Inflammatory mediators and the cardiovascular profile of young South Africans: The African-PREDICT study

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

Preface

This thesis is presented in article format in accordance with the guidelines of the North-West University, and consists of six chapters that are outlined below. All articles in this thesis (Chapters 3-5) were either published or submitted for publication in international peer-reviewed journals at the time of submission for examination.

Chapter layout of thesis:

Chapter 1: Background, Motivation and Literature Overview

Chapter 2: Methodology

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Chapter 4: Inflammation and salt in young adults: The African-PREDICT study

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Chapter 6: Summary, Conclusion and Recommendations Proposed for Future Studies

Author Contributions

Miss SH Crouch

Ms Crouch conducted the literature search and proposed the design of each of the research articles. She wrote the proposal for the PhD. She completed an ethics application for this sub-study as part of the African-PREDICT study. Ms Crouch conducted an in-depth literature review and contributed to data collection within the Hypertension Clinic as well as biochemical analyses of urine and serum samples as a postgraduate student. She performed the statistical analyses, interpreted the results for each research article, and wrote each article and the PhD thesis as a whole.

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PhD promoter and principal investigator of the African-PREDICT study. She contributed to the supervision and guidance of all aspects of this study. She contributed to the funding applications, statistical analyses, interpretation of results, critical evaluation of each research article and gave intellectual input throughout.

Doctor S Le Roux

PhD co-promoter. She contributed to the supervision and guidance. Additionally, to statistical analyses, interpretation of results, critical evaluation of each research article and thesis as well as intellectual input.

Professor C Delles

Co-author of all research articles. He contributed to the statistical analyses, arranging biochemical analyses, interpretation of results, as well as critically evaluation and intellectual input on each research article.

Doctor LA Graham

Co-author of research articles**.** She contributed to the critical evaluation of each research article and biochemical sample analyses of the multiplex data.

Statement by authors

The following is a statement by the co-authors confirming their individual roles in this study and giving their permission that the research articles may form part of this thesis:

Hereby, I declare that I approved the aforementioned research articles and that my role in this study, as stated above, is representative of my actual contribution. I also give my consent that these research articles may be published as part of this PhD thesis.

Professor Aletta E Schutte

Doctor Shani Le Roux

Professor Christian Delles

Doctor Lesley A Graham

Publications and Conferences

Publications resulting from this thesis

Crouch SH, Botha-le Roux S, Delles C, Graham LA, Schutte AE. Inflammation and salt in young adults: The African-PREDICT study. Eur J Nutr 2020. doi: 10.1007/s00394-020-02292-3.

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Other publications by the student:

Woodiwiss AJ, Kruger R, Norton GR, Schutte AE, Vally M, Da Silva Fernandes D, Masiu M, Kolkenbeck-Ruh A, Gafane-Matemane LF, Ahiante BO, Phalane E, **Crouch SH**, Beaney T, Xia X, Poulter NR. May Measurement Month 2017: an analysis of blood pressure screening results in South Africa—Sub-Saharan Africa. Eur Heart J Supplements. 2019;21(Supplement_D):D104-D106. doi: 10.1093/eurheartj/suz069.

Crouch SH, Ware LJ, Gafane-Matemane LF, Kruger HS, Van Zyl T, Van der Westhuizen B, Schutte AE. Dietary sodium intake and its relationship to adiposity in young black and white adults: The African-PREDICT study. J Clin Hypertens. 2018;20(8):1193-1202. doi: 10.1111/jch.13329

Conferences

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Crouch SH, Botha-le Roux S, Delles C, Graham LA, Schutte AE. Inflammation and salt in young adults: The African-PREDICT study. Eur J Nutr 2020. *Accepted as an oral presentation at the joint European Society of Hypertension – International Society of Hypertension 2020 meeting that was to be held on 30 May-1 June 2020 in Glasgow, Scotland. The meeting was now postponed to 11-14 April 2021 due to the COVID-19 pandemic.*

Crouch SH, Botha-Le Roux S, Delles C, Graham LA, Schutte AE. Distinct inflammatory mediator patterns in young black and white adults: The African-PREDICT study. *Physiology Society of Southern Africa annual conference. East London, Eastern Cape, South Africa. 18-21 August 2019. *Oral Presentation and winner of the Johan Koeslag Award for Integrative Human Physiology.*

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Summary

Inflammatory mediators and the cardiovascular profile of young South Africans: The African-PREDICT study

Motivation: Cardiovascular disease (CVD) remains a leading cause of death, accounting for approximately 17.9 million deaths annually, with 75% occurring in low-and middle-income countries. A number of studies have implicated inflammation in the development of CVD. However, the majority of studies have focused on only a few well-known inflammatory mediators, such as C-reactive protein (CRP). The potential role of numerous other mediators in CVD remains largely unexplored. While many questions remain, a pattern has emerged suggesting that lifestyle behaviours, such as dietary salt or fruit and vegetable intake, contribute to the release of inflammatory mediators. This may result in changes in cardiovascular structure and function, potentially leading to the development of CVD. Even in the early phases of CVD development, raised levels of known inflammatory markers have been linked with changes in cardiovascular function such as raised blood pressure (BP). It is therefore important to explore the relationship between a detailed range of inflammatory mediators and measures of BP, sodium and potassium intake at a young age.

Aim: The central aim of this study was to present a detailed inflammatory mediator profile and describe how it relates to sodium and potassium excretion and the cardiovascular profile of young, healthy South African adults.

Methods: This study used data from the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT). Data was collected using standard procedures and included a demographic questionnaire, body composition, accelerometry data, cardiovascular measurements, as well as biochemical analyses of all relevant biomarkers, including the multiplex analysis of 22 inflammatory mediators. A sub-sample of the total baseline cohort was followed over 4.5 years. In Manuscript 1 we analysed data for participants who took part in the baseline phase of the

study (n=1202). Participants using anti-inflammatory medication or with missing biochemical analyses data were excluded, resulting in a population size of n=1189. In Manuscript 2 participants with missing sodium and potassium data were additionally excluded, resulting in a baseline population size of n=991. In Manuscript 3, data from the first 407 participants included in both the baseline and the follow–up phase of the study was analysed. Participants with missing ambulatory blood pressure data were excluded, resulting in a population size of n=358.

Statistical Analyses

Variables with non-Gaussian distributions were logarithmically transformed and interactions of sex as well as black and white ethnicity were investigated. Groups were compared using dependent and independent t-tests, Chi-square and McNemar tests. Factor analyses of the multiple inflammatory mediators were performed to identify clusters of inflammatory mediators using the factor function of SPSS. Pearson, partial and multivariable-adjusted regression analyses were used to determine associations.

Results and Conclusions:

In Manuscript 1, we determined how a detailed range of inflammatory mediators related to blood pressure. Due to interactions of ethnicity, we also compared inflammatory profiles between young black and white adults. For pro-inflammatory mediators, the black adults reflected higher C-reactive protein, interferon-inducible T-cell alpha chemoattractant, and macrophage inflammatory protein 3 alpha (all $p \leq 0.008$), but lower interferon-gamma, interleukin (IL)-1β, IL-8, IL-12, IL-17A, and tumour necrosis factor alpha (all $p \leq 0.048$) than the white adults. For anti-inflammatory mediators the black group reflected lower levels of IL-5, IL-10 and IL-13 (all $p \leq 0.012$)), resulting in generally higher pro-to-anti-inflammatory ratios in black than white adults ($p \leq 0.001$). In mediators with both pro- and anti-inflammatory functions, the black group reflected lower granulocyte-macrophage colony-stimulating factor and IL-6 (both $p \leq 0.010$). These patterns were confirmed when participants were stratified according to

hypertensive status, sex and socio-economic status. Numerous measures of BP differed significantly between black and white populations. However, no relationship was found between inflammatory mediators and BP.

In Manuscript 2, we investigated the relationships between inflammatory mediators and 24-hour urinary potassium. The black and white adults were stratified according to low, middle and high salt intake (sodium tertiles). No differences were seen in plasma concentrations of inflammatory mediators between the sodium tertiles in either the black or white groups. In multivariable-adjusted regression analyses in white adults, we found that K^+ associated negatively with the pro-inflammatory mediators IFN-γ, IL-7, IL-12, IL-17A, IL-23 and TNF-α (all $p \leq 0.046$), but only in the lowest Na^+ tertile. No associations were seen in the black group.

In Manuscript 3, we determined whether individual or clusters of inflammatory mediators from a large biomarker panel were associated with change in BP over 4.5 years. We identified three factors from factor analyses which each included different mediators. Factor 1 included Interferon-gamma, IL-4, IL-7, IL-10, IL-12, IL-17A, IL-21, IL-23, macrophage inflammatory protein (MIP)-1α, MIP-1β, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF); Factor 2 included IL-5, IL-6, IL-8, IL-13 and Factor 3 included CRP, IL-1β, IL-2, MIP-3α. In multivariable-adjusted regression analyses in the total cohort, percentage change in 24-hour systolic BP associated positively with Factor 1 and Factor 2. Change in daytime systolic BP associated positively with Factors 1, 2 and 3. Subgroup analyses found that these findings were limited to white study participants, despite the increase in BP over time seen mainly in black participants.

Conclusion:

Black and white ethnic groups each consistently presented with distinct inflammatory mediator patterns. Although BP in black participants increased significantly across 4.5 years, this was not associated with inflammation, as seen in the white group. We found multiple associations between inflammatory mediators and change in BP, as well as a protective anti-inflammatory

association with potassium in those with a low daily salt intake in the white population. These findings suggest that inflammation does play a role in BP, but in a young black population early changes in BP appear to be driven by other factors.

Key Words: Inflammation, blood pressure, ethnicity, African, sodium, potassium, salt.

List of Abbreviations

ABPM	Ambulatory blood pressure
AEE	Activity energy expenditure
African-PREDICT	African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension
BMI	Body mass index
BP	Blood pressure
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
eGFR	Estimated glomerular filtration rate
GBD	Global Burden of Disease study
GGT	Gamma-glutamyltransferase
GMCSF	Granulocyte-macrophage colony-stimulating factor
HART	Hypertension in Africa Research Team
HDL-C	High density lipoprotein cholesterol
HIV	Human immunodeficiency virus
IFN-γ	Interferon gamma
IL-1 β	Interleukin 1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6

IL-7	Interleukin 7
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-17A	Interleukin 17A
IL-21	Interleukin 21
IL-23	Interleukin 23
ITAC	Interferon-inducible T-cell alpha chemoattractant
K^+	Potassium
LDL-C	Low density lipoprotein cholesterol
MIP-1α	Macrophage inflammatory protein 1-alpha
MIP-1β	Macrophage inflammatory protein 1-beta
MIP-3α	Macrophage inflammatory protein 3-alpha
MMPs	Matrix metalloproteinases
Na^+	Sodium
NCDs	Non-communicable diseases
NF-κB	Nuclear factor *kappa B*
NRF	National Research Foundation
p38/MAPK	Mitogen activated protein kinase *p38*
REDCap	Research Electronic Data Capture
ROS	Reactive oxygen species

SAMRC	South African Medical Research Council
SARChI	South African Research Chairs Initiative
SASCO	South African Standard Classification of Occupation
SBP	Systolic blood pressure
SGK1	Serine/threonine-protein kinase
TEE	Total energy expenditure
TNFα	Tumour necrosis factor alpha
WHO	World Health Organisation
ΔBP	Change in blood pressure

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Supplementary Information

Chapter 1

Background, Motivation and Literature Overview

1. Introduction

South Africa has a diverse and rapidly urbanising population. As of 2019 South Africa's population is estimated at 58.7 million, of which approximately 47.4 million are black, 4.65 million white, 5.18 million coloured and 1.5 million Indian or Asian.[1] The average life expectancy of South Africans is 61.5 and 67.7 years for men and women respectively.[1] Death, as a result of non-communicable disease (NCDs), remains a growing concern. Historically, concerns relating to the burden of NCDs have mainly focused on high-income countries. Studies have shown that the burden of NCDs is growing in low- and middle-income countries.[2] In 2016 NCDs accounted for approximately 60% of deaths in South Africa.[3]

The 2017 Global Burden of Disease Study (GBD) found the top five NCDs-related causes of absolute risk-attributable disability-adjusted life-years were ischaemic heart disease, intracerebral haemorrhage, ischaemic stroke, chronic obstructive pulmonary disease and type 2 diabetes,[4] all of which form part of, or are closely associated with, cardiovascular disease (CVD).[5, 6] The World Health Organization (WHO) has found CVD as the leading cause of death, accounting for 17.9 million deaths annually (31% of all deaths),[7] with a third of deaths as a result of CVD occurring in individuals under the age of 70 years and 75% taking place in low-and middle-income countries.[7]

Despite recent efforts in the prevention and treatment of CVD, it remains the most common cause of hospitalisation in the western world.[8] With recent advances in understanding CVD development, several contributing factors have been identified. One of these identified factors is the role of immunity and inflammation.[9-13] Despite the traditional understanding that inflammatory mediators protect during illness and injury,[14] there has been an awakened interest in inflammation and its potential contribution to the development of CVD. This contribution is through its role in changes to structure and function of the cardiovascular system.[15-18] However, it remains unclear precisely which inflammatory mediators and mechanisms are involved.[9-13]

2. Inflammatory Mediators

'Cytokine' is the general term used to refer to lymphokines and monokines, which are produced by lymphocytes and monocytes, respectively.[19] Cytokines also include chemokines, which have chemotactic activities, and interleukins, which are produced by leukocytes and can act on other leukocytes.[19] Cytokines are thus proteins that control a wide range of biological functions (**Figure 1**) including immunity, cell repair and proliferation via extracellular signalling, functioning largely in a paracrine fashion.[20]

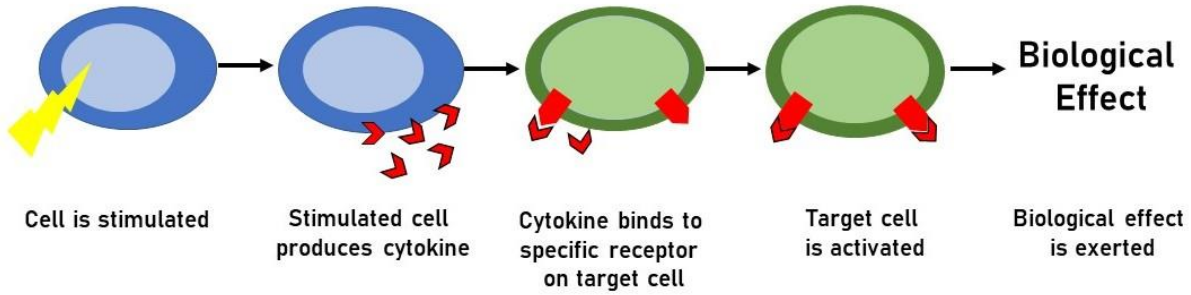

Figure 1. Cytokines mode of action. (Adapted from Testar.)[21]

A number of cytokines may be produced by multiple types of immune cell (e.g. interferon gamma may be produced by NK cells, B cells, as well as T helper cells),[22] the cytokines included in **Table 1** can all be produced by T cells and will be the focus of this PhD thesis.[23] C-Reactive protein (CRP), which is not classified as a cytokine, will also be included in this study, and henceforth these biomarkers will be referred to collectively as inflammatory mediators. It is, however, important to acknowledge that there are numerous other cytokines belonging to different cytokine families (**Table 2**) produced by different cells.

