

Leptin: a bi-ethnic approach to unravel its role in cardiovascular disease. The SABPA study

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- Above all, glory to God for the opportunities and abilities He has blessed me with.

“Everyone can rise above their circumstances and achieve success if they are dedicated to and passionate about what they do.” – Nelson Mandela

PREFACE

The article format as approved and outlined by the North-West University for postgraduate doctoral studies was used for the presentation of this thesis. This thesis consists of peer-reviewed published or submitted articles. The first chapter encloses an introduction, motivation and literature overview of the applicable topics investigated in the separate research articles, followed by the overall aim, objectives and hypotheses. Chapters 2, 3 and 4 contain the individual manuscripts in the form of original research articles for submission to peer-reviewed journals. The promoter and co-promoter were included as co-authors in each manuscript. The Ph.D. candidate as first author was responsible for literature searches, statistical analyses and the interpretation of results as well as writing of the research articles. All co-authors gave their approval for the research articles to be submitted for publication and for inclusion in this thesis.

The first article was published in the *Journal of Hypertension* (2014; 32:826-833), the second was published in *Hypertension Research* (2015; 38:507-512) and the third is under review at *Nutrition, Metabolism and Cardiovascular Diseases*. References are listed at the end of Chapter 1 and 5 according to the Vancouver referencing style. The references of the respective research articles (Chapters 2, 3 and 4) are listed according to the instructions for authors as specified by the applicable journal.

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SUMMARY

Motivation

The prevalence of cardiovascular disease is on the increase in sub-Saharan Africa largely owing to lifestyle changes associated with urbanisation. Traditional diets are being replaced with diets high in saturated fat and sugar. In addition to the nutritional transition, urbanisation in developing African countries also contributes to a more sedentary lifestyle. Together these trends contribute to a higher prevalence of obesity and hypertension that are major risk factors for the development of cardiovascular disease. Adipose tissue is now widely recognised as an endocrine organ that secretes numerous inflammatory mediators as well as adipocytokines such as leptin. The primary role of leptin is to induce satiety after a meal and to suppress appetite. However, in recent years the role of leptin in the development of obesity-related cardiovascular disease has gained increasing attention and interest. Furthermore, leptin levels not only differ with regard to gender but also ethnicity. Africans have higher leptin levels than Caucasians due to higher subcutaneous fat in Africans. Furthermore, the prevalence of hypertension and stroke are also greater in the African population. Taken together, it is important to investigate mechanisms by which elevated leptin may contribute to the development of cardiovascular disease, especially in cardiovascular disease-prone Africans.

Aim

The general aim of this study is to increase our understanding of the role of leptin in cardiovascular disease development by investigating associations of leptin with markers of sympathetic activity, endothelial dysfunction, and cardiovascular reactivity and recovery in Africans and Caucasians.

Methodology

Data from the SABPA (Sympathetic activity and Ambulatory Blood Pressure in Africans) study was used and presented in the original research articles described in Chapter 2, 3 and 4. This study included 409 African and Caucasian schoolteachers working in the Potchefstroom district in the North West Province of South Africa. Groups were stratified by ethnicity, gender and

ethnicity or obesity in order to demonstrate potential differences. We performed cardiovascular measurements and determined levels of leptin, renin, cortisol, plasminogen activator inhibitor-1 (PAI-1), von Willebrand factor (vWF) and urinary albumin-to-creatinine ratio (ACR). Independent t-tests were done to compare means between groups and Chi-square tests to compare proportions. Pearson's correlations were determined to investigate associations as well as partial correlations after minimal adjustment for potential confounders. Multiple regression analyses were performed to investigate independent associations of leptin with cardiovascular and biochemical markers according to the specific focus of each research manuscript.

Results and conclusions of the individual manuscripts

- Leptin may contribute to obesity-related hypertension through its sympatho-activating effects. In the first research article (Chapter 2), we compared mean leptin levels and markers of autonomic activity between Africans and Caucasians. We also investigated associations between markers of autonomic activity and leptin. Africans had higher leptin, body mass index, blood pressure and heart rate compared to Caucasians. Furthermore, Africans also demonstrated reduced heart rate variability that is indicative of autonomic imbalance. Markers of autonomic activity that collectively reflected sympathetic overactivity associated with leptin in both Africans and Caucasians, independent of significant covariates and confounders including body mass index. These findings suggest that leptin may contribute to the development of hypertension by inducing autonomic dysfunction.
- Leptin exerts direct vascular effects and may thereby contribute to increased cardiovascular disease risk in the obese. We therefore investigated associations between circulating markers of endothelial dysfunction (PAI-1, vWF and ACR) and leptin in lean and obese groups, irrespective of ethnicity (Chapter 3). As expected, leptin and plasminogen activator inhibitor-1 antigen levels were higher in the obese group. We found no differences for von Willebrand factor antigen and urinary albumin-to-creatinine

ratio. In the obese group, all markers of endothelial dysfunction were positively associated with leptin in univariate analysis. However, after full adjustment in multiple regression analyses, only the association with plasminogen activator inhibitor-1 remained significant. Higher leptin levels in the obese may possibly induce endothelial dysfunction through mechanisms related to thrombotic vascular disease.

- Greater cardiovascular reactivity to stress and prolonged recovery thereafter associates with increased cardiovascular disease risk. In the final research article (Chapter 4), we therefore investigated the relationship between cardiovascular reactivity and recovery to acute stress, induced by the cold pressor test, and leptin in Africans and Caucasians. Africans demonstrated greater cardiovascular reactivity compared to Caucasians. Associations of blood pressure, stroke volume, cardiac output, total peripheral resistance and arterial compliance reactivity with leptin were investigated during the stressor application and 1, 3 and 5 minutes post-stressor. There were no independent associations between cardiovascular reactivity and leptin during the stressor, and a few correlations at 1 and 3 minutes post-stressor. Associations were mostly evident at 5 minutes post-stressor and in Africans. We argue that higher leptin levels relate to impaired post-stress recovery and thereby could contribute to hypertension development in Africans.

General conclusion

Elevated leptin relates to sympathetic overactivity, vascular damage and delayed post-stress recovery, and thereby could contribute to increased cardiovascular disease risk.

Keywords: Africans, autonomic imbalance, black populations, cardiovascular reactivity, cold pressor test, obesity, sympathetic nervous system, vascular damage

AUTHOR CONTRIBUTIONS

The contribution of each author is specified in the table below.

AUTHOR	CONTRIBUTION
Ms. C Pieterse	Responsible for writing the complete thesis, proposal, literature searches for the individual chapters in this thesis and the collection of ambulatory as well as continuous blood pressure measurements with the Finometer apparatus. Other responsibilities included statistical analyses, the design and planning of the articles as well as the interpretation of findings. Writing of each article and all the other chapters in this thesis.
Prof. R Schutte (Promoter)	Assisted with data collection, advice and guidance with regard to statistical procedures and analyses. Supervised the writing of the research articles and critical appraisal of the individual articles and thesis.
Prof. AE Schutte (Co-Promoter)	Involved in data collection, provided advice and recommendations during the writing of the articles and ensured the proper evaluation of findings. Critical assessment of the complete thesis.

By signing this document, the co-authors verify their individual role in this study as stated above. They also give their consent that the research articles may be published as part of the Ph.D. thesis of Ms. C Pieterse.



Prof. R Schutte



Prof. AE Schutte

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ABBREVIATIONS

AMPK	adenosine monophosphate-activated protein kinase
ABPM	ambulatory blood pressure measurements
ACR	albumin-to-creatinine ratio
BMI	body mass index
BRS	baroreflex sensitivity
CI	confidence interval
CLT	clot lysis time
CRP	C-reactive protein
Cwk	arterial compliance
DBP	diastolic blood pressure
ECG	electrocardiogram
ECLIA	Electro-chemiluminescence immunoassay
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular regulated kinase
FMD	flow-mediated dilation
GGT	gamma-glutamyl transferase
HDL	high-density lipoprotein
HF	high frequency
HR	heart rate
HRV	heart rate variability
HRVti	heart rate variability triangular index
JAK/STAT	Janus kinase and signal transducer and activator of transcription pathway
kDA	kilodalton
LDL	low-density lipoprotein
LF	low-frequency
MAP	mean arterial pressure

MMPs	matrix metalloproteinases
NADPH	nicotinamide adenine dinucleotide phosphate
NPY	neuropeptide Y
Ob-Ra	short form receptor
Ob-Rb	long form receptor
Ob-Re	soluble receptor
PAI-1	plasminogen activator inhibitor-1
POMC	pro-opiomelanocortin
PTP1B	protein tyrosine phosphatase 1B
ROS	reactive oxygen species
SE	standard error
SABPA	sympathetic activity and ambulatory blood pressure in Africans
SBP	systolic blood pressure
SD	standard deviation
SOCS-3	suppressor of cytokine signalling-3
Std β	standardised β
TC:HDL	total cholesterol-to-high density lipoprotein
TNF-α	tumor necrosis factor- α
tPA	tissue-plasminogen activator
TPR	total peripheral resistance
vWF	von Willebrand factor
WHtR	waist-to-height ratio
α-MSH	α -melanocyte-stimulating hormone
χ^2	Chi-square

CHAPTER 1

INTRODUCTION AND LITERATURE STUDY

1. INTRODUCTION

Hypertension is an established independent risk factor for the development of cardiovascular disease and stroke.¹ During the year 2010, hypertension prevalence rates reached 30.5% and 28.5% respectively among men and women of the United States.² However, the global hypertension burden is not limited to developed countries, but also occurs in developing countries.³ In fact, cardiovascular disease, as well as diabetes and obesity, are reaching epidemic proportions in sub-Saharan Africa.⁴

Urbanisation is rapidly increasing worldwide, especially in sub-Saharan Africa where the fastest annual rate is found.⁵ Urbanisation is often associated with decreased physical activity and a shift towards an energy-rich, high-fat diet.⁵ Subsequently, these lifestyle changes contribute to the development of obesity, which is recognised as a chronic disease.⁶ Obesity is frequently accompanied by conditions such as type 2 diabetes, hypertension and dyslipidaemia.⁶ Data from the national demographic and health survey of South Africa showed that 25% of men and 26% of women have hypertension.⁷ Ethnic differences in hypertension prevalence rates were also observed, where Africans showed the highest percentage compared to Caucasians, Indians and Asians.⁷ In addition, individuals within the obese category show hypertension prevalence rates of 46.6% among men and 38.5% among women.⁸ The obesity trend in South Africa is a cause for concern as 57% of women and 29% of men are overweight or obese.⁹ It is therefore important to establish underlying mechanisms linking obesity with cardiovascular disease.

Adipose tissue is recognised as an endocrine organ which secretes several adipokines such as leptin.¹⁰ Leptin's primary function is to regulate the body's energy reserves by decreasing food intake and stimulating energy expenditure.¹¹ Leptin correlates with whole-body adipose tissue mass and it is well known that obese subjects have higher leptin levels than their lean counterparts.¹² Leptin levels not only differ between obese and lean subjects, but gender^{13,14} and ethnic differences also exist.¹⁴⁻¹⁶ Apart from leptin's metabolic functions, an independent role thereof in the development of cardiovascular disease has also been established.¹⁷ Even

though obese individuals are more likely to present higher leptin levels, a reduction in food intake and weight is absent.¹⁸ This suggests that obese individuals develop a state of selective leptin resistance with inadequate or no response towards leptin's metabolic functions.¹⁸ However, actions of leptin not related to metabolism, such as sympathetic nervous system activation are retained.¹⁹ Considering all of the above, it is important to investigate potential underlying mechanisms by which leptin contributes to the development of cardiovascular disease especially in an understudied, high-risk African population.

Data from the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study was used for the purpose of this thesis. This study was designed to assess sympathetic nervous system responses and associated lifestyle diseases in an urbanised cohort. The study included 409 African and Caucasian school teachers from the Potchefstroom district in the North West Province of South Africa. The mean leptin levels and cardiovascular profile of African and Caucasian participants were compared. The central focus of this study is to shed light on possible mechanisms by which leptin contributes to the development of cardiovascular disease.

The following sections of this chapter are composed of a literature overview with the relevant background on leptin and potential mechanisms linking leptin to cardiovascular disease development. This is followed by a brief motivation, aims and hypotheses for the individual research articles included in the subsequent chapters.

2. THE DISCOVERY OF LEPTIN

More than half a century ago two mouse strains, *ob/ob* and *db/db*, both characterised by severe obesity, hyperphagia, insulin resistance and infertility were identified.^{20,21} After a series of parabiosis experiments with *ob/ob* and *db/db* mice it was concluded that the *db/db* mice strain overproduced a blood-borne satiety factor but failed to respond to it. Contrastingly, the *ob/ob* mice strain did not produce this factor but were able to recognise and respond to it.²² Despite this finding, many still believed that obesity was entirely attributable to behavioural factors such

as a lack of discipline.²³ However, the identification of a satiety factor encoded by the *ob/ob* gene pointed to a physiological mechanism of body weight regulation.²² In 1994, positional cloning of *ob/ob* mice led to the identification of the satiety hormone, leptin,^{22,24} named after the Greek word 'leptos' meaning thin.^{24,25} Leptin injected into mice resulted in a reduction in body weight, body fat and food intake.²⁶ However, the possibility of leptin as an anti-obesity hormone was abolished after the failure of leptin to reverse obesity in obese animals and humans.^{12,27} These studies changed the field of obesity research by demonstrating that adipose tissue secreted a satiety hormone leptin; that leptin receptors were present in the hypothalamus; and that adipose tissue functioned as an endocrine organ.²² We now know that the effects of leptin extend beyond the regulation of food intake and energy expenditure, and it is especially its role in cardiovascular disease development that has increasingly gained interest since its discovery.^{10,19,28,29}

3. THE PHYSIOLOGY OF LEPTIN: STRUCTURE, FUNCTION AND SECRETION

Leptin is a 16-kDA, four-helix bundle protein which contains an N-terminal and a C-terminal with a disulphide bond.³⁰ The N-terminal plays a fundamental role during receptor binding and the C-terminal strengthens the binding activity.³⁰ Mutations around the N-terminal impair receptor activation, whereas mutations around the C-terminal do not affect receptor binding but moderately impair signalling.³¹

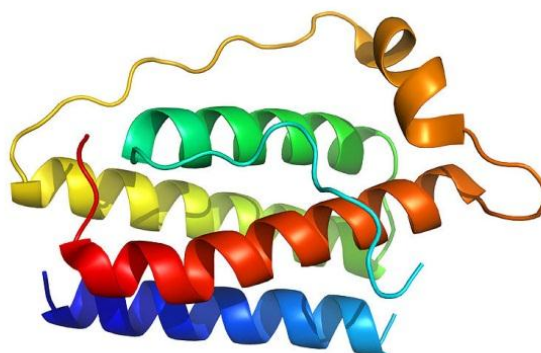


Figure 1: Structure of human leptin illustrating the four-helix bundle. Figure taken from the internet.³²

White adipose tissue is the main site of leptin production and the concentration thereof is positively associated with the amount of fat tissue.^{10,24} Leptin secretion is about two-fold higher in obese individuals compared to their lean counterparts.³³ This is mainly due to larger fat cells that release more leptin as well as an increased number of fat cells in the obese.³³ Other sites of leptin secretion include brown adipose tissue, the stomach and placenta.³⁴ Leptin levels rise after a meal and decrease during periods of fasting.^{35,36} The effect of nutrition and fasting on leptin levels seems to be related to changes in insulin secretion.³⁷ Insulin treatment stimulates leptin secretion whereas a reduction in leptin is seen in a low insulin induced environment.³⁷ In cultured human adipose tissue, insulin stimulates leptin production. On the other hand, when placed in a medium without insulin, leptin gene expression decreases by more than 50%.³⁸ Leptin secretion is also affected by hormones which include estradiol,³⁹ tumor necrosis factor- α (TNF- α),⁴⁰ catecholamines³³ and thyroid hormones.⁴¹ Thyrotrophin stimulates leptin secretion by human adipose tissue *in vitro*. This may explain the pulsatility of leptin due to the strong circulating pulsatility exhibited by thyrotrophin.⁴² Long term exposure to catecholamines chronically decreases leptin expression and secretion.³³ Leptin concentrations peak in the early morning, between 01:00 and 02:00, and are at its lowest mid-afternoon and early evening.⁴³ It is suggested that the nocturnal increase in leptin levels are purely related to appetite suppression during sleep and of no relevance with regard to obesity.⁴³

Gender differences also exist. Women have higher leptin levels than age-matched men^{13,44} at any given body fat mass.^{12,45} The increased production of leptin in women may be indicative of lower leptin sensitivity.⁴⁶ Another possible explanation includes the presence of more subcutaneous fat in women compared to men.^{47,48} Subcutaneous adipose tissue express more leptin mRNA^{49,50} and associates more strongly to increased leptin levels than visceral adipose tissue.⁵¹ Furthermore, women are more sensitive to hormones influencing leptin secretion such as insulin.⁴⁷ Sex hormones may also regulate leptin levels.⁴⁴ Estrogen administration increases leptin levels in rats³⁹ and women *in vivo*.⁵² Furthermore, ovariectomy decreases leptin levels in rats.⁵² On the other hand, testosterone is inversely related to leptin, independent of body mass index.⁵³

Ethnic differences in leptin levels are also displayed, being higher in Africans than Caucasians.^{14,54-56} This may also be attributed to higher subcutaneous adipose tissue in Africans compared to Caucasians with similar body mass indices.⁵⁷⁻⁵⁹

The structure of the leptin receptor is similar to class 1 cytokine receptors and act through the Janus kinase and signal transducer and activator of transcription (JAK/STAT) pathway (Figure 2).⁶⁰ Leptin receptors extend across the cell membrane and consist of an extracellular- and intracellular domain and to date six different receptor isoforms have been identified.⁶¹ However, the long form receptor (Ob-Rb), which is largely found in the hypothalamus, regulates most of leptin's actions, such as appetite regulation, thermogenesis and sympathetic nervous system activity.²⁸

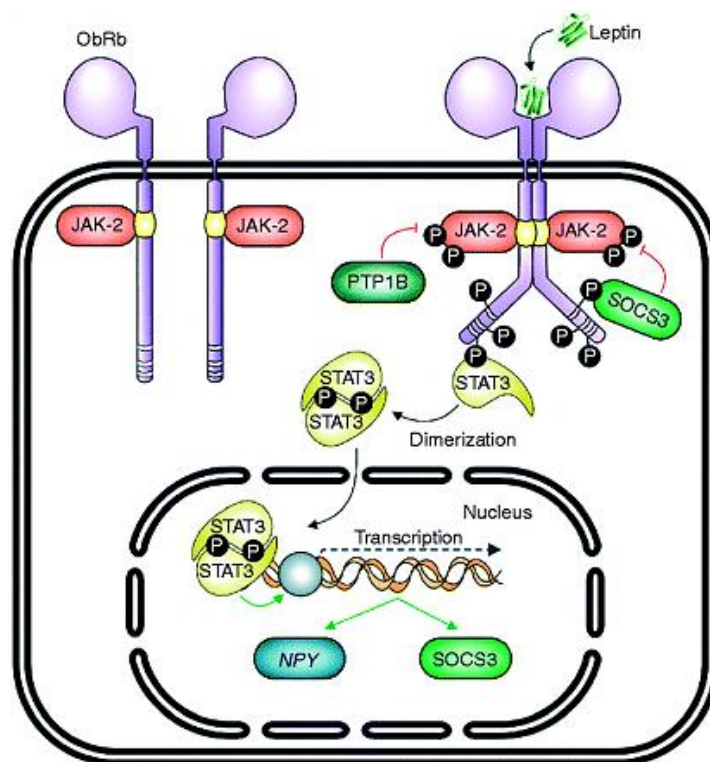


Figure 2: Leptin signalling via the Janus kinase and Signal Transducer and Activator of Transcription (JAK/STAT) pathway. Figure taken from Marroqui *et al.*⁶²

In order for leptin to interact with central nervous system receptors it crosses the blood-brain barrier via a saturable system.⁶³ The leptin short form receptors (Ob-Ra) regulate leptin transport across the blood brain barrier. This is supported by experimental studies in mice which show normal leptin transport across the blood-brain barrier despite lacking the long form receptor.^{61,64,65} The primary function of leptin is to inhibit food intake and to increase energy expenditure by binding to its Ob-Rb receptors, situated in the hypothalamus.^{25,66,67} These receptors are mainly expressed in the arcuate, ventromedial and dorsomedial nuclei of the hypothalamus.⁶⁷ Leptin acts directly on arcuate nucleus neurons and thereby regulate energy homeostasis by two distinct pathways.⁶⁷ Leptin binds to Ob-Rb receptors expressed by pro-opiomelanocortin neurons which produce α -melanocyte-stimulating hormone. Thereupon, α -melanocyte-stimulating hormone binds to melanocortin receptors to promote satiety and inhibit food intake.⁶⁸ Further, leptin inhibits the release of appetite-stimulating neuropeptide Y (NPY) by binding to a different population of neurons situated in the arcuate nucleus.⁶⁸ Apart from the membrane bound leptin receptors, a soluble leptin receptor isoform (Ob-Re) has also been identified which regulates circulating leptin availability.⁶⁹ Leptin bound to Ob-Re is unable to bind to Ob-Rb and may therefore act as an inhibitor to Ob-Rb mediated actions of leptin on food intake and energy metabolism. In lean individuals most of leptin are in the bound form (60 – 98%), whereas in obese individuals most of leptin circulate in the free form (86 – 95%).⁷⁰

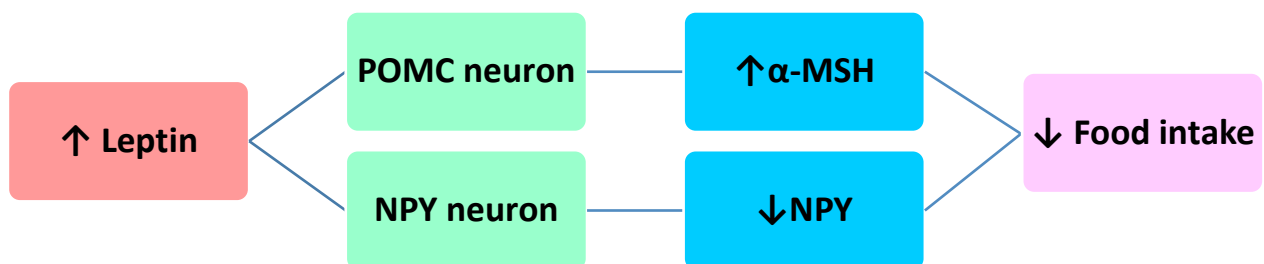


Figure 3: Appetite regulation by leptin in the hypothalamus.⁶⁸

POMC, pro-opiomelanocortin; NPY, neuropeptide Y; α -MSH, α -melanocyte-stimulating hormone.

