambulatory heart rate, 24-hour HRV LF/HF and DBP as well as negatively with HRV total power. Our

mediation and sensitivity analyses results suggest that leptin may influence (diastolic) blood pressure regulation via autonomic function in the men with moderate level of adiposity. Our finding therefore suggest an early influence of leptin in the regulation of blood pressure via autonomic modulation and was found to be mainly mediated by decreased 24-hour HRV total power. This finding was further supported by the mediation through increased 24-hour HRV LF/HF.

Leptin and autonomic activity

Gender-specific differences in the association between leptin and autonomic activity are controversial. Our study together with a previous study showed leptin's prominent effect on autonomic function in men rather than women [12]. Guerra et al. [27] demonstrated increased leptin receptor up-regulation in skeletal muscles of men, but not women, due to the influence of testosterone. This finding supports different functionality of leptin and its receptor in men and women. However, our findings are in disagreement with a study by Flanagan et al. [28] who observed a greater effect of leptin on sympathetic nervous system in women (N = 62) rather than men (N = 68).

Leptin and blood pressure

Leptin is a crucial contributory factor in obesity-related hypertension development in both human [24] and animal studies [29]. In a multi-ethnic group of lean and overweight/obese hypertensive men and women (aged >20 years of age) from the USA, leptin associated with hypertension prevalence after adjusting for BMI, ethnicity and other covariates [4]. Our study population were normotensive and the majority were not obese, yet we observed leptin's independent link with DBP in white men. Our finding was similar to a study in Japanese middle-aged men (34-65 years), where DBP also associated with leptin within normal ranges of blood pressure [30]. We, therefore, confirm the previously mentioned findings and extend them based on the outcome of our mediation and sensitivity analysis where both a decrease in 24-hour HRV total power and increase in 24-hour HRV LF/HF were significantly related to increased DBP, though the effect of 24-hour HRV total power seemed stronger. A reduction in total HRV has

been associated with increased risk of hypertension development (including pulmonary arterial hypertension in children [31]), as well as mortality following myocardial infarction [32, 33].

The link between 24-hour DBP and leptin was absent in young women who had a higher body fat percentage and greater circulating leptin concentration. The absence of this link cannot be fully explained by our study and further research is required. However, we could postulate that the vasoconstrictor effects of leptin on the blood pressure of women may either be attenuated due to their reduced susceptibility to central sympathetic neural activity drive [34] or because of the central protective effect of oestrogen against elevated blood pressure at middle age [35].

Not only was the link between 24-hour DBP and leptin absent in women, it was also absent in black men. A recent study found elevated DBP to be predictive for future CVD in white adults, whereas SBP was prominent in black adults [36]. Leptin may thus be an early contributor to elevated DBP in white men who also presented with higher BMI and SNA profile than black men. Apart from the effects of adiposity the renin-angiotensin system (RAS) is also eminent in obesity [37]. However, in non-obese normotensive German men (age 25 years), angiotensinogen associated positively with leptin [38], thus suggesting the potential role of RAS in the link between leptin and blood pressure in our study. However, black populations are known to have lower renin, angiotensin I and II levels compared to whites [39, 40], possibly explaining the lack of association between DBP and leptin in black men. More research is necessary to establish a link between leptin and the RAS system in Africans.

Leptin and endothelial function

An altered vascular function is one possible mechanism by which leptin may induce obesity-related hypertension [41]. Indeed, our findings support this notion with leptin being related to the chemokine, MCP-1 in the unadjusted analyses, further implicating obesity in altering vascular physiology, even at younger ages**.**

The findings of our study should be interpreted in the context of its strengths and limitations. We did not directly assess autonomic nerve activity by using methods such as norepinephrine spill

over or microneurography, but heart rate and HRV are well established measures reflecting autonomic nervous system activity [42]. Our mediation and sensitivity analysis may not be the ideal procedure for determining the pathophysiology by which leptin might influence diastolic blood pressure regulation, and a more direct measurement of autonomic activity is therefore recommended. We also did not perform correction for multiple testing, as this study is considered as hypothesis generating work. The study is limited to a cross-sectional study design, thus the observed associations cannot be used to interpret any related causality. However, the study was well-designed and conducted under well-controlled conditions.

6 Conclusion

In conclusion, although the majority of our young apparently healthy study population were not obese, leptin's independent association with autonomic neural activity, suggest an early influence of this adipokine on autonomic modulation and perhaps future blood pressure elevation. Considering that elevated DBP was reported to be predictive for future CVD, our study suggests a potential role that leptin may play in this process, especially men with a modest level of adiposity.

7 Financial support

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9 Supplementary materials

Table 1S. Interaction effect between leptin and either ethnicity or sex in their associations with some main dependent variables

***Independent variable*: Leptin (ng/ml)**		
Dependent variables:	**Ethnicity**	**Sex**
24-h heart rate (bpm)	p=0.19	**p<0.001**
24-h HRV LF (n.u)	p=0.19	**p<0.001**
24-h HRV HF (n.u.)	p=0.32	**p=0.001**
24-h HRV LF/HF	**P=0.041**	**p<0.001**
24-h HRV total power ($ms^{2)}$	**p<0.001**	p=0.24
24-h SBP (mmHg	**p<0.001**	p=0.36
MCP-1 (pg/ml)	**p<0.001**	**p<0.001**

P-values obtained with multiple regression analyses. HRV, heart rate variability; h, hour; LF, Low frequency; HF, high-frequency; SBP, systolic blood pressure; h, hour; MCP-1, monocyte chemo-attractantprotein-1 and p<0.05 were considered significant

Table 2S. Pearson correlations between leptin and markers of autonomic activity, endothelial cell activation and ambulatory blood pressure

	Black men (N = 168)	**White men (N = 169)**	**Black women (N = 263)**	**White women (N = 220)**
Autonomic activity				
24-h heart rate (bpm)	**r=0.21; p=0.006**	**r=0.46; p<0.001**	**r=0.20; p=0.001**	**r=0.29; p<0.001**
Day time heart rate (bpm)	r=0.14; p=0.084	**r=0.31; p<0.001**	**r=0.13; p=0.032**	**r=0.24; p<0.001**
Night time heart rate (bpm)	**r=0.26; p=0.001**	**r=0.48; p<0.001**	**r=0.27; p<0.001**	**r=0.31; p<0.001**
24-h HRV LF (n.u.)	**r=0.21; p=0.007**	r=0.12; p=0.14	r=0.04; p=0.54	r=0.06; p=0.39
24-h HRV HF (n.u.)	**r=-0.21; p=0.007**	r=-0.73; p=0.35	r=-0.64; p=0.31	r=-0.07; p=0.29
HRV 24-h LF/HF	**r=0.16; p=0.045**	**r=0.17; p=0.023**	r=0.06; p=0.35	r=0.09; p=0.18
HRV 24-h HRV total power (ms^2)	**r=-0.24; p=0.002**	**r=-0.45; p<0.001**	**r=-0.16; p=0.012**	**r=-0.28; p<0.001**
24-h HRV triangular index	**r=-0.19; p=0.014**	**r=-0.33; p<0.001**	**r=-0.20; p=0.001**	**r=-0.26; p<0.001**
24-h HRV SDNN (ms)	**r=-0.26; p=0.001**	**r=-0.39; p<0.001**	**r=-0.24; p<0.001**	**r=-0.27; p<0.001**
Endothelial cell activation				
ICAM-1 (ng/ml)	r=-0.04; p=0.60	r=0.11; p=0.16	r= 0.03; p=0.65	r=0.28; p<0.001
VCAM-1 (ng/ml)	**r=-0.26; p=0.001**	r=-0.15; p=0.054	**r=-0.17; p=0.005**	r=-0.02; p=0.77
Log MCP-1 (pg/ml)	r=-0.12; p=0.14	**r=0.17; p=0.026**	r=0.05; p=0.40	**r=0.18; p=0.007**
Ambulatory blood pressure				
24-h SBP (mmHg)	**r=0.26; p=0.001**	**r=0.28; p<0.001**	**r=0.43; p<0.001**	**r=0.52; p<0.001**

24-h DBP (mmHg)	**r=0.28; p<0.001**	**r=0.40; p<0.001**	**r=0.29; p<0.001**	**r=0.33; p<0.001**
24-h pulse pressure (mmHg)	r=0.07; p=0.36	r=-0.04; p=0.59	**r=0.36; p<0.001**	**r=0.45; p<0.001**

HRV, heart rate variability; HF (n.u.), high frequency measured at normalized unit; LF (n.u.), low frequency measured at normalized unit of the frequency domain; SDNN, standard deviation of normal RR intervals; h, hour; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemo-attractant protein-1; SBP, systolic blood pressure and p<0.05 were considered significant

Table 3S. Partial correlation coefficients between leptin and markers of autonomic activity, endothelial cell activation and ambulatory blood pressure after adjusting for age and body mass index

	Black men (N = 168)	**White Men (N = 169)**	**Black women (N = 263)**	**White women (N = 220)**
Autonomic activity				
24-h heart rate (bpm)	**r=0.29; p<0.001**	**r=0.35; p<0.001**	**r=0.15; p=0.015**	**r=0.24; p<0.001**
Day time heart rate (bpm)	**r=0.27; p<0.001**	**r=0.31; p<0.001**	**r=0.12; p=0.050**	**r=0.24; p<0.001**
Night time heart rate (bpm)	**r=0.25; p=0.001**	**r=0.36; p<0.001**	**r=0.17; p=0.007**	**r=0.23; p=0.001**
24-h HRV LF (n.u.)	r=0.11; p=0.18	**r=0.17; p=0.027**	r=0.02; p=0.70	r=-0.16; p=0.82
24-h HRV HF (n.u.)	r=-0.10; p=0.21	r=-0.15; p=0.06	r=-0.03; p=0.66	r=0.20; p=0.77
24-h HRV LF/HF	r=0.05; p=0.57	**r=0.23; p=0.003**	r=0.03; p=0.60	r=-0.01; p=0.92
24-h HRV total power (ms^2)	**r=-0.21; p=0.007**	**r=-0.38; p<0.001**	r=-0.09; p=0.14	**r=-0.14; p=0.001**
24-h HRV triangular index	r=-0.09; p=0.26	**r=-0.21; p=0.007**	r=-0.73; p=0.25	**r=-0.20; p=0.003**
24-h HRV SDNN (ms)	r=-0.15; p=0.061	**r=-0.19; p=0.013**	**r=-0.17; p=0.008**	**r=-0.18; p=0.007**
Endothelial cell activation				
ICAM-1 (ng/ml)	r=-0.11; p=0.18	r=-0.02; p=0.76	r=-0.02; p=0.70	r=0.004; p=0.95
VCAM-1 (ng/ml)	**r=-0.16; p=0.044**	r=-0.04; p=0.63	r=-0.05; p=0.42	r=-0.12; p=0.08
MCP-1 (pg/ml)	r=-0.04; p=0.59	r=0.01; p=0.93	r=-0.03; p=0.62	r=-0.67; p=0.33
Ambulatory blood pressure				
24-h SBP (mmHg)	r=-0.06; p=0.46	r=-0.12; p=0.14	r=0.06; p=0.13	r=0.78; p=0.25

24-h DBP (mmHg)	r=0.03; p=0.68	**r=0.30; p<0.001**	r=0.10; p=0.13	r=0.13; p=0.054
24-h pulse pressure (mmHg)	r=-0.10; p=0.21	**r=-0.36; p<0.001**	r=-0.15; p=0.82	r=-0.38; p=0.58

HRV, heart rate variability; HF (n.u.), high frequency measured at normalized unit; LF (n.u.), low frequency measured at normalized unit of the frequency domain; SDNN, standard deviation of normal RR intervals; h, hour; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemo-attractant protein-1; SBP, systolic blood pressure and $p<0.05$ were considered significant

Table 4S. Analysis of covariance after adjusting for age and social economic status

Variables	Black men	White men	p value	Black women	White women	p value
24-h HRV total power (m/s^2)	614 [559; 673]	633 [578; 693]	0.66	365 [338; 393]	436 [402; 473]	**0.003**
24-h DBP (mmHg)	70.4±6.15	69.8±6.11	0.40	68.3±5.97	67.9±5.92	0.47
Leptin (ng/ml)	3.29 [270; 401]	5.52 [553; 672]	**0.001**	34.3 [310; 381]	23.5 [210; 263]	**<0.001**

Data are adjusted means ± SD or -95% and +95% CI for logarithmically transformed variables. SD, standard deviation; CI, confidence inter

heart rate variability; h, hour; DBP, diastolic blood pressure and $p<0.05$ were considered significant

Table 5S. Independent association between 24 hours diastolic blood pressure and leptin after the inclusion of HRV total power to the model in white men (N = 169).

	24-h DBP (mmHg)	
Adjusted R^2 = 0.24; p_1<0.001	**Std β (95 % CI)**	**p_2**
Leptin (ng/ml)	0.23 (-0.03; 0.50)	0.090
Age (years)	0.13 (-0.06; 0.32)	0.19
SES status	0.07 (-0.12; 0.270	0.47
Body mass index (kg/m^2)	-0.04 (-0.30; 0.22)	0.78
Active energy expenditure (k/Cal)	0.24 (-0.14; 0.18)	0.77
Total cholesterol (mmol/l)	-0.0002 (-0.18; 0.18)	0.98
Triglyceride (mmol/l)	0.09 (-0.10; 0.290	0.37
C-reactive protein (mg/l)	-0.07 (-0.26; 0.120	0.49
gamma glutamyltransferase (U/l)	0.13 (-0.06; 0.32)	0.18
Glucose (mmol/l)	-0.11 (-0.28;	0.20
Self-reported alcohol intake (No/ yes)	0.07 (-0.09; 0.23)	0.42
Self-reported tobacco use (No/ yes)	-0.01 (-0.19; 0.17)	0.93
24-h HRV total power (m/s)	**-0.30 (-0.49; -0.10)**	**0.003**

Standardized β (Std β) represents the change in the dependent variable for every 1 SD change in the independent variable. β, partial regression coefficients, 95 % CI, 95 % confidence interval; Adjusted R^2, coefficient of determination of the total regression model; p_1, p value for Adjusted R^2; p_2, p value for Std β (95 % CI); DBP, diastolic- blood pressure; h, hour; SES, socio-economic status; All independent variables were included at the same time. P<0.05 were considered significant

Table 6S. Independent association between 24-hour diastolic blood pressure and leptin after the inclusion of 24-hour HRV LF/HF ratio to the model in white men (N = 169).