Table 1. Pro- and anti-inflammatory cytokines. (Compiled from Sochett *et al.*[18] and Moldoveanu *et al.*[24].)

Pro-inflammatory	Anti-inflammatory
• Fractalkine • Interferon Gamma (IFN-γ) • Interleukin 1-*beta* (IL-1 β) • Interleukin 2 (IL-2) • Interleukin 7 (IL-7) • Interleukin 8 (IL-8) • Interleukin 12 (IL-12) • Interleukin 17 A (IL-17A) • Interleukin 23 (IL-23) • Interferon-inducible T-cell alpha chemoattractant (ITAC) • Macrophage inflammatory protein *1-alpha* (MIP-1α) • Macrophage inflammatory protein *1-beta* (MIP-1β) • Macrophage inflammatory protein *3-alpha* (MIP-3α) • Tumour Necrosis Factor Alpha (TNF-α)	• Interleukin 4 (IL-4) • Interleukin 5 (IL-5) • Interleukin 10 (IL-10) • Interleukin 13 (IL-13)
Both pro- and anti-inflammatory	
• Granulocyte-macrophage colony-stimulating factor (GM-CSF) • Interleukin 6 (IL-6) • Interleukin 21 (IL-21)	

Table 2. Cytokine families and functions. (Compiled from Owen *et al.*,[25] Bixler *et al.*,[26] and Testar[21].)

Family	Representative members of the family	General function
Interleukin 1 family	IL-1α, IL-1β, IL-1 Ra, IL-18, IL-33	Variety of action
Interleukin 17 family	IL-17A, IL-17B, IL-17C, IL-17D, IL-17F	Promote neutrophil accumulation and activation
Interferon family	IFN-α, IFN-β, IFN-γ, IL-10, IL-19, IL-20, IL22, IL-24	Innate anti-viral response and modulate immune response
Chemokines	IL-8, CCL19, CCL21, RANTES, MCP-1, MIP-1α	Cell migration, adhesion and activation (chemoattractant functions)
Hematopoietin family	IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-12, IL-13, IL-15, IL-2, IL-23, GM-CSF, Growth Hormone, Prolactin, Erythropoietin	Cell differentiation and proliferation Chemotaxis
Tumour necrosis factor family	TNF-α, TNF-β, CD40L, Fas (CD95), BAFF, APRIL, LTβ	Immune system development, effector functions and homeostasis

There are two main groups of inflammatory mediators, namely pro- and anti-inflammatory.[24] Pro-inflammatory mediators do not always exhibit adverse effects (for example, they may rather act to initiate tissue repair following injury),[27] in the same way that anti-inflammatory

mediators do not always exhibit advantageous effects (for example, they may also inhibit the positive function of pro-inflammatory mediators).[28] The ratio between these two groups determines the net immune response.[29] An individual can, for instance, be young and appear to be healthy, despite elevated pro-inflammatory mediator levels, due to anti-inflammatory mediator levels which are satisfactory to compensate.[30] However, this compensation may become inadequate with progression in age.[31] It is important to note that the ridged classification of inflammatory mediators into pro- and anti-inflammatory categories may be an over simplification of an extremely complex system. [32] The ultimate effect of any inflammatory mediators may be significantly regulated by a number of factors including target cells, nature of neighbouring cells and the surrounding microenvironment.[32] Inflammatory mediators are increasingly being implicated in chronic diseases such as insulin resistance, impaired glucose tolerance and diabetes,[33] some cancers[34] and even Alzheimer's disease.[35] Additionally, inflammatory mediators have been identified as contributors to the development of CVD. However, the detailed physiological functions of some inflammatory mediators are not yet known.[9-13]

While the functions of some inflammatory mediators in CVD development remain vague, the association of others is clear. One study found CRP to associate with risk for coronary vascular disease independent of hyperlipidaemia,[36] while another found that even at levels once considered normal, CRP predicted future coronary events.[37] Fractalkine has been shown to predict the development of metabolic syndrome,[38] which is associated with the development of CVD.[39] A number of inflammatory mediators have all been found to be associated with measures of blood pressure (BP), as detailed in Section 3 below.[16, 18, 40-42] One study has suggested that fractalkine may contribute to the pathogeneses of atherosclerosis[43] and coronary plaque rupture.[44] IFN-γ, CRP, IL-6, IL-1β and TNF-α have also been shown to contribute to the development of atherosclerosis.[45-48] Furthermore, both CRP and IL-17A are associated with arterial stiffness.[18, 49] On the contrary, IL-10 has been shown to protect against atherosclerosis as it inhibits the production and release of IL-12, which, in turn, inhibits the

production IFN-γ,[50] as well as deactivates macrophages and some T cells.[47, 51] However, the activation of pro- and anti-inflammatory pathways does not occur in isolation, and interactions between inflammatory mediators can result in a cascading effect.[52] While IL-13 has not been found to link directly with CVD, it may elicit a protective effect through its inhibition of the production of IL-1β, IL-6, IL-8 and TNF-α.[53]

3. Raised Blood Pressure and Inflammatory Mediators

Hypertension or raised BP is a multi-factorial trait that develops as a result of both environmental and genetic factors such as age, stress, obesity, diet, physical activity and family history.[8] Hypertension is one of the most prominent contributing factors in the development of CVD.[54] In 2017, the GBD collaborators found that raised systolic BP (SBP) is the leading cause of death and accounts for 10.4 million deaths.[4] As of 2019, the WHO estimated that an approximate 1.13 billion individuals globally are hypertensive.[55] A systematic review reported an estimated 130.2 million cases of hypertension in Africa in 2010.[56] When looking specifically at a South African population, the 2016 South African Demographic and Health Survey found that 46% of women and 44% of men above the age of 15 were either hypertensive or receiving anti-hypertensive medication.[57] As is to be expected, the prevalence of hypertension was higher in older populations (**Figure 2**). [57] The South African Hypertension Society guidelines[58] for diagnosis and treatment are in line with those of both the European and International Society of Hypertension,[59, 60] defining hypertension as ≥140 and/or ≥90 mmHg.[58]

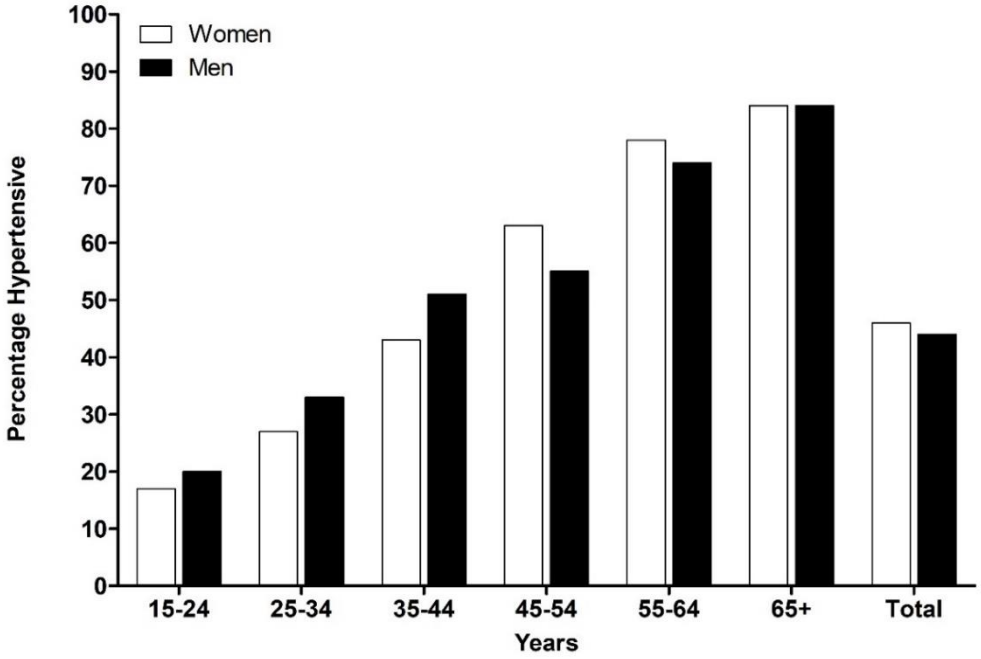

Figure 2. Percentage of South African men and women classified as hypertensive per age category. (Adapted from National Department of Health *et al.*)[57]

In recent years it has been suggested that inflammation may be one of the important contributors in the development and maintenance of hypertension (**Figure 3**). A number of pro-inflammatory mediators, namely CRP, IL-6, TNF-α, IL-8, IL-12 and IL-17A have all been found to correlate positively with measures of BP.[16, 18, 40-42] A study examining 506 healthy men found positive associations between IL-6 and BP.[61] Additionally, a study comparing 135 newly diagnosed and untreated hypertensive participants with 40 healthy controls found higher CRP concentrations in the hypertensive group than in the control group.[62] Furthermore, a study comparing 15 subjects demonstrating arterial hypertension with 15 healthy control subjects found higher plasma levels of CRP, IL-6, TNF-α and monocyte-chemoattractant-protein-1 in those with arterial hypertension. In contrast, other inflammatory mediators such as GM-CSF and IL-10 have been found to correlate negatively with BP.[16, 18] Research into the effect of IFN-γ on BP has produced contradictory results.[16, 18]

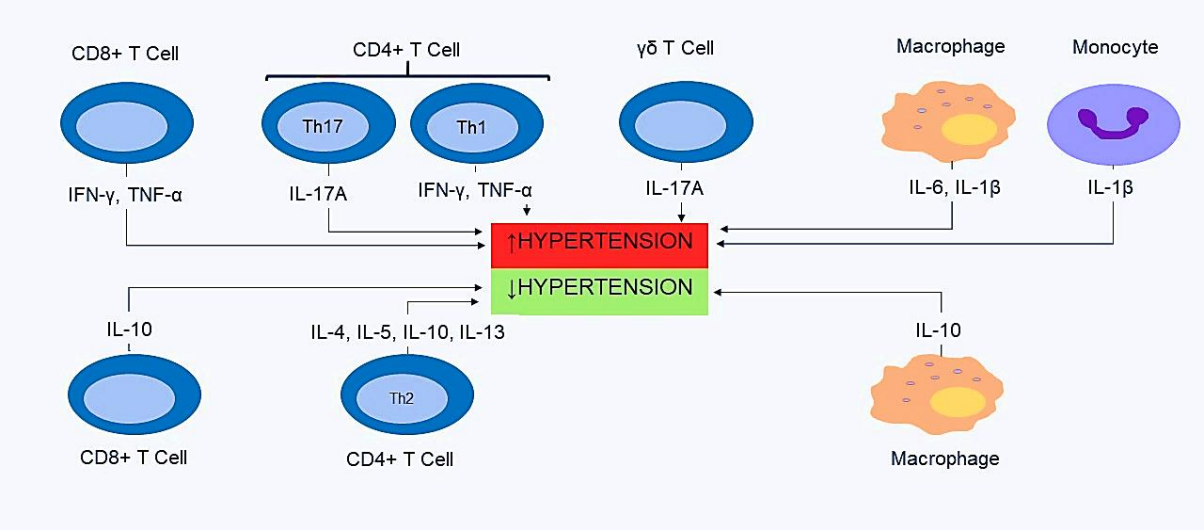

Figure 3. Inflammatory mediators and hypertension. (Adapted from Norlander *et al*.)[63]

3.1 Potential Mechanisms

There are a number of suggested mechanisms through which inflammation may contribute to the development of hypertension. It has been shown that both innate and adaptive immune responses contribute to the pathophysiology of hypertension.[40, 64] This is as a result of inflammatory changes that occur in the kidney, blood vessels and the brain.[40, 64] Inflammatory mediators may aid in the development of hypertension through their contribution to increased vascular permeability, as well as the release of cytokines, reactive oxygen species (ROS) and matrix metalloproteinases (MMPs).[65] Cytokine release leads to decreased lumen diameter of resistance vessels, the primary vessels involved in BP regulation via the neo-intima formation.[65] This is as a result of their contribution to neo-intima formations. In addition, cytokines can increase vascular resistance and stiffness via increased vascular fibrosis.[65] Cytokines are involved in the promotion of angiotensinogen and angiotensin II production, as well as sodium and volume retention in the kidneys, all of which lead to an increase in BP as a result of increased fluid retention.[40] Increased ROS production, as a result of inflammatory mediators, may contribute to the development of hypertension via a number of pathways such as nitric oxide depletion, increase sodium reabsorption, decreased glomerular filtration rate or increased efferent sympathetic activity.[66] In addition to its contribution to the development of

hypertension, ROS also leads to the development of vascular disease and dysfunction which may, in turn, worsen BP.[67] MMPs result in the development of hypertension via the degradation of the extracellular matrix, resulting in the infiltration of immune cells, apoptosis and collagen synthesis and ultimately target organ damage.[65] In addition, activation of specialised macrophages in the brain called microglial cells by cytokines increases sympathetic outflow resulting in hypertension.[68] However, what causes the initial release of inflammatory mediators? Trott *et al.*[69] describes a hypothesis of the process (**Figure 4**) in which an initial hypertensive stimulus leads to increase in BP (pre-hypertension), resulting in protein modifications creating neoantigens. These neoantigens, when processed by dendritic cells, promote T cell activation.[69] Activated T cells infiltrate the kidneys and vasculature, leading to changes in sodium handling and vascular remodeling which, in turn, results in overt hypertension.[69]