Another mechanism by which leptin promotes satiety, is by strengthening the central nervous system response to cholecystokinin and glucagon-like peptide 1 signals, which are produced in the stomach upon food intake.⁷¹ In addition to the regulation of energy balance, a role for leptin in glucose metabolism has also been established. The effect of leptin on increasing glucose uptake and turnover as well as decreasing glucose production seem to be mediated mainly through hypothalamic signalling pathways.^{72,73} Therefore, the presence of diabetes in leptin deficient *ob/ob* mice and *db/db* mice with defective leptin signalling comes as no surprise.⁶⁷ In leptin deficient mice, NPY is a key mediator in the development of diabetes. This further supports the role of leptin in maintaining glucose homeostasis, since leptin inhibits NPY secretion in the arcuate nucleus.⁶⁷ The administration of leptin to leptin-deficient mice attenuates their hyperglycemia as well as hyperinsulinemia.⁷⁴ Moreover, leptin therapy results in improved glycemia and dyslipidemia in patients with lipodystrophy.^{75,76} Leptin increases phosphatidylinositol-3-kinase signalling pathways in the arcuate nucleus and thereby improves peripheral insulin sensitivity.⁷¹ However, leptin may regulate glucose and fatty acid metabolism by directly targeting the pancreas, liver, skeletal muscle and adipocytes. This is supported by the expression of the Ob-Rb leptin receptor in the above-named peripheral tissues.⁷⁷ Numerous *in vitro* and *in vivo* studies demonstrate that leptin increases glucose uptake, glucose metabolism and fatty acid oxidation in adipose tissue and skeletal muscle.⁷⁷ Additionally, leptin directly activates 5' adenosine monophosphate-activated protein kinase (AMPK) which leads to increased glucose uptake, fatty acid oxidation and insulin sensitivity.^{77,78}

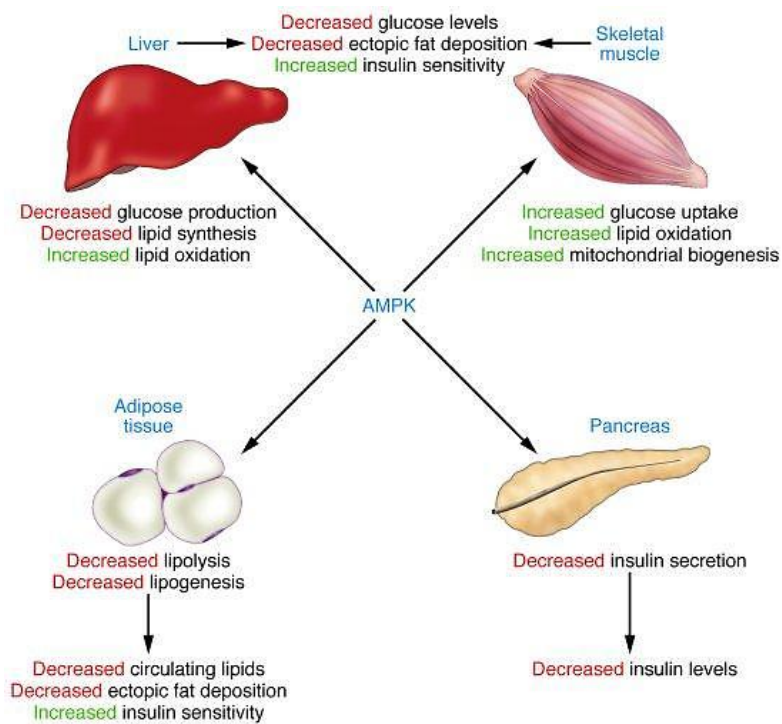


Figure 4: Role of 5' adenosine monophosphate-activated protein kinase (AMPK) in the regulation of whole-body glucose homeostasis. Figure taken from Long *et al.*⁷⁸

Leptin also plays a vital role in reproduction and the onset of puberty. In the interest of reproductive success, the body has to maintain sufficient energy supplies. Leptin receptors are expressed in neurons secreting gonadotropin-releasing hormone in the hypothalamus that stimulates the release of luteinizing hormone and follicle stimulating hormone from the pituitary.⁷⁹ Furthermore, evidence from rats suggests that leptin is able to directly stimulate the production and secretion of luteinizing hormone and follicle stimulation hormone from the pituitary.⁸⁰ Reproductive dysfunction has been shown in *ob/ob* and *db/db* mice as well as in obese humans. Low leptin levels exist in women who suffer from functional hypothalamic amenorrhea or amenorrhea due to strenuous exercise.⁸¹ Menstrual abnormalities are also seen in patients with anorexia nervosa where their leptin levels are much lower than healthy controls.⁸¹ An increase in serum leptin of 1 ng/ml can speed up the onset of menarche by one month,⁸² as evidenced by a large cross-sectional study showing that obese girls reached menarche earlier than their normal weight counterparts.⁸³

4. LEPTIN RESISTANCE

It seems logical that obese individuals would be leptin deficient. However, the opposite is true with obese individuals exhibiting higher than normal leptin levels.^{69,84} Despite elevated leptin levels, increased energy expenditure, weight loss and a suppressed appetite are absent in obese individuals.^{69,84} Similar observations are seen in animals fed a high-fat diet.⁸⁴ These studies have led researchers to the concept of leptin resistance.⁸⁵ It is believed that leptin resistance may stem either from inadequate leptin signalling or defective leptin transport across the blood-brain barrier.⁶⁸

Leptin transport across the blood-brain barrier is inhibited in mice receiving bovine milk, consisting of 98% triglycerides.⁸⁶ The ability of high triglycerides to reduce leptin transport across the blood-brain barrier is suggested to be an early adaptive mechanism to prevent starvation. Fasting or starvation increase triglyceride levels due to the movement of triglycerides from adipose tissue into the circulation.⁸⁷ In our modern day society, hypertriglyceridemia is more likely to be a consequence of obesity rather than starvation. This may then be misinterpreted by the blood-brain barrier as a starvation signal and thereby inhibit leptin transport into the cerebrospinal fluid and hypothalamus.⁸⁷ Diet-induced obese mice are widely used to study the pathogenesis of leptin resistance. Mice fed a high-fat diet, gradually become obese and hyperleptinemic due to increased adipose tissue mass.⁸⁸ Transport of leptin across the blood-brain barrier by Ob-Ra may also be disrupted by the soluble leptin receptor (Ob-Re) which acts as an antagonist of Ob-Ra activity.⁸⁹ Leptin resistance may also stem from inadequate leptin signalling mechanisms. Suppressor of cytokine signalling-3 (SOCS3) and protein tyrosine phosphatase 1B (PTP1B) inhibit Janus kinase activity and thereby provide a negative feedback mechanism.⁸⁹ Removal of SOCS3 as well as PTP1B increases leptin sensitivity by binding to Janus kinase. Furthermore, both SOCS3 and PTP1B are increased in the hypothalamus of leptin resistant animals.⁸⁹

Studies have also demonstrated that leptin resistance may be selective to leptin's appetite and weight reducing properties and that activation of the sympathetic nervous system remains

intact.⁸⁵ In agouti yellow mice, sympathetic nerve activity to the kidney remained the same for both obese and lean mice. On the other hand, intraperitoneal leptin administration reduced body weight and food intake only in lean mice compared to obese mice.⁹⁰ Similar results were found in a study by Rahmouni et al, when leptin was administered intracerebroventricular in normal and high-fat diet fed mice.⁹¹ In obese mice leptin retained the ability to stimulate the sympathetic nervous system via the dorsomedial hypothalamus.⁹² The subfornical organ, which lacks the blood-brain barrier, is also a potential site mediating leptin's central nervous system action.⁹³ It has been demonstrated that leptin signalling in the subfornical organ increases renal sympathetic nerve activity. Contrastingly, this had no effect on food intake due to systemic or centrally administered leptin targeted at the subfornical organ.⁹⁴ Therefore, site-specific leptin actions may partly explain selective leptin resistance and the presence of sympathetic overactivity in obese humans and animals.^{28,94}

An inhibitory effect of the inflammatory marker, C-reactive protein (CRP), on leptin's actions may also contribute to leptin resistance.⁹⁵ Firstly, incubation of leptin with human recombinant CRP for 30 minutes followed by immunoprecipitation and immunoblotting analysis confirms a physical interaction between leptin and CRP.⁹⁵ Secondly, it was demonstrated that incubation of leptin with CRP blocked endothelial nitric oxide synthase phosphorylation and reduced nitric oxide production.⁹⁵ Collectively, these results demonstrate that CRP may directly bind leptin and inhibit the physiological function thereof.

5. PATHOLOGICAL MECHANISMS LINKING LEPTIN AND CARDIOVASCULAR DISEASE

5.1 Hypertension

The hemodynamic determinants of blood pressure mainly include cardiac output (a product of heart rate and stroke volume) and total peripheral resistance.⁹⁶ The sympathetic and parasympathetic nervous system regulates heart rate,⁹⁷ whereas stroke volume is regulated by the contractile activity of the heart which is affected by the blood volume and venous return.⁹⁸ With regards to peripheral vascular resistance, multiple structural, mechanical and functional vascular changes that decrease the arteriolar radius will increase the resistance to blood flow.⁹⁹

These vascular changes may be subdivided into structural, mechanical and functional changes, however, they are all closely related to one another.⁹⁹ Hypertrophic remodelling is one such type of structural change characterised by media thickening, increase media-lumen ratio and cross-sectional wall area.⁹⁹ Mechanical changes are associated with increased arterial stiffness and decreased arterial compliance. Smooth muscle cell growth and proliferation as well as dysfunctional matrix metalloproteinase activity may result in structural and mechanical changes.⁹⁹ Additionally, reduced vasodilation and enhanced vasoconstriction are functional changes that contribute to increased peripheral vascular resistance and subsequently hypertension.¹⁰⁰⁻¹⁰²

The European Society of Hypertension defines hypertension as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg based on evidence which suggests that blood pressure reduction treatment is beneficial at these values.¹⁰³ Hypertension is one of the top three causes of mortality in developed countries¹⁰⁴ and associates with an elevated risk for heart disease, stroke, myocardial infarction and renal failure.¹⁰⁵ The aetiology of essential hypertension, which accounts for 95% of hypertensive cases, is complex and may be attributed to multiple genetic, environmental and behavioural factors.¹⁰⁶ In both obese children and adults, office and ambulatory blood pressure are higher than in their lean counterparts.¹⁰⁷ The sympathetic nervous system, endothelial dysfunction, impaired renal function and several hormones such as leptin are implicated in the pathophysiology of obesity-related hypertension.¹⁰⁷

In the Copenhagen City Heart Study, leptin was a predictor of new-onset hypertension independent of several cardiovascular risk factors after a ten year follow-up.¹⁰⁸ In a multi-ethnic sample of the Third National Health and Nutrition Examination Survey, leptin was positively associated with hypertension in men and women.¹⁷ Furthermore, in another multi-ethnic study, the association between leptin and hypertension was stronger in men than women.¹⁰⁹ In older men and women it was demonstrated that higher level of leptin increased the odds of having hypertension, independent of obesity, after a four year follow-up period.¹¹⁰ Animal studies have

shown that chronic hyperleptinemia in transgenic skinny and obese mice elevates blood pressure.¹¹¹ In contrast, lower arterial pressure is observed in obese leptin-deficient mice compared to lean controls with leptin.¹¹² On the contrary, transgenic skinny mice with hyperleptinemia show elevated systolic blood pressure and catecholamine levels.¹¹³ These studies support a possible role of leptin in the development of hypertension independent of adiposity.

5.2 Autonomic nervous system imbalance

The autonomic nervous system plays a fundamental role in regulating cardiovascular and energy homeostasis.¹¹⁴ The autonomic nervous system controls the activity of sympathetic and parasympathetic nerves which receive afferent nerve signals from the peripheral tissues and send efferent signals back in order to control numerous physiological processes.¹¹⁵ Under normal conditions blood pressure is maintained by both the sympathetic and parasympathetic branches of the autonomic nervous system. However, in patients with hypertension the sympathetic nervous system is often over-active.¹¹⁴ Sympathetic nerves innervate the heart, blood vessels and kidneys and thereby regulate heart rate, contractility, vasoconstriction as well as fluid balance.¹¹⁴ Weight gain increases sympathetic nerve activity and decreases parasympathetic activity in order to stimulate energy expenditure and promote weight loss.¹¹⁶ Obesity is therefore associated with elevated sympathetic nerve activity and studies suggest that leptin may be one of the links.¹¹⁴ The ability of leptin to stimulate sympathetic nerve activity to the kidney despite resistance to its metabolic actions in the hypothalamus of the obese lends support to a pathological role of leptin in hypertension development.¹¹⁷

Leptin administration in rats increased sympathetic nerve activity to the kidney, adrenal glands and brown adipose tissue.¹¹⁸ The ventromedial and dorsomedial hypothalamic nuclei have been identified as potential sites that regulate leptin's cardiovascular actions.^{119,120} Microinjections of leptin in the ventromedial nucleus increase catecholamine secretion,¹²⁰ mean arterial pressure and renal sympathetic nerve activity,¹¹⁹ whereas in the dorsomedial nucleus it results in

elevated mean arterial pressure as well as heart rate.¹¹⁹ Furthermore, binding of leptin to its long form receptors situated in the arcuate nucleus elevates renal sympathetic nerve activity.¹²¹

Elevated heart rate or tachycardia is a result of heightened sympathetic activity and reduced parasympathetic activity.¹²² Tachycardia is accompanied by increased myocardial oxygen consumption, reduced myocardial blood supply and decreased large artery compliance.¹²²⁻¹²⁴ Increased heart rate is associated with a higher risk of mortality and cardiovascular events such as myocardial ischemia.^{123,124} A reduction in artery compliance and distensibility may promote endothelial dysfunction and thereby facilitate atherosclerotic plaque formation.^{122,125}

Chronic infusion of leptin into the carotid arteries of male Sprague-Dawley rats elevated arterial pressure and heart rate.¹²⁶ This is consistent with the results of Carlyle *et al.*,¹²⁷ but they additionally demonstrated that α - and β -adrenergic blockade abolished the increases in heart rate and arterial pressure due to leptin administration. This study suggests a possible role of adrenergic activity in mediating the cardiovascular actions of leptin.¹²⁷ An independent association between leptin and 24 h heart rate was found in 60 hypertensive men and in apparently healthy men.¹²⁸ However, this association was not seen in women, therefore indicating that gender-specific mechanisms might be at work.¹²⁸ Furthermore, a relationship between leptin and heart rate was shown in heart transplant patients with cardiac denervation. Thus, the possibility of a direct action of leptin on increasing heart rate by binding to cardiac leptin receptors may exist.¹²⁹

Heart rate variability measures can be used to assess autonomic imbalance and a reduction thereof is associated with increased morbidity and mortality.¹³⁰ The clinical relevance of heart rate variability measures are demonstrated in heart failure patients or those who suffered myocardial infarction, where a reduction in heart rate variability is associated with increased mortality.¹³¹ The time intervals between heart beats are used to calculate measures of heart rate variability. An increase in sympathetic activity will shorten the time between heart beats and the opposite will occur during parasympathetic activation.¹³² In a US sample of 856 middle-aged

men and women, lower heart rate variability predicted coronary heart disease,¹³³ and in 633 normotensive men of the Framingham Heart Study, a lower heart rate variability predicted hypertension development after a 4-year follow-up period.¹³⁴ Similar results were seen in 7009 men and women after 9-years of follow-up, indicating that low heart rate variability may precede the development of hypertension.¹³⁵

Literature with regards to obesity and heart rate variability is scant. A small study of 10 obese subjects showed that body mass index correlated inversely with the total power component of heart rate variability.¹³⁶ In a small study of 25 men and women, frequency domain measures of sympatho-vagal balance were related to body mass index. This was reflected by an increase in the low frequency domain (index of sympathetic modulation) and decrease in the high frequency domain (index of parasympathetic modulation) when comparing the lower and upper body mass index tertile.¹³⁷ In 786 young men, increases in body mass index were significantly related to higher sympathetic activity as assessed by the ratio of the low-frequency/high-frequency ratio.¹³⁸ Further, in normotensive non-obese men, significant trend toward higher low frequency and a low-frequency/high-frequency ratio across leptin quartiles existed. This was independent of body fat content.¹³⁹

An increase in renal sympathetic nerve activity will stimulate renin release that may contribute to increased production of angiotensin II.¹⁴⁰ Renin is an enzyme that accelerates the conversion of angiotensinogen to angiotensin I. Angiotensin I is then converted into the active angiotensin II by angiotensin-converting enzyme.¹⁴¹ Angiotensin II increases vascular resistance as well as sodium and water retention, however its overactivity may also contribute to the development of hypertension.¹⁴⁰ A positive association between plasma renin activity and leptin was shown in 33 hypertensive individuals.¹⁴² Additionally, leptin increases angiotensin-converting enzyme activity in mice¹⁴³ and angiotensin II stimulates leptin release from rat adipocytes.¹⁴⁴

Stress exposure activates multiple physiological systems including the sympathetic nervous system, which is the system that responds most rapidly to stress.¹²⁵ Activation of the

sympathetic nervous system and other systems is necessary to adapt to stress, but under normal conditions these systems recover when the stressor is removed.¹²⁵ Sustained or repeated activation leads to the deregulation of multiple systems which over time may result in pathological conditions such as atherosclerosis.¹⁴⁵ The possibility exists that sympathetic nervous system hyperactivity as induced by elevated leptin levels, might contribute to the deregulation of the normal stress response. This may lead to conditions such as enhanced cardiovascular reactivity to stress. Studies investigating the relationship between cardiovascular reactivity and leptin are limited. Previous studies showed associations between stress-induced increases in heart rate, reductions in heart rate variability and leptin.^{146,147} Of note, in the study comparing men and women, the cardiovascular responses to stress were only seen in women who had elevated leptin levels compared to men.¹⁴⁷ Ethnic differences in cardiovascular reactivity to stress also exist, where Africans demonstrate greater reactivity than Caucasians.^{148,149} It is suggested that individual differences may originate from heightened central nervous system reactivity or alterations in peripheral tissues.¹⁵⁰ For instance, increased α -adrenergic vasoconstriction and reduced β -adrenergic vasodilation responses are seen in Africans compared to Caucasians.¹⁵¹

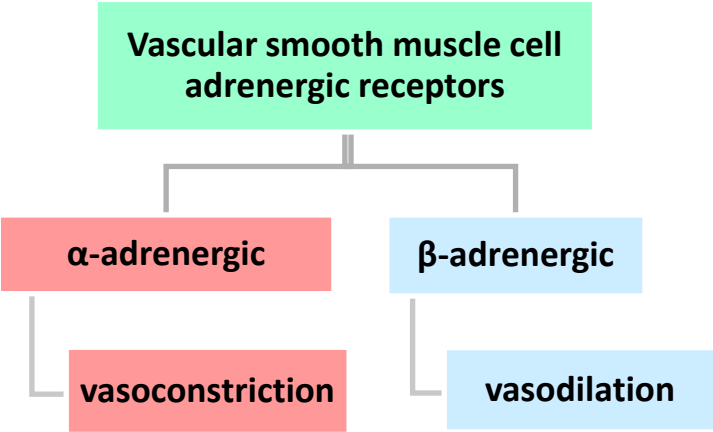


Figure 5: Vascular smooth muscle cell adrenergic receptors and their response to activation.^{152,153}

Robust associations between cardiovascular hyperreactivity, induced by a mental arithmetic test, and the development of hypertension were seen in a study with a follow-up period of 18-years.¹⁵⁴ Additionally, in people followed up for 45-years hyperreactivity to the cold pressor test

was predictive of future hypertension.¹⁵⁵ Heightened blood pressure responses associated with an increased stroke risk in men, especially due to thromboembolism and ischemia.¹⁵⁶ This is supported by a prospective study in Finnish men that found a relationship between increased systolic blood pressure reactivity to stress and carotid intima-media thickness.¹⁵⁷ In addition to heightened reactivity, delayed recovery to stress is also associated with increased carotid intima-media thickness in men and women.¹⁵⁸

5.3 Endothelial dysfunction

Repeated exposure to cardiovascular risk factors may result in endothelial activation and the inability of the endothelium to maintain vascular homeostasis.¹⁵⁹ This is broadly referred to as endothelial dysfunction.¹⁵⁹ Endothelial dysfunction is an early step in the development of cardiovascular disease,¹⁶⁰ and characterised by reduced vasodilation, altered haemostasis, increased secretion of vasoconstrictor substances, microalbuminuria, inflammation and oxidative stress.¹⁶¹ Furthermore, endothelial dysfunction associates with the presence of numerous cardiovascular risk factors such as hypertension, hypercholesterolemia, diabetes mellitus¹⁶² and obesity.¹⁶³

A single layer of endothelial cells forms a selective barrier which regulates the movement of nutrients and hormones between the blood and bordering vascular cells.¹⁶¹ The endothelium is constantly exposed to changes in blood flow or -composition and is therefore an important regulator of vascular homeostasis.¹⁶⁴ Homeostasis is maintained by the endothelial production and secretion of vasodilator and vasoconstrictor agents.¹⁶⁵ As in the case of exercise, increased blood flow will generate shear stress, which in turn activates the endothelial nitric oxide synthase enzyme. Thereupon, endothelial nitric oxide synthase will stimulate the oxidation of L-arginine to produce nitric oxide.¹⁶⁶ Apart from being an important regulator of flow-mediated dilation, nitric oxide also inhibits platelet adhesion and activation, inflammation, cell proliferation as well as thrombosis.^{165,166} Leptin has the ability to upregulate endothelial nitric oxide synthase expression and therefore stimulate endothelial nitric oxide production.^{29,167} However, in hyperleptinemic situations, the beneficial vasodilator effects of leptin may be opposed by leptin

induced reactive oxygen species production.²⁹ Reactive oxygen species bind to nitric oxide and thereby reduce endothelial nitric oxide bioavailability, which in turn will contribute to endothelial dysfunction.¹⁶⁸ Furthermore, experimental studies in rats suggest that leptin has little effect on nitric oxide production in resistance vessels and that leptin-induced nitric oxide production seen in other studies is due to a high pharmacological dose.¹⁶⁹

Stimulation of sympathetic nerve activity may be another mechanism by which leptin contributes to endothelial dysfunction.¹⁷⁰ It is suggested that sympathetic stimulation impairs flow-mediated dilation through an alpha-adrenergic receptor pathway.¹⁷¹ Endothelial alpha-adrenergic receptors promote cell growth in response to hypoxia-induced vascular injury.¹⁷² Furthermore, muscle sympathetic nerve activity was inversely related to endothelial function in ten individuals without cardiovascular disease.¹⁷³ In another study, angiotensin II receptor blockers inhibited sympathetic activation and improved acetylcholine-induced forearm vasodilation in patients with the metabolic syndrome.¹⁷⁴ Binding of angiotensin II to its type 1 receptor triggers multiple mechanisms which may lead to endothelial dysfunction such as vasoconstriction, inflammation, oxidative stress and stimulation of the sympathetic nervous system.¹⁷⁵ Experimental studies on leptin deficient ob/ob mice provide insight into the link between the renin-angiotensin system and leptin.¹⁷⁶ Both the angiotensin-I converting enzyme mRNA expression as well as the lung and plasma angiotensin-I converting enzyme activity are reduced in leptin deficient mice.¹⁷⁶ Acute and chronic leptin injection resulted in increased angiotensin-I converting enzyme activity in the ob/ob leptin deficient mice.¹⁷⁶

Flow-mediated dilation

In a study involving 35 weight gainers, weight gain impaired flow-mediated dilation (FMD), but associations between FMD and leptin were absent.¹⁷⁷ A lack of an association between FMD and leptin was also seen in 294 adolescents.¹⁷⁸ However, in non-diabetic and normotensive women, a negative association between leptin and FMD was seen, suggesting a potential role of leptin in inducing endothelial dysfunction.¹⁷⁹ In contrast, positive associations between FMD and leptin were observed in overweight diabetic patients.¹⁸⁰ These conflicting findings may

indicate that leptin's contribution to endothelial dysfunction is not limited to mechanisms related to impaired endothelial-induced vasodilation. Studies linking leptin with circulating markers of endothelial dysfunction are limited.

Plasminogen activator inhibitor-1

Plasminogen activator inhibitor-1 (PAI-1) inhibits tissue-type plasminogen activator and urokinase-type plasminogen activator that are both responsible for the conversion of plasminogen to plasmin. Plasmin is an important regulator of fibrin and extra cellular matrix degradation.¹⁸¹ PAI-1 is secreted by many cell types including endothelial cells and promotes thrombosis and vascular damage through cell adhesion, migration and proliferation.¹⁸² Upon secretion, it is released into the circulation either to act as an acute phase protein or to suppress fibrinolysis under normal concentrations.¹⁸² However, an overproduction of PAI-1 may contribute to vascular injury.¹⁸² A role of PAI-1 in the development of cardiovascular disease has been established.¹⁸³ An independent association between leptin, PAI-1 antigen and PAI-activity was shown in obese and non-obese women¹⁸⁴ and in hypertensive overweight participants.¹⁸⁵ A similar correlation was observed between PAI-1 antigen and leptin in men who experienced their first acute myocardial infarction.¹⁸⁶ An *in vitro* study showed that incubation of coronary endothelial cells with leptin induced PAI-1 expression at higher leptin levels (≥ 50 ng/ml).¹⁸⁷ Furthermore, stimulation of the sympathetic nervous system, increased inflammation, oxidative stress, epinephrine and angiotensin II stimulate PAI-1 secretion¹⁸² and may provide indirect mechanisms by which leptin induces PAI-1 secretion.