	24-hour DBP (mmHg)	
Adjusted R^2 = 0.23; p_1<0.001	**Std β (95 % CI)**	**p_2**
Leptin (ng/ml)	0.19 (-0.01; 0.37)	0.052
Age (years)	0.13 (0.01; 0.27)	0.036
SES status	0.06 (-0.09; 0.20)	0.45
Body mass index (kg/m^2)	-0.03 (-0.23; 0.17)	0.75
Active energy expenditure (k/Cal)	0.05 (-0.06; 0.17)	0.36
Total cholesterol (mmol/l)	-.0.06 (-0.20; 0.08)	0.42
Triglyceride (mmol/l)	0.09 (-0.05; 0.23)	0.21
C-reactive protein (mg/l)	-0.01 (-0.13; 0.13)	0.97
gamma glutamyltransferase (U/l)	0.28 (0.16; 0.40)	<0.001
Glucose (mmol/l)	-.0.3 (-0.16; 0.10)	0.66
Self-reported alcohol intake (No/ yes)	0.04 (-0.08; 0.15)	0.55
Self-reported tobacco use (No/ yes)	-0.03 (-0.15; 0.09)	0.63
24-HRV LF/HF	**0.16 (0.04; 0.28)**	**0.037**

Standardized β (Std β) represents the change in the dependent variable for every 1 SD change in the independent variable. β, partial regression coefficients, 95 % CI, 95 % confidence interval; Adjusted R^2, coefficient of determination of the total regression model; p_1, p value for Adjusted R^2; p_2, p value for Std β (95 % CI); DBP, diastolic- blood pressure; h, hour; SES, socio-economic status; All independent variables were included at the same time. P<0.05 were considered significant.

CHAPTER 4; Manuscript 2

Leptin and the Vasculature in Young adults: The African-PREDICT Study

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Leptin and the vasculature in young adults: The African-PREDICT study

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Abstract

Background and aim: Information regarding the effect of leptin on the vasculature in young healthy adults at risk for cardiovascular disease development is limited. We therefore examined the associations between measures of subclinical atherosclerosis (carotid intima-media thickness, carotid cross-sectional wall area),

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Summary of instruction to authors used for the preparation of manuscript 2

Journal Details:	
Title: of journal	European Journal of Clinical Investigation (EJCI):
Impact factor	3.086
Aims & scope	EJCI publishes original contributions from basic molecular sciences to applied clinical and translational research and evidence-based medicine across a wide range of subspecialties. The Journal considers topics on the following: genetic, molecular, cellular, or physiological basis of human biology and disease. The Journal also considers research that addresses the following: prevalence, diagnosis, course, treatment, and prevention of disease.
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Original paper	This article is an original article. Hence, the following guidelines were followed: All copy must be typed double-spaced with 1-inch margins and 12-point font size, do not justify lines. Do not use line numbering. World count limited to 2700 words (not including abstract, tables, figures, and references).
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	numbered consecutively in order of appearance. In text citations should cite references in consecutive order using Arabic superscript numerals), Acknowledgments, Funding Information, and Authors Contributions shoulsd be included, 5 tables and figures maximum
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Leptin and the Vasculature in Young Adults:

The African-PREDICT Study

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1 Abstract

Background and aim: Information regarding the effect of leptin on the vasculature in healthy young adults at risk for cardiovascular disease development is limited. We, therefore, examined the associations between measures of subclinical atherosclerosis (carotid intima-media thickness, carotid cross-sectional wall area), large artery stiffness (pulse wave velocity) and a measure of endothelial dysfunction (von Willebrand factor) with leptin in young, healthy men and women.

Methods: In a cross-sectional study in South Africa involving 820 normotensive individuals (337 men and 483 women) aged 20-30 years, we measured carotid intima-media thickness, carotid cross-sectional wall area, pulse wave velocity, von Willebrand factor from citrated plasma, and leptin from serum.

Results: Despite 7-fold higher leptin in women than men (p<0.001), only in healthy young men we observed negative, independent associations between measures of carotid wall thickness (carotid intima-media thickness: R^2=0.05; β=-0.20; p=0.036; carotid cross-sectional wall area: R^2=0.05; β=-0.20; p=0.035) with leptin in multivariable-adjusted regression analyses. When reviewing these associations across body mass index categories, we found an association to be evident only in overweight men (carotid intima-media thickness: R^2=0.15; β=-0.41; p=0.007; carotid cross-sectional wall area: R^2=0.21; β=-0.47; p=0.002). No association was observed in the women or between pulse wave velocity and von Willebrand factor with leptin.

Conclusion: In healthy young men we found a beneficial inverse association between measures of carotid wall thickness and circulating leptin, thereby supporting a potential vascular protective role of leptin.

Keywords: atherosclerosis, healthy, overweight, carotid intima-media thickness, endothelium and sex.

2 Introduction

Obesity is a growing public health concern worldwide [1] and is also now considered an important factor in the development of atherosclerotic cardiovascular disease (CVD), diabetes and other related metabolic disorders [2]. But the mechanisms by which adiposity contributes to alterations in both the anatomy and physiology of blood vessels remains only partially understood [2]. Accumulating evidence suggests a link between adipose tissue and the vasculature, implicating the product of the obesity (*ob*) gene, namely leptin [3].

Leptin is a pleiotropic [4] and vasoactive hormone [5], with a wide range of functions beyond the regulation of energy intake and expenditure [6]. The presence of leptin's receptor within the vasculature [7], also laid credence for leptin's involvement in the regulation of vascular function [8] which itself has yielded conflicting reports [9]. For example, leptin was shown to predict atherosclerosis, stroke, myocardial infarction and also coronary events in either overweight or obese individuals [10-12]. In contrast, other studies have reported a vascular protective role [8] of leptin against atherosclerosis in either overweight or obese humans or animals [13, 14]. Recently, a meta-analysis involving 4,257 participants with CVD and 26,710 controls showed that elevated leptin levels might not be associated with the risk of developing coronary heart disease and stroke in both men and women [15].

Suggested concepts which may explain the disparity between the beneficial and detrimental effects of leptin include the widespread cardiovascular and dose-dependent effects of leptin, as well as the concept of selective leptin resistance [16]. It is currently unknown whether leptin plays a role during the early phases of atherosclerosis development in young adults in the absence of overt CVD. Previous studies investigating the involvement of leptin in endothelial dysfunction and atherosclerosis development generally focused on older (mean age, 45 years) overweight or obese individuals, those with the metabolic syndrome (hypertension and diabetes), diseased (CVDs; myocardial infarction, coronary heart disease and stroke) or in Western populations [9, 10, 17-19]. However, the role of leptin on vascular health especially in young adults (20-30 years of age), and potential disparities in men and women at risk of obesity

and CVD development, is unknown. To address this, we investigated young healthy men and women, and determined whether measures of subclinical atherosclerosis, large artery stiffness and a marker of endothelial dysfunction (von Willebrand Factor (vWF) [20, 21] are associated with leptin.

3 Materials and methods

Study population

Participants were recruited as part of the larger African-PREDICT study (African Prospective study on the Early Detection and Identification of Cardiovascular disease and HyperTension). The African-PREDICT study is aimed at following up healthy young adults over a period of 10 years, in order to identify and track potential markers of early cardiovascular risk.

The current cross-sectional sub-study includes the first consecutive 820 participants (men N=337; women N=483; white N=389 and black N=431) enrolled in the African-PREDICT study, with measurements performed on the campus of the North-West University in South Africa. The study adhered to all applicable requirements of the Helsinki Declaration, and the African-PREDICT study and this present sub-study have been approved by the Health Research Ethics Committee of the North-West University. All subjects participated voluntarily in the study and also provided written informed consent. Inclusion criteria for eligible participants were the following: black and white men and women aged 20-30 years; normal clinic BP (BP <140/90 mmHg after 3 consecutive readings) and blood glucose; no known CVD; not using any antihypertensive medication; no chronic disease (or treatment thereof); HIV-free and not pregnant or breastfeeding.

Questionnaire data

Demographic and lifestyle questionnaires, as well as the global physical activity questionnaire (GPAQ) were used to determine medical history, lifestyle, socio-economic status, traditional risk factors and physical activity.

Body composition and physical activity

Weight ((kg) (SECA electronic scales, SECA, Birmingham, UK)), height ((cm) (SECA stadiometer, SECA)) and waist circumference were measured with a non-flexible tape measure (Holtain, Crymych, UK). Body Mass Index was calculated using the standard formula of weight (kg)/height (m^2). Bioelectrical impedance was used to assess lean body mass and body fat percentage (Bodystat 1500MDD dual-frequency analyser, Bodystat, Ltd, Ballakaap, British Isles). For the assessment of active energy expenditure (AEE; estimation of physical activity), each participant wore a combined heart rate (HR) and accelerometer, namely an ActiHeart device (CamNtech, Cambridge, UK) for a maximum of 7 consecutive days and data was collected at 60-s epochs. The AEE was further indexed by dividing AEE by the weight of the participants, to compensate for increased energy expenditure accompanied by an increase in body mass.

Biochemical measurements

In the early morning, a research nurse took a fasting blood sample, and samples were prepared on site and stored at -80 ^{0}C. We performed analyses of serum high-sensitivity C-reactive protein, total cholesterol, low density-lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides, gamma-glutamyltransferase, as well as glucose in sodium fluoride plasma (Cobas Integra 400plus, Roche, Basel, Switzerland). Serum cotinine was determined with a chemiluminescence method on the Immulite (Siemens, Erlangen, Germany). Serum leptin levels were determined in duplicate using an enzyme-linked immunosorbent assay kit (Quantikine, R&D Systems, MN, USA) and adiponectin levels were determined with the Human Adiponectin ELISA kit (BioCat GmbH, Heidelberg, Germany). We determined von Willebrand Factor (vWF) in citrate plasma using a sandwich ELISA. Polyclonal rabbit anti-vWF antibody and rabbit anti-vWF-horseradish peroxidase antibody (DAKO, Glostrup, Denmark) were used to perform the assay.

Cardiovascular measurements

Participants were fitted with a 24-hour ambulatory blood pressure and ECG apparatus (CardioXplore®, CE0120, Meditech, Budapest, Hungary), using an appropriately sized cuff on the participant's non-dominant arm. If less than 70% of the recordings were successful, the measurement was repeated the next day.

Carotid-intima media thickness (CIMT) was measured on the left and right common carotid artery (General Electric Vivid E9, GE Vingmed Ultrasound A/S, Horten, Norway), by a single medical technologist. Images from at least two optimal angles of the left and right common carotid artery were obtained. A single reader conducted measurements using a semi-automated program, namely the Artery Measurement Systems software (AMS) II v1.139 (Chalmers University of Technology, Gothenburg, Sweden). The cross-sectional wall area (CSWA) was calculated to confirm structural and not functional changes in luminal diameter: CSWA = π(d/2+ CIMT)^2- π(d/2)^2, where d denotes luminal diameter.

Carotid-femoral pulse wave velocity (PWV) was measured using the Sphygmocor® XCEL device (AtCor Medical Pty. Ltd, Sydney, Australia) according to the manufacturer's instructions. PWV was measured along the descending thoraco-abdominal-aorta using the foot-to-foot velocity method, while the participant was in a supine position. Prior to this test, participants were not allowed to eat at least 8 hours before the procedure. During this measurement, PWV was captured at the right carotid and femoral arterial pulse points. The femoral artery waveform was captured via an appropriately sized cuff placed around the thigh, and the carotid arterial waveform was captured simultaneously via applanation tonometry. The distances between the pulsated sites were measured using an infantometer, and 80% of these distances were used as the pulse wave travelled distance.

Statistical analyses

Variables that were not normally distributed were log transformed and represented as geometric mean with 5th and 95th percentiles. Normally distributed variables were presented as mean ±

standard deviation and categorical variables represented as percentages. We tested the interaction effects of either ethnicity or BMI for the associations between vWF, PWV, CIMT and CSWA with leptin. Independent t-tests were done to compare means of men and women, and Chi-square tests (χ^2) to compare frequencies. Linear regression analyses were conducted to determine the associations of vWF, PWV, CIMT and CSWA with leptin. These analyses were performed in the total group, in groups according to leptin tertiles, and in groups based on BMI categories. Regression models all included the following covariates: leptin, age, ethnicity, socio-economic score, body fat percentage, 24-h mean arterial blood pressure, LDL-cholesterol, C-reactive protein, glucose, gamma-glutamyltransferase and Moderate-Vigorous Physical activity.

4 Results

Characteristics of the study population

Aligned with our aim we grouped our participants into men and women, also due to reported differences in leptin concentrations with respect to sex [22, 23] (Table 1). When comparing men and women, we found that men had lower vWF (p=0.018), but higher PWV (p<0.001), CIMT (p=0.021) and CSWA (p<0.001) than women. Women had higher BMI (p=0.004), body fat percentage and leptin (all p<0.001) than men.

Table 1. Basic characteristics of young men and women

Number of participants	**Men (N = 337)**	**Women (N = 483)**	***p***
Ethnicity, black, N (%)	168 (49.9)	263 (54.45)	0.19
Age (years)	24.9±3.0	24.5±3.1	0.67
Socioeconomic status			
Low, N (%)	127 (37.7)	187 (38.7)	0.051
Middle, N (%)	81 (24.0)	129 (26.7)	
High, N (%)	129 (38.3)	167 (34.6)	
Body composition			
Body mass index (kg/m^2)	24.2 [17.8; 33.6]	25.3 [18.3; 37.8]	0.004
Waist circumference (cm)	82.0 [64.8; 108]	77.4 [63.0; 103]	<0.001
Body fat percentage (%)	17.5±6.73	32.0±8.63	<0.001
Lean body mass (kg)	61.5±11.7	45.7±6.61	<0.001
Biochemical variables			
Leptin (ng/ml)	4.27 [0.39; 32.5]	29.0 [6.58; 96.7]	<0.001
Von Willebrand factor (%)	80.7 [41.0; 173]	84.9 [39.0; 203]	0.018
Adiponectin (µg/ml)	3.07 [0.66; 10.1]	4.56 [1.13; 14.5]	0.008
Total cholesterol (mmol/l)	4.19 [2.81; 6.12]	4.09 [2.80; 5.97]	0.062
LDL-C (mmol/l)	2.73 [1.54; 4.57]	2.56 [1.45; 4.25]	0.11
HDL-C (mmol/l)	1.16 [0.75; 1.76]	1.33 [0.81; 2.10]	0.50
Triglycerides (mmol/l)	0.95 [0.45; 2.21]	0.76 [0.39; 1.65]	0.002
Glucose (mmol/l)	4.75±0.80	4.60±0.67	<0.001
C-reactive protein (mg/l)	0.76 [0.10; 6.72]	1.45 [0.14; 12.7]	0.14
Cardiovascular measurements			
24-h systolic BP (mmHg)	122±8.01	113±8.53	0.21
24-h diastolic BP (mmHg)	70.1±5.92	68.1±5.63	0.31
24-h mean arterial BP (mmHg)	90.7±5.94	86.1±6.34	0.19
Pulse wave velocity (m/sec)*	6.65±0.84	6.04±0.83	<0.001
Carotid intima-media thickness (mm)*	0.44±0.07	0.43±0.07	0.021
Carotid cross sectional wall area (mm^2)*	8.42±1.63	7.69±1.61	<0.001

Lifestyle			
Active energy expenditure (kCal)	328 [136; 672]	390 [147; 924]	0.035
AEE/body weight (kCal/kg)	36.5 [14.8; 74.7]	43.4 [16; 103]	0.035
MVPA (day)	53.8 [6.43; 406]	50.5 [7.14; 360]	0.51
Self-reported tobacco use, N min//total (**%**)	203/452 (44.91)	113/313 (36.10)	0.015
Cotinine (ng/ml)	7.60 [1.00; 411]	2.16 [1.00; 228]	<0.001
Gamma glutamyltransferase (U/l)	27.0 [12.6; 82.8]	18.7 [7.90; 54.8]	0.22
Self-reported alcohol use, N/total (**%**)	108/179 (60.3)	211/593 (35.6)	<0.001

Values are expressed as arithmetic mean ± standard deviation or geometric mean (5th to 95th percentile intervals) for logarithmically transformed variables, or number of participants and percentages (%).