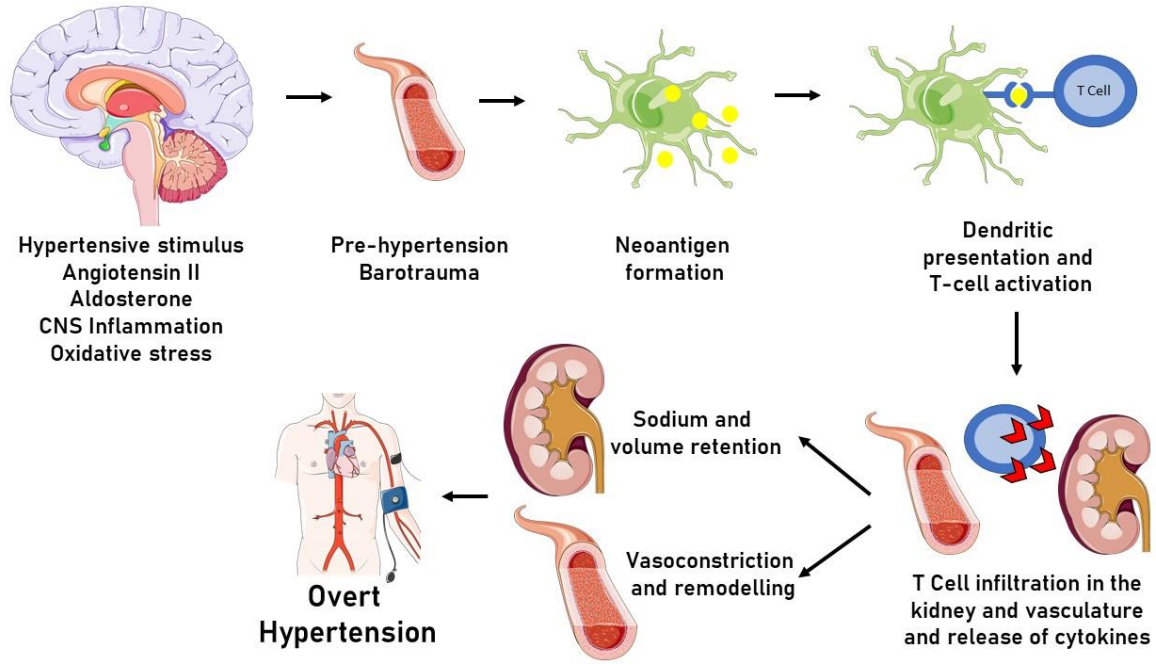

Figure 4. Role of immune cells in hypertension. (Adapted from Trott *et al.*)[69]

3.2 Anti-inflammatory Treatment

Keeping the above in consideration the question that remains is: if inflammation is mechanistically involved in increasing BP, would the inhibition of inflammatory mediators

(inflammatory targeting) or inflammatory diseases have positive effects in terms of lowering BP? A study performed by Czesnikiewicz-Guzik *et al.* investigated the effect of intense periodontitis treatment (n=50), a chronic inflammatory disease, versus a control periodontal treatment (n=51) on BP.[70] They found intense periodontitis treatment to result in an improvement in periodontal status in two months when compared to control periodontal treatment.[70] This was accompanied by a mean reduction of 11 mmHg in SBP which correlated with improvement in periodontal status. Reductions in DBP as well as IL-6 and IFN-γ were also seen.[70] The Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) evaluated whether inhibition of IL-1β, using canakinumab, would result in a reduction in BP. CANTOS randomised 10 061 patients with previous myocardial infarction and high-sensitivity CRP above 2 mg/L to canakinumab 50 mg, 150 mg, 300 mg or placebo.[71] Of the 9549 participants with follow-up BP readings, no reduction in BP or incident hypertension was seen.[71] However, a reduction in major cardiovascular events was found.[71]

On the contrary, the Cardiovascular Inflammation Reduction Trial (CIRT) evaluated 4786 patients with previous myocardial infarction or multivessel coronary disease as well as either type 2 diabetes or metabolic syndrome.[72] Participants were exposed to low-dose methotrexate at 15 to 20 mg weekly or a placebo dosage.[72] It was found that low-dose methotrexate did not result in lower IL-1β, IL-6, CRP, or cardiovascular incidence than the placebo dosage.[72] However, it did associate with reduced leukocyte and haematocrit levels, elevated liver-enzymes and incidence of non-basal-cell skin cancers when compared to the placebo.[72] A meta-analyses evaluating cardiovascular events in patients treated with anti-IL-12/23 found that those receiving anti-IL-12/23 treatments were at a potentially higher risk (OR=4.23 CI:1.07–16.75, p=0.04) for major adverse cardiovascular events than placebo groups,[73] highlighting the potential risks of anti-inflammatory therapies.

While the potential targeting of inflammatory mediators in BP and cardiovascular disease reduction remains elusive, the ultimate outcomes may be beneficial once a better mechanistic and pathophysiological understanding on the roles of specific mediators is obtained. As such,

is it imperative that the inflammatory mediators which may be involved in changes in BP and the development of cardiovascular disease are determined.

4. Non-modifiable Risk Factors and Inflammatory Mediators

4.1 Ethnicity

A large number of studies have shown vast differences in concentrations of cytokines between individuals of different ethnic groups.[74-80] While these differences have been found where numerous inflammatory mediators are concerned, findings remain contradictory.[78] For example, when examining IL-1β, Elkind *et al.*[75] found higher levels in black participants than their white counterparts. In contrast, Albandar *et al.*[76] found the highest levels of IL-1β in Hispanics, followed by white individuals, with black individuals reflecting the lowest levels of IL-1β . In terms of IL-6, Hong *et al.*[81] reported no ethnic differences in concentrations between black and white participants, while Elkind *et al.*[75] found black individuals to have higher levels than white individuals. In addition to the above studies, several studies conducted in South Africa found that black individuals presented with higher levels of inflammatory mediators than white individuals.[74, 80, 82, 83] A study examining 217 black and white women from the North West province between the ages of 20-50 years found that African women had higher levels of the inflammatory markers CRP, fibrinogen and leptin when compared to white women.[80] Another study comparing 398 urban black and white teachers from four districts in the North West province found that black participants of both sexes showed higher levels of CRP.[82] A study comparing 521 Africans and white individuals from South Africa from the North West province again found African participants to display higher levels of CRP as well as soluble urokinase plasminogen activator receptor than white participants.[83] Soluble urokinase plasminogen activator receptor elevation in African participants was independent of sex or smoking.[83] An additional study comparing black and white individuals from the Johannesburg area in South Africa found IL-1β levels to be higher in black individuals when compared to white individuals.[74] However, while findings comparing individuals of different ethnicities in South Africa appear

more consistent than other comparisons, the vast majority of these studies focussed on only a few of the known inflammatory mediators. This shortage of information with respect to the numerous under-explored inflammatory mediators does not allow for a true understanding of a detailed inflammatory mediator profile of black and white South Africans. The activation of inflammatory mediators does not occur in isolation and there are multiple intercorrelations between inflammatory mediators. As such, it is important to explore the larger inflammatory mediator profile of these ethnic groups to allow for better insight into the complex processes surrounding inflammation.[52]

A potential explanation for the ethnic differences may result from the variations in gene polymorphisms distribution relating to inflammation seen across ethnic groups.[84] It has been shown that there are a number of single nucleotide polymorphisms in the genes of inflammatory mediators.[84] This results in the alterations of transcriptional activity of genes or changes to the amino acid sequence of respective proteins.[84] Differences in the inheritance of the genotypes for IL-6 and IL-10 result in higher inflammatory mediator expression in black populations than in white populations.[84] Differences in genotypes between black and white populations have also been seen where IL-2 is concerned.[85] A study performed on a South Africa population found the IL-1 receptor allele to be significantly more prevalent in black South Africans than their white counterparts, while the IL-1 receptor antagonist allele was higher in white South Africans.[74]

4.2 Sex

In addition to ethnic differences, studies have reported differences in the inflammatory mediator profile of men and women.[86-88] Little is understood on how genetic and hormonal differences between men and women affect immune response.[86] A study evaluating the difference in CRP in prepubescent children aged younger than ten years, found CRP levels in girls to be approximately double that seen in age-matched boys,[86] suggesting a cause other than hormones, as hormonal differences are not significant at this age. With this said, sex

hormones have been shown to result in changes in inflammatory status; however, whether these changes are beneficial or not, remains unclear.[89] One study found that administering oestrogen resulted in an increased release of inflammatory mediators such as CRP.[90] Additionally, hormone replacement therapy was shown to increase CRP.[90] However, another study found hormone replacement therapy to reduce the levels of inflammatory mediators such as cell adhesion molecules E-selectin, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1, while having no effect on CRP or IL-6 concentrations.[91]

A study examining inflammation in individuals with a normal glucose tolerance, prediabetes and type 2 diabetes found that in individuals with a normal glucose tolerance, there were no significant differences in CRP or IL-1 receptor antagonist between men and women.[92] However, in individuals with prediabetes and type 2 diabetes, women showed significantly higher levels of both CRP and IL-1 receptor antagonist than men.[92] One potential explanation for the differences in inflammation between men and women in the prediabetic and type 2 diabetic groups is that of adiposity. Adiposity is closely associated with inflammation, in particular CRP.[93] Furthermore, it has been shown that this association is considerably stronger in women when compared to men.[93]

However, it has been found that men display increased levels of IL-6 following trauma when compared to the elevation seen in women.[94] Additionally, women have been found to have attenuated inflammatory responses to exercise, as indicated by the number of leukocytes released, when compared to the response seen in men.[95] This may be as a result of oestrogens which attenuate muscle disruption and/or delay muscle leukocyte infiltration in women.[96]

It is clear that the role of sex in inflammation, as a result of either genetic or hormonal influences, is complex and remains a matter of uncertainty.

4.3 Age

Jenny *et al.* describes biological aging not as a linear process but rather that *"multiple processes form a labyrinth network where loss of function in one system, whether from environmental insults and/or natural programmed phenomena, impacts all other systems, leading to and derived from inflammatory responses, and resulting in what is defined as aging."*[97]

It is well established that biological age and chronological age are not strictly comparable.[98] The Cardiovascular Risk Factors Affecting Vascular Age study has recently shown that vascular ageing is accelerated in the presence of cardiovascular risk factors.[99] Additionally, exposure to risk factors in early life plays a prominent role in the development of vascular structure and function and can be used to predict future changes in vascular stiffness in early adulthood.[99] One of the main mechanisms behind aging-related endothelial dysfunction is inflammation.[100] One study found that childhood environment affects the development of inflammatory phenotypes,[101] suggesting that the effects of a person's inflammatory mediator profile on their cardiovascular system may begin at a very early age.

In addition to changes in the inflammatory mediator profile that may occur during childhood, there are added changes in inflammation with age. It has been shown that inflammation may be the base of a number of disorders that develop with age, such as metabolic syndrome, which ultimately results in the development of CVD.[102] Older individuals have increased levels of pro-inflammatory mediators,[103] even when they are apparently healthy.[104] Older people present with chronic inflammation as opposed to inflammatory bursts following illness or injury that is seen in younger individuals; however, the reason for this switch is poorly understood.[103]

A study of 1411 black, white and Mexican Americans between the ages of 25-91 years, has shown that IL-6 and TNF-α receptor 1 increased with age, while IL-10, CRP and IL-1 receptor antagonists showed no significant increase in concentration with increasing age.[105] A second study examining 711 older individuals from the Framingham Heart Study (mean age 79 years)

and 21 healthy young individuals (mean age 39 years) found that IL-6 and IL-1 receptor antagonists were higher in the elderly, while IL-1β and TNF-α showed no differences. [106]

While research into inflammation is available, the vast majority of studies focus either on older individuals, children or a broad age range.[105-107] As mentioned above, individuals with increased age show elevated pro-inflammatory mediators even when healthy,[104] potentially leading to skewed results. It is therefore important to investigate the role of inflammation in CVD in young adults to allow for a better physiological understanding before age-related changes occur.

5. Modifiable Risk Factors and Inflammatory Mediators

5.1 Salt Intake

The WHO has recommended a daily dietary intake of no more than 5 g of salt or 1.7 g of sodium.[108] In 2010, the global average sodium consumption was 3.95 g/d per person, with approximately 99.2% of the adult population exceeding the WHO recommended daily sodium intake.[109] In South Africa, more than two-thirds of the population consume over 5 g/d of salt with an average intake around 7.2 g/d.[110, 111] Overall, in 2015, 69% of South African adults reflected salt intakes above that of the WHO recommendation.[111] In addition, 28% of the population was found to consume more than twice this level (>10 g/day), while 11% consumed at least three times the recommended level.[111] In the African-PREDICT study population, which will also be the target population of this thesis, it was found that in 79.9% of the population between the ages of 20-30 years, dietary sodium was above the recommended levels.[112] It has been found that 60% of salt consumed by South Africans is from nondiscretionary salt intake through processed foods.[113] Therefore, in June 2016 the South African Department of Health implemented legislation that regulates the sodium content of a range of processed foods.[114]

Increased dietary sodium intake has been linked to an increase in BP,[115, 116] as well as an increased risk for the development of CVD and stroke.[117, 118] One study evaluating the relationship between sodium and BP in 11095 adults found a positive association between sodium intake and both SBP and DBP in men and DBP in women.[119] Studies have also indicated a clear link between a reduction in sodium intake and reduced BP,[120] which will, in turn, translate to a reduced risk for the development of CVD.[121] One way a diet high in sodium may result in increased risk for hypertension and CVD is through its effect on the release of inflammatory markers.[122]

One study showed that a diet high in sodium may result in increased levels of inflammation, suggesting an increased release of pro-inflammatory cytokines without satisfactory compensation from anti-inflammatory cytokines.[123] However, the mechanisms by which increased dietary sodium intake results in increased levels of inflammation are complex. A study performed in rats found that a long-term diet high in sodium resulting in the development of hypertension, associated with significant changes in gene expression profiles of renal cytokines.[124] This resulted in an overall pro-inflammatory response.[124] A separate study revealed that IL-6 was involved in angiotensin II mediated hypertension.[125] Interestingly, neither IL-6 nor CRP were elevated in people on a low sodium diet which would result in the activation of the renin-angiotensin system.[125] This indicates that a diet low in sodium is non-inflammatory, despite the increased activity of the renin-angiotensin system.[125]

In addition to the potential effect of dietary sodium on inflammation, either directly or through the renal system, there is another potential indirect effect. A number of studies have suggested a relationship between dietary sodium intake and obesity.[123, 126-132] This suggests that the potential relationship seen between dietary sodium intake and inflammation may be due to dietary sodium resulting in increased obesity and the obesity, in turn, resulting in the increased levels of inflammation.