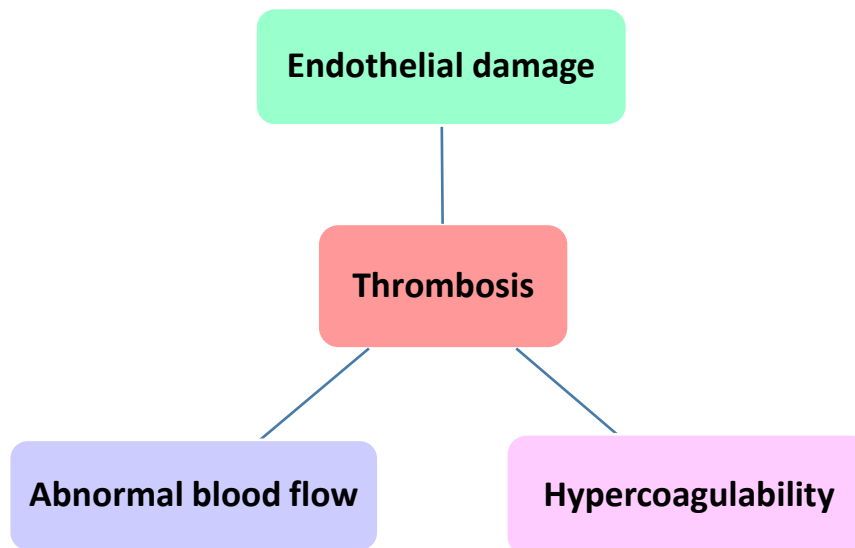


Figure 6: Virchow's triad: Three components that contribute to thrombus formation.¹⁸⁸

Von Willebrand factor

Von Willebrand factor (vWF) is another marker associated with endothelial dysfunction and damage.¹⁸⁹ Endothelial cells release vWF in response to vascular injury.¹⁹⁰ vWf promotes platelet aggregation and adhesion at sites of vascular injury especially in the arterial circulation where blood flows rapidly, whereas fibrinogen is mainly responsible for the formation of fibrin clots in vessels of the venous circulation where blood flow is relatively slow compared to the arterial circulation.^{191,192} Therefore, vWF is of particular relevance in high shear-induced thrombus formation, such as in vessels where atherosclerotic plaques obstruct the lumen.¹⁹¹

In a study comparing lean and obese women, obese women had higher leptin and vWF levels.¹⁹³ Furthermore, vWF was positively associated with leptin in obese women.^{193,194} In another study of obese men and women, leptin was positively associated with vWF in men only. The men had a higher body mass index, waist-to-hip ratio and visceral adipose tissue compared to the women but unexpectedly lower leptin levels.¹⁹⁵ A similar correlation was seen in men (n=3640) from The British Regional Heart study after adjusting for established cardiovascular risk factors except blood pressure.¹⁹⁶ That being said, a lack of association between blood

pressure and vWF was seen in men (22% hypertensive) and women (16% hypertensive) of the Framingham Offspring study.¹⁹⁷

Albumin-to-creatinine ratio

In addition to PAI-1 and vWF, albumin-to-creatinine ratio (ACR) is another well-established marker of endothelial dysfunction.¹⁹⁸ ACR is regarded as a risk marker rather than a risk factor for the development of endothelial dysfunction, because it is believed that microalbuminuria is preceded by endothelial dysfunction.¹⁹⁸ Therefore, the presence of microalbuminuria (urinary ACR of ≥ 2.5 mg/mmol in men and of ≥ 3.5 mg/mmol in women)¹⁹⁸ is likely to be indicative of established endothelial dysfunction as damage to the glomerular endothelial glycocalyx will lead to increased permeability and leakage of albumin into the urine.¹⁹⁸ Inflammation,¹⁹⁹ exposure to oxidised low-density lipoprotein²⁰⁰ and hyperglycemia²⁰¹ are some of the factors which could contribute to endothelial glycocalyx damage and endothelial dysfunction. A pathophysiological role of the endothelial glycocalyx in the initiation and progression of atherosclerosis has also been established.²⁰² An *in vitro* and *in vivo* study showed that leptin infusion resulted in glomerular endothelial cell proliferation.²⁰³

Inflammation

A pro-inflammatory role of leptin was identified *in vitro*, indicating that administration of leptin upregulates macrophage synthesis of cytokines namely, TNF- α , interleukin-6 and interleukin-12.²⁰⁴ Similarly, TNF- α and interleukin-1 increase circulating leptin and thus further enhance the inflammatory process in a positive feedback manner.²⁰⁴ In addition, leptin administration increases the expression of CRP in the endothelium and activates monocytes, macrophages, neutrophils and T lymphocytes.²⁰⁴ Leptin promotes monocyte recruitment and macrophage foam cell formation and may thereby contribute to atherosclerosis.²⁰⁵ An independent positive association between CRP and leptin was demonstrated in 100 apparently healthy men and women.²⁰⁶ This independent association was confirmed in a larger study including 1862 Finnish healthy men and women.²⁰⁷ Further, in patients with type 2 diabetes it was demonstrated that patients with both elevated leptin levels and CRP were at greater risk for cardiovascular disease

than those with only elevated leptin or CRP. Leptin remained associated with cardiovascular disease after adjusting for CRP in these patients, but the opposite did not hold true for CRP.²⁰⁸ An *in vitro* study showed that leptin-induced synthesis of CRP by coronary endothelial cells is mediated through reactive oxygen species production.²⁰⁹ The ability of leptin to induce interleukin-6 production²¹⁰ may act as an indirect mechanism which leads to CRP production in the liver.²¹¹ This may explain why the association between CRP and measures of atherosclerosis is attenuated by increasing body mass index.²¹² There is extensive evidence pointing to a role of CRP in the progression of atherosclerosis. CRP has the ability to bind to oxidised low-density lipoprotein (LDL), but not to unmodified LDL, and promotes the accumulation of oxidised LDL in macrophages.²¹³ CRP and oxidised LDL complexes are present in atherosclerotic lesions of diabetes mellitus patients.²¹³ In endothelial cell culture, CRP reduced nitric oxide bioavailability,²¹⁴ increased PAI-1²¹⁵ and increased the expression of adhesion molecules.²¹⁶

Oxidative stress

A reduction in nitric oxide bioavailability is characteristic of impaired endothelium-dependent vasodilation and may be attributed to elevated reactive oxygen species production (ROS).²¹⁷ Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase and nitric oxide synthase act as catalysts for ROS production. ROS such as superoxide anion interacts with nitric oxide and thereby reduces its availability.²¹⁷ Oxidative stress occurs when there is an increased production of ROS and reduced antioxidant capacity in the vasculature.²¹⁸ Oxidative stress is associated with endothelial dysfunction²¹⁹ and an increase in oxidative stress is seen in spontaneously hypertensive rats before the development of hypertension.²²⁰ However, it is also suggested that the presence of high blood pressure may further promote the generation of ROS.²¹⁹ In addition to endothelial dysfunction, other mechanisms linking ROS and hypertension include inflammation, cell growth and migration as well as vascular remodelling.²²¹ In 169 supposedly healthy individuals cysteine, a marker of oxidative stress, was independently associated with arterial stiffness as assessed by pulse wave velocity.²²²

Physiological as well as pathophysiological leptin concentrations increased ROS production in cultured endothelial cells.²²³ Leptin increases ROS production through pathways which may include NADPH oxidase expression and leptin stimulated endothelin-1 production.^{224,225} *In vitro* studies show that leptin upregulates endothelin-1 production in human endothelial cells²²⁶ as well as in rat cardiomyocytes.²²⁷ Further, it has been demonstrated that leptin induces cardiac hypertrophy through endothelin-1 mediated ROS production.²²⁷ Leptin treatment to male Wistar rats reduced paraoxonase 1 activity and increased oxidative stress.²²⁸ Paraoxonase 1 prevents oxidative stress by interacting with high-density lipoprotein (HDL) and promoting the antioxidant effects of HDL.²²⁹ In addition, paraoxonase 1 also prevents the oxidation of both LDL and HDL.^{230,231} Oxidised LDL contributes to endothelial dysfunction by inhibiting the production of nitric oxide and the subendothelial accumulation thereof plays an important role in the development of atherosclerosis.²³²

5.4 Atherosclerosis

Endothelial dysfunction is one of the initiating events leading up to the development of atherosclerosis.²³² Atherosclerosis is characterised by a chronic inflammatory state and the build-up of lipids in macrophages, which are commonly referred to as foam cells.²³² LDL diffuses passively from the blood into the subendothelial matrix and is taken up by macrophages once oxidised.²³² Oxidised LDL promotes the expression of adhesion molecules²³³ and monocyte chemoattractant protein-1 which facilitate monocyte adhesion as well as the subendothelial migration thereof.²³⁴ Besides oxidised LDL, factors such as hypertension,²³⁵ smoking,²³⁶ and inflammation²³⁷ also activate the dysfunctional endothelium to express adhesion molecules. Once the monocytes enter the intima they differentiate into macrophages which are able to engulf oxidized LDL and subsequently become foam cells.²³⁸ Very low-density lipoprotein also plays a proatherogenic role by either activating inflammation or by undergoing oxidative modification. Contrastingly, HDL plays a defensive role against atherosclerosis by opposing the inflammatory effects of oxidised lipids.²³⁹

Another key feature of atherosclerosis is the migration of vascular smooth muscle cells from the media to the intima and the proliferation thereof in the intima.²⁴⁰ Various cytokines and growth factors secreted by inflammatory cells within the intima stimulate vascular smooth muscle cell migration and proliferation.^{232,239} Furthermore, a role of protein-degrading matrix metalloproteinases (MMPs) in plaque formation and rupture has also been established.²⁴¹ Foam cells aggregate to form lesions known as fatty streaks which later on develop to become advanced lesions enclosed by a fibrous cap.²³² These advanced lesions are composed of foam cells that undergo apoptosis as well as smooth muscle cells.²³² The smooth muscle cells produce collagen to form the fibrous cap which can be degraded by MMPs in unstable plaques. A rapid increase in blood pressure may also contribute to plaque rupture by exerting stress on the weakened plaque.²⁴² Plaque rupture may lead to the formation of an arterial thrombosis and a potentially fatal stroke.²⁴³

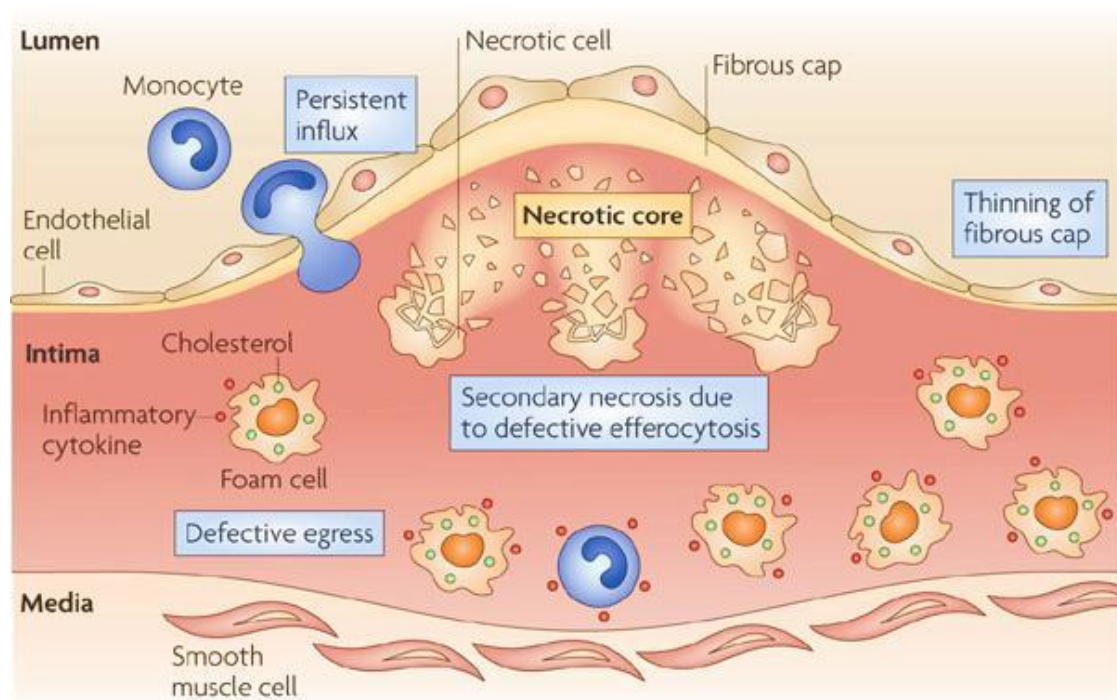


Figure 7: Plaque formation and disruption. Figure taken from Tabas.²⁴⁴

A role of leptin in the development of atherosclerosis has also been established. Firstly, leptin may drive the development of atherosclerosis by mechanisms related to endothelial dysfunction such as impaired nitric oxide-induced vasodilation, oxidative stress and inflammation.²²⁴

Paraoxonase 1 activity is reduced in tissues of hyperleptinemic rats and believed that it is dependent on the presence of oxidative stress.²⁴⁵ In obese humans, paraoxonase 1 activity is reduced compared to normal-weight individuals and inversely related to plasma leptin levels.²⁴⁶ Leptin also stimulates the migration and proliferation of human aortic vascular smooth muscle cells.²²⁴ Leptin stimulates the expression of MMP-2 and may thereby promote vascular smooth muscle cell migration and plaque rupture.²⁴⁷ Another potential mechanism by which leptin may induce vascular smooth muscle cell proliferation is by promoting endothelin-1 and transforming growth factor- β secretion from endothelial cells.^{226,248} Not only is endothelin-1 a powerful vasoconstrictor but it also stimulates smooth muscle cell proliferation.²²⁴

In line with the above leptin predicted acute cardiovascular events in a 5-year follow-up study which included over a 1000 men with hypercholesterolemia.²⁴⁹ In the Jackson Heart Study, leptin predicted stroke in African American women but not in men.²⁵⁰ Furthermore, in older European men leptin also predicted stroke, however measures of adiposity namely waist circumference and body mass index was not predictive.²⁵¹ Literature regarding the relationship between leptin and carotid intima-media thickness is controversial. In a study including 120 men and women, leptin was independently associated with carotid intima-media thickness. However, the significance was lost after adjusting for body mass index.²⁵² However, leptin was independently associated with intima-media thickness in a study involving patients with diabetes after adjusting for body mass index in multiple regression analysis.²⁵³ We demonstrated a similar result in a study which included supposedly healthy African and Caucasian men and women.²⁵⁴ On the contrary, a study by Bevan *et al.* showed that adiponectin, but not leptin, was associated with intima media thickness.²⁵⁵

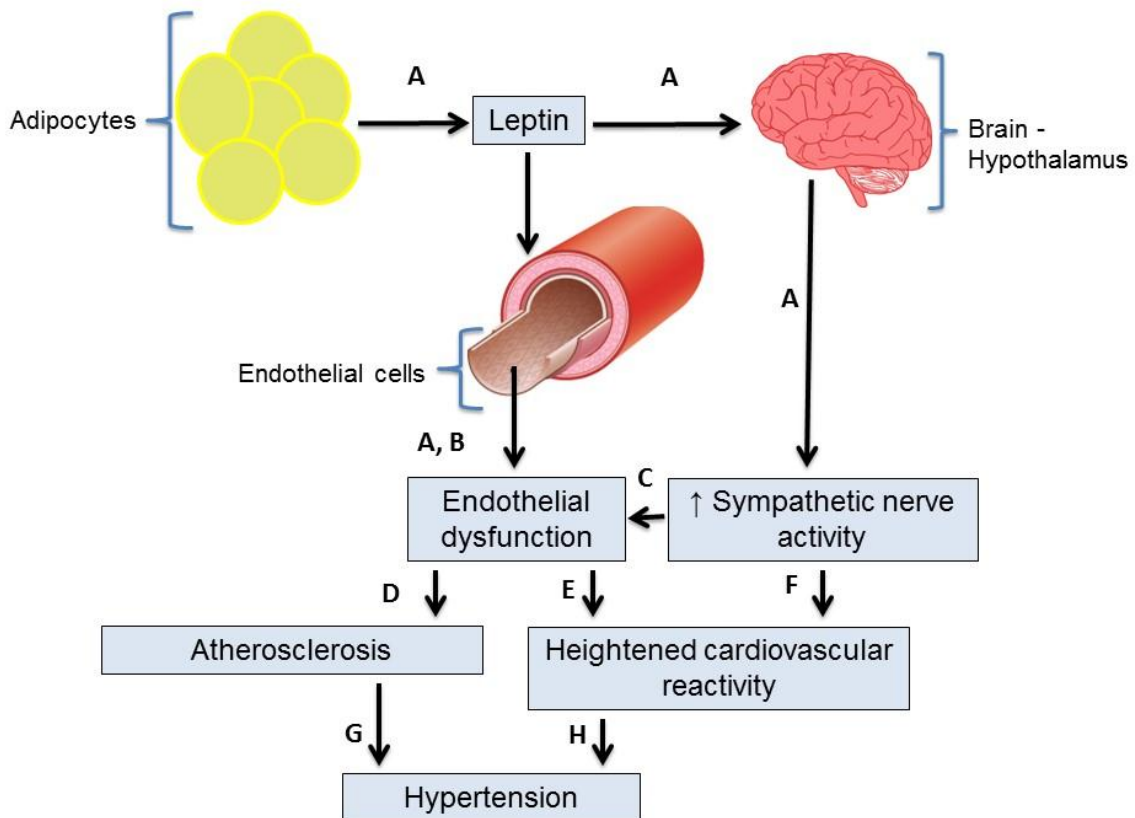


Figure 8: A summary of potential mechanisms linking leptin and the development of hypertension. (A,²⁸ B,²⁹ C,¹⁷⁰ D,²³² E,²⁵⁶ F,¹²⁵ G,⁹⁹ H,²⁵⁷)

6. CARDIOVASCULAR DISEASE IN AFRICANS

Cardiovascular and metabolic disease is increasing rapidly in sub-Saharan Africa.^{258,259} Early detection and prevention of hypertension remains a burning health issue in developing countries due to the human and economic cost involved.²⁶⁰ The global epidemic of hypertension has not left sub-Saharan Africa unaffected and lifestyle changes associated with urbanisation may largely be responsible for this.^{3,261,262} It is well documented that the prevalence of hypertension is higher in urban than rural populations.^{261,263,264} A steep rise in urbanisation is taking place in low-income countries such as Africa and Asia due to the greater economic growth, employment opportunities and availability of basic services in urban areas.²⁶⁵ Living in an urban environment may equip individuals with greater health benefits and facilities, but there are also several unfavourable health consequences related to urbanisation. Some of the unfavourable factors include a higher salt and fat intake, reduced physical activity and higher obesity prevalence

rates.^{260,261} Traditionally a rural diet consists of low-fat and high fibre foods whereas a typical urban diet is high in saturated meat- or dairy fat and sugary processed food.^{266,267} Low and middle income countries which were previously burdened by malnutrition and hunger are now facing increasing obesity prevalence rates.²⁶⁶ It is projected that 58% of the world adult population will be overweight or obese by the year 2030 if current trends continue.²⁶⁸ Furthermore, urbanisation is often accompanied by an increase in psychological stress which contributes to hypertension development.²⁶⁰

Hypertension, an established risk factor for the development of cardiovascular disease, is a serious health problem in South Africa and particularly in the African population.²⁶⁹ Ischaemic heart disease rarely occurs in Africans, whereas hypertension and related heart disease and stroke are highly prevalent amongst the African population.²⁶⁴ Results from a multi-ethnic study revealed that the prevalence of hypertension was higher in African-Americans (60%) compared to Caucasians (38%), Hispanics (42%) and Chinese (39%).²⁷⁰ Sub-Saharan Africa surveys also showed that Africans, especially urban Africans exhibited the highest prevalence rates.²⁶⁴ A possible reason for this includes the known higher salt-sensitivity in Africans.²⁶⁰ Furthermore, the sympathetic nervous system's response to a cardiovascular stressor was greater in African-American men compared to Caucasians.^{149,151} The reason for this may be due to increased α -adrenergic vasoconstrictor sensitivity¹⁵¹ and diminished β -adrenergic-mediated vasodilation in Africans.²⁷¹ In a study comparing normotensive African and Caucasian men and women, the sympathetic discharge rates were 20-40% higher in the African men compared to the African women and Caucasians.²⁷² It was also suggested that sympathetic overactivity in African women was dependent on adiposity.²⁷² In contrast, adiposity was not related to sympathetic overactivity in African men.²⁷² Urbanisation is also associated with increased total peripheral resistance in the African population.^{273,274} Collectively these studies demonstrate that Africans are a high-risk group for the development of cardiovascular morbidity and mortality.²⁶⁴

7. RECENT ADVANCES IN LEPTIN TREATMENT

The discovery of leptin sparked much curiosity surrounding its use as an anti-obesity drug. However, the potential of leptin as an anti-obesity hormone was soon abolished after the realisation that individuals with common obesity are hyperleptinemic and develop leptin resistance.²⁷⁵ Even though leptin therapy alone may not prove helpful to induce weight loss, leptin co-administered with amylin to restore leptin sensitivity shows promise in obese rodents and humans.²⁷⁶ The United States Food and Drug Administration has recently approved the use of Myalept® (recombinant methionyl human leptin) for the treatment of congenital or acquired lipodystrophy in patients with leptin deficiency.²⁷⁵ Larger randomised and placebo-controlled studies are needed to determine the longstanding safety and efficacy of leptin replacement therapy.²⁷⁷ The use of Myalept® has not yet been approved for the treatment of patients with HIV-related lipodystrophy.²⁷⁵ The metabolic effects of leptin replacement therapy include an increase in HDL and a reduction in triglyceride as well as glucose levels.²⁷⁵ In addition to its metabolic actions, leptin replacement therapy also leads to changes in cardiovascular disease biomarkers of inflammation, coagulation and fibrinolysis. The precise changes in cardiovascular disease biomarkers after leptin replacement needs further investigation, but it is suggested that leptin deficiency in the obese may protect against cardiovascular disease.²⁷⁵ Despite major scientific advances in leptin research, there still remains a number of unanswered questions. Once the central and peripheral actions of leptin are fully understood, leptin-based treatment options for HIV-related lipodystrophy, hypothalamic amenorrhea, non-alcoholic fatty liver disease, depression and dementia may be of potential therapeutic interest.²⁷⁵

8. MOTIVATION, AIMS AND HYPOTHESES

This section includes a brief motivation for the individual research articles which form part of this thesis as well as the detailed aims and hypotheses of each article.

The central aim of this study is to increase our understanding of the role of leptin in cardiovascular disease development through investigating associations of leptin with markers

reflecting sympathetic nervous system overactivity and endothelial dysfunction in Africans and Caucasians.

Research article 1

Motivation

Increased sympathetic nervous system activation to the heart and vasculature plays a fundamental role in the development of essential hypertension and heart failure.²⁷⁸ Autonomic imbalance is characterised by a hyperactive sympathetic nervous system and a hypoactive parasympathetic nervous system.¹³⁰ Reduced heart rate variability, which is indicative of sympathetic overactivity, is a predictor of mortality in post-myocardial infarction patients.²⁷⁹ Leptin increases sympathetic nerve activity²⁸ and is associated with hypertension development.¹⁰⁸ Hyperleptinemia may therefore contribute to the development of hypertension by inducing sympathetic hyperactivity.²⁷⁹ Sympathetic nervous system activity also seems greater in Africans compared to Caucasians.¹⁴⁸

Aims

Within this cross-sectional study including African and Caucasian schoolteachers, the aims were:

- To investigate ethnic differences in leptin levels and autonomic activity; and
- To determine associations of leptin with markers related to autonomic activity

Hypotheses

- Africans will have higher leptin levels and sympathetic overactivity compared to Caucasians; and
- Leptin associates with markers reflecting autonomic dysfunction in Africans.

Research article 2

Motivation

Endothelial dysfunction is one of the initial steps in the development of atherosclerosis²³² and is predictive of future cardiovascular events.²⁸⁰ *In vitro*^{29,281} and *in vivo*¹⁷⁹ studies demonstrate a potential role of hyperleptinemia in inducing endothelial dysfunction. An experimental study on mice suggests that leptin causes endothelial dysfunction through mechanisms related to sympathetic nervous system activation.¹⁷⁰ Endothelial dysfunction is not limited to impaired vasodilation, but also characterised by impaired coagulation and fibrinolysis as well as damage as reflected by albuminuria.^{161,282} However, the precise mechanisms whereby hyperleptinemia may induce endothelial dysfunction remain unknown.

Aim

In this bi-ethnic sample of lean and obese schoolteachers the aim was:

- To investigate associations between leptin and circulating markers of endothelial dysfunction namely PAI-1, vWF and ACR.

Hypothesis

- Leptin is positively associated with PAI-1, vWF and ACR in both Africans and Caucasians.

Research article 3

Motivation

Increased cardiovascular reactivity to stress and impaired post-stress recovery are associated with an increased risk of cardiovascular disease development.^{149,156,157,283} Stress exposure activates the sympathetic nervous system and hypothalamo-pituitary-adrenal axis.¹²⁵ In addition to stress, leptin also activates the sympathetic nervous system via receptors situated in the hypothalamus.²⁸ Leptin may therefore contribute to a state of sympathetic hyperactivity which may result in increased cardiovascular reactivity or delayed recovery to stress. The effects of

leptin on cardiovascular reactivity may therefore be more apparent in Africans who have higher leptin than Caucasians.^{16,54,56} Additionally, cardiovascular reactivity to stress is greater in Africans compared to Caucasians.^{149,151,273}

Aim

In this study including African and Caucasian schoolteachers the aim was:

- To investigate the relationship between leptin and cardiovascular reactivity and recovery to an acute stressor in Africans and Caucasians.

Hypothesis

- Higher leptin in Africans is associated with heightened cardiovascular reactivity and prolonged recovery to an acute stressor.

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CHAPTER 2

Autonomic activity and leptin in Africans and Caucasians: The SABPA study.

INSTRUCTIONS FOR AUTHORS ON THE PREPARATION AND SUBMISSION OF MANUSCRIPTS TO *JOURNAL OF HYPERTENSION*.

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Acknowledgements

Acknowledgements should be made only to those who have made a substantial contribution to the study.

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Autonomic activity and leptin in Africans and Caucasians: The SABPA study.

Short Title: Leptin and autonomic activity

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Abstract

Objectives: Evidence exists that leptin enhances sympathetic activity and may thereby contribute to the development of obesity-related hypertension. Sympathetic activation also seems more prominent in Africans than Caucasians. We compared leptin levels, and different markers of autonomic activity between Africans and Caucasians, and determined whether a relationship exists between leptin and autonomic activity.

Methods: The study included 409 African and Caucasian school teachers (aged, 44.6 ± 9.6 years). We determined leptin in serum and measured ambulatory blood pressure. Markers reflecting autonomic activity included renin, cortisol, baroreflex sensitivity, ambulatory heart rate and heart rate variability (HRV) components (assessed by 24 h ECG recordings in the frequency and geometric domain).

Results: Africans had higher leptin levels, body mass index (BMI), blood pressure and heart rate (all $P < 0.001$) as well as lower HRV triangular index and $-HRV$ total power ($P < 0.001$). After also adjusting for BMI in multivariate regression analyses, in African men, renin ($\beta = 0.228$; $P = 0.033$), nighttime heart rate ($\beta = 0.184$; $P = 0.034$), HRV triangular index ($\beta = -0.230$; $P = 0.010$) and HRV total power ($\beta = -0.221$; $P = 0.015$) associated with leptin. In Caucasian men, leptin associated with 24 h heart rate ($\beta = 0.376$; $P < 0.001$), as well as day and nighttime heart rate (both $P < 0.01$), HRV triangular index ($\beta = -0.335$; $P < 0.001$) and HRV total power ($\beta = -0.403$; $P < 0.001$). In African women, we observed an association of leptin with the total power component of HRV ($\beta = -0.214$; $P = 0.046$) and a borderline association with renin ($\beta = 0.219$; $P = 0.057$). No significant associations were apparent in the Caucasian women.

Conclusions: We found that leptin is independently associated with different markers of autonomic activity, especially in men.

Keywords: Autonomic nervous system, sympathetic activity, hypertension, ethnicity and leptin.

Introduction

Hypertension and obesity prevalence rates are increasing in sub-Saharan Africa largely due to Westernisation [1,2]. Changes in lifestyle such as over-nutrition and reduced physical activity results from this transition [3]. Obesity leads to diabetes [4] and hypertension, and therefore increased cardiovascular risk [5]. Experimental studies indicate that leptin, produced in adipose tissue, is one of the links between obesity and hypertension and is believed to do this through sympathetic activation, which is a common characteristic of obesity [6].

Leptin binds to the long form receptors (OB-Rb) located in the hypothalamus to reduce appetite and increase sympathetic nerve activity [7,8]. Animal studies demonstrate that leptin increases sympathetic nerve activity in a dose-dependent manner [9], which may lead to autonomic imbalance. Population studies indicate that autonomic imbalance characterised by elevated sympathetic nervous system activity is associated with cardiovascular morbidity and mortality [10]. Furthermore, such activation initiates and maintains hypertension with multiple mechanisms which take place in the vasculature or kidney [11,12]. We evaluated various markers of autonomic activity, each with their own strengths and weaknesses with regard to invasiveness, sensitivity and reproducibility [13].