*The values of carotid intima-media thickness, cross-sectional wall area as well as pulse wave velocity were adjusted for 24-hour mean arterial pressure (ANCOVA). BMI, body mass index; BP, blood pressure; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; AEE, active energy expenditure; MVPA, Moderate-Vigorous Physical activity and $p \leq 0.05$ were considered significant.

Pearson analysis

We performed Pearson correlation analyses between vWF, PWV, CIMT and CSWA with leptin in men and women, and found a negative association between CIMT and CSWA with leptin only in men (all $p<0.001$) (Figure 1). There were no significant associations between vWF and PWV in men, as well as between vWF, PWV, CIMT and CSWA with leptin women.

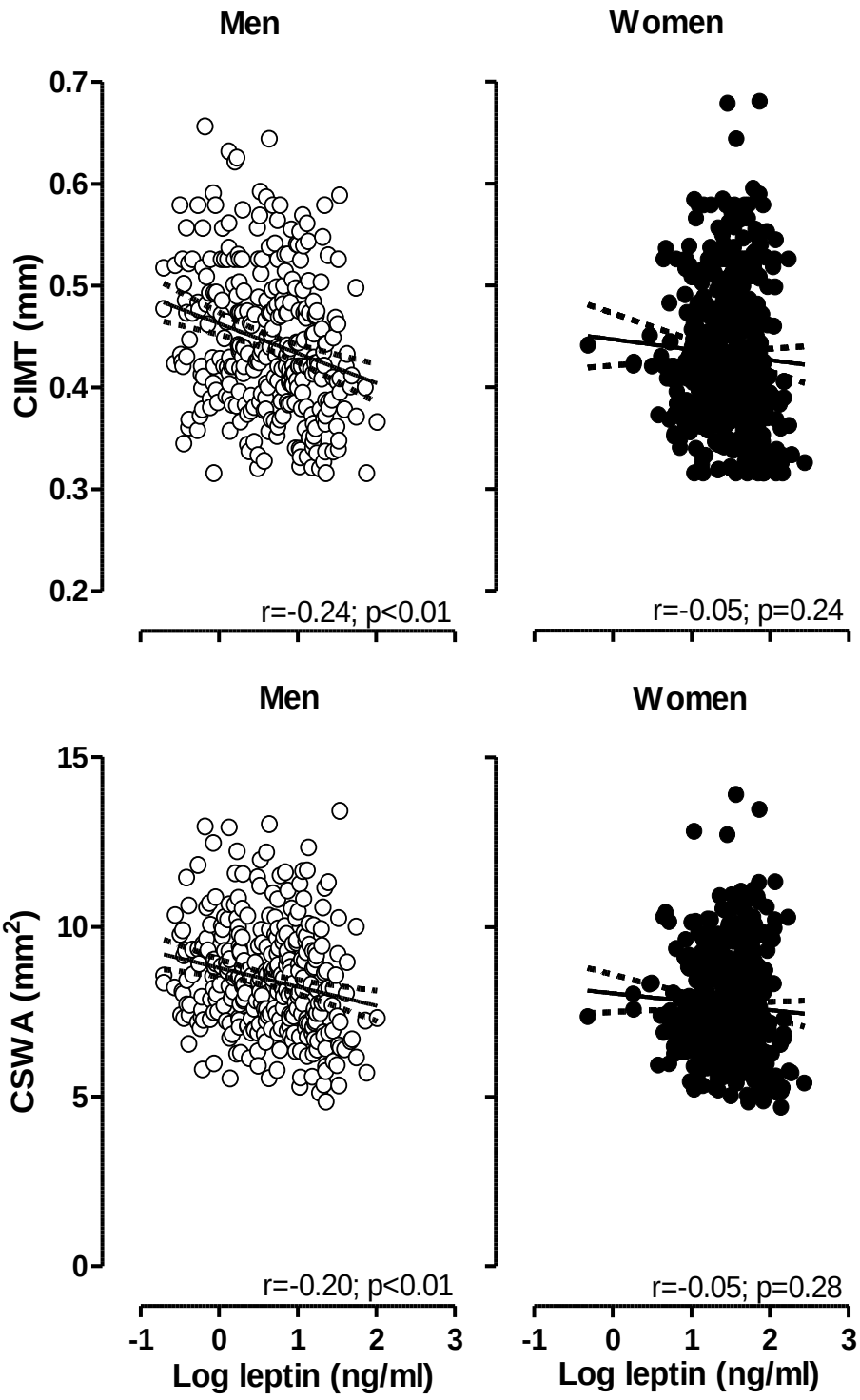

Figure 1: Leptin plotted against carotid intima-media thickness and cross-sectional wall area in both men and women. Solid and dashed lines represent the regression line and the 95% CI boundaries.

Multivariable adjusted regression analyses

We conducted a forward stepwise multiple variable adjustment analysis (Table 2) where we determined the associations between vWF, PWV, CIMT and CSWA with leptin in men and women. From the result, only men showed an independent inverse association between CIMT (Std β=-0.27; p<0.001; R^2= 0.07) and CSWA (Std β=-0.24; p=0.002; R^2= 0.06) with leptin.

Table 2. Forward stepwise multiple regression analysis between von Willebrand factor, pulse wave velocity, carotid intima-media thickness and cross-sectional wall area with leptin in men and women

	Men (N =337)				Women (N = 483)			
Variable	**Adj R^2**	**Adj *p***	**β (95% CI)**	***p***	**Adj R^2**	**Adj *p***	**β (95% CI)**	***p***
vWF (%)	**0.01†**	**0.04**	-		0.01	0.10	-	-
PWV (m/sec)	**0.12†**	**<0.001**	-		**0.10†**	**<0.001**	-	-
CIMT (mm)	**0.07†**	**<0.001**	**-0.27 [-0.41; -0.12]**	**<0.001**	**0.02†**	**0.016**	-	-
CSWA (mm^2)	**0.06†**	**0.003**	**-0.24 [-0.40; -0.09]**	**0.002**	**0.02†**	**0.016**	-	-

Standardized β (Std β) represents the change in the dependent variable for every 1 SD change in the independent variable. β, partial regression coefficients; 95 % CI, 95 % confidence interval; Adjusted R^2, coefficient of determination of each total regression model; Models for the regression were all included at once and included: leptin, age, ethnicity, socio-economic score, body fat percentage, 24-hour mean arterial blood pressure, LDL-cholesterol, C-reactive protein, glucose, gamma-glutamyltransferase and Moderate-Vigorous Physical activity. vWF, von Willebrand factor; PWV, pulse wave velocity; CIMT, carotid intima-media thickness; CSWA, cross sectional wall area; Adj R^2, adjusted R^2 and Adj p represents, p values for each regression model. Bold values indicate p≤0.05 and the symbol (**†**), indicates R^2 values at *p*≤0.05

Sensitivity analysis

We conducted a forward stepwise multiple regression analysis between vWF, PWV, CIMT and CSWA with leptin in the men and women after stratifying according to leptin tertiles and BMI categories. Based on leptin tertiles (Table 3), men showed an independent inverse association between PWV with leptin in the first tertile (Std β=-0.21; p=0.047; R^2=0.19) and women between vWF with leptin in the second tertile (Std β=-0.21; p=0.018; R^2=0.12). In the BMI categories (Table 4), only in overweight men did we observed a negative association between CIMT (Std β=-0.45; p<0.001; R^2=0.16) and CSWA (Std β=-0.43; p<0.001; R^2=0.23) associate negatively with leptin. No association was found in the lean men and in any of the BMI categories of women.

There was no interaction of ethnicity (Supplementary Table 1) between our variables of interest with leptin, but to further strengthen our findings and based on a previous study that has showed differences in leptin levels based on ethnicity [10], we also divided the subjects based on sex and ethnicity (Supplementary Table 2). Again, both the black and white men showed a negative association between CIMT (black men, Std β=-0.29; p=0.034; R^2=0.08; white men, Std β=-0.24; p=0.042; R^2= 0.07) and CSWA (black men, Std β=-0.22; p=0.038; R^2=0.10; white men, Std β=-0.25; p=0.022; R^2= 0.08) with leptin. There was no association between CIMT and CSWA with leptin in either the black or white women.

Table 3. Forward stepwise multiple regression analysis of von Willebrand factor, pulse wave velocity, carotid intima-media thickness and cross-sectional wall area with leptin after stratifying into leptin tertiles in men and women.

	Men				Women			
	Tertile 1 (N = 114)				**Tertile 1 (N = 161)**			
	Adj R^2	**Adj p**	**β (95% CI)**	***p***	**Adj R^2**	**Adj p**	**β (95% CI)**	***p***
vWF (%)	0.04	0.09	-	-	**0.07†**	**0.03**	-	-
PWV (m/sec)	**0.19†**	**0.002**	**-0.21 [-0.42; -0.01]**	**0.047**	**0.13†**	**<0.001**	-	-
CIMT (mm)	0.03	0.12	-	-	**0.06†**	**0.05**	-	**-**
CSWA (mm^2)	**0.07†**	**0.037**	-	-	**0.10†**	**0.01**	-	-
	Tertile 2 (N = 111)				**Tertile 2 (N = 161)**			
vWF (%)	0.01	0.26	-	-	**0.12†**	**0.002**	**-0.21 [-0.38; -0.04]**	**0.018**
PWV (m/sec)	**0.07†**	**0.04**	-	-	**0.07†**	**0.02**	-	-
CIMT (mm)	0.03	0.15	-	-	0.01	0.17	-	-
CSWA (mm^2)	0.04	0.14	-	-	0.01	0.18	0.09 [-0.01; 0.28]	0.29
	Tertile 3 (N = 112)				**Tertile 3 (N = 161)**			
vWF (%)	0.02	0.19	**-**	**-**	0.01	0.13	-	-
PWV (m/sec)	**0.10†**	**0.02**	-	-	**0.08†**	**0.04**	-	-
CIMT (mm)	**0.08†**	**0.040**	-0.15 [-0.39; 0.007]	0.18	0.04	0.06	-0.20 [-0.39; -0.02]	0.034
CSWA (mm^2)	**0.13†**	**0.011**	-0.15 [-0.36; 0.07]	0.19	0.05	0.07	-0.17 [-0.36; 0.02]	0.077

Standardized β (Std β) represents the change in the dependent variable for every 1 SD change in the independent variable. β, partial regression coefficients; 95 % CI, 95 % confidence interval; Adjusted R2, coefficient of determination of each total regression model; Models for the regression were all included at once and included: leptin, age, ethnicity, socio-economic score, body fat percentage, 24-hour mean arterial blood pressure, LDL-cholesterol, C-reactive protein, glucose, gamma-glutamyltransferase and Moderate-Vigorous Physical activity. vWF, von Willebrand factor; PWV, pulse wave velocity; CIMT, carotid intima-media thickness; CSWA, cross-sectional wall area; Adj R2, adjusted R2 and Adj p represents p values for each regression model. Bold values indicate p≤0.05 and the symbol (†), indicates R2 values at p≤0.05

Table 4. Forward stepwise multiple regression analysis of von Willebrand factor, pulse wave velocity, carotid intima-media thickness and cross-sectional wall area with leptin in lean and overweight men and women.

	Men				**Women**			
	Lean (N = 167)				**Lean (N = 225)**			
	Adj R^2	**Adj p**	**β (95% CI)**	***p***	**Adj R^2**	**Adj p**	**β (95% CI)**	***p***
vWF (%)	**0.03†**	**0.03**	-	-	**0.06†**	**0.01**	[-0.05; 0.27]	0.17
PWV (m/sec)	**0.16†**	**<0.001**	-	-	**0.17†**	**<0.001**	-	-
CIMT (mm)	0.01	0.16	-0.18 [-0.36; 0.01]	0.067	0.01	0.15	-	-
CSWA (mm^2)	0.03	0.13	-0.13 [-0.32; 0.07]	0.20	0.01	0.19	-	-
	Overweight (N = 96)				**Overweight (N = 124)**			
vWF (%)	0.02	0.22	-	-	0.04	0.06	**-**	**-**
PWV (m/sec)	**0.15†**	**0.01**	-	-	**0.12†**	**<0.001**	-	-
CIMT (mm)	**0.16†**	**0.003**	**-0.45 [-0.67; -0.22]**	**<0.001**	0.04	0.09	-0.15 [-0.36; 0.06]	0.17
CSWA (mm^2)	**0.23†**	**<0.001**	**-0.43 [-0.66; -0.20]**	**<0.001**	0.02	0.18	-	-
					Obese (N = 105)			
vWF (%)					0.01	0.24	-	-
PWV (m/sec)					**0.23†**	**0.003**	-	-
CIMT (mm)					0.02	0.28	-	-
CSWA (mm^2)					0.01	0.29	-	-

Standardized β (Std β) represents the change in the dependent variable for every 1 SD change in the independent variable. β, partial regression coefficients; 95 % CI, 95 % confidence interval; Adjusted R^2, coefficient of determination of each total regression model; Models for the regression were all included at once and included: leptin, age, ethnicity, socio-economic score, body fat percentage, 24-hour mean arterial blood pressure, LDL-cholesterol, C-reactive protein, glucose, gamma-glutamyltransferase and Moderate-Vigorous Physical activity. vWF, von Willebrand factor; PWV, pulse wave velocity; CIMT, carotid intima-media thickness; CSWA, cross sectional wall area; Adj R^2, adjusted R^2 and Adj p represents p values for each regression model. Bold values indicate p≤0.05 and the symbol (**†**), indicates R^2 values at $p \leq 0.05$

Footnote* We did not conduct regression analysis for underweight (N=24) and obese men (N=49) as well as in underweight women (N=29) due to their small sample sizes.