A study performed in healthy adolescents found a relationship between dietary sodium intake and obesity as well as inflammation independent of total energy intake and intake of sugar-sweetened beverages.[133] However, a previous study performed in this same population found no direct relationships between dietary sodium intake and numerous markers of obesity (with the exception of body surface area) after adjusting for various potential confounders.[112]

5.2 Potassium Intake

It is of importance to note one cannot investigate sodium without potassium. A number of reports have shed light on the importance of sodium-to-potassium ratio balance, in particular in relation to BP.[134-136] It has also been shown that the importance of the sodium-to-potassium ratio increases with age.[136] Potassium is an essential nutrient required for fluid and electrolyte balance as well as normal cellular functioning.[137] Potassium is available in an assortment of different foods such as fruits and vegetables.[138] However, the potassium content of food is generally reduced during food processing.[138] It has been recommended that the minimum daily potassium intake should be 90 mmol/day (3.51 g/day), although a higher intake is recommended in some countries.[139] One study has suggested the global population average potassium intake is below 70-80 mmol/day.[139] A separate study found that intake in countries such as China was as low as 1.7 g/day (approximately 44 mmol/day).[140] In terms of South Africa, two separate studies have shown that only 8% of the population met the minimum suggested potassium intake,[112, 113] with one study showing an average population intake of 34 mmol/day.[112] Both studies found intake to be lower in black participants than their white counterparts.[113] Individuals of mixed ancestry displayed lower levels than that of the white participants, but higher than the black participants.[113]

Potassium may have a protective effect in terms of CVD.[141, 142] This may be due to the role a diet high in potassium plays in counteracting the negative effects of sodium on BP.[141, 143] A diet high in potassium intake was shown to attenuate increases in BP in response to high salt intake.[144, 145] One study evaluating the relationship between sodium and BP in 11059 adults

found negative associations between potassium intake and DBP in men and SBP and DBP in women.[119] Furthermore, it found positive associations between sodium-to-potassium ratio and BP.[119] In addition to the direct effect of sodium on BP, it was shown that sodium results in increases in pro-inflammatory mediators. Therefore, as a diet high in potassium has a moderating effect on BP,[143] cardiovascular events and mortality,[142] potassium may have a similar protective effect on the modulation of inflammation.[146] This notion is supported by a study which indicated that potassium supplementation inhibited the production of IL-17A by human T lymphocytes induced by a salt load.[147] However, research on this matter is limited.

While it is currently unclear how this protective effect may occur, a number of potential mechanisms are proposed. A mechanism through which potassium may suppress inflammation is through its anti-oxidant effect.[147] Increases in extracellular potassium results in elevated membrane sodium pump activity.[148] This, in turn, leads to hyperpolarisation and ultimately reduced oxidase activity.[148] An additional proposed mechanism is via potassium inhibiting the effects of sodium on p38/MAPK, which, when activated, leads to an immune response.[147] Mitogen activated protein kinase *p38* (p38/MAPK) is phosphorylated in response to sodium, leading to the activation of SGK1 which is highly expressed in $CD4^+$ T cells.[149, 150] Serine/threonine-protein kinase (SGK1) is important in the polarisation of TH17 cells and initiation of IL-17A production.[151, 152] It has also been suggested that potassium may suppress nuclear factor *kappa B* (NF-κB) activation, potentially via the upregulation of Smad7, an inhibitor of NF-κB which regulate genes relating to inflammation in the kidneys[146, 153, 154] and an inhibition of NF-κB results in decreased inflammation.[155-157]

5.3 Other Confounders

5.3.1 Obesity

Obesity is a global trend that is continuing to increase and this upsurge is accelerating.[158] This could, in part, be attributed to global trade liberalisation, economic growth and rapid urbanisation.[159] In 2016, the South African Department of Health suggested that these obesity

figures among South Africans may be as high as 40% in men and 70% in women.[160] Increased levels of adiposity have been linked to numerous detrimental health effects, including its contribution both directly and indirectly to the development of CVD, including hypertension, coronary heart disease, atrial fibrillation, heart failure, sudden cardiac death and stroke.[161, 162] One such effect that has sparked recent interest is the relationship between increased levels of adiposity and elevated concentrations of inflammatory mediators. Adipose tissue, an endocrine organ, is responsible for the secretion of numerous factors responsible for systemic and vascular inflammation.[163] However, increased levels of adipose tissue may relate to the release of more than only adipokines. A study has suggested that total body fat is linked to a state of chronic low-grade inflammation in healthy adolescents[164] and that adiposity is linked to increased concentrations of IL-6, TNF-α and CRP.[165] In addition, a reduction in body fat results in a reduction in markers of vascular inflammation such as IL-6, IL-18 and CRP.[165] Regardless, the relationship between adiposity and inflammatory mediator concentrations may not be as straightforward as it seems.

5.3.2 Physical Activity

Globally, levels of physical inactivity have become alarming and diseases related to physical inactivity are now considered the fourth leading cause of death.[166] The WHO estimates that approximately 3.2 million deaths per annum result from physical inactivity-related diseases.[167] One study indicated that levels of physical inactivity in South Africa may be as high as 40-50%,[168] while another suggested 43.0% of men and 46.6 % of women are inactive.[169] Physical activity has been shown to provide significant benefits in the prevention of the development of CVD.[170] Physical activity is associated with the release of pro-inflammatory mediators (e.g. TNF-α, IL-1β, IL-6), followed by the release of anti-inflammatory mediators (e.g. IL-4 and IL-10) that act as regulators subsequent to physical activity,[24, 171] thus overall resulting in a reduced and favourable inflammatory profile.[172]

5.3.3 Tobacco Use

Tobacco use is associated with a wide range of chronic diseases.[173] Tobacco-related diseases kill up to half of those who use tobacco.[174] According to the 2016 South African Health and Demographic Survey, the prevalence of tobacco use in 2016 for men and women was 37% and 7%, respectively.[57] For many years it has been well established that tobacco is associated with the development of CVD.[175-177] One potential mechanism through which tobacco may result in the development of CVD is through its contribution to the development of an unfavourable inflammatory mediator profile.[178-180] It has been suggested that cigarette smoking has an effect on both the innate and adaptive immune responses.[178] One study found smoking to be linked to an increased release of the pro-inflammatory cytokines CRP and IL-6.[181] A separate study found that cigarette smoking enhanced the production of pro-inflammatory mediators such as TNF-α, IL-1, IL-6, IL-8 GM-CSF, while suppressing the production of anti-inflammatory mediators, for example IL-10.[178] This results in an overall less favourable inflammatory mediator profile.

5.3.4 Alcohol Consumption

Excessive alcohol consumption is a major risk factor for both chronic disease and physical injury.[182] Young individuals such as those in this PhD study are at particular risk. In individuals between the ages of 20-39 years, an alarming 25% of deaths were alcohol-related.[183] In South Africa, 28% of men over the age of 15 years exhibited risky drinking behaviour, while only 4.8% of women displayed the same behaviour.[57] It is clear that alcohol consumption is related to numerous health risks;[184-187] however, its relationship with CVD, in terms of the amount of intake, remains an ongoing matter of debate.[188-190] At least 50% of the protective effect seen with moderate alcohol consumption is believed to be as a result of increased high-density lipoprotein-cholesterol.[191] A second potential mechanism may be as a result of the effect that alcohol plays in systemic inflammation as a whole.[191] McCarty has suggested moderate alcohol intake to inhibit the production of IL-6.[192] A separate study found alcohol intake to show

a U-shaped association with CRP which highlights the fact that heavy alcohol consumption may result in negative effects.[193]

6. Motivation and Problem Statement

In some studies, positive relationships were found between inflammatory mediators and CVD development. A large number of these studies specifically focused on only a few well-known markers. While an array of other inflammatory mediators have been discovered, the potential role thereof in cardiovascular disease development remains largely unexplored.[15, 16, 40, 78, 194] While many questions remain, a pattern has emerged suggesting that lifestyle behaviours, such as dietary intake, contribute to the release of both pro- and anti-inflammatory cytokines. This, in turn, results in changes in cardiovascular structure and function, and potentially in the development of CVD over time, thereby instigating further release of inflammatory mediators. Additionally, it has been shown that low levels of inflammation, also referred to as low-grade inflammation, are associated with CVD.[102, 195] Even in the early phases of CVD development, disturbed inflammatory mediator profiles are linked with changes in cardiovascular function such as raised BP.[40] It is therefore important to explore the relationship between inflammatory mediators and measures of BP at this early stage of life. Furthermore, it should be investigated how this relationship may be influenced by lifestyle behaviours and ethnic differences.

In order to explore and understand these relationships more clearly, data from a high sensitivity analysis, namely the MILLIPEX Map Human High Sensitivity T Cell Magnetic Bead Panel, was used. This Panel involves low-level detection of a variety of cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, IL-21, IL-23, ITAC, MIP-1α, MIP-1β, MIP-3α, and TNFα), ensuring that possible relationships are not overlooked based on low concentrations. A number of these cytokines are poorly researched or understood, especially in terms of their relation to cardiovascular structure and function. As such, a pertinent focus of this study was to generate hypotheses for future research.

7. Aims, Objectives and Hypotheses

The central aim of this study was to present a detailed inflammatory mediator profile and describe how it relates to sodium and potassium excretion and the cardiovascular profile of young, healthy South African adults.

Manuscript 1:

Aim: To evaluate a detailed inflammatory mediator profile and its relationships with BP in young black and white adults.

Objective 1: To describe and compare a detailed panel of pro- and anti-inflammatory mediators between black and white adults.

Objective 2: To investigate the relationship between inflammatory mediators and measures of blood pressure, including 24-hour brachial SBP and diastolic BP (DBP), night-time brachial SBP and DBP and clinic central SBP.

Hypothesis 1: The inflammatory mediator profile will differ between black and white groups, with black adults presenting with a more pro-inflammatory profile than the white group.

Hypothesis 2: Measures of BP will be adversely related to pro-inflammatory mediators and beneficially related to anti-inflammatory mediators in both black and white participants.

Manuscript 2:

Aim**:** To explore the relationship between inflammation, sodium and potassium in young black and white adults.

Objective**:** To investigate the relationships between a detailed range of 22 pro- and anti-inflammatory mediators with 24-hour urinary sodium and potassium, respectively, in young black and white adults.

Hypothesis 1**:** Inflammatory mediators will associate adversely with sodium in both black and white adults.

***Hypothesis 2*:** Inflammatory mediators will be beneficially associated with potassium in both black and white adults.

Manuscript 3:

Aim: To evaluate the role of inflammation in the change in BP over time in young adults.

Objective: To investigate the relationship between a detailed range of 22 pro- and anti-inflammatory mediators, both individual mediators and clusters, and changes in BP over 4.5 years in young adults.

Hypothesis 1: Pro-inflammatory mediators will associate adversely with changes in BP in both black and white adults.

Hypothesis 2: Anti-inflammatory mediators will associate beneficially with changes in BP in both black and white adults.

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Chapter 2

Methodology

1. Study Design and Participants

This PhD study forms part of the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT). This study was started and executed by the Hypertension in Africa Research Team (HART) at the North-West University, with numerous collaborators. The African-PREDICT study is a longitudinal study that aims to gain new knowledge on early cardiovascular disease related pathophysiology as well as identify early markers or predictors of cardiovascular disease in young, apparently healthy South Africans. This will contribute to the implementation of successful prevention programs in the long term.

The African-PREDICT study aims to develop a detailed cardiovascular profile of young adults. These individuals will be followed every five years over a 10-year period (**Figure 1**). This will allow for the monitoring of changes in the cardiovascular profile with age. Measurements for the African-PREDICT study were collected and analysed by a multi-disciplinary team of national and international scientists.

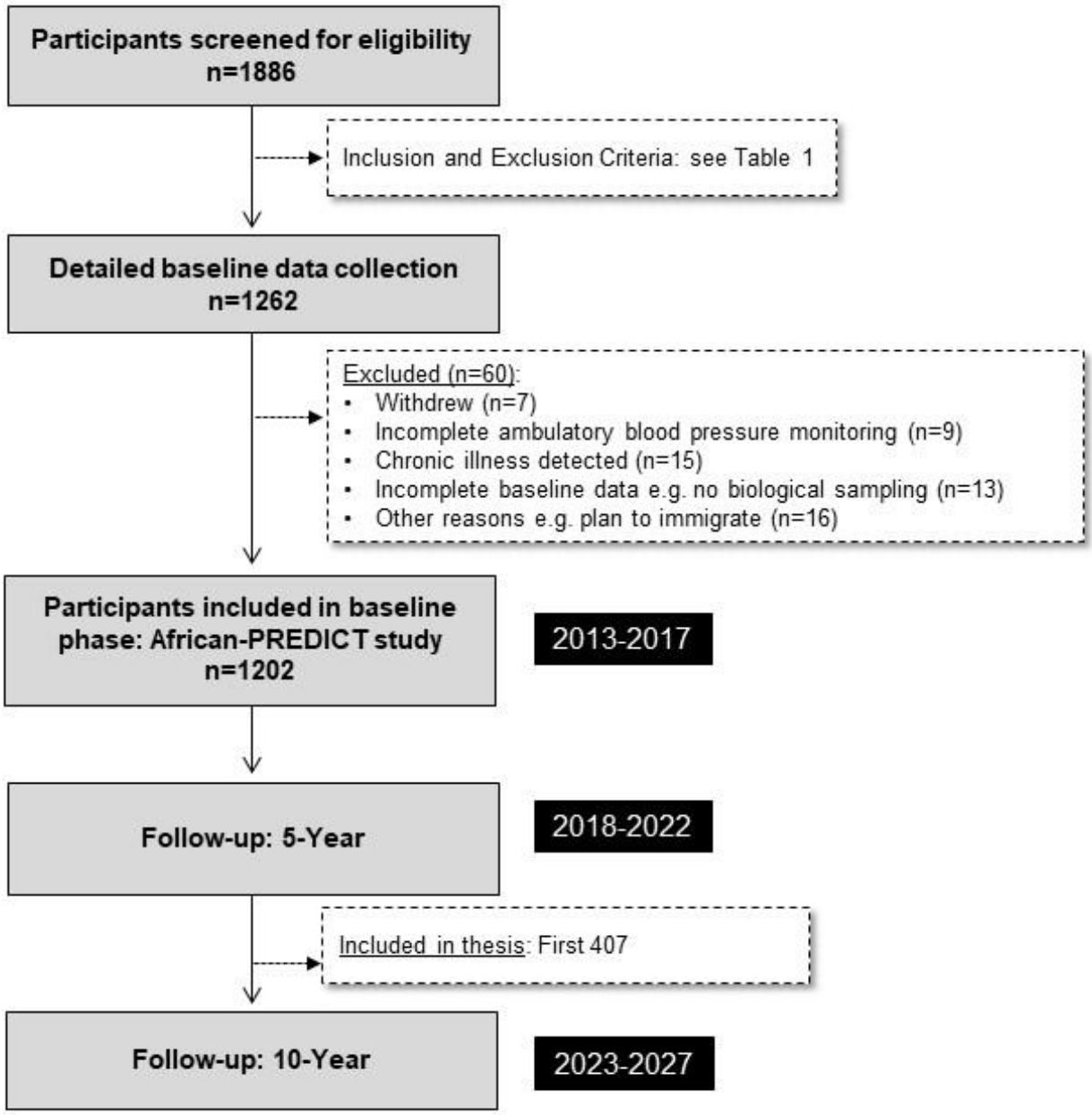

Figure 1. African-PREDICT study layout.