Both the prevalence of hypertension [1] and circulating leptin levels are higher in Africans than Caucasians [14]. We therefore investigated ethnic differences in leptin levels and autonomic activity, and determined whether leptin is related to measures of autonomic activity as reflected by renin, cortisol, baroreflex sensitivity and measures of heart rate variability.

Methods

Study population

This study forms part of the SABPA (Sympathetic activity and Ambulatory Blood Pressure in Africans) study, which included 409 African and Caucasian school teachers working in the Potchefstroom district in the North West Province of South Africa. The reason for the selection of this target population was to obtain a homogenous sample of participants from a similar socioeconomic class. Participants between the ages of 25 and 60 years were included. The exclusion criteria were an ear temperature above 37°C, psychotropic substance dependence or abuse, regular blood donors and individuals vaccinated in the past three months. Participants received detailed information about the procedures and objectives of the study prior to their recruitment. Participants requesting conveyance of information in their home language were assisted. All participants signed an informed consent form. The study complied with all applicable requirements and international regulations, including the Helsinki declaration of 1975 (as revised in 2008) for investigation of human participants. The study was approved by the Ethics Review Board of the North-West University (Potchefstroom Campus).

Cardiovascular measurements

Ambulatory blood pressure measurements (ABPM) were conducted during the working week. At approximately 08h00, an ABPM apparatus (Meditech CE120® Cardiotens; Meditech, Budapest, Hungary) and two-lead electrocardiogram (ECG) apparatus were attached on the participant's non-dominant arm at their workplace. The ABPM apparatus was programmed to measure blood pressure at 30 minute intervals during the day (08h00-22h00) and every hour during night-time. Participants received ambulatory diary cards and were requested to indicate abnormalities such as nausea, headache or stress experienced during their normal daily activities. At 16h30 participants were transported to the North-West University and admitted to the Metabolic Unit Research Facility. This facility consists of 10 bedrooms, two bathrooms, a living room and a kitchen. Participants received a standardised dinner and at 20h30 they received their last beverage (coffee/tea and two biscuits). Thereafter they relaxed by reading, watching television or social interaction and were encouraged to go to bed at 22h00.

Participants were requested to refrain from alcohol consumption, caffeine consumption and exercise. At 06h00 the ABPM apparatus was removed and subsequent measurements commenced.

Electrocardiogram and 24 h blood pressure data were downloaded onto a database using the CardioVisions 1.15.2 Personal Edition (Meditech, Budapest, Hungary). If less than 70% of the ABPM recordings for a particular participant were successful, the measurement was repeated the next day. The CardioVisions software automatically calculated the participants' heart rate variability. Heart rate variability (HRV) was assessed by using frequency domain analysis performed with fast Fourier transformation and by using the heart rate variability (HRV) triangular index, a geometric measure. By using HRV triangular index the effects of noise, artefact, missed beats and ectopic complexes are minimised [15,16]. The ambulatory software program (Cardiovisions 1.15.2) removed all ectopic beats. The frequency domain components allows for quantification of cardiovascular regulation by assessing spontaneous oscillations in heart rate. Two main spectral bands are usually considered: high-frequency oscillations (spectral band between 0.15 and 0.4 Hz) of heart rate relate to respiratory sinus arrhythmia and, therefore, to parasympathetic cardiovagal tone [17]. Low-frequency fluctuations (spectral band between 0.04 and 0.15 Hz) of heart rate are thought to reflect baroreflex-mediated adjustments to the sinus node encompassing sympathetic and parasympathetic fibres [17]. The low-frequency/high-frequency ratio (LF/HF) of heart rate variability allows for quantification of the relation between the two branches of the autonomic nervous system. The total power is a global measure of autonomic nervous system activity and represents the variance of the HRV over the measured time period [18].

The validated [19,20] Finometer device (Finapres Medical Systems, Amsterdam, the Netherlands) was connected, and a 7-min continuous measurement of resting cardiovascular parameters was carried out [21]. Spontaneous baroreflex sensitivity (BRS) was determined by the validated cross-correlation baroreflex sensitivity (xBRS) method [22], derived from the continuous BP measurement. xBRS computes the correlation between beat-to-beat SBP and R-

R interval, resampled at 1 Hz, over 10-sec sliding windows—a timespan sufficient to accommodate fully a 10-sec variability in rhythm, or several cycles at ventilatory frequencies [22]. It has been suggested that this method be used in clinical and experimental settings because of its lower within-patient variance than other BRS methods. The Actical® activity monitor (Mini Mitter Co., Inc., Bend, OR; Montreal, Quebec, Canada) was used to estimate physical activity. The monitor was fitted after all cardiovascular measurements were taken and worn around the waist by participants for 24 h. The physical activity index of participants was categorized according to high, moderate and low physical activity.

Anthropometric measurements

Height (stature) and weight of participants were measured while being in their underwear using calibrated instruments (Precision Health Scale, A & D Company, Tokyo, Japan; Invicta Stadiometer, IP 1465; Invicta, London, UK). Subsequently, the body mass index (BMI) was calculated for each participant. All measurements were taken in triplicate using standard methods [23].

Biochemical measurements

After the cardiovascular and anthropometric measurements were done, a registered nurse obtained a fasting blood sample with a sterile winged infusion set from the antebrachial vein branches. EDTA whole blood and serum were stored at $-80\text{ }^{\circ}\text{C}$. In serum, fasting samples for total cholesterol were analysed using the sequential multiple analyser computer (Konelab 20i TM, Thermo Scientific, Vantaa, Finland). Glucose was determined using a timed end point method and gamma-glutamyl transferase levels on an enzyme rate method [24] (Unicel DxC 800; Beckman and Coulter, Krefeld, Germany). Thyroid stimulating hormone was measured by an electrochemiluminescence immunoassay (ECLIA) and cortisol by using the Elecsys 2010 apparatus (Roche Diagnostics, Basel, Switzerland). We determined leptin levels using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine, R&D Systems, MN, USA). In plasma, the concentration of active renin was analysed by making use of a high-sensitivity radio-immunometric assay and cross-reaction with pro-renin below 0.4% (Renin III Generation,

CIS biointernational, Cedex, France). The source of reagents was mouse anti-human-active renin monoclonal antibody 125I in a Tris buffer (pH 7.9) containing horse serum and 0.1% sodium azide. The intra- and inter-assay coefficients of variation were 1.81 and 4.05%, respectively.

Statistical analysis

For database management and statistical analyses, we used Statistica software version 11.0 (Statsoft, Inc., Tulsa, OK, 2010). The distribution of leptin, glucose, thyroid stimulating hormone, gamma-glutamyl transferase and baroreflex sensitivity were normalised by logarithmic transformation. The central tendency and spread of these variables were represented by the geometric mean and the 5th and 95th percentile intervals. Independent t-tests were done to compare means between groups and the Chi-square test (χ^2) to compare proportions. We also tested interactions with ethnicity and gender for the association between leptin and autonomic activity by introducing appropriate interaction terms. Pearson's correlations were determined to investigate associations between markers of autonomic activity and leptin as well as partial correlations after adjustment for age. Sensitivity and exploratory analyses were carried out to confirm a possible link of leptin and autonomic activity with 24 h systolic blood pressure. Forward stepwise multiple regression analyses were performed to investigate independent associations between measures of autonomic activity and leptin, while adjusting for age, body mass index, 24 h mean arterial pressure, total cholesterol, glucose, smoking, gamma-glutamyl transferase, thyroid stimulating hormone, physical activity level and anti-hypertensive medication.

Results

Characteristics of study population

Table 1 lists the characteristics of the African (n=200) and Caucasian (n=209) participants. Africans had a higher body mass index (P<0.001) and leptin levels (P<0.001) but were of similar age (P=0.49). Africans with higher blood pressure (P<0.001), also had higher ambulatory heart rate (P<0.001) and lower baroreflex sensitivity (P=0.052). The African group displayed an unfavourable profile for all HRV measurements, with lower triangular index and total power (P<0.001). Renin data was not available for Caucasians. The number of normotensive and hypertensive participants stratified by gender and ethnicity is demonstrated in Supplementary Table S1 and Table S2.

Table 1 Characteristics of study population.

	Africans (n=200)	Caucasians	P
Age, years	44.3 ± 8.0	44.9 ± 10.9	0.49
Women, n (%)	99 (49.5)	108 (51.7)	0.66
Stature, cm	165 ± 9	174 ± 10	<0.001
Body mass, kg	81.4 ± 18.4	84.0 ± 21.1	0.21
Waist circumference, cm	93.6 ± 15.5	93.0 ± 16.1	0.71
Body mass index, kg/m ²	30.1 ± 7.0	27.6 ± 5.9	< 0.001
Biochemical measurements			
Leptin, ng/ml	23.6 (3.1 - 95.7)	12.8 (2.6 - 54.6)	< 0.001
Total cholesterol, mmol/l	4.60 ± 1.19	5.54 ± 1.28	< 0.001
Glucose, mmol/l	5.41 (4.04 - 10.43)	5.61 (4.70 - 6.90)	0.055
Renin, pg/ml	4.21 ± 2.61	-	
Cortisol, nmol/l	347.4 ± 139.0	379.4 ± 153.3	0.029
Thyroid stimulating hormone, uIU/ml	1.65 (0.65 - 4.20)	2.12 (0.85 - 5.33)	< 0.01
Cardiovascular measurements			
24 h Systolic blood pressure, mmHg	133 ± 16	124 ± 12	< 0.001
24 h Diastolic blood pressure, mmHg	84 ± 11	77 ± 8	< 0.001
24 h Mean arterial pressure, mmHg	103 ± 12	96 ± 9	< 0.001
24 h Heart rate, bpm	80 ± 11	74 ± 10	< 0.001
Daytime heart rate, bpm	84 ± 12	79 ± 11	< 0.001
Nighttime heart rate, bpm	72 ± 13	65 ± 10	< 0.001
BRS, ms/mmHg	7.94 (2.79 - 20.63)	8.93 (3.67 - 23.72)	0.052
24 h HRV triangular index	30.4 ± 11.3	37.8 ± 11.2	< 0.001
24 h HRV LF, n.u.	65.6 ± 12.7	73.0 ± 11.0	< 0.001
24 h HRV HF, n.u.	30.8 ± 11.0	24.8 ± 10.2	< 0.001
24 h HRV LF/HF	2.6 ± 1.7	3.7 ± 2.2	< 0.001
24 h HRV Total power	2741 ± 2169	3989 ± 2755	< 0.001
Lifestyle			
High level of physical activity, n (%)	11 (5.5)	26 (12.5)	< 0.001
Smoking, n (%)	34 (17.0)	29 (14.0)	0.39
Gamma glutamyltransferase, U/L	47.4 (20.1 - 183.8)	19.3 (7.0 - 76.0)	< 0.001
Antihypertensive medication, n (%)	43 (21.5)	18 (8.6)	< 0.001

Values are arithmetic mean ± SD, geometric mean (5 - 95th percentile interval), or number of participants
Abbreviations: BRS, baroreflex sensitivity; HRV, heart rate variability; LF, low frequency; HF, high
P denotes difference between Africans and Caucasians

Unadjusted analyses

In single regression analyses (Supplementary Table S3), we investigated associations between markers of autonomic activity and leptin in separate ethnic and gender groups. Leptin associated with several measures of autonomic activity in all groups, except for African women.

Adjusted analyses

After adjusting for age (Table 2), we again found significant results in all the groups except for African women. In African men, nighttime heart rate ($P=0.007$) correlated positively, and HRV triangular index ($P=0.002$) and HRV total power ($P=0.003$) negatively with leptin. In Caucasian men and women, we found associations with 24 h heart rate, daytime heart rate, nighttime heart rate and HRV triangular index (all $P<0.01$). Additionally, HRV total power correlated negatively with leptin in the Caucasian men ($P<0.001$).

Table 2 Partial Correlation Coefficients between markers of autonomic activity and leptin, adjusted for age.

	African men n=101	African women n=99	Caucasian men n=101	Caucasian women n=108
Plasma renin, pg/ml	$r = 0.22; P = 0.041$	$r = 0.20; P = 0.091$	-	-
Serum cortisol, nmol/l	$r = -0.10; P = 0.31$	$r = -0.02; P = 0.82$	$r = -0.04; P = 0.68$	$r = -0.16; P = 0.097$
24 h Heart rate, bpm	$r = 0.19; P = 0.058$	$r = 0.03; P = 0.79$	$r = 0.43; P < 0.001$	$r = 0.36; P < 0.001$
Daytime heart rate, bpm	$r = 0.15; P = 0.13$	$r = -0.02; P = 0.85$	$r = 0.41; P < 0.001$	$r = 0.30; P = 0.002$
Nighttime heart rate, bpm	$r = 0.27; P = 0.007$	$r = 0.14; P = 0.19$	$r = 0.34; P = 0.001$	$r = 0.44; P < 0.001$
BRS, ms/mmHg	$r = -0.06; P = 0.56$	$r = -0.12; P = 0.25$	$r = 0.05; P = 0.66$	$r = 0.08; P = 0.40$
24 h HRV triangular index	$r = -0.31; P = 0.002$	$r = -0.14; P = 0.17$	$r = -0.39; P < 0.001$	$r = -0.36; P < 0.001$
24 h HRV LF, n.u.	$r = 0.17; P = 0.10$	$r = -0.06; P = 0.59$	$r = -0.06; P = 0.57$	$r = -0.01; P = 0.89$
24 h HRV HF, n.u.	$r = -0.14; P = 0.16$	$r = -0.02; P = 0.87$	$r = 0.09; P = 0.38$	$r = 0.01; P = 0.92$
24 h HRV LF/HF	$r = 0.19; P = 0.06$	$r = 0.05; P = 0.65$	$r = -0.02; P = 0.88$	$r = 0.03; P = 0.74$
24 h HRV Total power	$r = -0.30; P = 0.003$	$r = -0.11; P = 0.27$	$r = -0.47; P < 0.001$	$r = -0.04; P = 0.68$

Abbreviations: BRS, baroreflex sensitivity; HRV, heart rate variability; LF, low frequency; HF, high frequency.

The independent associations between markers of autonomic activity and leptin in Africans and Caucasians are presented in Table 3. After adjusting for confounders we observed associations of renin ($\beta=0.228; P=0.033$), nighttime heart rate ($\beta=0.184; P=0.034$), HRV triangular index ($\beta=-0.230; P=0.010$), between HRV total power ($\beta= -0.221; P=0.015$) and leptin in African men. Furthermore, we found borderline significant associations with cortisol ($\beta=0.289; P=0.086$) and HRV LF ($\beta=0.186; P=0.070$). In African women, only a relationship between HRV total power

and leptin was evident ($\beta = -0.214$; $P = 0.046$) and a borderline significant association with renin ($\beta = 0.219$; $P = 0.057$). In Caucasian men, positive correlations of 24 h heart rate ($P < 0.001$), daytime heart rate ($P < 0.001$), nighttime heart rate ($P = 0.0051$) with leptin were observed, while HRV triangular index and total power ($P < 0.001$) correlated negatively with leptin. We found no associations in the Caucasian women. Multiple regression analyses were repeated in the men and women, additionally adjusting for ethnicity. Again, our results remained prominent in the men (Supplementary Table S4).

Exploratory analysis

We tested interactions of autonomic activity markers for the association between 24 h SBP and leptin by introducing appropriate interaction terms. In the total group, there was an interaction between leptin and nighttime heart rate ($P = 0.021$), and HRV LF/HF ($P = 0.082$) with 24 h SBP as dependent variable. Similarly, in the African men, an interaction existed with daytime heart rate ($P = 0.058$).

To confirm that our measures of autonomic activity are indeed related to blood pressure, we performed additional analyses for each ethnic group to determine whether associations exist between the markers of autonomic activity and 24 h SBP (while adjusting for gender) (Figure 1). In Africans, leptin ($P < 0.001$) and 24 h heart rate ($P = 0.011$) correlated positively, while HRV triangular index ($P < 0.001$) and HRV total power ($P = 0.001$) correlated negatively with 24 h SBP. In Caucasians we found similar associations of 24 h SBP with leptin, 24 h heart rate, HRV triangular index and HRV total power ($P < 0.001$). Additionally, daytime heart rate as well as nighttime heart rate correlated positively with 24 h SBP in Caucasians ($P < 0.001$).

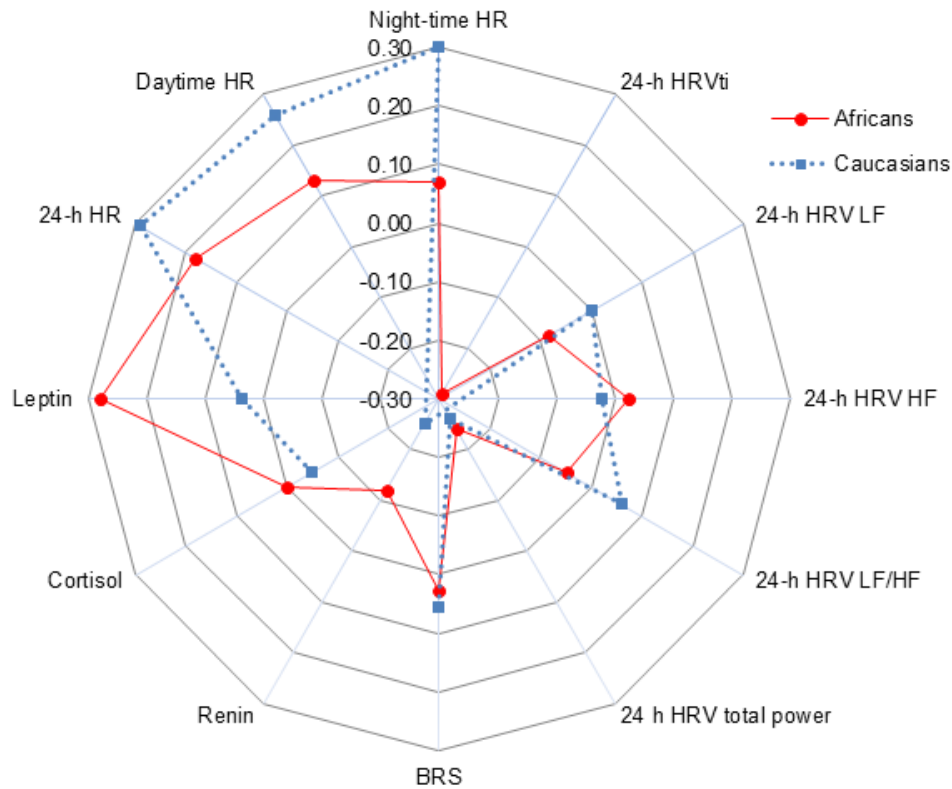


Figure 1 Correlations of 24 h systolic blood pressure with markers of autonomic activity, adjusted for gender. Abbreviations: HR, heart rate; HRVti, heart rate variability triangular index; LF, low frequency; HF, high frequency.

Sensitivity analyses

Sensitivity analyses were carried out due to the lack of associations between measures of autonomic activity and leptin in African women. In Supplementary Table S1 and Table S2 we compare leptin levels as well as measures of autonomic activity between normotensives and hypertensive individuals. In Table S1 we demonstrate that leptin levels and autonomic activity markers do not differ between normotensive and hypertensive African women. In the men (Table S2) and Caucasian women (Table S1), we observed higher leptin levels, nighttime heart rate and lower heart rate variability in the hypertensive groups. Additionally, in Caucasian men and women, daytime heart rate as well as 24 h heart rate was higher in the hypertensive groups.

Table 3 Independent associations between markers of autonomic activity and leptin.

Dependent variables	African men (n=101)				Caucasian men (n=101)			
	R ²	Adjusted R ²	Std β (95 % CI)	P	R ²	Adjusted R ²	Std β (95 % CI)	P
Plasma renin, pg/ml	0.163	0.121	0.228 (0.022 to 0.434)	0.033	-	-	-	
Serum cortisol, nmol/l	0.185	0.142	0.289 (-0.038 to 0.616)	0.086	0.104	0.056	N/S	
24 h Heart rate, bpm	0.249	0.209	N/S		0.308	0.255	0.376 (0.190 to 0.561)	< 0.001
Daytime heart rate, bpm	0.181	0.147	N/S		0.315	0.263	0.364 (0.180 to 0.548)	< 0.001
Nighttime heart rate, bpm	0.356	0.322	0.184 (0.015 to 0.353)	0.034	0.198	0.154	0.277 (0.087 to 0.467)	0.0051
BRS, ms/mmHg	0.036	0.016	N/S		-	-	N/S	
24 h HRV triangular index	0.348	0.314	-0.230 (-0.402 to -0.058)	0.010	0.316	0.271	-0.335 (-0.519 to -0.151)	< 0.001
24 h HRV LF, n.u.	0.111	0.063	0.186 (-0.120 to 0.384)	0.070	0.14	0.094	N/S	
24 h HRV HF, n.u.	0.071	0.041	N/S		0.172	0.146	N/S	
24 h HRV LF/HF	0.120	0.073	N/S		0.149	0.131	N/S	
24 h HRV Total power	0.337	0.309	-0.221 (-0.395 to -0.047)	0.015	0.321	0.284	-0.403 (-0.577 to -0.229)	< 0.001

Dependent variables	African women (n=99)				Caucasian women (n=108)			
	R ²	Adjusted R ²	Std β (95 % CI)	P	R ²	Adjusted R ²	Std β (95 % CI)	P
Plasma renin, pg/ml	0.077	0.051	0.219 (-0.002 to 0.440)	0.057	-	-	-	
Serum cortisol, nmol/l	0.186	0.137	N/S		0.085	0.049	N/S	
24 h Heart rate, bpm	0.231	0.185	N/S		0.293	0.265	N/S	
Daytime heart rate, bpm	0.192	0.144	N/S		0.259	0.229	N/S	
Nighttime heart rate, bpm	0.039	0.011	N/S		0.324	0.282	N/S	
BRS, ms/mmHg	0.116	0.077	N/S		-	-	N/S	
24 h HRV triangular index	0.187	0.126	N/S		0.243	0.221	N/S	
24 h HRV LF, n.u.	0.142	0.104	N/S		0.156	0.122	N/S	
24 h HRV HF, n.u.	0.107	0.081	N/S		0.120	0.168	N/S	
24 h HRV LF/HF	0.242	0.209	N/S		0.182	0.158	N/S	
24 h HRV Total power	0.191	0.124	-0.214 (-0.422 to -0.006)	0.046	0.236	0.198	0.237 (-0.011 to 0.485)	0.064

Standardised β (std β) reflects the change in the dependent variable for 1 SD change in the independent variable. A larger std β reflects greater strength of the association. Adjusted for age, body mass index, 24 h mean arterial pressure, total cholesterol, glucose, smoking, gamma-glutamyltransferase,

thyroid stimulating hormone, physical activity level and anti-hypertensive medication.

Abbreviations: BRS, baroreflex sensitivity; HRV, heart rate variability; LF, low frequency; HF, high frequency.

Discussion

We found that leptin levels were associated with indicators of autonomic imbalance indicating increased sympathetic activity. This was reflected by associations with ambulatory heart rate, heart rate variability (HRV) measures and renin in African and Caucasian men, of whom 79% and 69% were classified as being hypertensive, respectively. These findings were independent of several covariates, including body mass index, suggesting that higher leptin levels may contribute to the development of hypertension by inducing autonomic dysfunction, especially in men.

Experimental studies in obese humans and animals demonstrate increases in blood pressure, heart rate, sympathetic nervous system activity as well as stimulation of the renin-angiotensin-aldosterone system [7]. Leptin infusion in control Sprague-Dawley rats resulted in increased mean arterial pressure and heart rate. On the other hand, leptin administration in rats with α - and β -adrenergic receptor blockade led to decreased heart rate and no change in mean arterial pressure, emphasizing the role of the sympathetic nervous system in mediating leptin's effects [25]. Moreover, bulimia nervosa patients with low leptin levels exhibit sympathetic insufficiency as well as sympathovagal imbalance [26]. These studies suggest that a fine interplay exists between leptin and autonomic activity and under abnormal conditions may play an important role in autonomic dysregulation and possibly contribute to hypertension development. In a sample of 5599 men and women from the National Health and Nutrition Examination Survey study [27], leptin was positively associated with hypertension, independent of established cardiovascular risk factors. Although the authors speculated on the possible involvement of the sympathetic nervous and renin-angiotensin system, many of the mechanisms linking leptin to autonomic imbalance and hypertension remain unknown.

Autonomic imbalance as reflected by depressed HRV is associated with vascular and renal target organ damage [28] as well as with mortality in myocardial infarction patients [29]. Furthermore, reduced HRV total power is a measure that has been successfully used to discriminate between normal subjects and those with congestive heart failure [30]. We found

that the total power component of HRV correlated negatively with leptin in the Africans and Caucasian men. A reduction in total power is associated with tachycardia due to sympathetic activation [31], which is supported by associations between heart rate and leptin in the men from our study.

To the best of our knowledge, this is the first study to demonstrate that the triangular index of heart rate variability correlates negatively with leptin in men, independent of ethnicity. A reduction in the triangular index is indicative of sympathetic over-activity. This was demonstrated previously in a group of obese children [32], but it was uncertain whether leptin was the link. In another study in 120 non-obese normotensive men, leptin correlated positively with the low frequency component and low frequency/high frequency ratio component of heart rate variability, which reflects increased sympathetic activation [33]. Our results partially support this with the borderline association found between the low frequency component and leptin after adjusting for covariates.