* R^2 values at $p \leq 0.05$

5 Discussion

Our key finding is that in young healthy men leptin independently and negatively associated with measures of subclinical atherosclerosis, but not in women. This result was robust when performed separately in black or white men. Within BMI categories, we found this association to be evident specifically in overweight men. When reviewing leptin's relationship with other measures of arterial structure and function, leptin consistently showed beneficial inverse associations. Across leptin tertiles, we observed negative associations between arterial stiffness and leptin within the first leptin tertile for men and between von Willebrand Factor (as a measure of endothelial function) and leptin within the second tertile of women. Collectively these findings support the notion of potential beneficial vascular protective effects of leptin, especially in men.

Physiologically, leptin is suggested to be an important factor in the maintenance of vascular homeostasis and wall integrity [9]. However, many studies have shown leptin's detrimental effects on cardiovascular function, including atherosclerosis [24, 25]. This was reported especially in older overweight and obese individuals with conditions such as the metabolic syndrome, hypertension and type 2 diabetes [9, 10, 26, 27]. We observed a negative link between CIMT and CSWA with leptin in men. In contrast to the previously mentioned studies, the men in our study were young and healthy. Our finding may, therefore, suggest that the beneficial effects of leptin signalling previously reported in the vasculature [28] may be intact. Leptin is known to induce nitric oxide synthesis [29] and stimulate coronary artery vasodilation in humans [30]. Nitric oxide may be the potential mechanism underlying the observed negative associations between leptin and carotid wall thickness in our healthy young men, due to its important role in reducing platelet adhesion and vascular smooth muscle cell proliferation [31]. In fact, Rodríguez et al. [32], demonstrated leptin's ability to inhibit vasoconstriction (angiotensin II) and in turn reduce smooth muscle proliferation in an experimental animal model.

Additionally, Momin et al. [33] pointed out leptin's beneficial and direct effects on vascular smooth muscle tone regulation through hyperpolarization, independently of vascular endothelium-derived nitric oxide synthesis in humans with coronary artery disease. Other

studies have also shown leptin's potential to recruit beneficial vascular endothelial progenitor cells into the vasculature [8, 13, 16] – known to maintain vascular homeostasis and reduce plaque formation [34]. More so, not only is leptin suggested to be a beneficial factor in the regulation of myocardial metabolism as well as cardiac function [28], the injection of leptin in ob/ob mice has shown a significant reduction in the wall thickness and size of cardiac myocytes [28, 35]. This anti-hypertrophic potential of leptin on cardiac myocyte cells also suggests leptin as an important factor that may play a role in the reduction of neointima growth [36].

Our finding in the overweight healthy young men is aligned with a previous study [17] showing that leptin is independently associated with flow-mediated dilation in overweight patients with diabetes using insulin and not in the lean group. Also, Simiti et al. [37] showed that elevated leptin concentrations were associated with a better prognosis in overweight patients with coronary heart disease. Furthermore, leptin has been shown to reverse the positive association that existed between BMI and mortality in older overweight men with coronary heart disease and heart failure [38]. Importantly our finding of a beneficial association between leptin and carotid wall thickness was absent in lean men as well as in lean and obese women but was significant in overweight men. Circulating leptin concentrations in this group (8.95 ng/ml) is similar to the normal physiological level at which leptin is noted to modulate normal physiological responses, namely 8.70 ng/ml [9] and 10 ng/ml [16]. Yet, this finding was absent in lean men (2.17 ng/ml) and was thought to be related to the U-shaped relationship between leptin and cardiovascular risk, where leptin associated with increased risk both at low and high doses [17, 39]. A study by Wallace et al. [40] proposed a risk threshold for future coronary events in otherwise hypercholesterolemic men at risk of coronary artery disease, only from the 4^{th} and 5^{th} quintiles of leptin. In order to clarify the dose dependency of leptin in the young healthy adults, we investigated these associations within tertiles of leptin. Within our healthy young population, a U-shaped relationship between leptin and cardiovascular risk was not evident, as shown in previous studies [39, 40] such as one conducted in 392 elderly patients (62 years old) with atherosclerosis [39]. This may become evident as our study participants age [27], or develop an associated metabolic disorder such as dyslipidaemia or atherosclerosis [39,

40]. We rather observed leptin's negative association with large artery stiffness in the tertile with the lowest leptin concentrations, which was lost with increasing leptin tertiles. The observed negative associations between carotid wall thickness with leptin within the third tertile of leptin were not statistically significant but reflected a similar trend as observed in the overweight men (BMI category; $p<0.001$).

Importantly, our finding was more prominent in men than in women, who had higher body fat percentages and almost 7-fold higher leptin levels than men. Leptin may have different functionalities in men and women. Previous studies showed gender differences in the association between measures of autonomic function with leptin to be higher in men than women [23, 41]. Another study indicated that women show more resistance to the physiological effects of leptin than men [42]. More studies are recommended to explain the sex-specific effects of leptin. Although a study has shown that in either obese or lean healthy women without any metabolic complications, leptin did not predict either endothelial function or CIMT [43]. The negative association between leptin and von Willebrand factor – an established haemostatic risk factor for cardiovascular disease [21] – in young normotensive women (second leptin tertile), suggests a beneficial effect of leptin on endothelial function. This is in contrast to Guagnano et al. [19] that showed a positive association between von Willebrand factor and leptin in obese women compared to controls. Whether these contrasting findings could be explained by age differences or their states of adiposity requires more detailed investigation.

Our findings must be interpreted within the context of its limitations and strengths. As there is a lack of information on leptin's role in young, healthy populations, our findings shed light on a potential beneficial role of leptin. Secondly, the study is limited to a small sample size for the underweight men and women as well as in obese men prohibiting detailed analyses in these groups. Furthermore, the study is limited to a cross-sectional design, and we can only speculate on the possible mechanisms underlying the potential beneficial vascular effect of leptin in healthy young adults. Despite this, the study highlighted novel findings and was furthermore well designed and performed under highly controlled conditions in a Hypertension Research Clinic.

6 Conclusion

In conclusion, in young, healthy men measures of subclinical atherosclerosis were independently and negatively associated with circulating leptin, supporting the notion of vascular protective effects of leptin. The presence of these beneficial associations in specifically the healthy young overweight men shows that leptin is not associated with adverse vascular function in this group.

7 Acknowledgement

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8 Conflict of interest

The authors declared no conflict of interest

9 Financial information

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Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors, and therefore, the NRF does not accept any liability in this regard.

10 Author's contributions

AOB performed the literature search, data cleaning, statistical analyses, interpretation of data and writing of the draft manuscript. AES is the principal investigator of the African-PREDICT study, and AES, WS, LL were responsible for the research planning and design, acquisition of data, interpretation of data and revising article critically for intellectual content. All authors approved the final version.

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12 Supplementary materials

Table 1S. Interaction terms

***Independent variable*: Leptin (ng/ml)**		
Variables*:*	**Ethnicity**	**BMI (kg/m^2)**
vWF (%)	0.26	0.13
PWV (m/sec)	0.32	**<0.001**
CIMT (mm)	0.44	0.075
CSWA (mm^2)	0.36	**0.006**

Interaction term: p-values obtained with multiple regression analyses. von Willebrand factor; vWF, PWV, pulse wave velocity; CIMT, carotid intima-media thickness; CSWA, cross-sectional wall area. $p \leq 0.05$ were considered significant

Table 2S. Forward stepwise multiple regression analysis of von Willebrand factor, pulse wave velocity, carotid intima-media thickness and cross-sectional wall area with leptin in black and white men and women.

	Black men (N = 168)				**White men (N = 169)**			
	Adj R^2	**Adj p**	**β (95% CI)**	***p***	**Adj R^2**	**Adj p**	**β (95% CI)**	***p***
vWF (%)	0.01	0.29	**-**	**-**	0.02	0.19	-	-
PWV (m/sec)	**0.11†**	**0.003**	**-**	**-**	**0.17†**	**<0.001**	0.18 (-0.10; 0.45)	0.21
CIMT (mm)	**0.08†**	**0.006**	**-0.29 (-0.49; -0.10)**	**0.034**	**0.07†**	**0.03**	**-0.24 (-0.47; -0.01)**	**0.042**
CSWA (mm^2)	**0.10†**	**0.01**	**-0.22 -0.43; -0.01)**	**0.038**	**0.08†**	**0.02**	**-0.25 (-0.46; -0.04)**	**0.022**
	Black women (N = 263)				**White women (N = 220)**			
vWF (%)	0.01	0.11	-	-	0.01	0.14	-	-
PWV (m/sec)	0.08†	0.001	-	-	**0.12†**	**<0.001**	-	-
CIMT (mm)	0.02	0.08	-	-	**0.06†**	**0.01**	0.14 (-0.12; 0.40)	0.30
CSWA (mm^2)	0.02	0.08	-	-	**0.06†**	**0.02**	-	-

Standardized β (Std β) represents the change in the dependent variable for every 1 SD change in the independent variable. β, partial regression coefficients; 95 % CI, 95 % confidence interval; Adjusted R^2, coefficient of determination of each total regression model; Models for the regression were all included at once and included- : leptin, age, socio-economic score, body fat percentage, 24-h mean arterial blood pressure, LDL-cholesterol, C-reactive protein, glucose, gamma-glutamyltransferase and Moderate-Vigorous Physical activity. vWF, von Willebrand factor; PWV, pulse wave velocity;

CIMT, carotid intima-media thickness; CSWA, cross-sectional wall area; Adj R^2, adjusted R^2 and Adj p represents, p values for each regression model. Bold values indicate p≤0.05 and the symbol (**†**), indicates R^2 values at $p \leq 0.05$

CHAPTER 5; Manuscript 3

Leptin and the Retinal Microvasculature in Young Black and White Adults: The African-PREDICT Study

Leptin and the Retinal Microvasculature in Young Black and White Adults: The African-PREDICT Study

Manuscript 3 will be submitted to the European Journal of Endocrinology as an original article for publication

Summary of instruction to authors used for the preparation of manuscript 3

Journal Details	
Title:	European Journal of Endocrinology
Impact factor	4.333
Aims & scope	European Journal of Endocrinology publishes high-quality original clinical and translational papers and reviews in the following areas of research: paediatrics and adult endocrinology, as well as clinical practices guidelines, position statements and debates
Publisher	BioScientifica Ltd
Author Guidelines	
Original paper	Yes
Preparation of manuscripts	The following outlines were followed: **Page 1**: (a) title (maximum of 85 characters), (b) short running title (limit: 46 characters including spaces), (c) all authors and addresses (d) e-mail and postal addresses of the corresponding author **Page 2**: a) an abstract containing a maximum of 250 words and four keywords, (b) abstract must be subdivided into Objective, Design, Methods, Results and Conclusion. **Page 3 and onward:** Should include the following - Introduction, Materials and Methods, Discussion, Conclusion, Declaration of Interest, Funding and Acknowledgements.
References	(a) **In Text:** cite references in text in numerical order b) **List of References**: (a) use endnote (Vancouver style), (list references in the order they are cited in the text, (b) list all authors, list a maximum of ten authors and where there are more than ten authors, then list the first ten and then use et al, (c) maximum of 60 references
	For more information see: **https://eje.bioscientifica.com/page/140**

(Please note that some of the format was changed to ensure uniformity throughout the thesis)

Leptin and the Retinal Microvasculature in Young Black and White Adults: The African-PREDICT Study

Short running title: Leptin and the retinal microvasculature

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1 Abstract

Introduction

Leptin is a vasoactive peptide that has been linked to diseases associated with macrovascular deterioration. What is still uncertain is its involvement in the microvasculature. Since microvascular changes are presumed to precede macrovascular deterioration, we examined whether measures of the retinal microvasculature are associated with leptin in healthy young black and white individuals.

Method

We included 283 black and 289 white men and women (aged 20-30 years). We determined serum leptin, calculated central retinal artery and vein equivalents and arterio-venous ratio. We also measured retinal vessel responses to light flicker provocation.

Results

Black men were leaner and had lower leptin than white men, whereas black women had increased adiposity and leptin compared to white women (all $p<0.001$). Black groups had narrower retinal arteries, and greater maximum arteriolar and venular dilations in response to light flicker than the white groups ($p<0.001$). Arterio-venous ratio associated negatively with leptin (all $p\leq0.044$) in all groups (except black women), but was lost upon adjustment for body mass index and other covariates. We found an inverse association between maximal venular dilation and leptin only in black men in single and multiple regression analyses (Std $\beta=-0.22$; $R^2= 0.05$; $p=0.035$). No associations were found between other retinal measures with leptin in the other groups.

Conclusion

We found an independent negative association between retinal vein dilation with leptin in healthy young black men, suggesting a potential detrimental role for leptin in regulating

microvascular responses in a population group known to be at greater risk of cardiovascular disease development.

Keywords: Adipokines, healthy, ethnicity, microcirculation.

2 Introduction

Obesity is a significant risk factor in the development of hypertension [1]. The underlying mechanisms of hypertension development include morphological alterations to both the macro and microvasculature [2]. Studies suggest the involvement of the fat-derived vasoactive adipokine, leptin [3], in hypertension and other related cardiovascular disease development [1, 4]. This may involve macrovascular alterations which either occur dependently [5] or independently of obesity [4, 6]. Leptin was also shown to play a role in the microcirculation, by increasing the forearm blood flow in healthy men [7]. However, the potential involvement of leptin in the microvasculature is unclear.

The retinal microvasculature has close similarities with the cerebral and coronary circulation in terms of structure and function [8, 9], and may provide a unique opportunity to noninvasively study early cardiovascular deterioration [10]. Adverse changes in retinal vessel calibres are associated with cardiovascular risk [11, 12]. For example, retinal arteriolar narrowing is linked to increased blood pressure [13] and has been shown to associate with incident hypertension [11]. Venular widening is associated with an atherosclerotic profile - including markers of inflammation, higher body mass index (BMI), dyslipidaemia [14] and incident stroke [15]. Apart from reviewing retinal vessel calibres, microvascular responses to acute stimuli such as light flicker provocation are also informative indicating an indication of retinal vessel function and autoregulation [16]. Furthermore, reduced vessel dilation in response to light flicker provocation are related to untreated hypertension [17], diabetic retinopathy [18], obesity [19], coronary artery disease [20] and ocular pathologies [21, 22].

Leptin is a metabolic hormone [23] having both beneficial and detrimental cardiovascular effects. Leptin induces vasodilation [24, 25]. However, leptin also associates with hypertension and cardiovascular events such as stroke [26, 27] conditions that have been associated with retinal arteriolar narrowing and venular widening [15, 28]. Limited research has been done on leptin's potential association with retinal calibers. One study found leptin to be positively associated with the central retinal venular equivalent (CRVE) and negatively with arteriolar-

venular ratio (AVR) in obese German children. However, the association was dependent on BMI [29]. In contrast, in another study leptin associated positively with central retinal arteriolar equivalent (CRAE) in children and adolescents. In this study, the association between continuous serum leptin (before categorising into percentiles) with CRAE was dependent on body mass index z-score (zBMI) [30]. After dividing leptin, based on percentiles, the positive association between CRAE and leptin remained but was independent of zBMI at >90th percentile of leptin- a concentration defined as increased cardio-metabolic risk. This study suggests a direct vasodilatory role of leptin at a higher concentration independent of obesity.