The African-PREDICT study recruited participants from Potchefstroom and surrounding areas in the North West province, South Africa (**Figure 2**). Participants in this study were either black or white individuals, men and women, between the age of 20-30 years. The study included apparently healthy individuals with normotensive screening office blood pressures (BP) (<140/90 mmHg) and who were not diagnosed with the human immunodeficiency virus (HIV) or having had any other previous diagnosis of a chronic disease. Individuals from low, middle and high socio-economic status groups were specifically included.

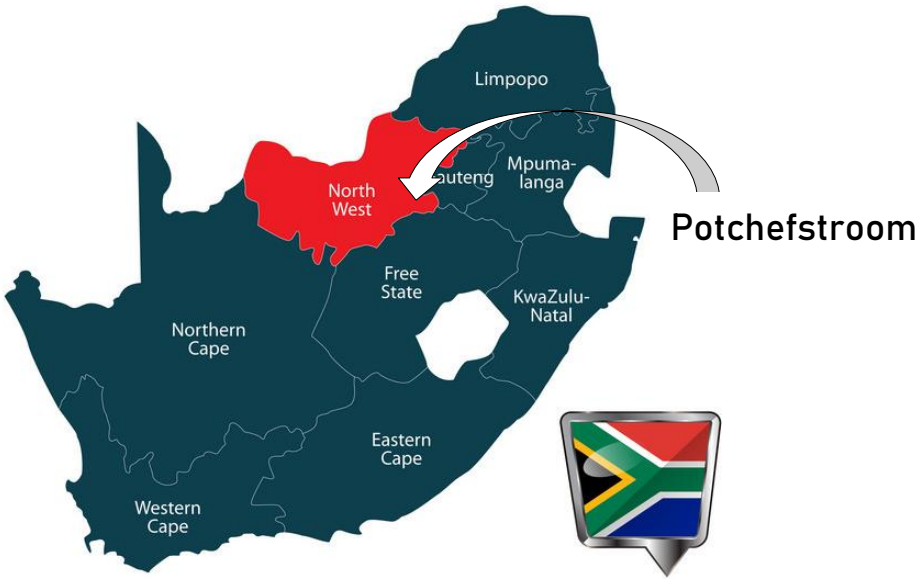

Figure 2. Map of South Africa, indicating the location of the North West province and Potchefstroom.[1]

2. Organisational Procedures

Participation in the African-PREDICT study involved two phases. In the first phase, participants were screened based on the criteria listed in **Table 1**. This screening procedure included completion of informed consent followed by a General Health and Demographic questionnaire, office BP measurement, anthropometric measurements, blood typing and an HIV test. A total of 1202 of the 1886 individuals who were screened were included in the study. Those participants eligible for participation in the study were then invited to take part in the African-PREDICT study and were provided with detailed information regarding procedures in advance.

It was requested that on the day of their measurement's participants fast for eight hours prior to their arrival at the location. Participants arrived at the Hypertension Research and Training Clinic (via their own transportation or the transportation provided to them) at 07h45, after which the organisational procedures were again explained and informed consent forms signed. A maximum of four participants were accommodated on a single day to maintain the quality of the measurements. Participants were firstly asked to provide a spot urine sample. Following

this, participants were taken to a private room where blood samples were drawn by a registered research nurse. Participants were then rotated around a number of research stations with the help of the nurse and research assistant. During the course of the morning participants completed a number of questionnaires. They also received a meal and refreshments upon completing the measurements that had required them to be fasting. All procedures were performed according to good clinical practice by a multi-disciplinary team of trained scientists or nurses. In addition, all measurements were performed in a temperature-controlled room, one participant at a time, while considering the privacy of each participant. Upon completion of all measurements, participants were transported back to their homes or workplaces.

Table 1. Inclusion and exclusion criteria of the African-PREDICT Study.

Inclusion criteria	Justification for inclusion/exclusion
1. Apparently healthy individuals 2. Age 20-30	1/2. The overall aim of the African-PREDICT is to track young healthy individuals over a period of 10-20 years to evaluate development of hypertension. Individuals in the age range of 20-30 years are ideal as these adults are considered to be in good cardiovascular health. This allows researchers to track changes in the cardiovascular and biochemical profile from the early changes of disease development.
3. Black or white ethnicity	3. HART and numerous others have shown black South Africans are at a higher are at risk for hypertension.[2] As such, black individuals were included in this study while white individuals were used as a comparison group.
4. Men and women	4. Both men and women are included to determine sex differences in CVD.
Exclusion criteria	
5. Office brachial BP ≥140mmHg and ≥90mmHg	5. Individuals with hypertension, based on office blood pressure, were excluded as this study aimed to track hypertension development.
6. HIV infected 7.Previous diagnosis or medication for a chronic disease	6/7. As this study aimed to track seemingly healthy individuals, participants with previous diagnosis of disease were therefore not included.
8. Pregnant or lactating	8. Hormones involved in pregnancy or lactation are a known influence of the cardiovascular system.
9. Not permanent resident of Potchefstroom or surrounding area	9. Due to the longitudinal nature of this study longitudinal study was imperative to recruit participants willing and able to return for the follow-up phase of the study.
10.Inability to read or understand English	10. Several questionnaires such as dietary, psychological and other questionnaires forming part of the study are in English.

3. Methodology of this PhD Study

This PhD study uses existing data that was collected during the African-PREDICT study and thus no additional measurements or data collection were performed. All procedures and protocols for this PhD study are consistent with those of the African-PREDICT study. As a research assistant and PhD candidate I have overseen significant parts of the follow-up phase of participants, with my specific role detailed in Section 6.

This PhD study utilised data from the first 1189 participants (599 black and 590 white individuals) who took part in the baseline data collection, after exclusion of those taking anti-inflammatory medications or with missing biological data. This study additionally used data of the first 407 participants who took part in both the baseline and follow-up phases of the study.

3.1 Demographic Questionnaire

Self-reported data with regards to demographic and lifestyle information was collected using a questionnaire. The questionnaire included information about age, sex, ethnicity, smoking, alcohol consumption, medication use, education, employment and household income. Apple iPads with a web-based program were used to complete this questionnaire. The questionnaire took approximately 15 minutes to complete, and assistance was available should any of the participants have had any questions. Socio-economic status was calculated using a point system that was adapted from Kuppuswamy's Socioeconomic Status Scale.[3] In this version, adapted for a South African population, participants were scored in the following three categories: education, household income and skill level. The South African Standard Classification of Occupation (SASCO) was used to classify skill level. These three factors were scored and then used to categorise participants into three socio-economic groups, namely low, middle and high. Socioeconomic status was scored as both a categorical and continuous variable.

3.2 Anthropometric Measurements

Trained anthropometrists took anthropometric measurements following the guidelines of the International Society for the Advancement of Kinanthropometry.[4] Weight (kg) and height (m) were measured to the nearest 0.01 (SECA electronic scales, SECA, Birmingham, UK). Waist, hip and neck circumferences were each measured three times (Holtain, Crymych, UK) and recorded to the nearest 0.1 cm. Subsequent analyses made use of the median of all three measurements. Body mass index (BMI) was calculated using the standard weight (kg)/height (m^2) calculation.

3.3 Physical Activity Measurements

Physical activity was measured using a compact, chest-worn accelerometric device that records heart rate, inter-beat-interval and physical activity in one combined unit. The ActiHeart device (CamNtech Ltd., England, UK) captures heart rate variability and then calculates total and activity energy expenditure (TEE, AEE). The device was worn for a minimum of four days and a maximum of seven days.

3.4 Brachial Blood Pressure

Duplicate brachial BP measurements were performed on both the left and right arms while the participant was in a seated position by using the Dinamap® Procare 200 Blood Pressure Monitor (GE Medical Systems, Milwaukee, WI, USA). A five-minute interval was observed and the measurements were then repeated. Participants were requested to fast for 8 hours and avoid tobacco and exercise for thirty-minutes prior to the measurement. The first measurement was performed after the participant was seated for a five-minute period.

3.5 Central Blood Pressure

The SphygmoCor XCEL (SphygmoCor XCEL, AtCor Medical, Sydney, Australia) device was used to non-invasively produce a central arterial waveform. A brachial cuff was placed on the right upper-arm while the participant was in a supine position. This technique records the

peripheral pressure waveforms and generates a corresponding central waveform.[5] This central arterial waveform provided an estimated central systolic BP reading, obtained from the peripheral arterial waveform via the built-in generalised transfer function. Some authors suggest that central systolic BP produces a better estimate of cardiovascular mortality than alternative measures of BP.[6]

3.6 Ambulatory Blood Pressure

Participants were fitted with a validated 24-hour ambulatory blood pressure (ABPM) and electrocardiogram apparatus (CardioXplore® CE120, Meditech, Budapest, Hungary). The apparatus was programmed to take measurements at 30-minute intervals during the day (from 06h00 to 22h00) and every hour throughout the night (from 22h00 to 06h00). An appropriately sized cuff was fitted to the participant's non-dominant arm at approximately the same time every day (late morning). Participants were provided with a diary card to record all activity that may influence BP during the 24-hour period.

3.7 Twenty-four-hour Urine Collection

A trained researcher explained the process for 24-hour urine sample collection and the necessary equipment (five-litre collection bottle, beaker and funnel) to the participants. Participants were asked to collect their 24-hour urine sample on a convenient day, which was noted. The first urine sample on the day of collection was to be discarded and all urine thereafter was collected. This included the first urine sample of the following morning (day two). The start and finish times were to be recorded. The protocol for 24-hour urine collection followed that of the Pan American Health Organization/World Health Organisation protocol for population level sodium determination in 24-hour urine samples.[7] Samples with a volume <300 ml per 24-hours and/or a 24-hour creatinine excretion <4 mmol or >25 mmol in women, and <6 mmol or >30 mmol in men were deemed incomplete.[8]

3.8 Blood Sampling and Biochemical Analyses

Venous blood samples were collected from the brachial vein branches, by a qualified nurse, early in the morning, using a sterile needle. This is an invasive procedure; however, it carries minimal risk to the participant. All participants were aware of the procedure prior to giving informed consent. The samples were prepared in accordance with standardised protocols. Following sampling, blood used for the preparation of serum was transported to the laboratory immediately and allowed to stand at room temperature for 30 minutes. Samples were then centrifuged using the Hettich Universal 320 centrifuge set at 3200 rpm at room temperature for 30 minutes. Urine samples were immediately transported to the laboratory at room temperature. Both urine and serum samples were then aliquoted and stored at -80°C until the time of analysis. All sample preparation was performed by trained postgraduate students and interns, who were dressed in protective laboratory clothing (i.e. nitrile gloves, lab coat, closed shoes and long trousers). Additionally, these individuals all had to provide proof of vaccination for Hepatitis B before they were permitted to work in the laboratory. All biochemical analyses were performed in the HART laboratory in Potchefstroom, with the exception of the multiplex analyses which were performed in Professor Delles' laboratory at the University of Glasgow.

Serum samples were analysed for high-sensitivity C-reactive protein (CRP), total cholesterol, low- and high-density lipoprotein cholesterol, glucose, and γ-glutamyltransferase (GGT) (Cobas Integra® 400plus, Roche, Basel, Switzerland). Creatinine concentrations were measured using the Creatinine Jaffé Gen.2 reagent (Roche, Basel, Switzerland). Cotinine was analysed using a chemiluminescence method on the Immulite (Siemens, Erlangen, Germany) apparatus. Estimated creatinine clearance was determined using the Cockroft Gault formula (Men [(140-age) * weight in kg * 1.23]/serum creatinine or Women [(140-age) * weight in kg * 1.04]/serum creatinine). Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology (CKD-EPI) formula.[9] The Cobas Integra® 400 plus (Roche, Basel, Switzerland) was used to measure urinary sodium, potassium, chloride and albumin by means of ion-selective electrode potentiometry. Daily urinary sodium and

potassium excretion were calculated by multiplying the sodium, potassium and creatinine concentrations (mmol/l) of the 24-hour urine by the total 24-hour volume of urine (in litres) resulting in the sodium, potassium and creatinine in mmol/day. Daily salt intake was then estimated from 24-hour urinary sodium excretion by converting sodium in mmol to mg: sodium (mmol) x 23= sodium (mg)[10] and then applying the conversion: 1g salt (sodium chloride) = 390 mg sodium.[10]

By using a MILLIPEX Map Human High Sensitivity T Cell Magnetic Bead Panel (EMD Millipore, Merck, Missouri, USA), a range of 21 inflammatory cytokines were measured. These include fractalkine, granulocyte-macrophage colony-stimulating factor, interferon gamma, interleukin 1 beta, interleukin 2, interleukin 4, interleukin 5, interleukin 6, interleukin 7, interleukin 8, interleukin 10, interleukin 12, interleukin 13, interleukin 17A, interleukin 21, interleukin 23, interferon-inducible T-cell alpha chemoattractant, macrophage inflammatory protein 1-alpha, macrophage inflammatory protein 1-beta, macrophage inflammatory protein 3-alpha and tumour necrosis factor alpha. This multiplex panel was analysed using Luminex xMAP technology on the Luminex 200™ analyser. This technology performed immunoassays on the surface of fluorescent-coded magnetic beads known as MagPlex-C microspheres. Microspheres were colour-coded with two fluorescent dyes. Through precise concentrations of these dyes, distinctly coloured bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres were created, each of which was coated with a specific capture antibody. Following analytes from a test sample that was captured by the bead, a biotinylated detection antibody was introduced. The reaction mixture was then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere. Samples determined to be below the detection limit (**Table 2**) were marked as missing data and not assigned a numerical value.

Table 2. Biochemical analyses lower detection limit, intra and inter assay variability.