Another finding of our study is the independent positive relation between active renin and leptin in African men. Previous studies among overweight normotensive and –hypertensive men and women demonstrated similar findings [34,35]. Higher renin levels are also found in obese subjects compared to their lean counterparts [36,37]. Lastly, cortisol as a stress hormone was not associated with leptin in our study population. A possible explanation comes from animal studies and in vitro studies suggesting that leptin reduces cortisol secretion by inhibiting the release of corticotropin-releasing factor from the hypothalamus and subsequently adrenocorticotrophic hormone secretion from the pituitary [38,39].

Leptin crosses the blood-brain barrier and binds to its long form receptors situated in several central nervous system regions such as the hypothalamus. Thereupon, it will increase sympathetic nerve activity and energy expenditure [7]. Intracerebroventricular administration of leptin results in higher blood pressure and renal sympathetic nerve activity as observed in rabbits fed a high-fat diet [40]. This is supported by the findings of Prior *et al*, which

demonstrated increases in heart rate and renal sympathetic nerve activity, especially in the high-fat fed animals compared to the controls. The authors speculate that greater leptin levels may centrally activate sympathetic pathways [41]. In addition, the activation of adrenergic receptors may be implicated in leptin mediated heart rate control. Hyperleptinemia increased heart rate in control rats whereas in the leptin infused α 1- and β -receptor blockade rats no changes were observed [25]. On the other hand, in 32 men with sympathetic and parasympathetic cardiac denervation there was an independent association between leptin and heart rate, suggesting a possible direct role of leptin on heart rate through cardiac leptin receptors [42].

It is necessary to point out that our result was more prominent in men, irrespective of ethnicity. This was unexpected, due to higher leptin levels compared to age- and BMI matched men that exist in women due to more naturally occurring subcutaneous fat (the source of leptin) [43]. However, as pointed out in the sensitivity analysis leptin levels and autonomic activity markers did not differ between hypertensive and normotensive African women suggesting that they might be less sensitive to the cardiovascular actions of leptin. Experimental studies in obese rodents demonstrate impaired leptin transport across the blood-brain barrier as well as impaired post-receptor signalling compared to lean rodents [44]. Therefore, hyperleptinemia may induce a state of global leptin resistance where there is resistance to leptin's satiety effects and sympathetic nervous system effects [7]. In addition, heightened leptin sensitivity is reported in individuals with lower adiposity and circulating leptin levels [45]. One could also speculate that the adverse effects of elevated leptin levels on the autonomic nervous system may influence men earlier and more prominently than women. Also of note is that 74% of the men in our study population were hypertensive compared to 43% of the women.

The prevention of the cardiovascular complications of obesity remains an important public health concern. Our results suggest that leptin may contribute to autonomic dysfunction which is believed to be a risk factor for morbidity and mortality [10]. Despite growing evidence regarding the unfavourable cardiovascular effects of leptin; leptin therapy is being considered as an

effective treatment option for insulin resistance and lipodystrophy in human immunodeficiency virus patients [46]. Furthermore, leptin injections are still being promoted as a safe and effective treatment of obesity and these injections are easily obtainable.

This study has to be interpreted within the context of its limitations and strengths. Although the contribution of leptin to the variability of the autonomic activity markers appears mild (4 – 21%), these associations were significant and independent of several confounders. Our results were statistically significant, but leptin may contribute to cardiovascular disease risk by interacting with other risk factors. We did not directly assess autonomic nerve activity by using methods such as norepinephrine spillover and microneurography. In addition, decreased heart rate variability is also associated with various cardiovascular risk factors such as hypertension, diabetes, hyperinsulinemia, dyslipidemia, work-related stress and lifestyle factors [10]. However, adjustments were applied for potential and available confounders in multiple regression analyses. This was a specific target population; therefore, results cannot be extrapolated to the general population. We cannot exclude the possibility that our associations were due to residual confounding. This was a cross-sectional study and therefore we cannot infer causality. Apart from this, we conducted a well-designed study under controlled conditions.

In conclusion, serum leptin levels were associated with markers that reflect sympathetic overactivity, independent of several cardiovascular risk factors, including body mass index. Effects of leptin on sympathetic nerve activity may not necessarily be secondary to obesity. Future epidemiological trials are needed to determine whether the use of leptin therapy, especially in conjunction with leptin sensitizers, will contribute to adverse cardiovascular outcomes.

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

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CHAPTER 3

Leptin links with plasminogen activator inhibitor-1 in human obesity: the SABPA study

INSTRUCTIONS FOR AUTHORS ON THE PREPARATION AND SUBMISSION OF MANUSCRIPTS TO *HYPERTENSION RESEARCH*.

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Scope

The journal publishes original clinical and experimental research that contributes to the advancement of knowledge in the field of hypertension and related cardiovascular diseases.

Conflicts of interest

Authors of all submitted papers should declare any conflict of interest (COI) with regard to the submitted work. This should follow the guidelines and detailed regulations set out by the Japanese Society of Hypertension (JSH) in 2012.

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The authors may provide any supplementary material that will aid the review process.

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Manuscripts should be accompanied by an abstract. Original Articles must not exceed 5000 words, including abstract but excluding references. The manuscripts should not include more than six tables and/or figures. The following sections should form part of the manuscript (each starting a new page): Title, abstract and keywords, text (introduction, methods, results and discussion), references, tables and figure captions.

Title page

The title page should give an informative title, first and last names of all authors, full contact details for the corresponding author, running title of no more than 50 characters

Abstracts

Abstracts should not exceed more than 250 words. The abstract should outline the purpose of the study and provide basic procedures as well as the conclusions. The abstract should be followed by 3-5 keywords.

Introduction

This should give a brief and clear account of the background and reasons for undertaking the study.

Methods

This section should contain sufficient detail so that all experimental procedures can be repeated by others in conjunction with cited references. This section may be divided into subheadings to assist the reader.

Results

The description of results should not simply reiterate results presented in tables and figures. Also, the same data should not be displayed in both tables and figures. The results section should be clear and follow a logical sequence.

Discussion

The significance of the results should be discussed against the background of existing knowledge, and aspects that are novel should be described.

Acknowledgements

All sources of financial support and personal assistance should be mentioned. Only those who made a significant contribution to the study should be mentioned.

References

Authors are responsible for the accuracy of the references. All authors should be quoted. References should be numbered consecutively and indicated by a superscript in the text. Each reference should be numbered individually and listed at the end of the manuscript.

Tables and Figures

These should be labelled sequentially as Table 1, Table 2, etc. Tables must be accompanied by a brief footnote that identifies all abbreviations used. Figures should be labelled sequentially as Figure 1, Figure 2, etc. Each figure should be accompanied by a figure legend.

Leptin links with plasminogen activator inhibitor-1 in human obesity: the SABPA study

Short title: Leptin and vascular damage.

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Abstract

The relationship between obesity and the development of cardiovascular disease is well established. However, the underlying mechanisms contributing to vascular disease and increased cardiovascular risk in the obese remain largely unexplored. Since leptin exerts direct vascular effects, we investigated leptin and the relationship thereof with circulating markers of vascular damage, namely plasminogen activator inhibitor-1 antigen (PAI-1_{ag}), von Willebrand factor antigen (vWF_{ag}) and urinary albumin-to-creatinine ratio (ACR). The study included a bi-ethnic population of 409 African and Caucasian teachers who were stratified into lean (<0.5) and obese (≥0.5) groups according to waist-to-height ratio. We obtained ambulatory blood pressure measurements and determined serum leptin levels, PAI-1_{ag}, vWF_{ag} and ACR, as markers of vascular damage. The obese group had higher leptin (P<0.001) and PAI-1_{ag} (P<0.001) levels and a tendency existed for higher vWF_{ag} (P=0.068). ACR did not differ between the two groups (P=0.21). In single regression analyses positive associations existed between leptin and all markers of vascular damage (all P<0.001) only in the obese group. After adjusting for covariates and confounders in multiple regression analyses, only the association between leptin and PAI-1_{ag} remained (R²=0.440; β=0.293; P=0.0021). After adjusting for gender, ethnicity and age, additional analyses indicated that leptin also associated with fibrinogen and clot lysis time in both lean and obese groups, which in turn is associated with 24 h blood pressure and pulse pressure. This result provides evidence that elevated circulating leptin may directly contribute to vascular damage, possibly through mechanisms related to thrombotic vascular disease.

Keywords: obesity; vascular damage; leptin

Introduction

The prevalence of obesity is increasing in sub-Saharan Africa.¹ This increase may be attributed to Westernization which usually coincides with unhealthy dietary habits and a sedentary lifestyle.² Obesity often predisposes the development of hypertension,³ and obesity-related hypertension is now considered a distinct hypertensive phenotype.⁴ Endothelial damage is one of many mechanisms linking obesity to cardiovascular disease⁵ and *in vitro* and *in vivo* studies suggest that leptin may be involved.^{6,7}

Physiological leptin levels promote nitric oxide production and endothelium-dependent relaxation by inducing nitric oxide synthase expression.⁸ Contrastingly, in pathological conditions such as obesity and related hyperleptinemia, the leptin-mediated production of nitric oxide seems impaired and thereby contributes to endothelial dysfunction and damage.^{6,9} Assessment of circulating biomarkers of vascular damage may provide valuable information of the mechanisms at work. A damaged endothelium promotes the secretion of pro-thrombotic factors such as plasminogen activator inhibitor-1¹⁰ and von Willebrand factor¹¹ and results in leakage of albumin in urine as reflected by the urinary albumin-to-creatinine ratio.¹²

We therefore investigated leptin and the associations thereof with plasminogen activator inhibitor-1, von Willebrand factor and albumin-to-creatinine ratio as markers of vascular damage in a bi-ethnic sample of 409 teachers.

Methods

Study design

This study forms part of the SABPA (Sympathetic activity and Ambulatory Blood Pressure in Africans) study, which included 409 African and Caucasian school teachers working in the Potchefstroom district in the North West Province of South Africa. The reason for the selection of this target population was to obtain a homogenous sample of participants from a similar socioeconomic class. Participants between the ages of 25 and 60 years were included. The exclusion criteria were a tympanic temperature above 37°C, psychotropic substance dependence or abuse, regular blood donors and individuals vaccinated in the past three months. Participants received detailed information about the procedures and objectives of the study prior to their recruitment. All participants signed an informed consent form. The study complied with all applicable requirements and international regulations, including the Helsinki declaration of 1975 (as revised in 2008) for investigation of human participants. The study was approved by the Ethics Review Board of the North-West University (Potchefstroom Campus).

Cardiovascular measurements

Ambulatory blood pressure measurements (ABPM) were taken during the working week. At approximately 08h00, an ABPM apparatus (Meditech CE120® Cardiotens; Meditech, Budapest, Hungary) and two-lead electrocardiogram (ECG) were attached on the participant's non-dominant arm at their workplace. The ABPM apparatus was programmed to measure blood pressure at 30 minute intervals during the day (08h00-22h00) and every hour during night-time. Participants received ambulatory diary cards and were requested to indicate abnormalities such as nausea, headache or stress experienced during their normal daily activities. At 16h30, participants were transported to the North-West University and admitted to the Metabolic Unit Research Facility. This facility consists of 10 bedrooms, two bathrooms, a living room and a kitchen. Participants received a standardised dinner and at 20h30 they received their last beverage (coffee/tea and two biscuits). Thereafter they relaxed by reading, watching television or social interaction and were encouraged to go to bed at 22h00. Participants were requested to refrain from alcohol consumption, caffeine consumption and exercise. At 06h00, the ABPM

apparatus was removed and subsequent measurements commenced. Electrocardiogram and 24 h blood pressure data were downloaded onto a database using the CardioVisions 1.15.2 Personal Edition (Meditech, Budapest, Hungary). If less than 70% of the ABPM recordings for a particular participant were successful, the measurement was repeated the next day. The Actical® activity monitor (Mini Mitter Co., Inc., Bend, OR; Montreal, Quebec, Canada) was used to estimate physical activity. The monitor was fitted after all cardiovascular measurements were taken and worn around the waist by participants for 24 h. The physical activity index of participants was categorised according to high, moderate and low physical activity.

Anthropometric measurements

Height (stature), weight and waist circumference of participants were measured while being in their underwear using calibrated instruments (Precision Health Scale, A & D Company, Tokyo, Japan; Invicta Stadiometer, IP 1465, London, UK). All measurements were taken in triplicate using standard methods.¹³ Subsequently, the body mass index and waist-to-height ratio (WHtR) were calculated for each participant. We used a WHtR value of 0.5 as cut off based on recent studies confirming the usefulness of this measurement.¹⁴

Biochemical measurements

After the cardiovascular and anthropometric measurements were completed, a registered nurse drew a fasting blood sample with a sterile winged infusion set from the antebrachial vein branches. EDTA whole blood and serum were stored at -80°C . In serum, fasting samples for total cholesterol, high-density lipoprotein cholesterol, glucose, γ -glutamyl transferase and high sensitivity C-reactive protein were analysed using two sequential multiple analysers (Konelab 20i TM, Thermo Scientific, Vantaa, Finland; Unicel DxC 800; Beckman and Coulter, Krefeld, Germany). We determined leptin levels using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine, R&D Systems, MN, USA) and citrate von Willebrand antigen (vWF_{ag}) levels with a “sandwich” ELISA assay. A polyclonal rabbit anti-vWF antibody and a rabbit anti-vWF-HRP antibody (DAKO, Johannesburg, South Africa) were used to form the assay. The 6th International Standard for vWF/FVII was used to create the standard curve against which the

samples were measured.¹⁵ Plasminogen activator inhibitor-1 antigen (PAI-1_{ag}) levels were determined with triniLIZE PAI-1 (Trinity Biotech, Bray, Ireland) antigen kit using ELISA. Plasma fibrinogen levels were determined with a viscosity-based clotting method using STAGO FIB kit (STAGO diagnostics, Asnières, France). Clot lysis time (CLT) was determined by studying the lysis of a tissue factor-induced clot by exogenous tissue-plasminogen activator (t-PA). Changes in turbidity during clot formation and lysis were monitored as described by Lisman et al.¹⁶ Tissue factor and t-PA concentrations were slightly modified to obtain comparable CLTs of about 60 minutes. The modified concentrations were 17 mmol/L CaCl₂, 60 ng/ml t-PA (Actilyse, Boehringer Ingelheim, Ingelheim, Germany) and 10 µmol/L phospholipids vesicles (Rossix, Mölndal, Sweden). Tissue factor was diluted 3000 times (Dade Innovin, Siemens Healthcare Diagnostics Inc., Marburg, Germany). CLT was de-fined as the time from the midpoint in the transition from the initial baseline to maximum turbidity, which is representative of clot formation, to the midpoint in the transition from maximum turbidity to the final baseline turbidity, which represents the lysis of the clot.

In urine, creatinine was determined with a calorimetric method and albumin with the measurement of immunoprecipitation enhanced by polyethylene glycol at 450 nm, with two sequential multiple analysers (Konelab 20i TM, Thermo Scientific, Vantaa, Finland Unicel DxC 800; Beckman and Coulter, Krefeld, Germany) with a coefficient variation of 1.7-3.3%. Albumin-to-creatinine ratio (ACR) measured in an 8 h overnight urine sample is highly correlated with 24 h urinary albumin excretion.^{17,18}

Statistical analysis

For database management and statistical analyses, we used Statistica software version 12.0 (Statsoft, Inc., Tulsa, OK, 2010). A WHtR of 0.5 was used to categorise our study population into lean (<0.5) and obese (≥0.5) individuals. A power calculation was performed and it was found that sample sizes of 127 and 282 participants are sufficient to determine biological differences of leptin and PAI-1_{ag} concentrations, at a significance level of P = 0.05 and a power of 99%. The distribution of leptin, ACR, glucose, C-reactive protein and γ-glutamyl transferase

were normalised by logarithmic transformation. The central tendency and spread of these variables were represented by the geometric mean and the 5th and 95th percentile intervals. Independent t-tests were done to compare means between groups and the Chi-square test (χ^2) to compare proportions. We used Pearson's correlations to investigate associations between markers of vascular damage and leptin and illustrated by scatterplots. We also performed partial correlations by adjusting for ethnicity, gender and age. Adjusted mean values of leptin were plotted by quartiles of PAI-1_{ag}. Multiple regression analyses were performed to investigate independent associations, while adjusting for ethnicity, gender, age, WHtR, 24 h systolic blood pressure, high-density lipoprotein-to-total cholesterol ratio, serum glucose, C-reactive protein, current smoking, γ -glutamyl transferase, physical activity level and the use of anti-hypertensive medication.

Results

Table 1 Characteristics of the study population stratified by a WHtR of 0.5

	Lean (n=127)	Obese (n=282)	P
Age, years	42 ± 10	46 ± 9	< 0.001
African, n (%)	50 (39.4)	150 (53.2)	< 0.01
Women, n (%)	72 (56.7)	135 (47.9)	0.099
Stature, cm	170 ± 10	169 ± 10	0.66
Body mass, kg	66 ± 11	90 ± 18	< 0.001
Waist circumference, cm	77 ± 7	101 ± 13	< 0.001
Waist-to-height ratio	0.46 ± 0.03	0.60 ± 0.07	< 0.001
Body mass index, kg/m ²	23.0 ± 2.78	31.5 ± 6.08	< 0.001
Biochemical measurements			
Leptin, ng/ml	9.18 (1.94 - 40.7)	23.4 (4.95 - 91.2)	< 0.001
TC:HDL	4.01 ± 1.66	5.07 ± 1.85	< 0.001
Glucose, mmol/l	5.14 (4.26 - 6.03)	5.62 (4.40 - 8.90)	< 0.001
C-reactive protein, mg/l	1.73 (0.45 - 9.25)	3.75 (0.99 - 26.9)	< 0.001
PAI-1, ng/mmol	24.9 ± 11.1	30.0 ± 10.2	< 0.001
von Willebrand factor, %	73.7 ± 23.8	78.6 ± 25.0	0.068
ACR, mg/mmol	0.52 (0.11 - 2.82)	0.60 (0.11 - 3.13)	0.21
Fibrinogen, g/L	2.89 ± 0.54	3.47 ± 0.78	< 0.001
Clot lysis time, min	70.8 ± 12.9	85.6 ± 17.1	< 0.001
Cardiovascular measurements			
24 h SBP, mmHg	120 ± 11	132 ± 15	< 0.001
24 h DBP, mmHg	76 ± 8	82 ± 10	< 0.001
24 h Pulse pressure, mmHg	45 ± 6	50 ± 9	< 0.001
Lifestyle			
High level of physical activity, n (%)	20 (15.8)	17 (6.1)	< 0.001
Smoking, n (%)	20 (15.8)	43 (15.3)	0.91
γ-glutamyltransferase, U/L	20.8 (7.00 - 93.3)	35.2 (11.0 - 134)	< 0.001
Cardiovascular disease history, n (%)	6 (4.7)	38 (13.5)	< 0.01
Hypertension prevalence, n (%)	48 (37.8)	213 (75.5)	< 0.001
Diagnosed diabetes, n (%)	1 (0.8)	11 (3.9)	0.084
Use of anti-hypertensive medication, n (%)	21 (16.5)	75 (26.6)	0.026
Use of statins, n (%)	0 (0.0)	11 (3.9)	0.024

Values are arithmetic mean ± SD, geometric mean (5 - 95th percentile interval), or number of participants (%). Abbreviations: WHtR, waist-to-height ratio; TC:HDL, total cholesterol-to-high-density lipoprotein ratio; PAI-1, plasminogen activator inhibitor-1; ACR, albumin-to-creatinine ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Comparison of lean and obese participants

Table 1 lists the characteristics of the study population stratified by WHtR. Leptin levels, 24 h systolic- and diastolic blood pressure (all $P < 0.001$) were all higher in the obese group compared to the lean group. The obese group had higher PAI-1_{ag} ($P < 0.001$) and a tendency for higher vWF ($P = 0.068$), but no difference existed between the lean and obese groups for ACR ($P = 0.21$).

Single and partial regression analyses

In single regression analyses (Figure 1), we found positive associations of PAI-1_{ag}, vWF_{ag} and ACR with leptin ($P < 0.001$) only in the obese group. After adjusting for ethnicity, gender and age, the association between PAI-1_{ag} and leptin ($r = 0.18$; $P = 0.002$) remained, while the significant associations with vWF_{ag} ($r = 0.03$; $P = 0.58$) and ACR ($r = 0.08$; $P = 0.20$) in the obese group were lost. In exploratory analyses (Figure 2), we plotted PAI-1_{ag} by quartiles of leptin after additionally adjusting for WHtR, confirming the positive association between PAI-1_{ag} and leptin (P for trend = 0.015) in the obese.

Multiple regression analyses

The independent associations between PAI-1_{ag}, vWF_{ag}, ACR and leptin in the respective WHtR groups are presented in Table 2. After adjusting for confounders, the above association between PAI-1_{ag} and leptin in the obese group was confirmed ($\beta = 0.293$; $P = 0.0021$). We also repeated the analyses in the obese Africans and Caucasians separately (Supplementary Table S1). By doing so, the relationship between PAI-1_{ag} and leptin remained in the obese Caucasians (Adj $R^2 = 0.198$; $\beta = 0.321$; $P = 0.042$) and a similar tendency was seen in the obese Africans (Adj $R^2 = 0.037$; $\beta = 0.303$; $P = 0.057$).

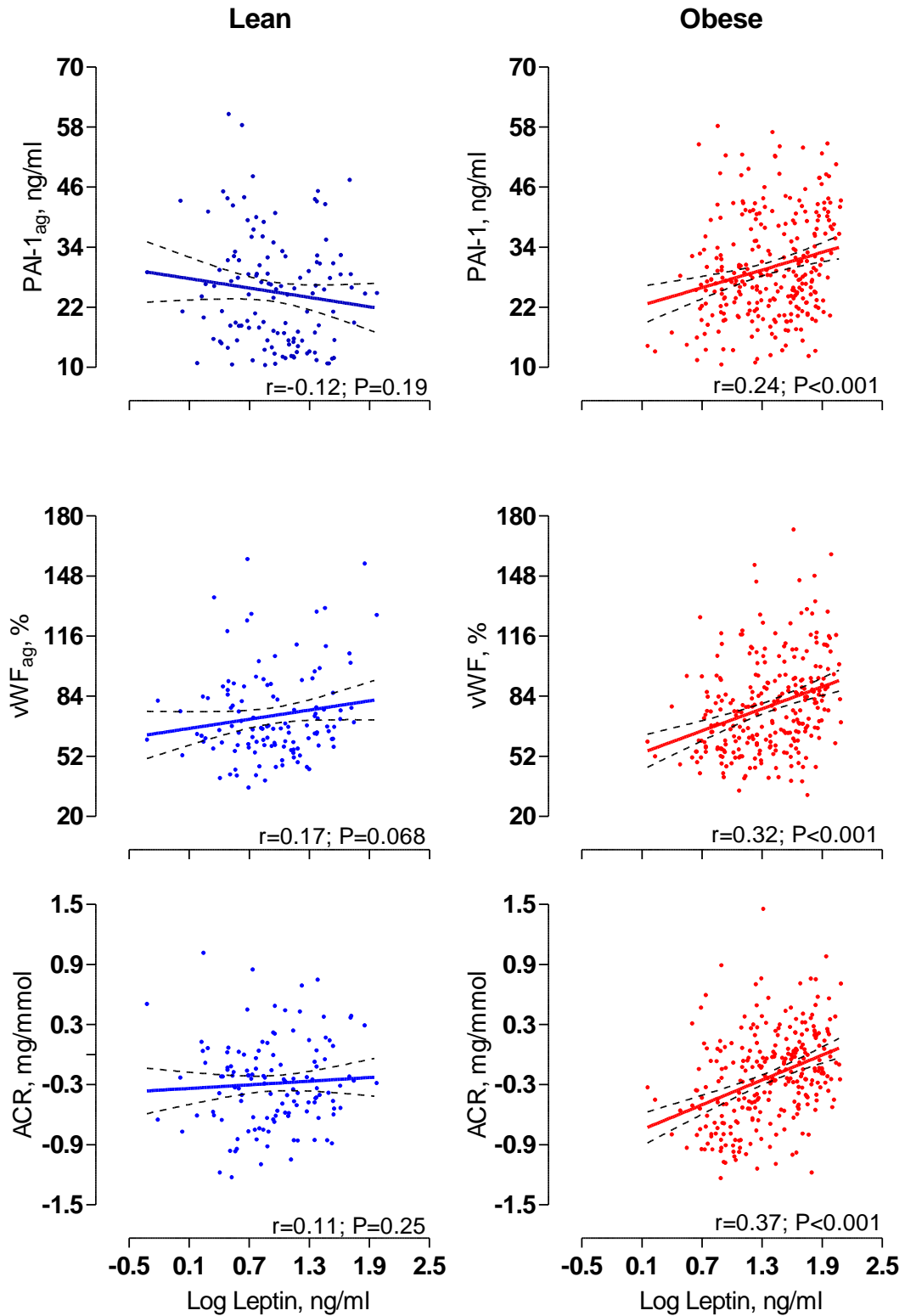


Figure 1 Single regression analyses of markers of vascular damage with leptin in the respective lean and obese groups. Solid and dashed lines represent the regression line and the 95% CI boundaries. PAI-1_{ag}, plasminogen activator inhibitor-1 antigen; vWF_{ag}, von Willebrand factor antigen; ACR, albumin-to-creatinine ratio.