Apart from these contradictory findings on the retinal calibres, no study has investigated the relationship between leptin with retinal vessel dilatory responses to a light flicker provocation, especially in young adults without overt cardiovascular disease. We, therefore, determined whether measurements of the retinal microvasculature are associated with leptin in healthy young black and white adults.

3 Materials and methods

Study population

Participants were recruited as part of the larger African-PREDICT study (African Prospective study on the Early Detection and Identification of Cardiovascular disease and HyperTension). The African-PREDICT study aims to follow healthy, young adults over ten years in order to identify and track potential markers of early cardiovascular risk. The study was conducted at the Hypertension Research Clinic on the Potchefstroom Campus of the North-West University, South Africa. In this cross-sectional substudy, we included a total of 572 young black (N = 283) and white (N = 289) adults from the baseline phase, with complete data on leptin and the retinal microvasculature. Participation in the study was voluntary and all participants provided prior written informed consent. Inclusion criteria for eligible participants were as follows: black and white men and women, aged 20-30 years; normal clinic BP (BP <140/90 mmHg after three consecutive readings) and blood glucose; not using any antihypertensive medication; no self-reported chronic disease (or treatment thereof); ocular trauma or pathology; HIV-free; and not

pregnant or breastfeeding. They were all from Potchefstroom and surrounding areas in South Africa.

This study was approved by the Health Research Ethics Committee of the North-West University and complied with the applicable requirements of the Helsinki Declaration for medical research involving human participants.

General Health and Demographic Questionnaire

Information such as age, sex, ethnicity, level of education, employment information, household income, smoking, alcohol consumption, medication use, and family history was collected with a General Health and Demographic Questionnaire.

Socio-economic status was calculated by a point system adapted from Kuppuswamy's Socioeconomic Status Scale 2010, in alignment with the South African environment [31]. Participants were then scored based on three categories namely: skill level, education and household income. Skill level was classified according to the South African Standard Classification of Occupation (SASCO) [32]. These three factors were scored and used to group the participants into low, middle and high socio-economic groups both as a categorical and continuous variable.

Body composition and physical activity

Calibrated instruments were used to assess weight ((kg) (SECA electronic scales, SECA, Birmingham, UK)), height ((cm) (SECA stadiometer, SECA)) and waist circumference with a non-flexible tape measure in cm (Holtain, Crymych, UK). Body Mass Index (BMI) was calculated using the standard formula of weight (kg)/height (m^2). Bioelectrical impedance was used to measure lean body mass and body fat percentage (Bodystat 1500MDD dual-frequency analyser, Bodystat, Ltd, Ballakaap, British Isles). For the assessment of activity energy expenditure (AEE; estimation of physical activity), each participant wore a combined heart rate (HR) and accelerometer, namely an ActiHeart device (CamNtech, Cambridge, UK) for a maximum of 7 consecutive days and data was collected at 60-s epochs.

Blood sampling and biochemical measurements

A research nurse took a blood sample with a sterile winged infusion set from the ante-brachial vein of the participants in the morning, after an overnight fast. Blood samples were centrifuged in specific tubes to obtain serum and sodium fluoride plasma. Aliquots were made and stored at -80 ^{0}C, until analyses. We determined serum high-sensitivity C-reactive protein, total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides, gamma-glutamyltransferase, as well as plasma glucose (Cobas Integra 400plus, Roche, Basel, Switzerland). Serum cotinine was determined with a chemiluminescence method on the Immulite (Siemens, Erlangen, Germany). Serum leptin levels were determined in duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine, R&D Systems, MN, USA) and adiponectin levels were determined with the Human High-Molecular-Weight Adiponectin ELISA kit (R&D systems, Minneapolis, MN USA) analysed on a Synergy H4 hybrid microplate reader (BioTek, Winooski, VT, USA).

Cardiovascular measurements

We assessed 24-hour ambulatory blood pressure with a Card(X)plore® device (CE0120, Meditech, Budapest, Hungary). An appropriately sized cuff was fitted to the participant's non-dominant arm and instructions given to each participant on how to ensure successful inflation rates. Participants completed an ambulatory diary card during the measurements and the mean group percentage successful inflation rate was 88.5%

The Retinal Vessel Analyzer (RVA, IMEDOS Systems, Jena, Germany) fitted with a Zeiss Fundus camera FF-450 was used to measure both the static and dynamic retinal vessel analysis. Thirty minutes before the measurement, a drop of Tropicamid (1% Alcon) was administered in the right eye (used the majority of the time) of the participants to induce a mydriatic condition. Dynamic vessel analysis recording with flicker provocation was firstly performed, followed by fundus photography for static vessel analysis. For the static vessel analysis, the central retinal artery and vein equivalents (CRAE, CRVE) and the subsequent AVR (CRAE/CRVE) were calculated using revised formulas described by Knudtson et al [33]. In

this calculation, only the six largest arteriolar and venular segments (the trunk) were used. Both CRAE and CRVE were measured in measuring units (MU). One MU correlates with a micrometre (1:1) if the measuring extent of the eye is equivalent to the normal Gullstrand eye. The arterio-venous ratio (AVR) was calculated as the ratio of CRAE/CRVE. Comprehensive and detailed procedures, as well as precautions used for this analysis are described by Strauss et al [34].

The dynamic retinal vessel analysis was performed to assess the functional response of the retinal microvasculature by measuring the arteriolar and venular dilations in response to flicker light-induced provocation (FLIP) [35]. In this measurement, a standard flicker protocol by IMEDOS Systems was used as discussed previously [19]. The camera was set at an angle of 30°degree and participants were told to focus on the tip of a fixated rod within the camera. Using RVA software (version 4.50), arteriolar and venular segments were selected in the upper or lower temporal quadrant of the retinal. The vessels were selected if they were at least between 0.5-2.0 optic disc diameters farther away from the margin of the optic disc. Arteriolar and venular median segment lengths were 1125 and 1255 MU, respectively in this study. The maximum retinal arteriolar and venular dilations expressed as a percentage of baseline in response to FLIP were determined as previously described by Kotliar et al. [36].

Statistical analyses

We conducted statistical analysis with Statistica Software Version 13.2 (TIBCO Software Inc. Palo Alto, CA 94304 USA). Variables that were not normally distributed (BMI, waist circumference, maximum vein dilation, leptin, adiponectin, and total cholesterol, and LDL-C, HDL-C, C-reactive protein, AEE and gamma-glutamyltransferase) were log transformed and represented as geometric mean with 5^{th} and 95^{th} percentiles. In the instance where cotinine as a variable was not normally distributed after log transformation, the Mann Witney U test was used to compare cotinine and was presented as median (25^{th} to the 75^{th} percentile). Normally distributed variables were presented as mean ± standard deviation and categorical variables represented as percentages. We tested the interaction effects of either ethnicity or sex on the

associations between retinal vessel calibers (CRAE, CRVE and AVR) and vessel responses to flickering light-induced provocation (arteriolar and venular maximum dilations) with leptin. Independent t-tests were done to compare the means of two different groups and Chi-square tests (χ^2) to compare frequencies. Pearson correlations and Spearman ranked test, and forward stepwise multivariable-adjusted regression analyses were conducted to determine the associations of retinal vessel parameters (CRAE, CRVE, AVR, arteriolar and venular dilations) with leptin. In the multiple regression analysis, the models were the same for each group and included: age, BMI, 24-hour mean arterial blood pressure, socio-economic score, C-reactive protein, gamma-glutamyltransferase, triglycerides and glucose. When CRAE was used as the dependent variable, CRVE was included as covariate and vice versa. Also, when maximum arteriolar or venular dilation was selected as the dependent variable, arteriolar or venular segment diameter was included in the model. These covariates were selected among many others using appropriate selection criteria and were based on their relationship with our main variables.

4 Results

Basic characteristics

We stratified our population based on ethnic differences in leptin levels, as well as the physiological differences that also exist in leptin levels between the men and women [4, 37]. This stratification was confirmed with interaction testing (Table 1). The basic characteristics of our study population are presented in Table 2. Black men showed lower values for all adiposity measures as well as leptin levels compared to the white men (all $p<0.001$). For the cardiovascular variables, black men had lower 24-h systolic blood pressure, smaller CRAE ($p=0.049$) and AVR ($p=0.003$), with a greater maximum artery ($p<0.001$) and vein dilation ($p<0.001$) in response to light flicker provocation than white men. Black women showed higher mean values for all adiposity measures (all ≤ 0.029) and leptin ($p<0.001$) than white women. In terms of the retinal microvasculature, black women showed smaller CRAE ($p<0.001$), larger CRVE ($p=0.011$), and larger maximum arteriolar and venular dilations ($p<0.001$).

Table 1. Interactions of each of ethnicity and sex on the association between measures of the retinal microvasculature with leptin

Independent variable: Leptin

Dependent variables	**Ethnicity**	**Sex**
CRAE (MU)	0.81	**0.015**
CRVE (MU)	0.80	0.68
AVR	0.98	**0.007**
Maximum arteriolar dilation (%)	0.54	**<0.001**
Maximum venular dilation (%)	**<0.001**	**<0.001**

Data represent the (interaction terms) *p-values* obtained with multiple regression analyses. CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent AVR, arterio-venous ratio

Table 2. Characteristics of participants stratified by ethnicity and sex (N=572)

	Black men (N = 108)	**White men (N = 126)**	***p***	**Black women (N = 175)**	**White women (N = 163)**	***p***
Age (years)	24.2±3.27	25.6±2.94	0.001	24.7±3.35	25.5±2.77	0.020
Socio-economic status						
Low	71 (65.7)	18 (14.3)	<0.001	98 (56.0)	15 (9.20)	<0.001
Middle	23 (21.3)	25 (19.8)		52 (29.7)	37 (22.7)	
High	14 (13.0)	83 (65.9)		25 (14.3)	111 (68.1)	
Anthropometry						
Body mass index (kg/m^2)	21.6 [17.4; 27.9]	27.1 [20.5; 36.4]	<0.001	25.9 [17.9; 39.7]	24.0 [18.6; 33.9]	0.001
Waist circumference (cm)	73.6 [63.1; 92.0]	90.3 [74.5; 117]	<0.001	78.1 [62.0; 103]	75.4 [63.4; 99.0]	0.029
Body fat (%)	14.7±5.71	19.2±7.28	<0.001	33.1±8.16	29.1±8.22	<0.001
Cardiovascular measurements						
24-h SBP (mmHg)	119±8.28	124±7.15	<0.001	113±8.62	113±8.37	0.64
24-h DBP (mmHg)	69.3±6.31	70.7±6.17	0.089	68.5±5.90	68.2±5.62	0.59
24-h MAP (mmHg)	89.4±6.37	92.1±5.66	0.001	86.5±6.56	86.1±6.22	0.96
CRAE (MU)	157±13.0	160±11.1	0.049	156±12.3	162±12.3	<0.001
CRVE (MU)	249±19.0	246±15.9	0.21	252±18.1	247±18.1	0.011
Arterio-venous ratio	0.63±0.05	0.65±0.05	0.003	0.62±0.05	0.66±0.05	<0.001
Maximum arteriolar dilation (%)	5.50±2.02	3.90±2.12	<0.001	5.70±1.83	3.34±1.93	<0.001

Maximum venular dilation (%)	4.81 [2.20; 10.2]	3.29 [1,10; 7.20]	<0.001	4.52 [1.91; 9.83]	3.67 [1.60; 7.35]	<0.001
Biochemical variables						
Leptin (ng/ml)	2.48 [0.36; 34.7]	7.36 [1.11; 33.2]	<0.001	34.8 [7.57; 112]	22.4 [5.90; 92.5]	<0.001
Adiponectin (µg/mL)	4.57 [1.22; 14.0]	2.70 [0.50; 9.27]	<0.001	4.75 [1.21; 15.2]	5.55 [1.36; 17.1]	0.061
Glucose (mmol/l)	4.21±0.86	5.11±0.47	<0.001	4.33±0.77	4.76±0.51	<0.001
Total cholesterol (mmol/l)	3.72 [2.71; 5.53]	4.81 [3.54; 6.32]	<0.001	3.77 [2.67; 5.50]	4.67 [3.30; 6.43]	<0.001
HDL-C (mmol/l)	1.29 [0.83; 1.83]	1.10 [0.70; 1.67]	0.036	1.24 [0.80; 1.90]	1.57 [1.01; 2.44]	0.001
LDL-C (mmol/l)	2.20 [1.38; 3.84]	3.22 [1.97; 4.87]	<0.001	2.30 [1.26; 3.90]	2.78 [1.74; 4.43]	<0.001
Triglycerides (mmol/l)	0.80 [0.39; 1.62]	1.15 [0.55; 2.68]	<0.001	0.70 [0.39; 1.30]	0.90 [0.42; 2.14]	<0.001
C-reactive protein (mg/l)	0.65 [0.10; 5.94]	0.93 [0.10; 8.08]	0.036	1.73 [0.20; 11.9]	1.05 [0.13; 11.0]	0.001
Lifestyle						
Activity energy expenditure (k/Cal)	337 [125; 674]	338 [144; 674]	0.95	416 [178; 983]	376 [137; 920]	0.20
Self-reported tobacco use (N/total %)	48/102 (47.1)	36/119 (30.3)	0.010	19/164 (11.6)	22/153 (14.4)	0.46
Cotinine (ng/ml)	1 [1.00; 232]	1 [1.00; 84.9]	0.064	1.00 [1.00; 1.00]	1.00 [1.00; 1.00]	0.77
Self-reported alcohol use (N/total %)	73/100 (46.8)	83/119 (53.2)	0.60	87/161 (47.3)	97/153 (53.2)	0.092
Gamma glutamyltransferase (U/l)	27.0 [13.0; 82.8]	25.9 [10.8; 64.5]	0.59	22.7 [10.3; 59.7]	14.2 [6.90; 35.9]	<0.001

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial blood pressure, CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol. Data are expressed as arithmetic mean ± standard deviation or geometric mean (5th to 95th percentile intervals) for logarithmically transformed variables, or number of participants and percentages (%) and $p<0.05$ were considered significant. *Cotinine was compared with the Mann Witney U test and presented as median (25^{th} to 75^{th} percentiles).

Pearson correlation analysis

We performed Pearson correlations between all retinal vessel measurements and leptin in black and white men and women (Supplementary Table 1S; Figures 1 and 2). In all groups, except black women, AVR correlated negatively with leptin (all $p \leq 0.044$), whereas in black women CRAE associated negatively with leptin ($r=-0.16$, $p=0.035$). In black women, we observed a positive association between maximum artery dilation with leptin ($r=0.21$; $p=0.009$**)**, and black men showed an inverse association between maximum vein dilation with leptin ($r=-0.23$; $p=0.020$).