	Lower Detection limit	Intra assay variability (CV)	Inter assay variability (CV)
CRP (mg/L)	0.10	1.3	3.5
Fractalkine (pg/mL)	7.75	<5%	<15%
INF-γ (pg/mL)	0.47	<5%	<20%
IL-1β (pg/mL)	0.14	<5%	<15%
IL-2 (pg/mL)	0.18	<5%	<15%
IL-7 (pg/mL)	0.43	<5%	<15%
IL-8 (pg/mL)	0.12	<5%	<15%
IL-12 (pg/mL)	0.16	<5%	<15%
IL-17 A (pg/mL)	0.31	<5%	<20%
IL-23 (pg/mL)	3.06	<5%	<20%
ITAC (pg/mL)	1.24	<5%	<15%
MIP-1α (pg/mL)	0.93	<5%	<15%
MIP-1β (pg/mL)	0.69	<5%	<15%
MIP-3α (pg/mL)	0.79	<5%	<20%
TNF-α (pg/mL)	0.16	<5%	<15%
IL-4 (pg/mL)	1.07	<5%	<15%
IL-5 (pg/mL)	0.10	<5%	<20%
IL-10 (pg/mL)	0.51	<5%	<20%
IL-13 (pg/mL)	0.24	<5%	<20%
IL-6 (pg/mL)	0.11	<5%	<20%
IL-21 (pg/mL)	0.14	<5%	<15%
GM-CSF (pg/mL)	0.33	<5%	<15%
Total Cholesterol (mmol/L)	0.10	0.51	1.90
HDL-C (mmol/L)	0.08	1.13	1.00
LDL-C (mmol/L)	0.10	1.50	1.90
Glucose (mmol/L)	0.24	1.80	2.10
γ-glutamyltransferase (U/L)	3.00	1.80	1.80

4. Data Management

The African-PREDICT study makes use of the REDCap (Research Electronic Data Capture, see http://project-redcap.org) system to capture data. REDCap is a free, secure online electronic database software. REDCap can be quickly and easily customised for collecting and tracking information and data from research studies and can additionally be used for participant scheduling.[11] Dr. Lisa Uys was appointed and trained as data manager for this study. When using this system, all laboratory specimens, evaluation forms, reports, data and other records are identified only by the participant number to maintain subject confidentiality. Apart from this system, the data manager ensures that regular backups of all data on

password-protected hard drives are made. The accuracy of the data used during statistical analysis is ensured by the data being imported directly into SPSS from automatically generated Microsoft® Excel spreadsheets.

A six-digit ID number is used: the first two digits correspond with a particular examination where, for example, "00" will refer to the baseline phase and "01" to the first follow-up; the next four digits denote the subject's unique four-digit number.

5. Ethical Considerations

The African-PREDICT study was authorised by the provincial and National Department of Health and was evaluated and approved by the North-West University's Health Research Ethics Committee (NWU-00001-12-A1) (**Appendix B**). The African-PREDICT study conformed to the Declaration of Helsinki and is registered at ClinicalTrials.gov (Identifier: NCT03292094). In addition, the African-PREDICT study is monitored continuously and approval is reissued every six months. This PhD study was additionally approved by the Health Research Ethics Committee of the North-West University (NWU-00058-18-A1) (**Appendix D**). While this PhD study has no direct benefit to the participants, the knowledge obtained during this study will add to the body of literature surrounding cytokine profiles in relation to health behaviours, BP and end-organ damage. In addition, participants will receive information about their cardiovascular health. The use of existing data from this study and the relevant above-mentioned methodology would not expose the participants to any additional mental, physical or emotional risks.

Prior to participation in the study, detailed information was conveyed individually to participants, with opportunities to ask questions. Voluntary participation included consent that personal and sensitive identifiable information would be captured for this study. However, the minimum amount of personally identifiable information was captured and only information that is directly pertinent to the aims of the study was captured. It was explained both orally and, in the participant informed consent leaflet, that this information would be stored; however, it

would be handled securely and coding would occur promptly. If the participants wished to participate, written informed consent was obtained.

This study made use of field workers who were able to convey and explain all the study procedures to participants in their home language to ensure that they fully understood what was required before informed consent was obtained. Field workers encouraged and helped participants to ask questions, which allowed for a two-way communication process.

6. Student Contributions

While this PhD study made use of existing data from the African-PREDICT study, I (Ms. S.H. Crouch) was involved in both the initial screening phase and the advanced research measurements of the African-PREDICT study. During my BSc Honours (2016), MHSc (2017), PhD (2018-2020) and as a research assistant (2018-2019), I was involved in numerous aspects of the African-PREDICT study.

For each of these I was responsible for explaining the procedure to the participants, applying the apparatus and performing the measurements. I was additionally involved in the downloading and checking of data for completion and accuracy before it could be added to the master dataset.

Screening:

- Urine dipstick analyses
- Glucose testing
- Cholesterol testing
- Office BP measurements
- Blood typing

Data Collection and Cleaning:

- Electrocardiography
- Pulse wave analyses and velocity
- Physical activity data
- Carotid sonography

Research:

- Questionnaire data collection
- Anthropometric measurement
- Urine sample collection (spot and 24-hour)
- Standard 12-lead electrocardiography
- Pulse wave analyses
- Pulse wave velocity
- Carotid sonography
- Office BP measurements
- Continuous BP measurements

I contributed to laboratory work for this study on a number of data collection days. Once blood and urine sample collection were completed on the research day, I was involved in ensuring that blood samples were centrifuged and aliquoted into cryovial tubes and correctly stored in -80°C biofreezers. I further contributed to the urine analysis of the 24-hour and spot urine samples, for the biochemical analysis of sodium, potassium and chloride making use of ion-selective electrode potentiometry on the Cobas Integra® 400 plus (Roche, Basel, Switzerland). I participated in measuring urinary creatinine using the Creatinine Jaffé Gen.2 reagent (Roche, Basel, Switzerland). Furthermore, I analysed serum samples for numerous analytes that form part of a clinical chemistry panel on the Cobas Integra® 400 plus (Roche, Basel, Switzerland). I was responsible for the sorting and packing of serum samples for shipping to the University of Glasgow for Multiplex analyses.

As a research assistant, I additionally contributed to telephoning and e-mailing participants to update their contact details and enquire about their willingness to remain in the study, as well as scheduling participants for their follow-up appointments. I was also responsible for controlling the flow of participants between measurements on the research day and capturing participant data. In addition to my contributions to the African-PREDICT study, I contributed to numerus facets of another study conducted by the research group, namely the EndoAfrica study. I was further involved in the sorting and preparation of samples for shipping for multiple other studies.

7. Statistical Analyses

IBM®, SPSS® version 25 and 26 (IBM Corporation, Armonk, New York) was used for the data analysis of this study. GraphPad Prism 5.03 (GraphPad Software, San Diego) was used for graphical designs. Continuous variables were inspected for normality and variables with a non-Gaussian distribution were logarithmically transformed.

The detailed statistical analyses for each of the research manuscripts (Chapters 3-5) are indicated in the Methods section of each chapter.

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Chapter 3

Distinct inflammatory mediator patterns in young black and white adults: The African-PREDICT study

Cytokine			
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Publisher	Elsevier		
Citation	Crouch SH, Botha-Le Roux S, Delles C, Graham LA, Schutte AE. Distinct inflammatory mediator patterns in young black and white adults: The African-PREDICT study. Cytokine. 2020;126:154894. https://doi.org/10.1016 /j.cyto.2019.154894 		
Aims & Scope	*Cytokine* is devoted exclusively to the study of the molecular biology, biochemistry, immunology, diagnostic and clinical applications of all known interleukins, hematopoietic factors, growth factors, cytotoxins, interferons, and new cytokines, *Cytokine* provides comprehensive coverage of cytokines and their receptors, 12 times a year, by publishing original high quality refereed scientific papers from prominent investigators in both the academic and industrial sectors.		
Author Instructions *Cytokine makes use of the Your Paper Your Way submission style*			
Language	Not specified.	**Font**	Arial, Helvetica, Times New Roman, Times, Symbol, Courier.
Spacing	Not specified.	**Margins**	Not specified.
Word count	Not specified.	**Tables& figures**	Not specified.
References	Not specified.	**Alignment**	Not specified.
Manuscript	There are no strict formatting requirements but all manuscripts must contain the essential elements needed to convey your manuscript, for		

	example Abstract, Keywords, Introduction, Materials and Methods, Results, Conclusions, Artwork and Tables with Captions.
Title page	• Title. • Author names and affiliations. • Corresponding author. • Present/permanent address.
Abstract	A concise and factual abstract is required.
Keywords	Max 6 keywords.
Text	Introduction, Methods, Results & Discussion.
Acknowledgements	Include acknowledgements.
Conflict of interest	Include statement declaring conflict of interest.
Funding	Include funding.
Ethical considerations	Work involving human subjects should be in accordance with Declaration of Helsinki, Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals and aim for the inclusion of representative human populations (sex, age and ethnicity). The terms sex and gender should be used correctly.
References	References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the article number or pagination must be present. Use of DOI is highly encouraged. *Reference to a journal publication:* [1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59. https://doi.org/10.1016/j.Sc.2010.00372.
Tables	• Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. • Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. • Avoid using vertical rules and shading in table cells.
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Distinct inflammatory mediator patterns in young black and white adults: The African-PREDICT study

Running title: Inflammatory mediator profile of bi-ethnic adults.

Key Words: Inflammatory mediators, inflammation, ethnicity, blood pressure.

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Abstract

Objective: Inflammatory mediators have been implicated in the early stages of cardiovascular disease development, including hypertension. Since global reports reflect a higher hypertension prevalence in black than white populations, we hypothesise the involvement of specific inflammatory mediators. We therefore compared a detailed range of 22 inflammatory mediators between young black and white adults, and determined the relationship with blood pressure. **Approach and Results:** We included 1197 adults (20-30 years; 50% black; 52% female) with detailed ambulatory blood pressures. Blood samples were analysed for 22 inflammatory mediators. For pro-inflammatory mediators, the black adults had higher C-reactive protein, interferon-inducible T-cell alpha chemoattractant, macrophage inflammatory protein 3 alpha (all p≤0.008), but lower interferon-gamma, interleukin (IL)-1β, IL-8, IL-12, IL-17A, and tumour necrosis factor alpha (all p≤0.048). For anti-inflammatory mediators the black group consistently had lower levels (IL-5, IL-10 and IL-13 (all p≤0.012)), resulting in generally higher pro-to-anti-inflammatory ratios in black than white adults (p≤0.001). In mediators with pro- and anti-inflammatory functions, the black group had lower granulocyte-macrophage colony-stimulating factor and IL-6 (both p≤0.010). These patterns were confirmed after adjustment for age, sex and waist circumference, or when stratifying by hypertensive status, sex and socio-economic status. Multi-variable adjusted regression analyses and factor analysis yielded no relationship between inflammatory mediators and blood pressure in this young healthy population. **Conclusions:** Black and white ethnic groups each consistently presented with unique inflammatory mediator patterns regardless of blood pressure, sex or social class. No association with blood pressure was seen in either of the groups.

Abbreviations

CVD	Cardiovascular disease
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IFN-γ	Interferon gamma
IL-1 β	Interleukin 1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-17A	Interleukin 17A
IL-23	Interleukin 23
ITAC	Interferon-inducible T-cell alpha chemoattractant
MIP-1α	Macrophage inflammatory protein 1-alpha
MIP-1β	Macrophage inflammatory protein 1-beta
MIP-3α	Macrophage inflammatory protein 3-alpha
TNFα	Tumour Necrosis Factor Alpha

Introduction

Inflammatory mediators have been implicated in the development of chronic diseases,[1-3] including hypertension and cardiovascular disease (CVD).[4] However, the physiological mechanisms through which this occur, are not all completely understood.[5-9] A number of pro-inflammatory mediators, such as CRP,[10] IL-6,[10-12] IL-17A,[12, 13] and TNF-α[12] contribute to an increase in blood pressure. In contrast, others such as IL-10, GM-CSF and IFN-γ show inverse associations with blood pressure.[12, 14] The functions of numerous other pro- and anti-inflammatory mediators such as IL-21,[15] and ITAC[16] are not yet clearly known, and neither is their association with blood pressure and CVD. Pro- and anti-inflammatory mediators are mainly produced by helper T cells and macrophages but also by other cell populations such as monocytes and certain nonimmune cells.[17]

Globally it has been reported that black populations have higher blood pressure than white populations.[18, 19] Many mechanisms have been proposed to explain this, such as a suppressed renin-angiotensin-aldosterone-system and salt sensitivity in black populations.[20-22] Based on our previous work in South Africa,[23, 24] another possible mechanism could be related to inflammation. Differences in pro- and anti-inflammatory mediator concentrations between ethnic groups have indeed been reported, e.g. in populations of African, European and South American descent.[24-30] Findings remain contradictory;[29] for example Mwantembe *et al.*[25] found that IL-1β levels were higher in black than in white adults, whereas Albandar *et al.*[27] found levels to be highest in Hispanics, followed by white individuals, while black participants exhibited the lowest levels. With regards to IL-6, Hong *et al.*[31] reported no ethnic differences between black and white participants, while Elkind *et al.*[26] found black individuals to have higher levels than white individuals. In South Africa, it was consistently shown that black individuals display higher levels of pro-inflammatory markers than whites.[23, 24, 32]

In this study we therefore compared a detailed range of 22 pro- and anti-inflammatory mediators and numerous blood pressure measurements between young black and white

adults. We also investigated the relationships between blood pressure and the pro- and anti-inflammatory mediator profile. This unique young disease-free sample allowed us to examine ethnic differences without interference from overt cardiovascular disease.

Methodology

Study population

This study forms part of the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT).[33] We recruited young black and white, men and women, between the ages of 20-30 years. African-PREDICT included apparently healthy individuals who were HIV uninfected; had a screening office brachial blood pressure of <140 mmHg systolic and <90 mmHg diastolic; had no self-reported previous diagnosis or used any medication for a chronic disease; and, if female, were not currently pregnant or lactating. Although individuals with office brachial BP of ≥140 and/or ≥90 were excluded during screening, there was an average two-week period between the screening and research phases. Some participants were classified as being hypertensive based on 24-hour ambulatory blood pressure during the research phase and were included in this study. This study analysed data for participants included in the baseline phase of the study (n=1202). Participants on anti-inflammatory medication or with missing biochemical analyses data were additionally excluded, resulting in a total population size of n=1189. The study was approved by the Health Research Ethics Committee (HREC) of the North-West University (NWU-00058-18-A1), adheres to the Declaration of Helsinki and all participants in the study provided written informed consent prior to participation.

General Measurements

Self-reported data with regards to demographic and lifestyle information were collected using a questionnaire. Socio-economic status was calculated using a point system that was adapted from Kuppuswamy's Socio-economic Status Scale[34] for a South African environment. Height, weight and waist circumference were measured using standard methods.[33] Body mass index

(BMI) was calculated using weight (kg) / height(m)^2. A compact, chest-worn accelerometric device (Actiheart4 CamNtech Ltd and CamNtech Inc, UK) was used to objectively measure physical activity over a maximum period of 7 days.