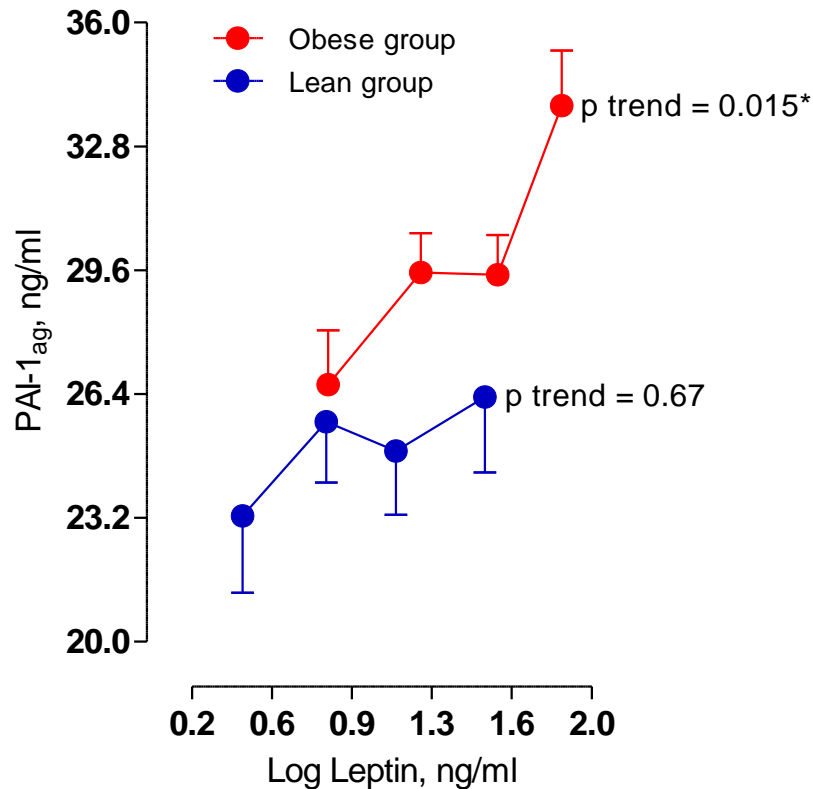


Figure 2 PAI-1_{ag} by quartiles of leptin levels in the lean and obese groups adjusted for ethnicity, gender, age and WHtR. Values are arithmetic mean \pm S.E. P denotes significance for trend; *P<0.05 (quartile1 vs quartile4). PAI-1_{ag}, plasminogen activator inhibitor-1 antigen; WHtR, waist-to-height ratio.

Additional analyses

Due to the fact that we only found associations between PAI-1_{ag} and leptin, we investigated whether leptin associates with markers of thrombotic vascular disease. After adjusting for ethnicity, gender and age, we found positive associations of fibrinogen and clot lysis time with leptin in the lean ($r = 0.33$; $P < 0.001$, $r = 0.28$; $P = 0.002$) and obese groups ($r = 0.24$; $P < 0.001$, $r = 0.32$; $P < 0.001$). Additionally, fibrinogen ($r = 0.20$; $P = 0.030$) and clot lysis time ($r = 0.19$; $P = 0.045$) were positively associated with 24 h pulse pressure in the lean group, whereas positive associations were seen between clot lysis time, 24 h systolic blood pressure ($r = 0.17$; $P = 0.006$) and 24 h pulse pressure ($r = 0.14$; $P = 0.028$) in the obese group.

Table 2 Independent associations between markers of vascular alterations and leptin.

	Lean			Obese		
	PAI-1, ng/mmol	vWF, %	ACR, mg/mmol	PAI-1, ng/mmol	vWF, %	ACR, mg/mmol
R ²	0.526	0.399	0.454	0.440	0.422	0.449
Adjusted R ²	0.469	0.325	0.388	0.413	0.392	0.423
	Std β (95 % CI)	Std β (95 % CI)	Std β (95 % CI)	Std β (95 % CI)	Std β (95 % CI)	Std β (95 % CI)
Leptin, ng/ml	0.028 (-0.199 to 0.255)	0.143 (-0.1818 to 0.404)	-0.062 (-0.307 to 0.183)	0.293 (0.109 to 0.477)‡	0.091 (-0.097 to 0.279)	0.129 (-0.051 to 0.309)
Ethnicity (0,1)	-0.648 (-0.852 to -0.444)§	-0.553 (-0.786 to -0.320)§	-0.609 (-0.830 to -0.388)§	-0.539 (-0.662 to -0.416)§	-0.656 (-0.783 to -0.529)§	-0.483 (-0.605 to -0.361)§
Gender (0,1)	-0.162 (-0.294 to 0.030)	0.177 (-0.121 to 0.475)	0.262 (-0.018 to 0.542)	-0.258 (-0.430 to -0.086)‡	0.029 (-0.149 to 0.207)	0.062 (-0.109 to 0.253)
Age, years	0.025 (-0.124 to 0.174)	0.094 (-0.078 to 0.266)	0.293 (0.130 to 0.456)§	-0.028 (-0.124 to 0.068)	0.099 (-0.003 to 0.201)	0.113 (0.017 to 0.209)‡
WHtR	-0.045 (-0.204 to 0.114)	-0.024 (-0.208 to 0.160)	-0.116 (-0.288 to 0.056)	-0.085 (-0.205 to 0.035)	-0.043 (-0.166 to 0.080)	-0.035 (-0.153 to 0.083)
24 h SBP, mmHg	0.036 (-0.127 to 0.199)	0.045 (-0.141 to 0.231)	0.137 (-0.037 to 0.311)	-0.036 (-0.142 to 0.070)	0.005 (-0.105 to 0.115)	0.211 (0.107 to 0.315)§
TC:HDL, mmol/l	0.043 (-0.131 to 0.263)	0.026 (-0.174 to 0.226)	0.003 (-0.185 to 0.191)	0.007 (-0.099 to 0.113)	0.038 (-0.072 to 0.148)	-0.092 (-0.198 to 0.014)
Serum glucose, mmol/l	-0.017 (-0.184 to 0.150)	0.071 (-0.119 to 0.261)	-0.139 (-0.319 to 0.041)	0.174 (0.072 to 0.276) §	0.018 (-0.086 to 0.122)	-0.014 (-0.114 to 0.086)
C-reactive protein, mg/l	-0.001 (-0.152 to 0.149)	0.003 (-0.172 to 0.177)	0.047 (-0.118 to 0.212)	-0.099 (-0.217 to 0.019)	0.138 (0.016 to 0.259)†	0.009 (-0.109 to 0.127)
Smoking (0,1)	0.020 (-0.121 to 0.161)	-0.064 (-0.225 to 0.097)	-0.096 (-0.247 to 0.055)	0.039 (-0.057 to 0.135)	-0.013 (-0.111 to 0.085)	-0.011 (-0.105 to 0.083)
GGT, U/L	-0.018 (-0.216 to 0.180)	0.131 (-0.096 to 0.358)	-0.005 (-0.219 to 0.209)	0.082 (-0.034 to 0.198)	-0.090 (-0.210 to 0.030)	-0.096 (-0.210 to 0.018)
Physical activity (0,1,2)	-0.046 (-0.187 to 0.095)	0.081 (-0.082 to 0.244)	0.097 (-0.056 to 0.250)	-0.046 (-0.138 to 0.046)	0.012 (-0.084 to 0.108)	-0.045 (-0.137 to 0.047)
Anti-hypertensive medication (0,1)	0.036 (-0.105 to 0.177)	-0.086 (-0.247 to 0.075)	0.046 (-0.107 to 0.199)	0.057 (-0.041 to 0.155)	0.047 (-0.055 to 0.149)	-0.075 (-0.171 to 0.021)

Standardised β (std β) reflects the change in the dependent variable for 1 SD change in the independent variable. A larger std β reflects greater strength of the association.

Abbreviations: WHtR, waist-to-height ratio; PAI-1, plasminogen activator inhibitor-1; vWF, von Willebrand factor; ACR, albumin-to-creatinine ratio; SBP, systolic blood pressure; GGT, gamma-glutamyl transferase.

*0.1 \geq P \geq 0.05; †P \leq 0.05; ‡P \leq 0.01; §P \leq 0.001

Discussion

We explored the relationship between PAI-1_{ag}, vWF_{ag}, ACR and leptin, and found a positive association between PAI-1_{ag} and leptin in obese participants, independent of waist-to-height ratio and blood pressure. Additional analyses indicated that instead of relating to vWF_{ag} and ACR as more structural markers of endothelial damage, leptin rather related, in addition to PAI_{ag}, to fibrinogen and clot lysis time, which in turn related to 24h systolic blood pressure and pulse pressure, suggesting a potential role of leptin in thrombotic vascular disease.

Few studies addressed the relationship between leptin and circulating markers of vascular damage. Previous studies which support the association between PAI-1_{ag} and leptin are limited and lack adjustment for ambulatory blood pressure¹⁹ and C-reactive protein,²⁰ which are known to be related to PAI-1_{ag} and leptin.²¹ In 74 hypertensive overweight individuals, leptin correlated positively with PAI-1, but the association disappeared after correcting for body mass index.²² In agreement with our finding, a study by Mertens *et al*, consisting of 280 overweight and obese participants, found a positive association between leptin and PAI-1 activity independent of visceral- and subcutaneous adipose tissue, percentage fat mass and insulin resistance.²³ An independent association between PAI-1_{ag} and leptin was also demonstrated in 61 non-diabetic women.²⁴ A study on obese children showed no relationship between PAI-1_{ag} and leptin, however this relationship may not be established yet.²⁵

The absence of associations of vWF_{ag} and ACR with leptin contradicts previous studies. The study by Mertens *et al*.²³ also showed an association between vWF_{ag} and leptin, but only in men. Furthermore, positive associations between leptin and vWF_{ag} were found in 3640 overweight men²⁶ and 51 obese women.²⁷ In the latter study it was suggested that inflammation may be the mechanistic link between leptin and vWF_{ag}. However, the potential confounding effect of inflammation and blood pressure was not taken into consideration by the authors. When we included C-reactive protein as inflammatory marker in multiple

regression analyses the relationship between vWF_{ag} and leptin did indeed seem confounded by C-reactive protein.

Regarding albuminuria, a positive association between urinary albumin and leptin was observed in chronic kidney disease patients.²⁸ Results from the Framingham Heart Study showed that visceral adipose tissue was associated with microalbuminuria in men of which 30% were obese, but the authors did not investigate leptin.²⁹ In a study on non-diabetic black Africans, leptin as well as ACR levels were higher in participants with four or more components of the metabolic syndrome compared to those without.³⁰ However, associations between leptin and ACR were not reported.

Our main finding is therefore the independent association between $PAI-1_{ag}$ and leptin in obese individuals. The pathway(s) by which leptin may induce $PAI-1$ secretion remains undefined. Experimental studies provide evidence of a possible direct stimulatory effect of leptin. Incubation of human endothelial cells with leptin at concentrations greater than 50 ng/ml stimulates $PAI-1$ protein expression.³¹ Singh *et al.*³¹ suggested that the extracellular signal-regulated kinase (ERK)1/2 pathway may be involved. We recently demonstrated in this population group that a hyperleptinemic state is associated with sympathetic overactivity.³² Stimulation of sympathetic nerve activity by leptin may be a potential indirect pathway by which leptin promotes $PAI-1$ secretion.⁶ Stimulation of the sympathetic nervous system activates $PAI-1$ gene expression through the α -adrenergic receptor.³³ In addition, leptin may also stimulate $PAI-1$ secretion by inducing oxidative stress^{34,35} and inflammation.³⁶ Additional analyses after adjusting for ethnicity, gender and age, also demonstrated that leptin is associated with fibrinogen and clot lysis time and these hemostatic variables in turn associated with 24h systolic and pulse pressure. The lack of associations with vWF_{ag} , and ACR may suggest that leptin does not directly induce vascular damage *per se*, but rather a prothrombotic state leading to thrombotic vascular diseases. The independent association in this study and the *in vitro* findings of Singh *et al.*³¹ suggests

that leptin may directly stimulate PAI-1 secretion, but this seems to be activated only in obese and/or hyperleptinemic conditions. Consequently, PAI-1 may then contribute to vascular disease by inhibiting fibrinolysis.³⁶

One could speculate on the absence of associations between vWF_{ag}, ACR and leptin. vWF is secreted by endothelial cells either to act as an acute phase protein³⁷ or to initiate platelet adhesion at sites of vascular injury.³⁸ Endothelial damage will also contribute to vascular permeability and not the other way around.³⁹ Therefore, albuminuria is preceded by a damaged endothelium and may not be as a direct result of elevated leptin. We therefore propose that leptin may directly contribute to changes by inducing PAI-1 secretion and a resultant prothrombotic pathway. However, if obesity is left unopposed, chronic exposure of endothelial cells to leptin and other cardiometabolic risk factors will take effect, and vascular damage may progress to the extent where the damaged endothelium secretes vWF³⁸ and permit the movement of macromolecules such as albumin through the endothelial layer.³⁹

Globally, obesity and related cardiovascular disease are reaching alarming proportions. Given the disruptive effect of obesity on the cardiovascular and endocrine systems,^{40,41} studies aimed at identifying the mechanistic links between leptin and vascular alterations in the obese are of clinical relevance. These studies may provide the necessary information to appropriately treat and delay vascular alterations which is regarded as an early step in the development of cardiovascular disease.

This study has to be interpreted within the context of its limitations and strengths. This cross-sectional study investigated the associations between leptin and markers of vascular damage, and we therefore cannot infer causality. While our results were consistent after multiple adjustments, we cannot exclude the possibility that our associations were due to residual confounding. The possibility exists that vascular alterations could occur due to obesity and that leptin is only elevated as a consequence of obesity? However, we adjusted

for a marker of obesity in order to demonstrate a potential direct effect of leptin on vascular alteration or damage independent of obesity. This was a specific target population study; therefore, results cannot be extrapolated to the general population. We did not account for gene mutations and genetic factors such as ABO blood groups which influence vWF levels.⁴² Further, we were unable to correct for muscle mass and lack sufficient dietary data to report on factors which may influence creatinine excretion. Additionally, we do not have the necessary data to investigate the potential influence of the menstrual cycle on circulating levels of leptin, PAI-1_{ag}, vWF_{ag} and ACR. However, our main result remains robust after additional adjustment for estrogen and progesterone in multiple regression analyses ($P < 0.01$). In general, we conducted a well-designed study under controlled conditions.

To conclude, our results show a positive association between PAI-1_{ag} and leptin in obese humans, alluding to the mechanistic link between cardiovascular disease and obesity, possibly through leptin promoting thrombotic vascular disease. Given the global burden of cardiovascular disease, experimental studies should be aimed at the benefits of treating leptin resistance in the obese to decrease cardiovascular risk.

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

Conflict of interest

The authors declare no conflict of interests.

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CHAPTER 4

Leptin relates to prolonged cardiovascular recovery after acute stress in Africans: the SABPA study

INSTRUCTIONS FOR AUTHORS ON THE PREPARATION AND SUBMISSION OF MANUSCRIPTS TO THE *AMERICAN JOURNAL OF HYPERTENSION*.

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Scope

The American Journal of Hypertension is a peer-reviewed journal that publishes high-quality original research in the field of hypertension and cardiovascular disease.

Original contributions

Word Limit: 3,000 words maximum excluding abstract, references, tables, and figures

Abstract: 250 words maximum

References: 40 maximum

Figures/Tables: 4 figures, 4 tables maximum

Manuscript format

Manuscripts must be typed in English and double spaced.

- The title page should display the following: Manuscript title of no more than 150 characters, running head of no more than 50 characters, all authors' names (listed as first and middle initials followed by last name) and affiliations, the name, address, telephone, and fax numbers and email address of the corresponding author, a conflict of interest statement and keywords.
- Abstracts should contain the following headings: Background, methods, results and conclusions.
- Manuscript headings: Introduction, Methods, Results, Discussion and Disclosure.

Ethics

Indicate whether the study was conducted in accordance to with the Helsinki Declaration of 1975.

Abbreviations

Define abbreviations at the first mention.

Disclosure

The disclosure should be included on the title page and at the end of the manuscript under the heading "Disclosure". If the authors have no conflict of interest to declare, this should be stated in the "Disclosure" section.

Acknowledgments

Any sources of support, including federal and industry support should be mentioned in this section.

References

References should be typed double-spaced and numbered in the order of citation within the article. Citations in the main text should appear as superscript Arabic numerals. All authors must be listed. References should be formatted in Vancouver style.

Tables and Figures

Each table should be accompanied by a brief title of no more than 15 words for each and explanatory matter should be placed in the footnotes. Figures should be labeled sequentially, numbered, and cited in the text. Figure legends: Legends should be brief. All abbreviations should be explained in the figure legend.

Supplementary information

Supplementary information is posted on the journal's web site and linked to the article when the article is published. The printed article must be complete and self-explanatory without the supplementary information.

Leptin relates to prolonged cardiovascular recovery after acute stress in Africans: the SABPA study

Short title: Leptin and cardiovascular reactivity

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Keywords: Stress, peripheral resistance, cold pressor test, ethnicity

(This paper is under review at the *American Journal of Hypertension*)

Abstract

Background

Heightened cardiovascular reactivity and delayed recovery to stress are associated with an increased risk of cardiovascular disease. Africans, who are more prone to develop hypertension, show greater cardiovascular reactivity to stress. However, causal factors underlying individual and ethnic differences in stress reactivity and recovery remain largely unexplored. Leptin, which is known for its sympatho-activating effects, is higher in Africans compared to Caucasians for any given body mass index.

Methods

We compared how cardiovascular reactivity and recovery relate to leptin in African (n=200) and Caucasian (n=209) teachers. We measured leptin in serum and cardiovascular baseline and reactivity continuously with the Finometer device during the cold pressor test for 1 minute, and recovery at intervals of 1, 3 and 5 minutes.

Results

Africans had higher body mass index, leptin and blood pressure (all $P < 0.001$). After full adjustment in multiple regression analyses, associations were seen mainly at the 5 minute recovery interval. In Africans, cardiac output reactivity ($\beta = -0.335$; $P=0.0018$) and arterial compliance- ($\beta = -0.241$; $P = 0.048$) associated negatively and total peripheral resistance- ($\beta = 0.227$; $P = 0.047$) positively with leptin. In Caucasians, diastolic blood pressure correlated positively with leptin ($\beta = 0.200$; $P = 0.015$).

Conclusions

In Africans, higher circulating leptin levels associated with prolonged cardiovascular recovery after exposure to stress which could explain their increased vulnerability to hypertension development.

Introduction

Urbanized Africans are more prone to develop hypertension possibly due to health behaviors such as higher susceptibility to psychological stress and obesity.¹ Exaggerated cardiovascular reactivity and prolonged recovery to stress associate with an increased risk of hypertension development.^{2,3} Additionally, impaired post-stress recovery is associated with increased carotid intima-media thickness.⁴

Physiological, environmental or psychological stressors activate either the sympathetic nervous system and/or the hypothalamo-pituitary-adrenal axis.⁵ Activation of these pathways plays an important role in adaptation to stress, but if activated for extended periods, cardiovascular pathology ensues.⁵ The inability of some individuals to inactivate the stress response has led to the concept of allostatic load or chronic wear and tear.⁶ The allostatic load concept implies that multiple systems contribute to stress-related dysregulation which include cardiovascular, neuroendocrine, metabolic and inflammatory systems.⁷ Leptin is known to elicit central as well as peripheral cardiovascular responses,⁸ where central actions include the activation of the sympathetic nervous system upon binding to receptors in the hypothalamus,⁹ and activation of peripheral receptors situated on endothelial cells.¹⁰ Africans have higher leptin levels compared to Caucasians for a given body mass index^{11,12} and displays a distinct hyperreactive profile to a stressor.^{13,14} Furthermore, it is suggested that the sympatho-activating effects of leptin will be more evident under stressful conditions.¹⁵ Hyperleptinemic conditions together with the exposure to daily stressors may therefore possibly explain why Africans are more prone to develop hypertension.

We therefore investigated the relationship between serum leptin and cardiovascular reactivity and recovery to an acute stressor, namely the cold pressor test, in Africans and Caucasians.

Methods

Study design

This study forms part of the SABPA (Sympathetic activity and Ambulatory Blood Pressure in Africans) study, which included 409 African and Caucasian school teachers working in the Potchefstroom district in the North West Province of South Africa. The reason for the selection of this target population was to obtain a homogenous sample of participants from a similar socioeconomic class. Participants between the ages of 25 and 60 years were included. The exclusion criteria were a tympanic temperature above 37°C, psychotropic substance dependence or abuse, regular blood donors and individuals vaccinated in the past three months. Participants received detailed information about the procedures and objectives of the study prior to their recruitment. All participants signed an informed consent form. The study complied with all applicable requirements and international regulations, including the Helsinki declaration of 1975 (as revised in 2008) for investigation of human participants. The study was approved by the Ethics Review Board of the North-West University (Potchefstroom Campus).

Cardiovascular measurements

Each participant was exposed to the standardized cold pressor test which is believed to mimic everyday life stress, invoking sympathetic activation.¹⁶ The cold pressor test was performed by immersing the participant's right foot in ice water with a temperature of 4°C. The participants' were asked to maintain their normal breathing rhythm and to avoid straining. The cold pressor test predominantly leads to sympathetically mediated α -adrenergic vasoconstriction,¹⁷ consequently evoking increases in total peripheral resistance and diastolic blood pressure. The validated^{18,19} Finometer device (FMS, Finapres Measurement Systems, Amsterdam, The Netherlands) was connected, and after a 10 minute resting period, a 5 minute continuous measurement of resting cardiovascular parameters was carried out. During the recording, after 2 minutes, a return-to-flow systolic calibration was performed to provide an individual subject-level adjustment of the finger arterial pressure with the brachial artery pressure.¹⁹ The highest precision in cardiovascular measurements is obtainable only after this calibration,¹⁹ and blood

pressure levels met the requirements of the Association for the Advancement of Medical Instrumentation.²⁰ Thereafter, the participant was exposed to the cold pressor test for 1 minute, followed by measurements during the 5 minute recovery period. We obtained the average of the last 2 minutes of the resting recordings and the average of the last 15 s of the stressor and recovery recordings. The cardiovascular reactivity and recovery were calculated for each participant as the percentage change from the resting value.

Anthropometric measurements

Height (stature) and weight of participants were measured while being in their underwear using calibrated instruments (Precision Health Scale, A & D Company, Tokyo, Japan; Invicta Stadiometer, IP 1465, London, UK). All measurements were taken in triplicate using standard methods.²¹ Subsequently, the body mass index was calculated for each participant.

Biochemical measurements

After the cardiovascular and anthropometric measurements were completed, a registered nurse drew a fasting blood sample with a sterile winged infusion set from the antebrachial vein branches. EDTA whole blood and serum were stored at -80°C . In serum, fasting samples for total cholesterol, high-density lipoprotein cholesterol, glucose, γ -glutamyl transferase and high sensitivity C-reactive protein were analyzed using two sequential multiple analyzers (Konelab 20i TM, Thermo Scientific, Vantaa, Finland; Unicel DxC 800; Beckman and Coulter, Krefeld, Germany). We determined leptin levels using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine, R&D Systems, MN, USA).

Statistical analysis

For database management and statistical analyses, we used Statistica software version 12.0 (Statsoft, Inc., Tulsa, OK, 2010). The distribution of leptin, glucose, C-reactive protein and γ -glutamyl transferase were normalized by logarithmic transformation. The central tendency and spread of these variables were represented by the geometric mean and the 5th and 95th

percentile intervals. Independent t-tests were done to compare means between groups and the Chi-square test (χ^2) to compare proportions. Pearson's correlations were determined as well as partial correlations after adjustment for age, sex and body mass index. Adjusted mean values of cardiovascular reactivity markers were plotted to compare ethnicities over the time course of 5 minutes. Forward stepwise multiple regression analyses were performed to investigate independent associations, while adjusting for age, sex, body mass index, high-density lipoprotein-to-total cholesterol ratio, glucose, current smoking, γ -glutamyl transferase, C-reactive protein, anti-hypertensive medication and baseline cardiovascular variables. All values of $P < 0.05$ were regarded as significant.

Results

Characteristics of the study population

Table 1 lists the characteristics of the study population stratified by ethnicity. Compared to Caucasians, Africans had higher body mass index ($P < 0.001$) and almost double the leptin levels (23.4 ng/ml vs 12.6 ng/ml; $P < 0.001$). When comparing baseline cardiovascular measures, Africans had higher blood pressure and heart rate, and lower arterial compliance (all $P < 0.001$). There were no differences in stroke volume and total peripheral resistance ($P \geq 0.15$).

Table 1 Characteristics of the study population.

	Africans (n=200)	Caucasians (n=209)	P
Age, years	44 ± 8	45 ± 11	0.49
Women, n (%)	99 (49.5)	108 (51.7)	0.66
Stature, m	1.65 ± 0.09	1.74 ± 0.09	< 0.001
Body mass, kg	81 ± 18	83 ± 21	0.21
Body mass index, kg/m ²	30.1 ± 7.01	27.6 ± 5.94	< 0.001
Biochemical measurements			
Leptin, ng/ml	23.4 (3.09 – 95.5)	12.6 (2.57 – 56.2)	< 0.001
TC:HDL	4.48 ± 2.05	4.99 ± 1.62	< 0.01
Glucose, mmol/l	5.37 (4.07 – 10.47)	5.62 (4.68 – 6.92)	0.055
C-reactive protein, mg/l	4.37 (0.56 – 33.1)	2.04 (0.99 – 8.91)	< 0.001
Baseline cardiovascular measurements			
SBP, mmHg	141 ± 18	131 ± 14	< 0.001
DBP, mmHg	81 ± 10	77 ± 8	< 0.001
Heart rate, bpm	69 ± 11	66 ± 11	0.030
MAP, mmHg	106 ± 12	100 ± 10	< 0.001
Stroke volume, ml	101.5 ± 27.5	97.7 ± 24.3	0.15
Cardiac output, l/min	6.85 ± 1.91	6.43 ± 1.94	0.028
TPR, mmHg/ml/s	1.01 ± 0.38	1.04 ± 0.52	0.58
Cwk, ml/mmHg	1.87 ± 0.41	2.09 ± 0.53	< 0.001
Lifestyle			
Antihypertensive medication, n (%)	69 (34.5)	27 (12.9)	< 0.001
Smoking, n (%)	34 (17.0)	29 (13.9)	0.39
γ-glutamyltransferase, U/L	47.9 (19.9 – 182)	19.5 (7.08 – 75.9)	< 0.001

Values are arithmetic mean ± SD, geometric mean (5 - 95th percentile interval), or number of participants (%).