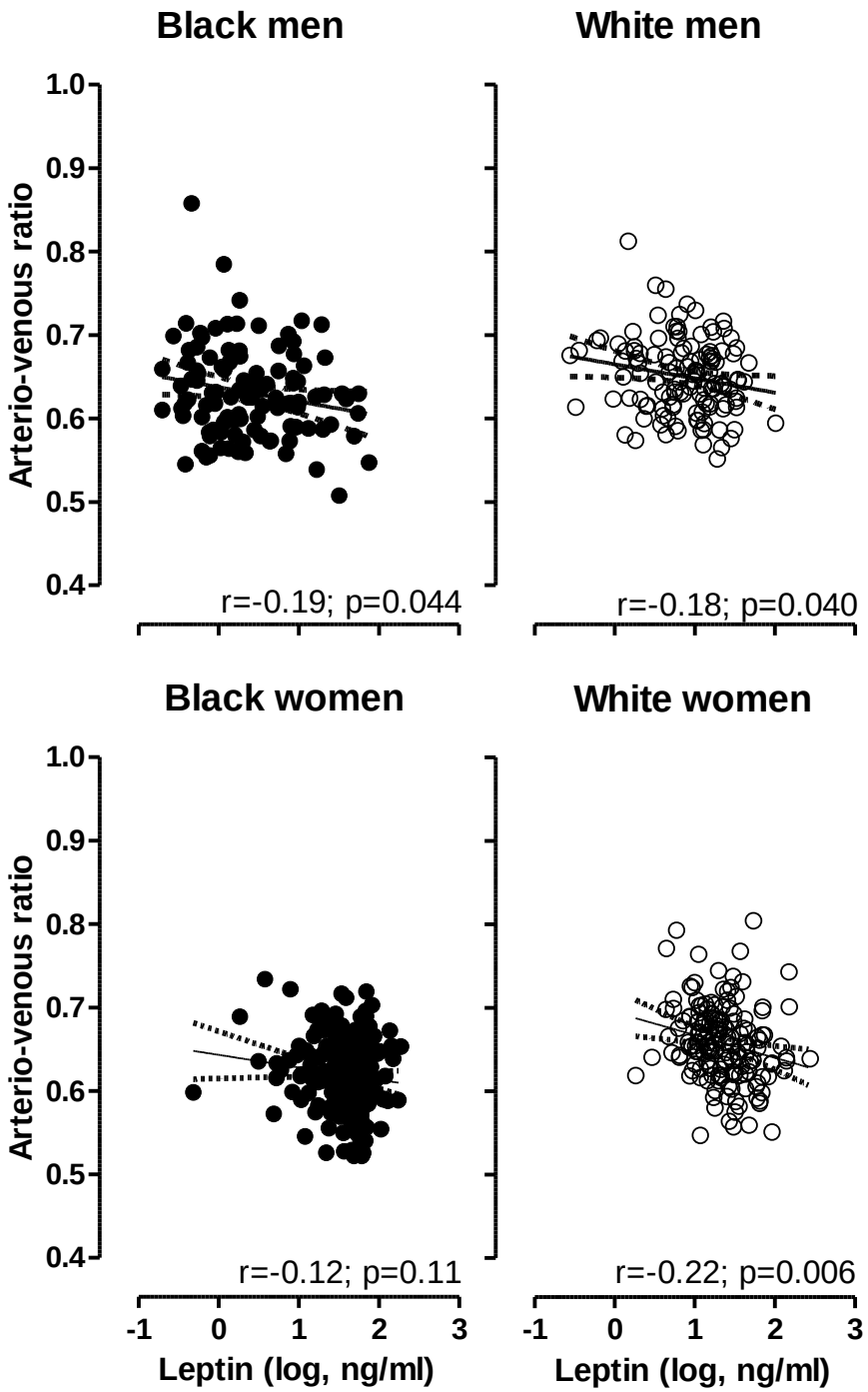

Figure 1: Leptin plotted against arterio-venous ratio in black and white men and women. Solid and dashed lines represent the regression line and the 95% CI boundaries.

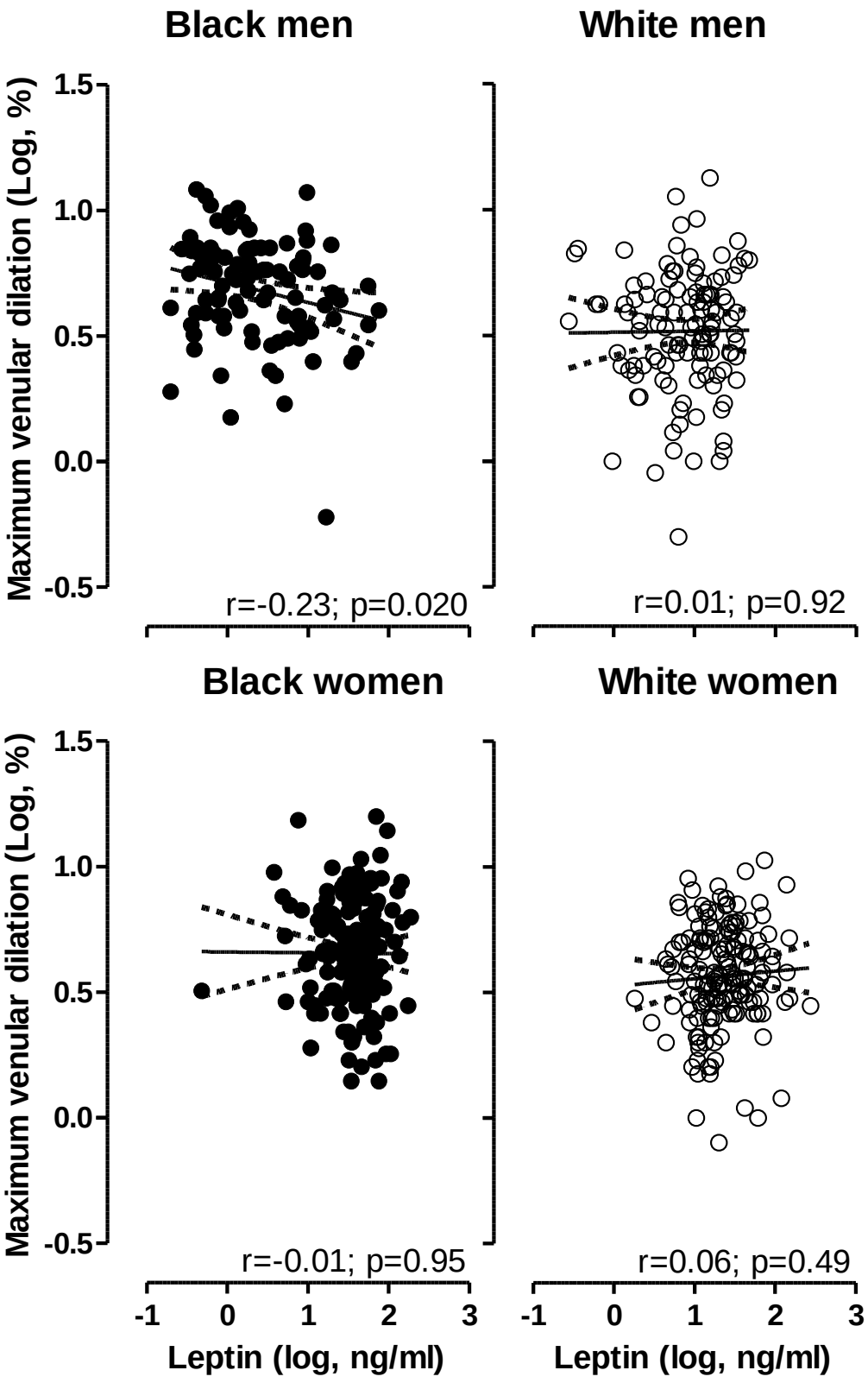

Figure 2: Leptin plotted against retinal maximum venular dilation in black and white men and women. Solid and dashed lines represent the regression line and the 95% CI boundaries.

Sensitivity analysis

We observed a potential outlier in maximum vein dilation in black men (Figure 2) and therefore used a Spearman's rank correlation analysis to determine the association between maximum vein dilation with leptin in black men. The first association was confirmed (r_s=−0.23; p<0.024).

Forward stepwise multiple regression analysis

We conducted a multivariate-adjusted regression analysis between all our dependent variables (retinal vessel calibers and vessel responses to light flicker provocation) and leptin. The original negative association between maximum venular dilation with leptin in black men was confirmed (R^2=0.05; Std β=-0.22; p=0.035) (Table 3). Leptin also showed a non-significant negative association with maximum venular dilation in the black women, where BMI was positively associated with maximum venular dilation (R^2=0.07; Std β=0.30; p=0.012). No association was found between other retinal vessel calibers or vessel responses to light flicker provocation with leptin in any of the other groups. We also repeated the multiple regression analysis between maximum venular dilation and leptin in black men, after removing the single previously mentioned outlier. The association between the maximum venular dilation and leptin remained (R^2= 0.03; Std β=-0.24; p=0.028).

Table 3. Forward stepwise multiple regression analyses between leptin and retinal maximum venular response to flicker light provocation with leptin in young black and white men and women

	Maximum venular dilation (%)							
	Black men (N=98)		**White men (N=116)**		**Black women (N=157)**		**White women (N=160)**	
	(Adj R^2) 0.05†		**(Adj R^2)** 0.02		**(Adj R^2) 0.07†**		**(Adj R^2)** 0.03	
	β (95% CI)	***p***	**β (95% CI)**	***p***	**β (95% CI)**	***p***	**β (95% CI)**	***p***
Leptin (ng/ml)	**-0.22 [-0.41; -0.03]**	**0.035**	-	-	-0.15 [-0.38; 0.08]	0.19	-	-
Age (years)	**-**	**-**	-	-	**-0.21 [-0.37; -0.05]**	**0.011**	-0.01 [-0.17; 0.15]	0.27
SES	**-**	**-**	-0.16 [-0.18; -0.14]	0.090	-	-		
BMI (kg/m^2)	**-**	**-**	-		**0.30 [0.07; 0.53]**	**0.012**		
24-h MAP (mmHg)	**-**	**-**	-	-	-	-		
CRP (mg/l)	**-**	**-**	0.12 [-0.07; 0.31]	0.24	-	-	0.16 [-0.01; 0.33]	0.063
GGT (U/l)	**-**	**-**	-	-	-		-0.14 [-0.31; 0.03]	0.10
Triglyceride (mmol/l)	**-**	**-**	-0.13 [-0.32; 0.06]	0.20	**-0.19 [-0.35; -0.03]**	**0.020**		
Glucose (mmol/l)	-0.12 [-0.41; 0.07]	0.23	-	-	-	-	0.12 [-0.04; 0.28]	0.13
VSD (MU)	**-**	**-**	-0.10 [-0.28; 0.08]	0.27	-	-		

*Standardized β (Std β) represents the change in the dependent variable for every 1 SD change in the independent variable. β, partial regression coefficients; 95 % C.I, 95 % confidence interval; (R^2) Adjusted R^2 is the coefficient of determination of each total regression model; Same model was used for each group and included the following covariates: leptin, age, body mass index (BMI), socio-economic score (SES), 24-hour mean arterial blood pressure (24-h MAP), C-reactive

protein (CRP), gamma-glutamyltransferase (GGT), triglyceride and glucose and vein segment diameter (VSD). All the covariates were included at the same time. Bold values indicate p≤0.05 and the symbol (**†)**, indicates significant R^2 values at p<0.05.

5 Discussion

The study aimed to determine whether measurements of the microvasculature (retinal vessel calibers and vessel responses to light flicker provocation) are associated with leptin in healthy young black and white adults. We demonstrated that the black group presented with a more vulnerable retinal caliber profile than the white group, exhibiting a narrower arteriolar diameter, a measure known to relate to future hypertension development [11, 13]. Notwithstanding these narrower calibers, black men and women displayed significantly greater arteriolar and venular dilations in response to light flicker stimulation, than whites. Overall there were limited associations between retinal measures and leptin in our young participants. We found in black men, white men and white women a consistent negative association between AVR and leptin, suggesting that leptin or obesity is related to a higher risk of cardiovascular disease development [29]. However, upon adjustment for obesity and other covariates, the association with leptin was lost.

Regarding the functional response of the retinal vessels to a light stimulus, we found a persistent inverse association between maximal retinal venular dilation with leptin in young black men. A similar trend was also observed in the black women but was, however, not statistically significant, irrespective of their greater retinal arteriolar and venular dilations compared to their white counterparts. A recent study by Wentzel et al. [38] in older black and white adults of the Sympathetic Activity and Ambulatory Blood Pressure in Africa study, showed that black adults equally displayed greater arteriolar and venular dilations after flicker light induced stimulation than whites in despite their detrimental cardiovascular profile [39]. The higher arteriolar and venular dilations in the black individuals may be related to their higher nitric oxide synthesis capacity than the white population in spite of their detrimental cardiovascular profile [39].

Although not yet used clinically, studies have shown that diminished retinal arteriolar and venular vasodilations to light flicker stimulation may have clinical importance in systemic disease [16, 19]. Reduced retinal vasodilations have been linked to several systemic diseases

including obesity, hypertension and coronary artery disease [17, 19, 20]. This study is the first to ascertain the potential role of leptin on retinal vessel responses to light flicker provocation. Our study participants were young and healthy, yet the negative association between leptin and retinal venular dilation in black men may suggest a harmful role of leptin by playing a role in inhibiting microvascular vasodilation and ultimately, blood flow in response to an external stimulus. It is noteworthy that our finding refers to venular dilation, and not arteriolar dilation. Dynamic vessel analysis following acute light stimulation has also shown reduced retinal venular dilation in patients with central serious chorioretinopathy (CSCR) [21] and hypercholesterolemia [35]. To speculate on the interpretation of this finding, it is worth mentioning that the venules are more susceptible to vascular inflammatory infiltration than arterioles [40]. Venules also seem more receptive to metabolic alterations in the microcirculation compared to the arterioles [35].

In a study by Reimann et al. [35], LDL-apheresis in hypercholesterolemic patients showed an improvement in retinal venular dilation (but not arteriolar dilation) after flicker light-induced stimulation. They found that the improvement in retinal venular dilation was not related to the reduction in serum cholesterol or triglycerides, but suggested that the reduction of oxidative stress in the retinal microcirculation through the removal of pro-oxidative agents and events after LDL apheresis may be the mechanism explaining improved retinal venular dilation. We have shown previously that black men have higher levels of reactive oxygen species (ROS) [41] and fibrinogen than white men [42]. The contribution of these factors in explaining our finding would be speculative but is indeed an area for further research. Previous studies reported that leptin might contribute to altered vascular function through the activation of reactive oxygen species in the endothelium of vessels [43].