Blood pressure

Brachial blood pressure

Duplicate brachial blood pressure measurements were done on the left and right arms, with a 5-minute interval in-between, in a seated and resting state. We used the Dinamap® Procare 200 Blood Pressure Monitor (GE Medical Systems, Milwaukee, WI, USA).

Central blood pressure

cSBP was shown to provide a better estimate of cardiovascular mortality than other measures of blood pressure.[35] We therefore performed pulse wave analysis using the SphygmoCor XCEL (AtCor Medical, Sydney, Australia) device. This technique records the peripheral pressure waveforms and generates a corresponding central waveform.[36]

Ambulatory blood pressure

Participants were fitted with a validated 24-hour brachial ambulatory blood pressure (ABPM) monitor (Card(X)plore® CE120, Meditech, Budapest, Hungary). The apparatus was programmed to record every 30 minutes during the day (06h00 to 22h00) and every hour during the night (22h00 to 06h00).[37] Participants had a mean successful recording rate of 88%.

Biological sampling and biochemical analyses

Participants fasted overnight for at least eight hours prior to attending the day of research measurements. Blood samples were collected from the median cubital vein. The samples were prepared according to standardised protocols and stored at -80°C until the time of analysis.

Serum samples were analysed for high-sensitivity C-reactive protein (CRP), total cholesterol, low- and high-density lipoprotein cholesterol, glucose and γ-glutamyltransferase (GGT) (Cobas Integra® 400plus, Roche, Basel, Switzerland). Serum creatinine concentrations were

measured using the Creatinine Jaffé Gen.2 reagent (Roche, Basel, Switzerland). Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology (CKD-EPI) formula, without race in the equation as this is not appropriate for a South African population.[38, 39] Serum cotinine was analysed using a chemiluminescence method on the Immulite (Siemens, Erlangen, Germany) apparatus. EDTA whole blood samples were analysed for white blood cell, neutrophil, basophil, eosinophil, lymphocyte and monocyte count (Coulter AcT5 diff OV Hematology analyzer, Beckman Coulter, Brea, CA, US).

A MILLIPEX Map Human High Sensitivity T Cell Magnetic Bead Panel (EMD Millipore, Merck, Missouri, USA) was used to analyse 21 cytokines. This multiplex panel was analysed using Luminex xMAP technology on the Luminex 200™ analyser. This technology performed immunoassays on the surface of fluorescent-coded magnetic beads known as MagPlex-C microspheres. Microspheres were colour-coded with two fluorescent dyes. Through precise concentrations of these dyes, distinctly coloured bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres were created, each of which was coated with a specific capture antibody. A biotinylated detection antibody was introduced, and the mixture was then incubated in a streptavidin-phycoerythrin (PE) conjugate reporter. The fluorescent intensity of the reporter was determined which correlates with the concentration of a given analyte in solution. A standard curve was generated to calculate final analyte concentration.

Statistical analyses

IBM®, SPSS® version 24 (IBM Corporation, Armonk, New York) was used for data analysis. GraphPad Prism 5.03 (GraphPad Software, San Diego) was used all for graphics. Continuous variables were inspected for normality using QQ plots as well as inspection of skewness and kurtosis. Variables with non-Gaussian distributions were logarithmically transformed. To evaluate if data should be presented and analysed independently by sex or ethnicity, we investigated the interactions of these variables on the relationship between cSBP, 24-hour

systolic blood pressure (SBP) and the full range of pro- and anti-inflammatory mediators. We divided our groups by ethnicity based on the interactions found (**Table S1**). Pro- to anti-inflammatory ratios were calculated based on literature,[40, 41] and new ratios were suggested based on instances where pro-inflammatory mediators were higher and anti-inflammatory mediators were lower in the black and white groups. T-tests and Chi-square tests were used to compare the profiles of black and white participants. Analyses of covariance, adjusting for age; sex; and waist circumference, were used for ethnic comparisons of pro- and anti-inflammatory mediator concentrations. This was also done in normotensive and hypertensive groups, and groups categorised according to socio-economic status. The relationships between measures of blood pressure as the dependent variables and pro- and anti-inflammatory mediators as the main independent variables were explored using Pearson, partial and multiple regression analyses. Factor analyses were performed using the factor function of SPSS. Principal component analyses were used and factors with an eigenvalue >1 were retained. Varimax rotation was used to obtain independent interpretable factors. A factor loading of ≥0.3 was used to interpret the factor patterns. Double loading was handled by placing the variable in the factor with the strongest loading factor. Factor scores with a cumulative percentage of >50 were subsequently used for multiple regression analyses to determine the relationship between measures of blood pressure and factor scores.

Results

The general characteristics of the participants (n=1202) are shown in **Table 1.** The overall mean blood pressures of all participants were in the optimal blood pressure ranges.[37] Compared to the white group, black individuals had lower 24-hour and night SBP readings (all $p \leq 0.027$), but higher office diastolic blood pressure (DBP) ($p<0.001$) and central SBP ($p<0.001$). With regards to body composition, the black group had a lower BMI and waist circumference.

Black participants showed higher levels of the pro-inflammatory mediators CRP, ITAC, MIP3-α (all p≤0.008), but lower levels of IFN-γ, IL-1β, IL-8, IL-12, IL-17A, and TNF-α (all p≤0.048). Regarding anti-inflammatory mediators, black individuals had lower levels of IL-5, IL-10 and IL-13 (all p≤0.012). In terms of mediators that display both pro- and anti-inflammatory functions, we observed lower levels of both GM-CSF and IL-6 (all p≤0.010) in the black group. Black participants generally had higher pro-to-anti-inflammatory ratios than their white counterparts (p≤0.001). The black group exhibited lower white blood cell, neutrophil, monocyte, eosinophil and basophil counts (all p≤0.013).

We also performed analyses of covariance comparing black and white individuals (**Table 2**), adjusting for age, sex and waist circumference. Findings were largely similar (**Figure 1**).

Additionally, we compared ethnic groups, stratified by hypertensive status, sex and the middle socio-economic status group to account for the role these factors may play in determining inflammatory mediator status (**Table II-VI**). Similar to the ethnic comparisons in the total group, nearly identical ethnic profiles were seen, independent of hypertension status, sex and socio-economic status. **Figure 1** summarises the pro- and anti-inflammatory mediator profiles of black and white groups based on **Table 3** and **Tables I-VI.**

Multiple regression analyses were performed to determine whether blood pressure (24-hour SBP, 24-hour DBP, cSBP and nighttime SBP) is related to pro- and anti-inflammatory mediator concentrations within each ethnic group. In our young, healthy populations, the multiple regression analyses yielded no consistent statistically significant results (**Figures 2-3 and Figures I-II**)**.**

Factor analyses were performed with the pro- and anti-inflammatory mediator data to determine factor scores (**Table VII-IX**). Factor scores were subsequently used for multiple regressions analyses to determine whether blood pressure (24-hour SBP, 24-hour DBP, cSBP and nighttime SBP) is related to the pro- and anti-inflammatory mediator factors; this yielded no statistically significant results (**Table X**).

Table 1. Characteristics of young black and white adults.

	Black (n=599)	**White (n=590)**	***P***
Age, years	24.5± 3.17	24.6 ± 3.06	0.55
Male, n (%)	296 (49.0)	281 (47.4)	0.58
Socio-economic Status			
Low, n (%)	356 (58.9)	118 (19.9)	**<0.001**
Middle, n (%)	163 (27.0)	182 (30.7)	
High, n (%)	85 (14.1)	293 (49.4)	
Office BP (mmHg)			
Brachial SBP	117 ± 12.0	116 ± 12.6	0.16
Brachial DBP	79.4 ± 8.37	77.7 ± 8.12	**<0.001**
Central SBP	110 ± 9.58	105 ± 9.69	**<0.001**
Ambulatory BP (mmHg)			
24-hour SBP	116 ± 8.99	118 ± 9.82	**<0.001**
24-hour DBP	68.8 ± 5.93	68.5 ± 5.86	0.45
Night SBP	107 ± 10.1	109 ± 10.9	**0.027**
Night DBP	59.6 ± 6.77	59.1 ± 6.70	0.14
Hypertensive status, n (%)	50 (8.40)	78 (13.2)	**0.009**
Body Composition			
Body mass index (kg/m^2)	24.0 (17.6; 35.3)	25.0 (18.8; 35.1)	**<0.001**
Waist circumference (cm)	77.0 (63.0; 97.0)	81.5 (64.9; 107)	**<0.001**
Male	75.9 (64.3; 92.3)	88.5 (74.0; 110)	**0.007**
Female	78.2 (62.0; 102)	75.7 (63.5; 99.5)	**<0.001**
Inflammatory Markers			
Pro-Inflammatory			
CRP (mg/L)	1.01 (0.09; 11.1)	0.78 (0.08; 7.89)	**0.002**
Fractalkine (pg/mL)	28.0 (9.02; 71.7)	29.3 (9.82; 74.3)	0.24
IFN-γ (pg/mL)	6.75 (1.65; 21.5)	7.82 (163; 22.0)	**0.002**
IL-1β (pg/mL)	0.98 (0.20; 3.74)	1.08 (0.27; 3.66)	**0.033**
IL-2 (pg/mL)	0.78 (0.13; 4.11)	0.85 (0.16; 3.91)	0.21
IL-7 (pg/mL)	5.63 (1.34; 18.7)	5.65 (1.12; 18.8)	0.95
IL-8 (pg/mL)	1.74 (0.45; 5.97)	1.91 (0.47; 7.88)	**0.048**
IL-12 (pg/mL)	1.75 (0.35; 6.40)	1.97 (0.45; 6.72)	**0.024**
IL-17 A (pg/mL)	3.19 (0.64; 13.5)	3.57 (0.67; 14.1)	**0.044**
IL-23 (pg/mL)	119 (13.4; 600)	133 (12.9; 668)	0.10
ITAC (pg/mL)	4.70 (1.45; 19.9)	3.67 (1.40; 11.6)	**<0.001**
MIP-1α (pg/mL)	9.57 (2.74; 26.3)	10.3 (2.85; 27.0)	0.089
MIP-1β (pg/mL)	7.06 (2.70; 15.6)	7.28 (2.88; 16.4)	0.33
MIP-3α (pg/mL)	2.17 (0.54; 8.12)	1.90 (0.48; 6.61)	**0.008**
TNF-α (pg/mL)	1.61 (0.41; 5.29)	1.79 (0.49; 5.73)	**0.016**
Anti-Inflammatory			
IL-4 (pg/mL)	42.62 (7.33; 155)	43.85 (7.92; 154)	0.61
IL-5 (pg/mL)	0.89 (0.20; 3.88)	1.02 (0.26; 3.99)	**0.012**
IL-10 (pg/mL)	4.38 (0.89; 21.1)	5.42 (1.14; 21.2)	**<0.001**
IL-13 (pg/mL)	3.88 (0.58; 23.2)	5.04 (0.69; 30.3)	**<0.001**
Pro- and Anti-Inflammatory			
IL-6 (pg/mL)	1.81 (0.25; 9.84)	2.35 (0.30; 12.8)	**<0.001**
IL-21 (pg/mL)	1.34 (0.21; 5.53)	1.49 (0.26; 6.53)	0.074
GM-CSF (pg/mL)	7.23 (1.22; 32.5)	8.47 (1.22; 38.1)	**0.010**
Pro-to-Anti Inflammatory Ratios			
IL-6 to IL-10	0.41 (0.10; 2.03)	0.43 (0.12; 2.02)	0.39
IL-1β to IL-10	0.22 (0.07;0.73)	0.19 (0.07; 0.52)	**0.001**

	Black (n=599)	White (n=590)	*P*
TNF-α to IL-10	0.37 (0.17; 0.98)	0.33 (0.15; 1.01)	**<0.001**
CRP to IL-10	0.23 (0.01; 3.80)	0.14 (0.01; 2.40)	**<0.001**
MIP-*1α* to IL-10	2.13 (0.71; 7.72)	1.82 (0.59; 5.76)	**<0.001**
ITAC to IL-4	0.11 (0.02; 0.90)	0.08 (0.02; 0.67)	**<0.001**
ITAC to IL- 5	5.33 (1.10; 32.5)	3.60 (0.79; 17.7)	**<0.001**
ITAC to IL-10	1.08 (0.27; 6.65)	0.68 (0.21; 3.12)	**<0.001**
ITAC to IL-13	1.22 (0.17; 9.92)	0.73 (0.10; 5.66)	**<0.001**
Biochemical Markers and White Blood Cell Counts			
Total Cholesterol (mmol/L)	3.47 ± 0.97	4.04 ± 1.33	**<0.001**
HDL-C (mmol/L)	1.14 ± 0.38	1.17 ± 0.46	0.28
LDL-C (mmol/L)	2.07 (0.99; 3.71)	2.45 (1.20; 4.42)	**<0.001**
Triglycerides (mmol/L)	0.64 (0.31; 1.37)	0.80 (0.33; 2.11)	**<0.001**
Glucose (mmol/L)	3.94 ± 1.03	4.25 ± 1.09	**<0.001**
eGFR (ml/min/1.73m^2)	118 ± 14.5	106 ± 16.1	**<0.001**
White blood cell counts (x10^9/L)	5.21 (3.10; 8.40)	5.79 (3.80; 9.36)	**<0.001**
Neutrophils (x10^9/L)	2.43 (1.03; 5.49)	2.93 (1.35; 5.95)	**<0.001**
Lymphocytes (x10^9/L)	2.02 (1.26; 3.24)	1.98 (1.29; 3.01)	0.26
Monocytes (x10^9/L)	0.30 (0.14; 0.57)	0.42 (0.20; 0.79)	**<0.001**
Eosinophils (x10^9/L)	0.14 (0.05; 0.41)	0.15 (0.06; 0.39)	**0.008**
Basophils (x10^9/L)	0.04 (0.01; 0.11)	0.04 (0.02; 0.10)	**0.013**
Health Behaviours			
Serum cotinine (ng/ml)	4.04 (1.00; 341)	3.22 (1.00; 308)	0.081
Self-Reported Tobacco use n (%)	154 (25.5)	132 (22.3)	0.18
γ-glutamyltransferase (U/L)	22.2 (8.40; 66.2)	14.8 (5.40; 46.6)	**<0.001**
Self-Reported Alcohol use, n (%)	333 (55.8)	330 (55.7)	0.99
Hormonal Contraceptive Use, n (% of women)	143 (47.2)	130 (41.9)	0.19

Abbreviations: SBP, Systolic blood pressure; DBP, Diastolic blood pressure; BMI, Body mass index; HDL-C High density lipoprotein cholesterol; LDL-C, Low density lipoprotein cholesterol. Bold values indicate P<0.05. Data presented as mean ± SD; or geometric mean 95 C.I. Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interferon gamma (IFN-γ), Interleukin 1 beta (IL-1β), Interleukin 2 (IL-2), Interleukin 4 (IL-4), Interleukin 5 (IL-5), Interleukin 6 (IL-6), Interleukin 7 (IL-7), Interleukin 8 (IL-8), Interleukin 10 (IL-10), Interleukin 12 (IL-12), Interleukin 13 (IL-13), Interleukin 17A (IL-17A), Interleukin 21 (IL-21), Interleukin 23 (IL-23), Interferon-inducible T-cell alpha chemoattractant (ITAC), Macrophage inflammatory protein *1-alpha* (MIP-1α), Macrophage inflammatory protein *1-beta* (MIP-1β), Macrophage inflammatory protein 3-*alpha* (MIP-3α) and Tumour Necrosis Factor Alpha (TNFα).