Abbreviations: TC:HDL, total cholesterol-to-high-density lipoprotein ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; TPR, total peripheral resistance; Cwk, arterial compliance.

Exploratory analysis

After adjusting for resting values (Figure 1), we additionally investigated ethnic differences in cardiovascular reactivity during and after application of the cold pressor test. Cardiovascular reactivity values of mean arterial pressure ($P = 0.048$), cardiac output ($P < 0.001$), total peripheral resistance ($P < 0.001$) and arterial compliance ($P = 0.023$), were higher and more prolonged in response to stress in Africans at 5 minutes post-stressor.

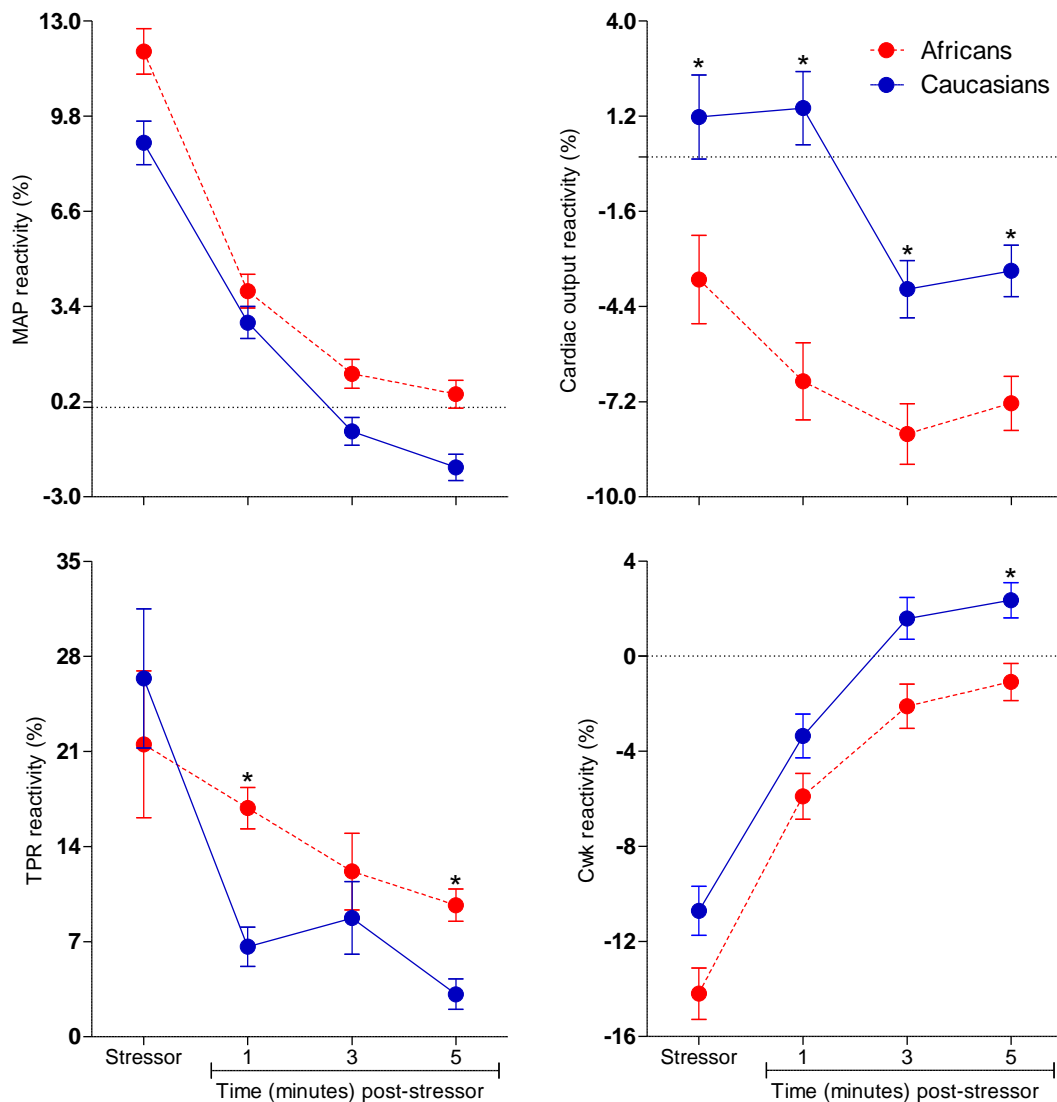


Figure 1 Cardiovascular reactivity measures of Africans and Caucasians during and after stressor application, adjusted for baseline values. Values are arithmetic mean \pm S.E.; * $P < 0.05$. MAP, mean arterial pressure; TPR, total peripheral resistance; Cwk, arterial compliance.

Single and partial regression analyses

Single regression analyses are presented in the Supplementary Table S1. In the Africans, there were negative associations of heart rate ($r = -0.15$; $P = 0.047$) and cardiac output ($r = -0.20$; $P = 0.007$) as well as positive associations of total peripheral resistance reactivity ($r = 0.16$; $P = 0.025$) with leptin at 1 minute post-stressor. Additionally, associations between heart rate reactivity ($r = -0.17$; $P = 0.020$), cardiac output reactivity ($r = -0.16$; $P = 0.028$) and leptin were seen 5 minutes post-stressor. After adjusting for age, sex and body mass index (Table 2), a negative association emerged between cardiac output reactivity and leptin during the stressor ($r = -0.16$; $P = 0.031$) in Africans. No associations between markers of cardiovascular reactivity and leptin were seen at 1 minute and 3 minutes post-stressor. However, the Africans demonstrated a negative association of cardiac output ($r = -0.21$; $P = 0.005$) and positive association of total peripheral resistance reactivity ($r = 0.16$; $P = 0.027$) with leptin at 5 minutes post-stressor. In the Caucasian group, there were negative associations between heart rate reactivity and leptin at 1 minute- ($r = -0.17$; $P = 0.016$) and 3 minutes post-stressor ($r = -0.15$; $P = 0.039$).

Table 2 Partial correlation coefficients between cardiovascular reactivity markers and leptin, adjusted for age, sex and BMI.

Africans (n = 200)				
	Reactivity during	1 minute post-stressor	3 minutes post-stressor	5 minutes post-stressor
SBP, %	$r = -0.09$; $P = 0.23$	$r = -0.07$; $P = 0.34$	$r = 0.03$; $P = 0.73$	$r = 0.07$; $P = 0.35$
DBP, %	$r = -0.04$; $P = 0.56$	$r = -0.02$; $P = 0.80$	$r = 0.04$; $P = 0.57$	$r = 0.07$; $P = 0.36$
Heart rate, %	$r = -0.14$; $P = 0.053$	$r = -0.11$; $P = 0.16$	$r = -0.08$; $P = 0.26$	$r = -0.15$; $P = 0.051$
MAP, %	$r = -0.05$; $P = 0.47$	$r = -0.01$; $P = 0.86$	$r = 0.04$; $P = 0.58$	$r = 0.07$; $P = 0.30$
Stroke volume, %	$r = -0.07$; $P = 0.35$	$r = -0.08$; $P = 0.26$	$r = -0.08$; $P = 0.26$	$r = -0.11$; $P = 0.14$
Cardiac output, %	$r = -0.16$; $P = 0.031$	$r = -0.13$; $P = 0.081$	$r = -0.13$; $P = 0.091$	$r = -0.21$; $P = 0.005$
TPR, %	$r = 0.06$; $P = 0.43$	$r = 0.07$; $P = 0.38$	$r = 0.07$; $P = 0.35$	$r = 0.16$; $P = 0.027$
Cwk, %	$r = 0.03$; $P = 0.73$	$r = 0.004$; $P = 0.96$	$r = -0.04$; $P = 0.61$	$r = -0.07$; $P = 0.36$
Caucasians (n = 209)				
SBP, %	$r = -0.05$; $P = 0.50$	$r = -0.06$; $P = 0.39$	$r = -0.04$; $P = 0.58$	$r = -0.002$; $P = 0.99$
DBP, %	$r = 0.02$; $P = 0.81$	$r = 0.04$; $P = 0.55$	$r = 0.05$; $P = 0.44$	$r = 0.09$; $P = 0.21$
Heart rate, %	$r = -0.12$; $P = 0.095$	$r = -0.17$; $P = 0.016$	$r = -0.15$; $P = 0.039$	$r = -0.12$; $P = 0.098$
MAP, %	$r = 0.01$; $P = 0.94$	$r = 0.02$; $P = 0.74$	$r = 0.04$; $P = 0.55$	$r = 0.06$; $P = 0.37$
Stroke volume, %	$r = 0.03$; $P = 0.70$	$r = -0.02$; $P = 0.83$	$r = 0.002$; $P = 0.98$	$r = -0.03$; $P = 0.64$
Cardiac output, %	$r = -0.07$; $P = 0.33$	$r = -0.13$; $P = 0.076$	$r = -0.08$; $P = 0.24$	$r = -0.09$; $P = 0.18$
TPR, %	$r = 0.05$; $P = 0.45$	$r = 0.10$; $P = 0.17$	$r = 0.06$; $P = 0.44$	$r = 0.09$; $P = 0.23$
Cwk, %	$r = 0.01$; $P = 0.88$	$r = -0.02$; $P = 0.81$	$r = -0.01$; $P = 0.89$	$r = -0.08$; $P = 0.28$

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; TPR, total peripheral resistance; Cwk, arterial compliance. Bold text denotes statistical significance, $P < 0.05$.

Multiple regression analyses

The independent associations between cardiovascular reactivity (5 minutes post-stressor) and leptin are demonstrated in Table 3. After full adjustment, associations between cardiovascular reactivity and leptin were mainly evident 5 minutes post-stressor. No independent associations between cardiovascular reactivity and leptin were seen during the stressor, 1 minute post-stressor and 3 minutes post-stressor in any ethnic group (data not shown). The only results found for these time intervals were the negative association between cardiac output reactivity and leptin in Africans during the stressor ($\beta = -0.276$; $P = 0.011$) and at 3 minutes post-stressor ($\beta = -0.247$; $P = 0.032$). At 5 minutes post-stressor in Africans, we found negative associations of cardiac output reactivity ($\beta = -0.335$; $P = 0.0018$) and arterial compliance reactivity ($\beta = -0.241$; $P = 0.048$), and a positive association of total peripheral resistance reactivity ($\beta = 0.227$; $P = 0.047$) with leptin. In Caucasians, a positive association existed between diastolic blood pressure and leptin ($\beta = 0.200$; $P = 0.015$).

Table 3 Independent associations between 5 minute post-stressor cardiovascular reactivity markers and leptin.

Africans (n = 200)				
Dependent variables	R ²	Adjusted R ²	Std β (95 % CI)	P
SBP, %	0.090	0.070	N/S	
DBP, %	0.142	0.128	N/S	
Heart rate, %	0.081	0.061	-0.197 (-0.406 to 0.012)	0.067
MAP, %	0.097	0.082	N/S	
Stroke volume, %	0.154	0.125	N/S	
Cardiac output, %	0.216	0.190	-0.335 (-0.543 to -0.127)	0.0018
TPR, %	0.129	0.094	0.227 (0.004 to 0.450)	0.047
Cwk, %	0.113	0.072	-0.241 (-0.478 to -0.004)	0.048
Caucasians (n=209)				
SBP, %	0.171	0.149	N/S	
DBP, %	0.124	0.110	0.200 (0.041 to 0.359)	0.015
Heart rate, %	0.156	0.142	N/S	
MAP, %	0.117	0.103	N/S	
Stroke volume, %	0.272	0.245	N/S	
Cardiac output, %	0.365	0.345	N/S	
TPR, %	0.239	0.215	N/S	
Cwk, %	0.071	0.052	N/S	

Standardised β (std β) reflects the change in the dependent variable for 1 SD change in the independent variable. A larger std β reflects greater strength of the association. Adjusted for age, sex, body mass index, high-density lipoprotein-to-total cholesterol ratio, glucose, current smoking, γ -glutamyltransferase, C-reactive protein, anti-hypertensive medication and baseline cardiovascular variables.

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; TPR, total peripheral resistance; Cwk, arterial compliance.

Discussion

We investigated ethnic differences for the relationship between leptin, cardiovascular reactivity to stress and post-stressor recovery. Independent associations between cardiovascular reactivity and leptin were mostly evident in Africans at 5 minutes post-stressor, suggesting that elevated leptin levels may contribute to impaired post-stress recovery, especially in Africans who also had higher leptin.

Previous studies investigating ethnic differences in cardiovascular reactivity to stress showed heightened responses in Africans compared to Caucasians.^{14,22} Africans also demonstrated greater reactive reductions in stroke volume and arterial compliance.²² These reactivity measures also associated with end-organ damage such as carotid wall thickening and left ventricular hypertrophy.²² Exaggerated stress responses of Africans may be attributed to increased α -adrenergic vasoconstrictor sensitivity and reduced β -adrenergic vasodilation²³ as well as impaired endothelium-dependent vasodilation.²⁴ Moreover, urbanized Africans show greater total peripheral resistance reactivity than rural Africans,²⁵ suggesting that factors accompanying urbanization, such as obesity, may further contribute to the observed ethnic differences in cardiovascular reactivity. Leptin, secreted by adipose tissue, is known to increase blood pressure through sympathetically mediated adrenergic activation.⁹ It therefore seems plausible that hyperleptinemia, as seen in Africans, may induce a state of sympathetic hyperactivity and thereby enhance responsiveness to stress.

Our results are in line with previous studies^{14,22} indicating greater cardiovascular reactivity in Africans. During rest, Africans had higher mean arterial pressure and cardiac output than Caucasians, but they had a similar mean stroke volume and total peripheral resistance. However Africans demonstrate significantly greater reductions in cardiac output- and arterial compliance reactivity, as well as greater increases in total peripheral resistance reactivity at 5 minutes post-stressor (Figure 1). Of note, when investigating associations between leptin, cardiovascular reactivity to stress and post-stressor recovery, the associations were mostly evident at 5 minutes post-stressor. This points to a potential role of elevated leptin and the

observed ethnic differences in cardiovascular recovery to stress. In Africans, we found a positive association of total peripheral resistance, and negative associations of arterial compliance as well as cardiac output with leptin. On the other hand, diastolic blood pressure associated positively with leptin in the Caucasians. These associations reveal that elevated leptin may augment sympathetically mediated vascular responses induced by stress. This is characterized by vascular α -adrenergic activation that leads to increased total peripheral resistance, as well as reduced compliance and β -adrenergic responsiveness of the heart which leads to a reduction in cardiac output.^{26,27} This transition is seen in patients with established hypertension, and with structural cardiac and vascular changes.²⁸

Physiological or emotional stress activates the sympathetic nervous system and hypothalamo-pituitary-adrenal axis to adapt and maintain stability.⁵ The cold pressor test activates the sympathetic nervous system and causes predominantly α -adrenergic vasoconstrictive effects, which consequently increases total peripheral resistance, diastolic blood pressure and decreases arterial compliance.^{17,29} In addition to stress, there are numerous studies supporting a role of leptin in sympathetic nervous system activation.^{9,30} Sympathetic activation further impairs endothelial-mediated vasodilation through mechanisms related to α -adrenergic activation.³¹ This is supported in a study of apparently healthy men and women, which showed mental stress to be associated with impaired flow-mediated vasodilation.³² In wild-type mice, leptin also induced endothelial dysfunction through mechanisms similar to sympathetic nervous system activation.³³ Leptin therefore also acts directly on receptors situated on the endothelium to promote endothelial and smooth muscle cell proliferation.³⁴ Furthermore, leptin upregulates endothelin-1 production in endothelial cells *in vitro*.³⁵ It is therefore possible that leptin contributes to enhanced cardiovascular reactivity to stress or impaired post-stress recovery through mechanisms related to sympathetic activation and endothelial dysfunction.

Regarding our finding that mainly cardiovascular reactivity 5 minutes post-stressor was associated with leptin - and not during or after the stressor at 1 or 3 minutes recovery - we could

speculate that elevated leptin contributes to impaired post-stress recovery rather than heightened reactivity responses during stress. Sustained or inefficient inactivation of the sympathetic nervous system after stress leads to allostatic load or the wear and tear of multiple systems, which include the cardiovascular system.³⁶ Results from a meta-analysis suggest that greater reactivity to stress as well as impaired post-stress recovery predict hypertension and increased carotid intima-media thickness.³⁷ Additionally, the associations between cardiovascular reactivity and leptin were mainly evident in the African group, implying that higher leptin levels of Africans may be responsible for the observed ethnic differences. Higher leptin levels in Africans may be attributed to the presence of more subcutaneous fat³⁸ which produces more leptin than visceral fat compartments.³⁹

The following limitations of our study must be noted. Firstly, we cannot eliminate the possibility that our results were due to residual confounding. The cross-sectional nature of this study also restricts us from drawing any causal conclusions. Future follow-up studies are therefore warranted to determine if these results can be extrapolated to the general population. Lastly, the stress assessment was carried out only once and the duration of the stressor may not have been adequate to demonstrate ethnic differences. Despite these limitations, strengths of this study include a homogenous sample with regard to socioeconomic status, seeing that lower socioeconomic status is known to be associated with impaired post-stress recovery.⁴⁰ This study was well-designed and the necessary care was taken to conduct the study under controlled conditions.

In conclusion, elevated leptin is associated with prolonged cardiovascular recovery to stress in Africans. This may potentially explain why Africans, who have higher leptin than Caucasians, are at increased risk for developing hypertension. These results suggest that hyperleptinemia may potentiate the cardiovascular responses to stress exposure and thereby contribute to increased cardiovascular risk.

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

Disclosure

The authors declare no conflict of interest.

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CHAPTER 5

GENERAL FINDINGS, RECOMMENDATIONS AND CONCLUSIONS

1. INTRODUCTION

The main findings of the separate research articles included in this thesis are summarised in this chapter. The main results of each manuscript are discussed according to the hypotheses as set in Chapter 1. The results were also interpreted with reference to the literature in order to draw conclusions. Potential factors which may have influenced the results are also mentioned. Recommendations are made for investigators with a similar research interest.

2. SUMMARY OF THE MAIN FINDINGS

Chapter 2 - Autonomic activity and leptin in Africans and Caucasians: The SABPA study

Leptin may contribute to obesity-related hypertension through sympathetic nervous system activation. Therefore, leptin levels and markers of autonomic activity were compared and associations thereof were investigated in Africans and Caucasians. Africans with higher leptin also had higher ambulatory blood pressure and heart rate compared to Caucasians. Africans also demonstrated lower heart rate variability, as reflected by the total power and triangular index, which is indicative of sympathetic overactivity. Several independent associations between leptin and markers of autonomic activity were found after full adjustment in multiple regression analyses in both the Africans and Caucasians. In African men, positive associations of renin and heart rate and negative associations of heart rate variability with leptin were observed. In Caucasian men, ambulatory heart rate correlated positively and heart rate variability correlated negatively with leptin. In African women, only a negative association between the heart rate variability total power component and leptin was evident. No significant associations between leptin and markers of autonomic activity were seen in the Caucasian women.

Hypotheses as stated in Chapter 1

- *Africans will have higher leptin levels and sympathetic overactivity compared to Caucasians; and*
- *Leptin associates with markers reflecting autonomic dysfunction in Africans.*

The first hypothesis is accepted. Africans had higher leptin levels and sympathetic overactivity compared to Caucasians. The second hypothesis is partially accepted, since leptin associated with markers reflecting sympathetic overactivity in both African and Caucasian men. However, in women, there were no independent associations in Caucasians.

Chapter 3 - Leptin links with plasminogen activator inhibitor-1 in human obesity: the SABPA study

Leptin may induce endothelial dysfunction and thereby contribute to increased cardiovascular disease risk. We therefore investigated associations of leptin with circulating markers of endothelial dysfunction namely, plasminogen activator inhibitor-1, von Willebrand factor and albuminuria in lean and obese groups separately. There were no ethnic differences for the associations between leptin and markers of endothelial dysfunction. Therefore, we did not divide the study population according to ethnicity. The obese group had higher leptin and plasminogen activator inhibitor-1, whereas von Willebrand factor and the albumin-to-creatinine ratio did not differ. In single regression analyses, all markers of endothelial dysfunction correlated positively with leptin in the obese group. However, after adjusting for ethnicity, gender and age only the association between leptin and plasminogen activator inhibitor-1 remained. This association remained even after adjusting for waist-to-height ratio and blood pressure in multiple regression analyses.

Hypothesis as stated in Chapter 1

- *Leptin is positively associated with PAI-1, vWF and ACR in both Africans and Caucasians.*

The hypothesis is partially accepted. An independent association between leptin and plasminogen activator inhibitor was found only in the obese Africans and Caucasians. Leptin did not correlate with von Willebrand factor and albuminuria in either the lean or obese groups.

Chapter 4 - Leptin relates to prolonged cardiovascular recovery after acute stress in Africans: the SABPA study

Heightened cardiovascular reactivity and delayed recovery to stress is associated with increased cardiovascular disease risk. The aim of this study was to investigate whether leptin relates to cardiovascular reactivity and recovery to acute stress in Africans and Caucasians. Africans had higher leptin and baseline blood pressure compared to Caucasians. Cardiovascular reactivity was compared between Africans and Caucasians during stressor application and after the stressor at 1, 3, and 5 minutes. Differences in the cardiovascular response to stress were mostly evident 5 minutes post-stressor. Africans demonstrated greater increases in total peripheral resistance and reductions in cardiac output and arterial compliance. After adjusting for age, sex and body mass index, associations between cardiovascular reactivity and leptin were demonstrated in Africans during the stressor and at 5 minutes post-stressor. In Caucasians, associations were found at 1 and 3 minutes post-stressor. After full adjustment, associations were mainly evident 5 minutes post-stressor in Africans, where total peripheral resistance correlated positively and cardiac output and arterial compliance correlated negatively with leptin.

Hypothesis as stated in Chapter 1

- *Higher leptin in Africans is associated with heightened cardiovascular reactivity and prolonged recovery to an acute stressor.*

Higher leptin was related to delayed recovery after stress in both Africans and Caucasians, but associations were mostly evident in the Africans. We therefore partially accept the hypothesis.

3. DISCUSSION OF MAIN FINDINGS

The results obtained in this study add to current literature demonstrating a role of leptin in the development of cardiovascular disease.¹ Increased sympathetic nervous system activation is probably the most evidenced mechanism implicating leptin in cardiovascular disease development. Experimental animal studies confirm the sympathetically-mediated cardiovascular actions of leptin.¹ Leptin administered to Sprague-Dawley rats increased sympathetic nerve activity to brown adipose tissue, the kidney and adrenal gland.² Adrenergic blockade in Sprague-Dawley rats abolished the effect of leptin infusion on increasing mean arterial pressure and heart rate.³ Injection of leptin into the hypothalamus of rats caused increase in heart rate and blood pressure.⁴ Moreover, leptin correlated positively with muscle sympathetic nerve activity in 37 healthy men⁵ and in hypertensive patients, and those with higher leptin also had faster heart rates.⁶ Moreover, low sympathetic tone is reported in leptin deficient humans.⁷

Our results support these previous studies by showing independent associations between leptin and markers reflecting sympathetic activation. The positive association between leptin and renin is consistent with other smaller studies (n=33)⁸ and (n=23)⁹ which included hypertensive patients. Stimulation of renal sympathetic nerves results in renin secretion,¹⁰ and our result therefore suggests that leptin may induce renal sympathetic nerve activation. Furthermore, we showed that leptin is related to a reduction in heart rate variability, suggesting an autonomic imbalance through sympathetic overactivity.¹¹ Reduced heart rate variability is associated with end-organ damage¹² as well as all-cause mortality.¹¹ Additionally, positive associations were obtained between leptin and ambulatory heart rate measurements in this study. Previous studies showed similar results but short-term heart rate recordings were obtained.^{6,13} The associations between leptin and autonomic activity markers were mostly evident in the men from both ethnic groups. It seems that the adverse sympathetically-mediated effects of leptin may be more apparent in men. This is supported by a multi-ethnic study that showed associations between leptin and hypertension to be more prominent in men.¹⁴

Exploratory univariate analyses were performed to establish whether the markers reflecting autonomic activity were indeed related to 24 h systolic blood pressure. In both the Africans and Caucasians, leptin and 24 h heart rate correlated positively and heart rate variability negatively with 24 h systolic blood pressure. These results not only support the role of leptin in sympathetic activation, but also point to a potential mechanistic link whereby hyperleptinemia may contribute to hypertension development.

Leptin also contributes to endothelial dysfunction through mechanisms related to sympathetic nervous system activation.¹⁵ Endothelial dysfunction is often seen in the presence of hypertension¹⁶ and is the initial step in the development of atherosclerosis.¹⁷ The endothelium plays a vital role in the maintenance of vascular tone by balancing vasodilation and vasoconstriction.¹⁸ A dysfunctional endothelium induces vasoconstriction as well as a pro-thrombotic and pro-inflammatory state which increases cardiovascular risk.¹⁸ It is suggested that leptin induces endothelial dysfunction through mechanisms related to sympathetic activation.¹⁵ Further, *in vitro* studies showed that leptin promotes endothelin-1 secretion¹⁹ and reactive oxygen species production in human endothelial cells.^{20,21} A study on Japanese women also showed an inverse association between leptin and flow-mediated dilation,²² suggesting that leptin affects nitric oxide-mediated and endothelium-dependent vasodilation.²³ However, endothelial dysfunction is not limited to impaired vasodilation, and we therefore investigated the relationship between leptin and circulating markers reflecting endothelial dysfunction and damage.