It was interesting to note that we observed our finding in the venular and not arteriolar retinal vessel. With static vessel analysis, it is well known that retinal arteriolar and venular calibers relate differently to cardiovascular risk factors. For instance, inflammatory markers have been consistently associated with wider vein and not artery [44, 45]. Although not yet confirmed, it is feasible then that the arteriolar and venular responses to light flicker provocation may also

differentially relate to various cardiovascular risk factors. Contrary to our study in apparently healthy participants, some studies have also observed a reduction in both the artery and venular dilations after a light-induced stimulation in individuals with systemic and metabolic-related disorders such as obesity, diabetes mellitus and hypercholesterolemia [18, 19]. Studies have suggested the reduced responses in either the single vascular bed or the combination of the retinal arteriolar and venular beds to several mechanisms including impaired neurovascular coupling and autoregulation, as well as endothelial dysfunction [35, 46]. Tomasso et al [21] who also observed a reduced retinal venular dilation in response to photic stimulus in CSCR individuals - a disorder commonly seen in young to middle-aged men and also linked to autonomic imbalance [47], stroke [48] and coronary heart disease [49], suggested increased sympathetic tone to the choroid as a factor that may be responsible for the reduced vein dilation. Previous studies, including ours [50] conducted in the African-PREDICT population, showed that leptin associates with autonomic function with a potential to increase sympathetic activity especially in men [51]. Leptin may partly interfere negatively with vascular responses to light flicker provocation indirectly through increased sympathetic activity.

Leptin's role in the microcirculation is not yet clear. Microvascular actions of leptin have been demonstrated in a previous study (assessed with a laser Doppler flow-meter), where leptin increases perfusion rate during a microvascular provocation with temperature elevation. [52]. Against our observation is the role of leptin in the improvement of forearm blood flow in normotensive young Japanese men (n=10) [7]. Further studies aimed at investigating the role of leptin and other adipokines and their microvascular actions are therefore recommended.

Another prominent finding of our study is that the majority of our young population (black men, white men and women) showed a negative association between arterio-venous ratio and leptin – albeit an association dependent on obesity which is consistent with a previous study [29]. Decreased arterio-venous ratio has been associated with elevated blood pressure [53], and cardiovascular events [54]. Apart from this consistent association, there were no associations between retinal vessel calibers with leptin in our study. The limited studies that observed a relationship were either conducted in obese German children (10-11 years old) [29] or Flemish

children and adolescents (7-16 years old) [30]. Our study population were older (20-30 years old) and the majority not obese and may serve as an explanation for the differences in the observation between leptin and vessel calibers between our study and the above studies.

The findings of our study should be interpreted within the context of its strengths and limitations. The study was conducted under controlled conditions, and included a unique young healthy bi-ethnic population. The cross-sectional study design limits interpretation of causality. Our sample size was relatively small albeit larger than most comparable studies [29, 30]. We also recognise that the majority of the covariates included in our model did not contribute to retinal vein dilation. We recommend future studies with larger sample size to investigate sympathetic activity, pro-inflammatory and thrombotic factors as well as nitric oxide bioavailability to better inform the physiological mechanisms explaining the association between retinal responses with leptin in our study.

6 Conclusion

Our results with venular dilation suggest a potential detrimental role of leptin in regulating microvascular responses. This may provide insight into the understanding of early cardiovascular risk especially in black men; a population already suggested being at a hiher risk of cardiovascular disease development [41, 55].

7 Conflict of interest

There was no conflict of interest among the authors

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10 Supplementary material

Table 1S. Pearson correlations between retinal vessel calibers and vessel responses to flicker light provocation with leptin

	Black men (N = 108)	**White men (N = 126)**	**Black women (N = 175)**	**White women (N = 163)**
CRAE (MU)	r=0.07; p=0.48	r=-0.17; p=0.051	**r=-0.16; p=0.035**	r=-0.13; p=0.10
CRVE (MU)	r=0.16; p=0.11	r=0.01; p=0.90	r=-0.03; p=0.70	r=0.08; p=0.31
AVR	**r=-0.19; p=0.044**	**r=-0.18; p=0.040**	r=-0.12; p=0.11	**r=-0.22; p=0.006**
Maximum arteriolar dilation (%)	r=-0.09; p=0.40	r=0.18; p=0.055	**r=-0.21; p=0.009**	r=0.15; p=0.055
Maximum venular dilation (%)	**r=-0.23; p=0.020**	r=0.01; p=0.92	r=-0.01; p=0.95	r=0.06; p=0.49

CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; AVR, arterio-venous ratio; $p \leq 0.05$ was considered significant

CHAPTER 6

Summary of Main Findings and Conclusion

1 Introduction

This concluding chapter consists of a summary of the main findings and references to the original hypotheses. This is then followed by a model suggesting the potential role of leptin on the cardiovascular health of young adults based on the findings from this Ph.D. study, and the contributions of the study. Finally, recommendations are made followed by the conclusion of the study.

2 Summary of main findings and references to the original hypotheses.

2.1 Summary of main findings

Based on previous reports in older, obese, diseased populations [1-4] highlighting a potential role for leptin in the development of obesity-related complexities, the central aim of this study was to determine whether relationships already exist between measures of autonomic activity, endothelial activation, blood pressure, large artery and microvascular structure and function with leptin in a healthy young bi-ethnic population. This study is the first to examine the role of leptin on various cardiovascular indices in young Africans who may be at risk of obesity and hypertension development [5-7]. The study was conducted in a unique population consisting of young black and white men and women who are void of any chronic diseases, including hypertension and other related cardiovascular diseases (self-reported).

Below is an overview of the main findings from the three manuscripts of this thesis (Figure 1). We explored the associations that exist between measures of various cardiovascular indices with leptin in healthy young black and white adults in the following order:

Manuscript 1: Investigated the associations that exist between measures of autonomic activity, endothelial cell activation and blood pressure in 820 healthy young black and white men and women.

Manuscript 2: Determined the associations that exist between the measures of subclinical atherosclerosis, large artery stiffness and a measure of endothelial dysfunction (von Willebrand factor) (macrovasculature) with leptin in 820 healthy young men and women.

Manuscript 3: Examined whether any relationship exists between measures of the retinal microvasculature with leptin in 572 healthy young

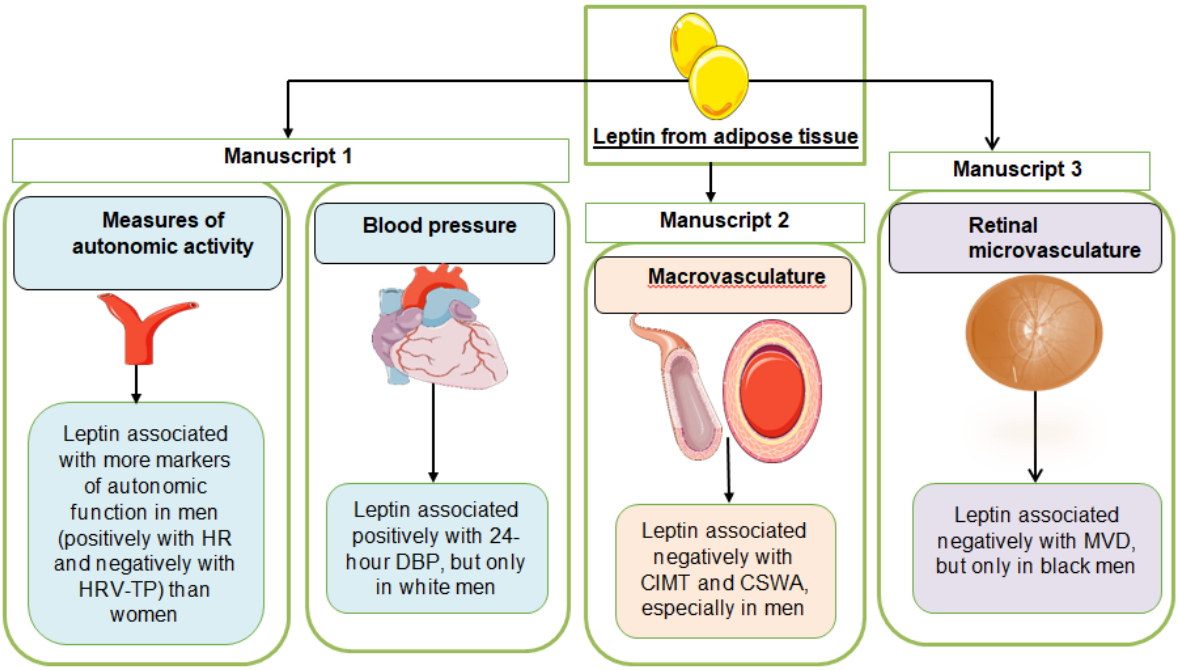

Figure 1. Diagrammatic representations of the summary of main findings from the individual manuscripts (1, 2 and 3).

*HR, heart rate; HRV, heart rate variability; TP, total power; DBP, diastolic blood pressure; CIMT, carotid intima-media thickening; CSWA, cross-sectional wall area and MVD, maximum venular dilation. *Images obtained from Servier Medical art.*

It is also important to highlight our negative findings. Leptin did not associate independently and significantly with any measure of endothelial activation (manuscript 1), pulse wave velocity or von Willebrand factor (manuscript 2) and retinal vessel calibres and arteriolar dilation (manuscript 3) in any of the study groups irrespective of gender and ethnicity.

2.2 References to the original hypotheses

2.2.1 Manuscript 1 (Chapter 3), published in *Hormone and Metabolic Research*:

Title: Leptin and its Relation to Autonomic Activity, Endothelial Cell Activation and Blood Pressure in a Young Black and White Population: The African-PREDICT Study

Brief motivation and aim

Leptin has been implicated in obesity-associated hypertension development [8, 9]. In order for us to better understand leptin's contributions towards early cardiovascular deterioration, we, therefore, investigated leptin and its associations with measures of autonomic activity, endothelial activation and blood pressure in young, healthy black and white men and women.

Hypotheses (Chapter 1, page 23)

In a group of healthy young black and white men and women, we made the following hypotheses:

- Leptin will associate negatively with heart rate variability (HRV) high frequency (HF), 24-hours total power, triangular index, standard deviation of normal to normal R-R intervals (SDNN), and positively with, low frequency (LF), LF/HF ratio and ambulatory heart rates

This hypothesis is partially accepted since leptin did not show an association with all the measures of autonomic activity. Also, associations observed were inconsistent within genders. We observed consistent associations between markers of autonomic activity (such as 24-hour heart rate, day and night-time heart rate as well as heart rate variability total power) with leptin in both white (all $p \leq 0.001$) and black men (all $p \leq 0.040$). These findings were absent or less prominent in women, despite their almost 10-fold higher leptin levels than men.

- We also hypothesised that leptin will associate positively with markers of endothelial activation (monocyte chemo-attractant protein-1 (MCP-1), soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular adhesion molecule-1 (sVCAM-1))

This hypothesis is rejected since leptin did not associate with any measure of endothelial activation and function in any of the groups.

- Leptin will associate positively with 24-hour blood pressure, and the above relationships will be observed in all the groups.

This hypothesis is partially accepted because leptin displayed an association with only 24-hour diastolic blood pressure, and the association was also only observed in the white men (Std β = 0.37; p=0.006). No association was found with other components of blood pressure, or in any other group (black men and women and white women).

2.2.2 Manuscript 2 (Chapter 4), published in the European Journal of Clinical Investigation*:*

Title: Leptin and the Vasculature in Young Adults: The African-PREDICT Study

Brief motivation and aim

The role of leptin on the macrovasculature especially in healthy young adults and potential disparities in men and women at risk of cardiovascular disease development is unknown. The aim of this manuscript was to determine whether measures of subclinical atherosclerosis (carotid intima-media thickness, carotid cross-sectional wall area), large artery stiffness (pulse wave velocity) and a marker of endothelial dysfunction (von Willebrand factor; vWF [10, 11]) are associated with leptin in healthy young men and women.

Hypothesis (Chapter 2, page 24)

- Leptin will associate positively with large artery structure and function (pulse wave velocity (PWV), carotid intima-media thickness (CIMT) and cross-sectional wall area (CSWA) and a marker of endothelial dysfunction (vWF), and the above relationships will be observed in all the groups.

We reject this hypothesis since leptin was negatively associated with measures of subclinical atherosclerosis (CIMT: β=-0.20; p=0.036; CSWA: β=-0.20; p=0.035) in men. These associations were absent in women, and there were also no associations between PWV and vWF with leptin in either the men or women.

2.2.3 Manuscript 3 (Chapter 5) will be submitted to the *European Journal of Endocrinology:*

Title: Leptin and the Retinal Microvasculature in Young Black and White Adults: The African-PREDICT Study

Brief motivation and aim

Considering that microvascular deterioration may precede macrovascular pathologies, which have also been associated with leptin [12-14], we, therefore, investigate the role of leptin in the microvasculature using the retinal microvasculature as an ideal site for non-invasive investigation of the microvasculature. Studies have shown that the retinal microvasculature bears resemblance with the cerebral and coronary bed [15, 16], and any alteration may signify vascular pathology or increased cardiovascular risk [17-19]. The study aimed to determine whether measurements of the retinal microvasculature (CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; AVR, arteriolar-venular ratio, as well as maximum arteriolar and venular dilations) are associated with leptin in healthy young black and white individuals.

Hypothesis (Chapter 2, page 24)

- Leptin will relate positively to central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE) and negatively to arterio-venous ratio (AVR).

This hypothesis is partially accepted as leptin only associated with AVR. Also, the association was present in all groups except black women as in the fully adjusted model this was not significant.

- Leptin will relate positively to maximum arteriolar and venular dilations following a light flicker provocation, and the above relationships will be observed in all the groups.

We reject this hypothesis because leptin associated negatively with maximum venular dilation in black men only. Apart from this, there was no association between leptin and maximum arteriolar dilation in any of the groups.

2.3 Conceptualisation of the potential cardiovascular role of leptin in the young adults and contributions of the study

2.3.1 The potential cardiovascular role of leptin in young adults

This Ph.D. study contributes to the existing body of knowledge on the potential role of leptin on the cardiovascular profile of healthy young adults, including autonomic activity, endothelial activation, blood pressure, large artery structure and function and the microvasculature. In a healthy young adult, leptin may be involved in several cardiovascular functions as shown below.