In this study population, leptin associated with plasminogen activator inhibitor-1 in the obese. Similarly, De Mitrio *et al*²⁴ demonstrated that leptin is associated with plasminogen activator inhibitor-1 in women, independently of adiposity. An *in vitro* study showed that leptin upregulates plasminogen activator inhibitor-1 expression in coronary artery endothelial cells.²⁵ However, we observed no associations between von Willebrand factor, albuminuria and leptin. This is contradictory to a study in older men (aged 60-79), showing a positive association

between leptin and von Willebrand factor.²⁶ This may be due to the fact that the authors failed to consider the confounding effects of blood pressure and inflammation. With regard to the lack of association between leptin and albuminuria, leakage of albumin will only occur at advanced stages of endothelial damage resulting in protein permeability. It is therefore plausible that leptin is not directly related to albuminuria, but rather other circulating markers, such as plasminogen activator inhibitor-1, which directly contribute to endothelial dysfunction. We speculate that hyperleptinemia induces endothelial changes which promote thrombotic vascular disease as indicated by the positive association with plasminogen activator inhibitor-1 in the obese.

It is possible that leptin, through mechanisms related to sympathetic nervous system activation^{27,28} and endothelial dysfunction,^{19,29} may contribute to heightened cardiovascular reactivity and prolonged recovery to stress. Prospective studies showed that heightened cardiovascular reactivity³⁰ and impaired recovery³¹ to stress associates with increases in blood pressure. Additionally, delayed recovery to stress also associates with increases in carotid-intima media thickness.³² It is well known that stress activates the sympathetic nervous system.³³ It therefore seems reasonable to assume that leptin may contribute to heightened and prolonged reactivity to stress through sympathetic overactivity. Ethnic differences in cardiovascular reactivity exists where Africans demonstrate higher cardiovascular reactivity to stress compared to Caucasians,^{34,35} mostly displaying increased α -adrenergic vasoconstriction and reduced β -adrenergic vasodilation.³⁶

In this study, greater cardiovascular reactivity to stress was indeed seen in Africans and it is therefore in agreement with previous studies.³⁴⁻³⁶ When comparing the cardiovascular reactivity during stressor application and post-stressor, it became evident that leptin was associated with reactivity mostly in Africans 5 minutes post-stressor. This result indicates a potential role of leptin in inducing a state of sympathetic overactivity which may lead to prolonged recovery to stress and increased cardiovascular risk, especially in Africans.

4. CHANCE AND CONFOUNDING

Potential factors which may contribute to the misinterpretation of the main results and lead to incorrect study conclusions must be mentioned. With regard to the study design, results were obtained in a target population with a similar socio-economic status and from the same urban area. It is therefore unknown whether similar results will be obtained in the general population. The cross-sectional design of this study also restricts us from assigning causality.

Incorrect statistical procedures may lead to the over- or underestimation of significance. For instance, standard multiple regression analyses can only determine linear relationships between dependent and independent variables. If the relationship is not linear, multiple regression analyses will underestimate the result. Even though we adjusted for covariates and confounders in multiple regression analyses, poor measurement of confounding variables will lessen their effect during adjustment and lead to residual confounding. We ensured that the study sample size is adequate to reduce the possibility of our results from being due to chance and random variation. The P value is an arbitrary value which indicates statistical significance and does not imply that the findings are of any physiological importance. However, it does indicate that differences were not simply due to chance.

Regardless of factors which might have contributed to chance and confounding, this was a well-designed study and measurements were conducted in a controlled overnight facility. In addition to the controlled environment, measurements were taken with validated apparatus and the necessary care was taken to ensure that high-quality blood samples were obtained.

5. RECOMMENDATIONS

The following recommendations may prove helpful to future researchers investigating the link between leptin and cardiovascular disease.

- Most previous studies focused mainly on Caucasians. Even though, we provide some insight into potential ethnic differences, future studies in Africans are needed to confirm our findings.
- The use of heart rate variability as a non-invasive measure of autonomic activity may not reflect sympathetic overactivity of the entire cardiovascular system. Therefore, it remains to be determined whether leptin directly stimulates sympathetic drive to the vasculature. Sympathetic nerve activity recorded by microneurography may be more reliable.
- Prospective studies are needed to establish the long term effects of leptin, especially in the development of end-organ damage and cardiovascular events. Results from these studies may support the use of leptin as a predictive marker of cardiovascular disease risk.
- Intervention studies where weight loss and lowering leptin in the obese translates into reduced cardiovascular disease risk could prove particularly valuable.
- Our findings need to be confirmed in larger randomised population studies.

6. CONCLUSIONS

Leptin is associated with markers reflecting sympathetic overactivity in both Africans and Caucasians. Leptin also associated with prolonged reactivity to stress, which was mostly evident in Africans who also had higher leptin levels. Hyperleptinemia may therefore induce a state of sympathetic overactivity that could impair cardiovascular recovery to stress. Leptin also associated with plasminogen activator inhibitor-1 in obese Africans and Caucasians, suggesting that leptin contributes to endothelial alterations related to thrombotic vascular disease, which is of particular relevance in Africans who are at greater cardiovascular risk. Together these findings support a role of leptin in the development of cardiovascular disease through mechanisms related to sympathetic activation and vascular damage. Adipose tissue mass is the most important determinant of leptin and obesity is reaching epidemic proportions. Considering this, the promotion of weight loss remains an important preventative measure to reduce cardiovascular disease risk in the obese.

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APPENDICES

Original Article

Autonomic activity and leptin in Africans and whites: the SABPA study

Chiné Pieterse, Rudolph Schutte, and Aletta E. Schutte

See editorial comment on page 738

Objectives: Evidence exists that leptin enhances sympathetic activity and may thereby contribute to the development of obesity-related hypertension. Sympathetic activation also seems more prominent in Africans than whites. We compared leptin levels, and different markers of autonomic activity between Africans and whites, and determined whether a relationship exists between leptin and autonomic activity.

Methods: The study included 409 African and white school teachers (aged, 44.6 ± 9.6 years). We determined leptin in serum and measured ambulatory blood pressure. Markers reflecting autonomic activity included renin, cortisol, baroreflex sensitivity, ambulatory heart rate and heart rate variability (HRV) components (assessed by 24-h ECG recordings in the frequency and geometric domain).

Results: Africans had higher leptin levels, BMI, blood pressure and heart rate (all $P < 0.001$) as well as lower HRV triangular index and HRV total power ($P < 0.001$). After also adjusting for BMI in multivariate regression analyses, in African men, renin ($\beta = 0.228$; $P = 0.033$), night-time heart rate ($\beta = 0.184$; $P = 0.034$), HRV triangular index ($\beta = -0.230$; $P = 0.010$) and HRV total power ($\beta = -0.214$; $P = 0.046$) associated with leptin. In white men, leptin associated with 24-h heart rate ($\beta = 0.376$; $P < 0.001$), as well as day and night-time heart rate (both $P < 0.01$), HRV triangular index ($\beta = -0.335$; $P < 0.001$) and HRV total power ($\beta = -0.403$; $P < 0.001$). In African women, we observed an association of leptin with the total power component of HRV ($\beta = -0.221$; $P = 0.015$) and a borderline association with renin ($\beta = 0.219$; $P = 0.057$). No significant associations were apparent in the white women.

Conclusion: We found that leptin is independently associated with different markers of autonomic activity, especially in men.

Keywords: autonomic nervous system, ethnicity and leptin, hypertension, sympathetic activity

Abbreviations: ABPM, ambulatory blood pressure measurement; BRS, baroreflex sensitivity; HF, high frequency; HRV, heart rate variability; LF, low frequency; Std β , standardised β

INTRODUCTION

Hypertension and obesity prevalence rates are increasing in sub-Saharan Africa largely due to Westernisation [1,2]. Changes in lifestyle such as over-nutrition and reduced physical activity results from this transition [3]. Obesity leads to diabetes [4] and hypertension, and therefore increased cardiovascular risk [5]. Experimental studies indicate that leptin, produced in adipose tissue, is one of the links between obesity and hypertension and is believed to do this through sympathetic activation, which is a common characteristic of obesity [6].

Leptin binds to the long form receptors (OB-Rb) located in the hypothalamus to reduce appetite and increase sympathetic nerve activity [7,8]. Animal studies demonstrate that leptin increases sympathetic nerve activity in a dose-dependent manner [9], which may lead to autonomic imbalance. Population studies indicate that autonomic imbalance characterised by elevated sympathetic nervous system activity is associated with cardiovascular morbidity and mortality [10]. Furthermore, such activation initiates and maintains hypertension with multiple mechanisms, which take place in the vasculature or kidney [11,12]. We evaluated various markers of autonomic activity, each with their own strengths and weaknesses with regard to invasiveness, sensitivity and reproducibility [13].

Both the prevalence of hypertension [1] and circulating leptin levels are higher in Africans than whites [14]. We therefore investigated ethnic differences in leptin levels and autonomic activity, and determined whether leptin is related to measures of autonomic activity as reflected by renin, cortisol, baroreflex sensitivity and measures of heart rate variability.

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ORIGINAL ARTICLE

Leptin links with plasminogen activator inhibitor-1 in human obesity: the SABPA study

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The relationship between obesity and the development of cardiovascular disease is well established. However, the underlying mechanisms contributing to vascular disease and increased cardiovascular risk in the obese remain largely unexplored. Since leptin exerts direct vascular effects, we investigated leptin and the relationship thereof with circulating markers of vascular damage, namely plasminogen activator inhibitor-1 antigen (PAI-1_{ag}), von Willebrand factor antigen (vWF_{ag}) and urinary albumin-to-creatinine ratio (ACR). The study included a bi-ethnic population of 409 African and Caucasian teachers who were stratified into lean (<0.5) and obese (≥0.5) groups according to waist-to-height ratio. We obtained ambulatory blood pressure measurements and determined serum leptin levels, PAI-1_{ag}, vWF_{ag} and ACR, as markers of vascular damage. The obese group had higher leptin ($P < 0.001$) and PAI-1_{ag} ($P < 0.001$) levels and a tendency existed for higher vWF_{ag} ($P = 0.068$). ACR did not differ between the two groups ($P = 0.21$). In single regression analyses positive associations existed between leptin and all markers of vascular damage (all $P < 0.001$) only in the obese group. After adjusting for covariates and confounders in multiple regression analyses, only the association between leptin and PAI-1_{ag} remained ($R^2 = 0.440$; $\beta = 0.293$; $P = 0.0021$). After adjusting for gender, ethnicity and age, additional analyses indicated that leptin also associated with fibrinogen and clot lysis time in both lean and obese groups, which in turn is associated with 24-h blood pressure and pulse pressure. This result provides evidence that elevated circulating leptin may directly contribute to vascular damage, possibly through mechanism related to thrombotic vascular disease.

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INTRODUCTION

The prevalence of obesity is increasing in sub-Saharan Africa.¹ This increase may be attributed to Westernization which usually coincides with unhealthy dietary habits and a sedentary lifestyle.² Obesity often predisposes the development of hypertension,³ and obesity-related hypertension is now considered a distinct hypertensive phenotype.⁴ Endothelial damage is one of many mechanisms linking obesity to cardiovascular disease⁵ and *in vitro* and *in vivo* studies suggest that leptin may be involved.^{6,7}

Physiological leptin levels promote nitric oxide production and endothelium-dependent relaxation by inducing nitric oxide synthase expression.⁸ Contrastingly, in pathological conditions such as obesity and related hyperleptinemia, the leptin-mediated production of nitric oxide seems impaired and thereby contributes to endothelial dysfunction and damage.^{6,9} Assessment of circulating biomarkers of vascular damage may provide valuable information of the mechanisms at work. A damaged endothelium promotes the secretion of pro-thrombotic factors such as plasminogen activator inhibitor-1¹⁰ and von Willebrand factor¹¹ and results in leakage of albumin in urine as reflected by the urinary albumin-to-creatinine ratio.¹²

We therefore investigated leptin and the associations thereof with plasminogen activator inhibitor-1, von Willebrand factor and albumin-to-creatinine ratio as markers of vascular damage in a bi-ethnic sample of 409 teachers.

METHODS

Study design

This study forms part of the Sympathetic activity and Ambulatory Blood Pressure in Africans study, which included 409 African and Caucasian school teachers working in the Potchefstroom district in the North-West Province of South Africa. The reason for the selection of this target population was to obtain a homogenous sample of participants from a similar socioeconomic class. Participants between the ages of 25 and 60 years were included. The exclusion criteria were a tympanic temperature above 37°C, psychotropic substance dependence or abuse, regular blood donors and individuals vaccinated in the past three months. Participants received detailed information about the procedures and objectives of the study prior to their recruitment. All participants signed an informed consent form. The study complied with all applicable requirements and international regulations, including the Helsinki declaration of 1975 (as revised in 2008) for investigation of human participants.

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Appendix C: Supplementary Tables – Article 1

Supplementary Table S1 Unadjusted associations between markers of autonomic activity and leptin.

	African men n=101	African women n=99	Caucasian men n=101	Caucasian women n=108
Age, years	r = 0.26; P = 0.009	r = 0.17; P = 0.086	r = 0.06; P = 0.95	r = -0.09; P = 0.34
Body mass index, kg/m ²	r = 0.81; P < 0.001	r = 0.70; P < 0.001	r = 0.77; P < 0.01	r = 0.69; P < 0.001
Plasma renin, pg/ml	r = 0.21; P = 0.052	r = 0.21; P = 0.070	-	-
Serum cortisol, nmol/l	r = -0.12; P = 0.25	r = -0.03; P = 0.79	r = 0.04; P = 0.68	r = -0.17; P = 0.085
24 h Heart rate, bpm	r = 0.23; P = 0.023	r = 0.02; P = 0.84	r = 0.42; P < 0.001	r = 0.37; P < 0.001
Daytime heart rate, bpm	r = 0.18; P = 0.064	r = -0.04; P = 0.72	r = 0.41; P < 0.001	r = 0.32; P = 0.001
Nighttime heart rate, bpm	r = 0.31; P = 0.002	r = 0.14; P = 0.17	r = 0.34; P < 0.001	r = 0.45; P < 0.001
BRS, ms/mmHg	r = -0.081; P = 0.43	r = -0.15; P = 0.13	r = -0.04; P = 0.72	r = 0.02; P = 0.81
24 h HRV triangular index	r = -0.37; P < 0.001	r = -0.16; P = 0.12	r = -0.39; P < 0.001	r = -0.35; P < 0.001
24 h HRV LF, n.u.	r = 0.15; P = 0.13	r = -0.10; P = 0.34	r = -0.05; P = 0.60	r = -0.04; P = 0.67
24 h HRV HF, n.u.	r = -0.14; P = 0.17	r = 0.02; P = 0.87	r = 0.08; P = 0.43	r = 0.04; P = 0.67
24 h HRV LF/HF	r = 0.18; P = 0.080	r = 0.02; P = 0.87	r = -0.01; P = 0.91	r = 0.003; P = 0.98
24 h HRV Total power	r = -0.38; P < 0.001	r = -0.12; P = 0.24	r = -0.46; P < 0.001	r = -0.03; P = 0.77

Abbreviations: BRS, baroreflex sensitivity; HRV, heart rate variability; LF, low frequency; HF, high frequency.

Supplementary Table S2 Leptin levels and autonomic activity markers of normotensive and hypertensive women.

	African women (n=99)			Caucasian women (n=108)		
	Normotensive (42%)	Hypertensive (58%)	P	Normotensive (70%)	Hypertensive (30%)	P
Leptin, ng/ml	54.9 (23.6 - 96.4)	54.9 (22.5 - 109.4)	0.99	18.2 (4.9 - 55.8)	29.5 (11.4 - 72.9)	0.002
Plasma renin*, pg/ml	4.1 ± 2.1	3.6 ± 2.6	0.32	-	-	-
Serum cortisol, nmol/l	337.9 ± 157.9	315.0 ± 138.6	0.45	381.5 ± 168.2	360.6 ± 164.2	0.56
24 h Heart rate, bpm	81 ± 9	80 ± 11	0.74	74 ± 8	79 ± 11	0.002
Daytime heart rate, bpm	85 ± 9	84 ± 12	0.48	78 ± 9	83 ± 10	0.010
Nighttime heart rate, bpm	74 ± 9	71 ± 18	0.29	65 ± 7	72 ± 12	< 0.001
BRS, ms/mmHg	8.05 (2.18 - 19.59)	8.75 (3.18 - 24.66)	0.53	9.38 (3.97 - 22.91)	9.16 (4.42 - 15.78)	0.82
24 h HRV triangular index	31.0 ± 9.4	28.1 ± 10.0	0.15	39.1 ± 8.6	31.1 ± 9.1	< 0.001
24 h HRV LF, n.u.	63.7 ± 11.7	59.6 ± 12.8	0.12	69.3 ± 10.7	70.6 ± 12.2	0.56
24 h HRV HF, n.u.	32.9 ± 10.8	35.2 ± 10.5	0.31	28.6 ± 10.1	26.5 ± 10.6	0.33
24 h HRV LF/HF	2.4 ± 2.0	2.0 ± 1.0	0.12	2.8 ± 1.4	3.4 ± 2.2	0.093
24 h HRV Total power	2593.7 ± 1676.3	2399.7 ± 2026.0	0.62	3641.2 ± 2370.8	2249.7 ± 1216.8	0.002

Values are arithmetic mean ± SD, geometric mean (5 - 95th percentile interval), or number of participants (%). Abbreviations: BRS, baroreflex sensitivity; HRV, heart rate variability; LF, low frequency; HF, high frequency.

* Only in Africans

Supplementary Table S3 Leptin levels and autonomic activity markers of normotensive and hypertensive men.

	African men (n=101)			Caucasian men (n=101)		
	Normotensive (21%)	Hypertensive (79%)	P	Normotensive (31%)	Hypertensive (69%)	P
Leptin, ng/ml	5.9 (1.8 - 22.6)	12.3 (2.9 - 43.7)	0.002	5.4 (1.1 - 19.1)	8.9 (2.5 - 38.0)	0.008
Plasma renin*, pg/ml	4.4 ± 1.9	4.6 ± 3.0	0.71	-	-	-
Serum cortisol, nmol/l	366.3 ± 93.6	370.1 ± 136.4	0.90	357.1 ± 151.3	395.1 ± 133.1	0.21
24 h Heart rate, bpm	76 ± 8	80 ± 12	0.16	67 ± 11	74 ± 11	0.002
Daytime heart rate, bpm	83 ± 10	85 ± 13	0.45	71 ± 11	80 ± 12	< 0.001
Nighttime heart rate, bpm	65 ± 9	72 ± 12	0.013	59 ± 10	64 ± 11	0.043
BRS, ms/mmHg	7.33 (3.40 - 20.18)	7.50 (2.76 - 19.28)	0.88	7.35 (3.06 - 25.18)	9.14 (3.67 - 26.92)	0.11
24 h HRV triangular index	37.2 ± 11.6	30.0 ± 12.5	0.018	46.0 ± 12.6	35.7 ± 11.4	< 0.001
24 h HRV LF, n.u.	70.9 ± 12.6	69.4 ± 11.4	0.61	74.5 ± 10.3	77.9 ± 8.7	0.099
24 h HRV HF, n.u.	26.9 ± 11.0	27.7 ± 10.2	0.76	23.1 ± 9.1	20.3 ± 8.4	0.14
24 h HRV LF/HF	3.2 ± 1.6	3.1 ± 1.7	0.71	3.9 ± 1.9	4.7 ± 2.6	0.11
24 h HRV Total power	6338.4 ± 3385.6	4167.0 ± 2651.8	< 0.001	6338.4 ± 3385.6	4167.0 ± 2651.8	< 0.001

Values are arithmetic mean ± SD, geometric mean (5 - 95th percentile interval), or number of participants (%). Abbreviations: BRS, baroreflex sensitivity; HRV, heart rate variability; LF, low frequency; HF, high frequency.

* Only in Africans

Supplementary Table S4 Independent associations between markers of autonomic activity and leptin.

Dependent variables	Men (n=202)				Women (n=207)			
	R ²	Adjusted R ²	Std β (95 % CI)	P	R ²	Adjusted R ²	Std β (95 % CI)	P
Plasma renin, pg/ml	-	-	-		-	-	-	
Serum cortisol, nmol/l	0.069	0.039	N/S		0.089	0.070	N/S	
24 h Heart rate, bpm	0.317	0.287	0.228 (0.101 to 0.355)	< 0.001	0.242	0.209	N/S	
Daytime heart rate, bpm	0.273	0.242	0.218 (0.087 to 0.349)	0.0012	0.190	0.165	N/S	
Nighttime heart rate, bpm	0.356	0.328	0.196 (0.073 to 0.319)	0.002	0.142	0.128	0.316 (0.166 to 0.466)	< 0.001
BRS, ms/mmHg	0.012	0.007	N/S		0.050	0.030	N/S	
24 h HRV triangular index	0.351	0.330	-0.284 (-0.407 to -0.161)	< 0.001	0.292	0.266	N/S	
24 h HRV LF, n.u.	0.172	0.136	N/S		0.144	0.131	N/S	
24 h HRV HF, n.u.	0.164	0.132	N/S		0.142	0.124	N/S	
24 h HRV LF/HF	0.169	0.152	N/S		0.172	0.150	N/S	
24 h HRV Total power	0.366	0.343	-0.325 (-0.448 to -0.202)	< 0.001	0.144	0.121	N/S	

Standardised β (std β) reflects the change in the dependent variable for 1 SD change in the independent variable. A larger std β reflects greater strength of the association. Adjusted for age, body mass index, 24 h mean arterial pressure, total cholesterol, glucose, smoking, gamma-thyroid stimulating hormone, physical activity level, anti-hypertensive medication and ethnicity.

Abbreviations: BRS, baroreflex sensitivity; HRV, heart rate variability; LF, low frequency; HF, high frequency.

Appendix D: Supplementary Table – Article 2

Supplementary Table S1 Independent associations between markers of vascular alterations and leptin in Africans and Caucasians, separately.

	Obese Africans			Obese Caucasians		
	PAI-1, ng/mmol	von Willebrand factor, %	ACR, mg/mmol	PAI-1, ng/mmol	von Willebrand factor, %	ACR, mg/mmol
R ²	0.119	0.118	0.127	0.274	0.111	0.348
Adjusted R ²	0.037	0.035	0.045	0.198	0.018	0.279
	Std β (95 % CI)	Std β (95 % CI)	Std β (95 % CI)	Std β (95 % CI)	Std β (95 % CI)	Std β (95 % CI)
Leptin, ng/ml	0.303 (-0.016 to 0.622)*	0.159 (-0.160 to 0.478)	-0.016 (-0.333 to 0.301)	0.321 (0.011 to 0.631)†	-0.017 (-0.360 to 0.326)	0.196 (-0.098 to 0.490)
Gender (0,1)	-0.307 (-0.623 to 0.009)	-0.090 (-0.405 to 0.225)	-0.075 (-0.388 to 0.238)	-0.211 (-0.534 to 0.112)	0.200 (-0.157 to 0.557)	0.211 (-0.095 to 0.517)
Age, years	-0.003 (-0.179 to 0.173)	0.130 (-0.048 to 0.308)	0.049 (-0.127 to 0.225)	-0.020 (-0.204 to 0.164)	0.127 (-0.076 to 0.330)	0.237 (0.064 to 0.409)‡
WHtR	-0.116 (-0.322 to 0.311)	-0.076 (-0.281 to 0.129)	0.029 (-0.174 to 0.411)	0.018 (-0.231 to 0.267)	0.030 (-0.246 to 0.306)	0.028 (-0.207 to 0.263)
24 h SBP, mmHg	-0.070 (-0.262 to 0.122)	-0.008 (-0.200 to 0.184)	0.207 (0.016 to 0.397)†	-0.039 (-0.223 to 0.145)	0.065 (-0.139 to 0.269)	0.254 (0.079 to 0.428)‡
TC:HDL, mmol/l	-0.057 (-0.243 to 0.129)	0.021 (-0.165 to 0.207)	-0.059 (-0.245 to 0.127)	0.221 (0.025 to 0.417)†	0.008 (-0.210 to 0.226)	-0.187 (-0.373 to -0.001)
Serum glucose, mmol/l	0.222 (0.037 to 0.406)†	0.062 (-0.122 to 0.246)	0.045 (-0.139 to 0.229)	0.185 (0.005 to 0.365)†	-0.160 (-0.360 to 0.040)	-0.118 (-0.289 to 0.053)
C-reactive protein, mg/l	-0.139 (-0.335 to 0.057)	0.211 (0.013 to 0.409)†	0.016 (-0.180 to 0.212)	-0.099 (-0.303 to 0.105)	-0.016 (-0.243 to 0.111)	0.003 (-0.191 to 0.197)
Smoking (0,1)	0.111 (-0.089 to 0.275)	-0.014 (-0.196 to 0.168)	0.025 (-0.156 to 0.212)	-0.013 (-0.176 to 0.149)	-0.029 (-0.209 to 0.151)	-0.027 (-0.180 to 0.126)
GGT, U/L	0.074 (-0.116 to 0.287)	-0.134 (-0.324 to 0.056)	-0.216 (-0.406 to -0.026)†	0.178 (-0.012 to 0.368)	0.032 (-0.179 to 0.243)	-0.013 (-0.193 to 0.167)
Physical activity (0,1,2)	-0.094 (-0.266 to 0.078)	0.132 (-0.040 to 0.304)	-0.035 (-0.207 to 0.137)	-0.063 (-0.224 to 0.098)	-0.083 (-0.261 to 0.095)	-0.061 (-0.212 to 0.090)
Anti-hypertensive medication (0,1)	0.023 (-0.155 to 0.201)	0.180 (0.002 to 0.364)†	-0.198 (-0.374 to -0.022)	0.167 (-0.004 to 0.338)	-0.153 (-0.519 to 0.213)	-0.005 (-0.168 to 0.158)

Standardised β (std β) reflects the change in the dependent variable for 1 SD change in the independent variable. A larger std β reflects greater strength of the association.

Abbreviations: WHtR, waist-to-height ratio; PAI-1, plasminogen activator inhibitor-1; ACR, albumin-to-creatinine ratio; SBP, systolic blood pressure; GGT, gamma-glutamyl transferase.