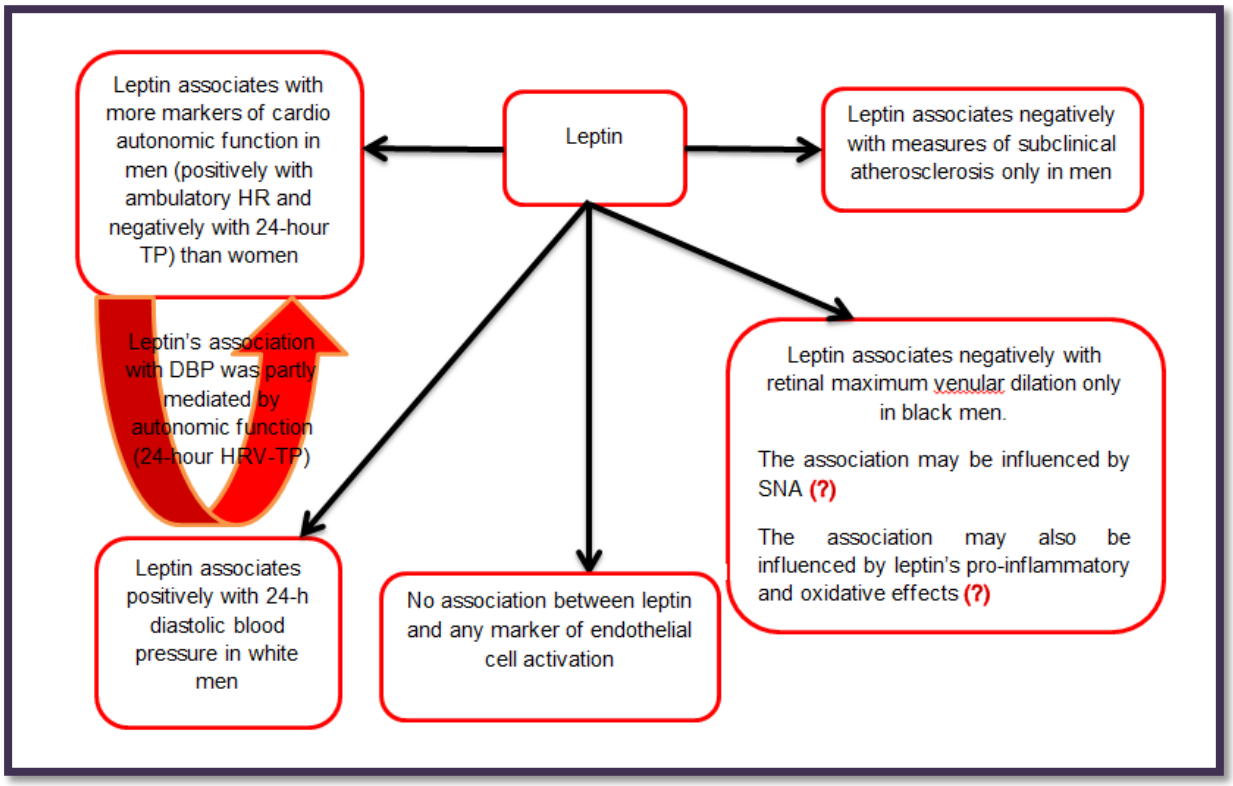

Figure 2. A model showing the potential cardiovascular role of leptin in healthy young adults. HR, heart rate; HRV, heart rate variability and TP, total power.

2.3.2 Contributions of the study

- This Ph.D. study implicates the possible role of autonomic dysfunction (reduced HRV) in future blood pressure elevation, especially in young men. Previous studies suggest increased sympathetic activity as the underlying mechanism in obesity-associated hypertension and cardiovascular disease development in older individuals [20-23]. However, due to the associations observed in this study – between leptin with autonomic activity (ambulatory heart rates and depressed heart rate variability) and blood pressure, the study suggests a potential role for leptin in future blood pressure elevation, especially in men. The study also suggests that at a younger age, leptin may physiologically influence blood pressure by increasing diastolic blood pressure – a proposed risk factor for cardiovascular disease development in white individuals [24].
- In the healthy young adults, leptin showed a potential beneficial vascular protective role by associating negatively with surrogates of subclinical atherosclerosis, especially in men. This further suggests that the physiological effect of leptin in the macrovasculature of these young individuals is still intact. Contrary to this finding, a study conducted in the older obese and overweight population (mean age 45 years) at a higher risk of cardiovascular disease development (some of the participants already developed diabetes and hypertension), showed a positive association between the carotid cross-sectional area with leptin [1]. This may become evident in young adults as they grow in age and also develop an associated metabolic disorder [13, 25, 26].
- Leptin's negative association with retinal venular dilation in response to light flicker provocation, suggests a vital role of leptin in adverse microvascular function even at a younger age; especially in black individuals have also been suggested to be at a greater risk of cardiovascular disease development [27-29]. This potential adverse role of leptin in the microvasculature of black men is occurring irrespective of their high vasodilation observed in response to external stimulus.
- The study observed the differential role of leptin in the micro and macrovasculature, by associating negatively with the retinal venular dilation (potential detrimental role) and

measures of subclinical atherosclerosis (potential vascular protective role). It has been reported that microvascular endothelial dysfunction occurs earlier before any alteration in the macrovasculature [30, 31], and reduced retinal dilations following a light stimulus, including venular dilation, may suggest endothelial dysfunction [19, 32]. Although we did not observe any associations between leptin and markers of endothelial activation, this further suggests the retinal microvasculature as an important marker for the evaluation of early cardiovascular risk especially in black individuals - who also have been reported to be at a greater risk of cardiovascular disease development [30, 31]. According to Szűcs et al [33], carotid intima-media thickening of the large artery lumen may occur with advances in ages and after a prolonged disease period. Apart from this, leptin may influence the micro and the macrovasculature through different mechanisms, since leptin plays multiple roles and also have different biological pathways in the endothelium [34]. Previous studies have also suggested that sensitivity to leptin's effects may vary and also depend on the different cell types [35-37].

- In both the black and white population, leptin showed a potential detrimental cardiovascular role by associating with different cardiovascular measures. In black men, leptin associated negatively with maximum venular dilation and positively with 24-hour diastolic blood pressure in white men. This, therefore, suggests a role for leptin in cardiovascular disease development and may occur differently in the black and white populations.
- The cardiovascular role of leptin as observed in this study seems to be sex-specific as the majority of our findings were observed in the men rather than women. Leptin's association with several markers of cardiovascular function, such as autonomic activity and diastolic blood pressure only in men, gives an indication that men may have a greater leptin-induced cardiovascular risk [38]. This may be due to women being less responsive to the effect of leptin than men [39]. However, in the older obese black South African women (mean age 41 years), leptin associated positively with systolic blood pressure, thereby suggesting the potential effects of aging in leptin's involvement in cardiovascular deterioration [1, 40]. For the micro and macrovasculature, leptin associated with markers of subclinical atherosclerosis and

retinal venular dilation in men and not women, suggesting higher sensitivity to the vascular effect of leptin in men than women. This specific finding therefore warrants further investigation.

- Leptin showed no association with any measure of endothelial cell activation, suggesting that other mechanisms (such as autonomic function) may be more important at this young age of 20-30 years, with respect to leptin's involvement in CVD development.

3 Strengths and limitations of the study

- The study participants were recruited from Potchefstroom and surrounding areas of the North-West Province, as such do not represent the entire population of young adults in Africa and South Africa in particular. Apart from this**,** the African-PREDICT study deployed and used a unique population consisting of black and white men and women with roughly equal distribution.
- This study made use of a cross-sectional study design; therefore, the observed associations cannot be used to determine cause and effects. Also, due to the nature of the study design used, the study could only suggest possible mechanisms that might be responsible for the above relationships. However, the study was well-designed and conducted under well-controlled conditions.
- The study used a non-invasive technique in the assessment of the cardiovascular variable of interest, including measures of autonomic activity, blood pressure, arterial stiffness, subclinical atherosclerosis and retinal microvasculature. However, the study made use of well-established techniques and procedures for the in direct assessments. Also, trained scientists took the measurements following standard protocols, and all the equipment were well calibrated and validated
- We also did not perform a correction for multiple testing, as this study is considered as hypothesis generating work.

4 Chances and confounding

With regard to the result, this Ph.D. study considers the possibility of chance findings. Adjustments for confounders were made when required and in the multiple regression analysis, a rule was followed which allows for one covariate per 10 number sample size. Furthermore, the results that were obtained from this study were also interpreted from a physiological point of view, and hence we relied on statistical significance while taking physiological significance into account.

During the investigation of the association between variables of interest with leptin in this Ph.D. study, we adjusted for several covariates (see the statistical analysis section of each manuscript. The covariates were selected among many others using appropriate selection criteria and were based on their interrelationships with our main variables, including leptin, measure of autonomic activity, endothelial activation, blood pressure, large artery structure and function and retinal microvasculature.

It is also necessary to acknowledge the fact that there are other potentially interfering (confounding) variables that were not accounted for statistically or included in the model and may have had an influence on the results. Examples of such variables are: diet – particularly dietary salt in-take, as there may be an inter-relationship between obesity, leptin and salt sensitivity [41], psychological stress, and actual amount of subcutaneous fat tissue. More so, appropriate adjustment for socio-economic status and genetic predisposition was difficult to determine. Lastly, when reviewing the association between leptin with blood pressure, we did not account for the typical low renin phenotype in black individuals or high renin in the white population [42, 43].

Overall, the sample size estimation of the study confirmed that a population of 800 participants produces adequate statistical power (80% power) to accurately assume the probability for the proposed research question.

5 Recommendations

Based on the findings of this study, the following recommendations are made for future studies:

- Longitudinal studies are required where healthy young adults will be followed over time to observe detailed cardiovascular changes and how these are related to leptin concentrations.
- More studies are required to confirm the involvement of the renin-angiotensin system (RAS) in the association between blood pressure and leptin in the white and also to establish a link between leptin and the RAS system in Africans. We suggest the possible role of RAS in the observed association between leptin and 24-hour DBP in the young white men. It is also well established that black individuals have lower renin, Angiotensin II and I levels than their white counterparts [42, 43].
- Studies investigating other cardiovascular risk factors, such as pro-inflammatory and oxidative stress markers, in the association between the retinal microvasculature and leptin are encouraged and may shed light on understanding current findings.
- We recommend future studies with larger sample size and with more direct techniques to better inform the physiological mechanisms explaining the associations between the macro and microvascular structure and function with leptin, especially in African populations. Such techniques could include better estimation of endothelial function using flow-mediated dilation, retinal wall-to-lumen ratios, or performing intervention studies such as weight loss - to determine the effect on leptin concentrations and related vascular responses. The study should also aim at investigating any differences in the mechanism of action of leptin between these vessels.

6 Final conclusions

In young adults without any overt cardiovascular disease between the ages of 20-30 years, leptin levels were observed to be higher in women than men. Despite this, only men showed a consistent association between markers of autonomic activity with leptin, accompanied by an independent positive association between leptin and diastolic blood pressure, only in white men.

Furthermore, leptin showed a potential beneficial role on the vasculature by associating negatively with carotid intima-media thickness in men. Irrespective of this possible beneficial association between leptin and carotid wall thickness, leptin's inverse association with retinal maximum venular dilation in black men suggest a greater risk of cardiovascular disease development in black men. Overall, leptin's possible role in the cardiovascular health of the healthy young adults seems to depend on different cell types, ethnicity and gender.

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ANNEXURES

ANNEXURE A

Ethics Approval

NORTH-WEST UNIVERSITY
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Faculty of Health Sciences
Health Sciences Ethics Office for Research, Training and Support
Health Research Ethics Committee (HREC)

Tel: 018-285 2291
Email: Wayne.Towers@nwu.ac.za

27 July 2016

Prof AE Schutte
Physiology

Dear Prof Schutte

APPROVAL OF YOUR APPLICATION BY THE HEALTH RESEARCH ETHICS COMMITTEE (HREC) OF THE FACULTY OF HEALTH SCIENCES

Ethics number: NWU-00066-16-S1

Kindly use the ethics reference number provided above in all correspondence or documents submitted to the Health Research Ethics Committee (HREC) secretariat.

Study title: Exploring leptin from endothelial activation to subclinical organ damage in young black and white adults: The African-PREDICT study

Study leader/supervisor: Prof AE Schutte

Student: BO Ahiante

Application type: Single study

Risk level: Minimal

You are kindly informed that your application was reviewed at the meeting held on 13/07/2016 of the HREC, Faculty of Health Sciences, and was approved on 27/07/2016.

The commencement date for this study is 27/07/2016 dependent on fulfilling the conditions indicated below. Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation up to a maximum period of three years when extension will be facilitated during the monitoring process.

After ethical review:

Translation of the informed consent document to the languages applicable to the study participants should be submitted to the HREC, Faculty of Health Sciences (if applicable).

The HREC, Faculty of Health Sciences requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the proposal or other associated documentation must be submitted to the HREC, Faculty of Health Sciences prior to implementing these changes. Any adverse/unexpected/unforeseen events or incidents must be reported on either an adverse event report form or incident report form at Ethics-HRECIncident-SAE@nwu.ac.za.

A monitoring report should be submitted within one year of approval of this study (or as otherwise stipulated) and before the year has expired, to ensure timely renewal of the study. A final report must be provided at completion of the study or the HREC, Faculty of Health Sciences must be notified if the study is temporarily suspended or terminated. The monitoring report template is obtainable from the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-Monitoring@nwu.ac.za. Annually a number of studies may be randomly selected for an external audit.

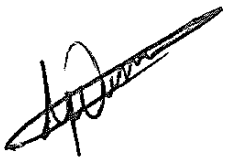
Please note that the HREC, Faculty of Health Sciences has the prerogative and authority to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.

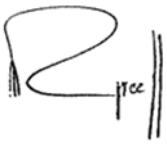
Please note that for any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the HREC, Faculty of Health Sciences. Ethics approval is required BEFORE approval can be obtained from these authorities.

The HREC, Faculty of Health Sciences complies with the South African National Health Act 61 (2003), the Regulations on Research with Human Participants (2014), the Ethics in Health Research: Principles, Structures and Processes (2015), the Belmont Report and the Declaration of Helsinki (2013).

We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-HRECApply@nwu.ac.za.

Yours sincerely





Dr Wayne Towers

HREC Chairperson

Prof Minrie Greeff

Ethics Office Head

Current details: (13210572) C:\Users\13210572\Documents\HREC\HREC - Applications\2016 Applications\Applications 06 - 13 July 2016\NWU-00066-16-S1 (AE Schutte-BO Ahiante)\NWU-00066-16-S1(AE Schutte-BO Ahiante)-AL\NWU-00066-16-S1(AE Schutte-BO Ahiante)-AL.docm
27 July 2016

File reference: 9.1.5.3

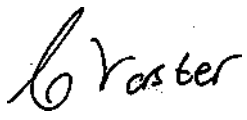
ANNEXURE B

Declaration of Language Editing

DECLARATION

I, C Vorster (ID: 710924 0034 084), Language editor and Translator and member of the South African Translators' Institute (SATI member number 1003172), herewith declare that I did the language editing of a thesis, written by BO Ahiante from the North-West University.

Title of the thesis: Exploring leptin from endothelial activation to subclinical organ damage in young black and white adults: The African-PREDICT study



14 March 2019

_____ _____

C Vorster Date

cvlanguage.editing@gmail.com

ANNEXURE C

Turnitin Report

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