Table 2. A comparison of cytokine concentrations between black and white individuals, adjusted for age, sex and waist circumference.

	Black (n=599)	**White (n=590)**	*p*	Difference: Black minus White
Inflammatory Markers				
Pro-Inflammatory				
CRP (mg/L)	1.16 (1.05; 1.29)	0.67 (0.61; 0.75)	**<0.001**	0.49
Fractalkine (pg/mL)	27.9 (26.4; 29.3)	29.5 (28.1; 31.1)	0.12	-1.60
IFN-γ (pg/mL)	6.68 (6.25; 7.14)	7.93 (7.40; 8.47)	**0.001**	-1.25
IL-1β (pg/mL)	0.97 (0.91; 1.04)	1.09 (1.02; 1.17)	**0.021**	-0.12
IL-2 (pg/mL)	0.77 (0.71; 0.85)	0.86 (0.78; 0.93)	0.12	-0.09
IL-7 (pg/mL)	5.62 (5.25; 6.01)	5.66 (5.28; 6.07)	0.86	-0.04
IL-8 (pg/mL)	1.72 (1.61; 1.84)	1.93 (1.81; 2.07)	**0.015**	-0.21
IL-12 (pg/mL)	1.74 (1.62; 1.87)	1.98 (1.85; 2.13)	**0.013**	-0.24
IL-17 A (pg/mL)	3.16 (2.92; 3.40)	3.61 (3.34; 3.90)	**0.016**	-0.45
IL-23 (pg/mL)	118 (107; 130)	135 (122; 149)	0.059	-17.0
ITAC (pg/mL)	4.70 (4.42; 5.00)	3.67 (3.45; 3.90)	**<0.001**	1.03
MIP-1α (pg/mL)	9.51 (8.95; 10.1)	10.4 (9.75; 11.0)	0.051	-0.89
MIP-1β (pg/mL)	7.24 (6.71; 7.35)	7.31 (7.00; 7.66)	0.20	-0.07
MIP-3α (pg/mL)	2.15 (2.01; 2.31)	1.91 (1.79; 2.06)	**0.022**	0.24
TNF-α (pg/mL)	1.59 (1.50; 1.70)	1.80 (1.69; 1.92)	**0.008**	-0.21
Anti-Inflammatory				
IL-4 (pg/mL)	42.5 (39.4; 45.9)	44.2 (40.1; 47.8)	0.49	-1.70
IL-5 (pg/mL)	0.88 (0.82; 0.95)	1.03 (0.96; 1.11)	**0.004**	-0.15
IL-10 (pg/mL)	4.32 (4.00; 4.66)	5.48 (5.08; 5.92)	**<0.001**	-1.16
IL-13 (pg/mL)	3.83 (3.48; 4.20)	5.11 (4.65; 5.61)	**<0.001**	-1.28
Pro- and Anti-Inflammatory				
IL-6 (pg/mL)	1.79 (1.63; 1.96)	2.38 (2.17; 2.62)	**<0.001**	-1.37
IL-21 (pg/mL)	1.32 (1.21; 1.43)	1.51 (1.39; 1.64)	**0.024**	-0.59
GM-CSF (pg/mL)	7.18 (6.58; 7.82)	8.55 (7.83; 9.33)	**0.006**	-0.19
Pro-to-Anti Inflammatory Ratios				
IL-6 to IL-10	0.41 (0.38; 0.45)	0.43 (0.40;0.47)	0.38	-0.02
IL-1β to IL-10	0.22 (0.21; 0.23)	0.19 (0.18; 0.20)	**0.001**	0.03
TNF-α to IL-10	0.37 (0.36; 0.39)	0.33 (0.32; 0.35)	**<0.001**	0.04
CRP to IL-10	0.27 (0.24; 0.31)	0.12 (0.11; 0.14)	**<0.001**	0.15
MIP-*1α* to IL-10	2.14 (2.01; 2.29)	1.81 (1.70; 1.93)	**<0.001**	0.33
ITAC to IL-4	0.11 (0.10; 0.12)	0.08 (0.08; 0.09)	**<0.001**	0.03
ITAC to IL- 5	5.38 (4.98; 5.82)	3.57 (3.30; 3.86)	**<0.001**	1.81
ITAC to IL-10	1.10 (1.02; 1.18)	0.67 (0.62; 0.72)	**<0.001**	0.43
ITAC to IL-13	1.24 (1.12; 1.36)	0.77 (0.65; 0.79)	**<0.001**	0.47

Bold values indicate P<0.05.
Normotensive status determined using 24-hour ambulatory blood pressure.

	Total		NT		HT		M		F		Mid SES	
	B	**W**	**B**	**W**	**B**	**W**	**B**	**W**	**B**	**W**	**B**	**W**
Black, n	599		536		50		307		307		162	
White, n	590		507		78		311		311		180	
Pro-Inflammatory												
CRP (mg/L)												
Fractalkine (pg/mL)												
IFN-γ (pg/mL)												
IL-1β (pg/mL)												
IL-2 (pg/mL)												
IL-7 (pg/mL)												
IL-8 (pg/mL)												
IL-12 (pg/mL)												
IL-17 A (pg/mL)												
IL-23 (pg/mL)												
ITAC (pg/mL)												
MIP-1α (pg/mL)												
MIP-1β (pg/mL)												
MIP-3α (pg/mL)												
TNF-α (pg/mL)												
Anti-Inflammatory												
IL-4 (pg/mL)												
IL-5 (pg/mL)												
IL-10 (pg/mL)												
IL-13 (pg/mL)												
Pro- and Anti-Inflammatory												
IL-6 (pg/mL)												
IL-21 (pg/mL)												
GM-CSF (pg/mL)												
Pro-to-Anti Inflammatory Ratios												
IL-6 to IL-10												
IL-1β to IL-10												
TNF-α toIL-10												
CRP to IL-10												
MIP-1α to IL-10												
ITAC to IL-4												
ITAC to IL- 5												
ITAC to IL-10												
ITAC to IL-13												

Figure 1. Comparison of cytokines between black and white individuals.

Cytokine concentrations that differ between black and white participants are indicated in colour (P<0.05). A darker shade indicates a higher value. Adjusted for: age, sex, waist circumference. Based on Tables 3 and II-VI. Black (B), White (W), Men (M), Women (F), Normotensive (NT), Hypertensive (HT), Middle Socio-economic Status (Mid SES)

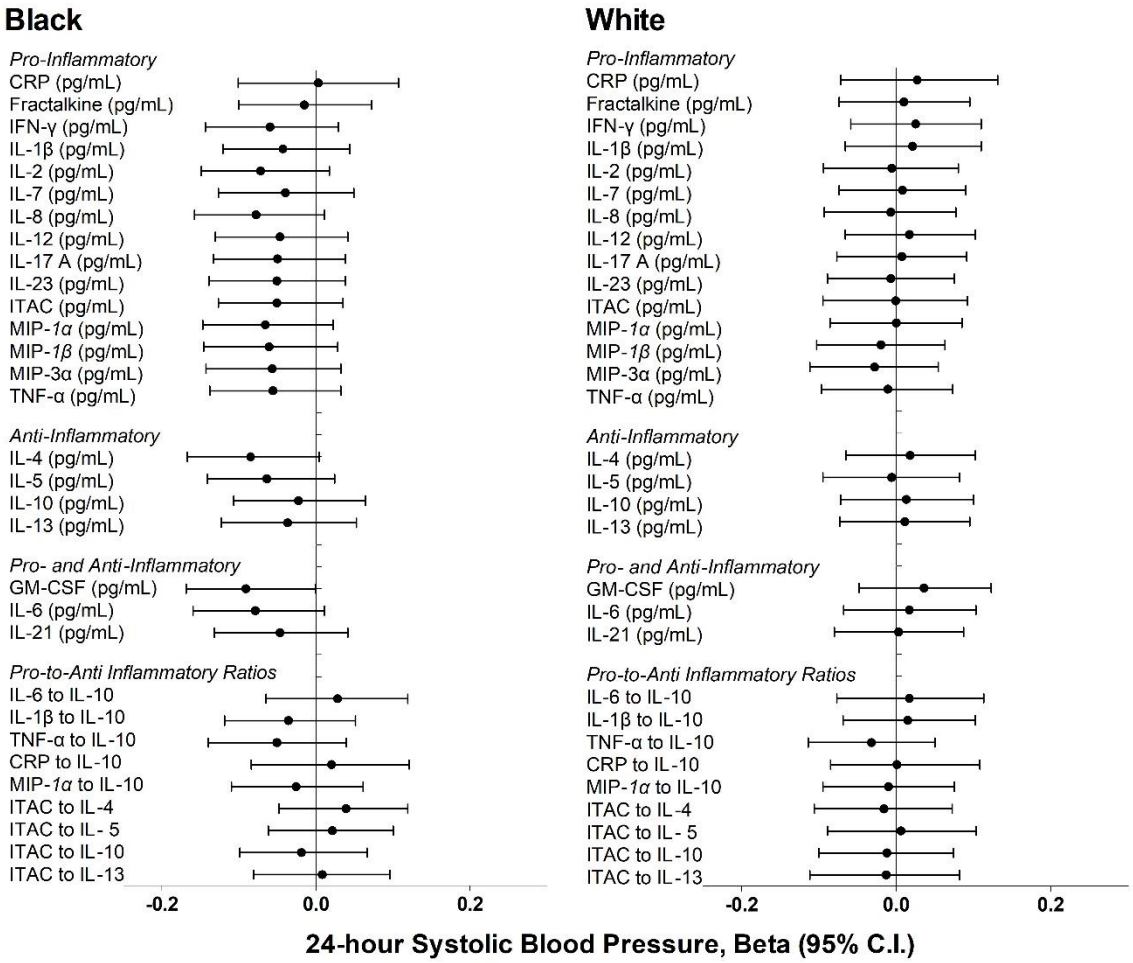

Figure 2. Multiple regression analyses showing the relationship between cytokine concentrations and 24-hour systolic blood pressure in black and white adults, respectively.

Adjusted for: sex, socio-economic status, waist circumference, total cholesterol, glucose, gamma glutamyltransferase, cotinine, estimated glomerular filtration rate, activity energy expenditure.

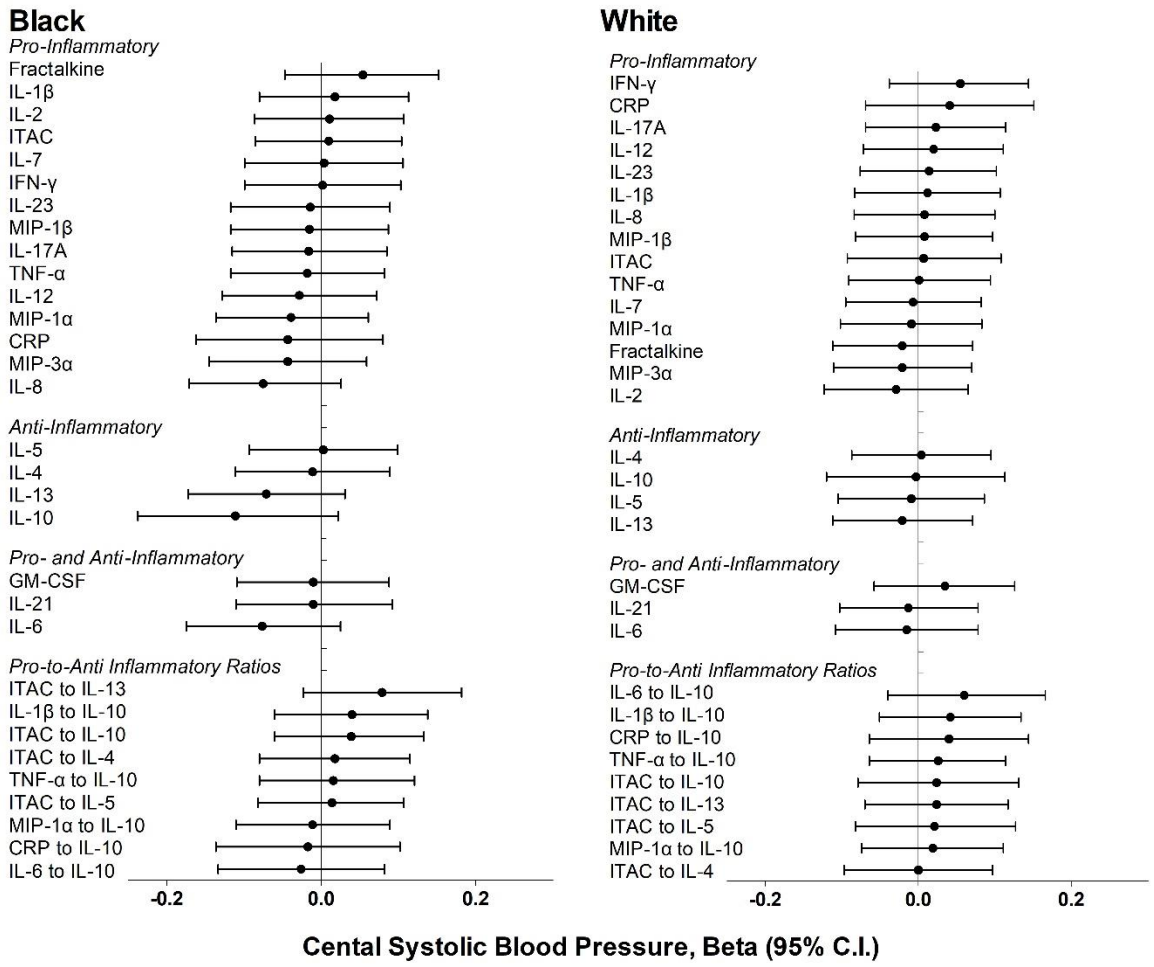

Figure 3. Multiple regression analyses showing the relationship between cytokine concentrations and central systolic blood pressure in black and white adults, respectively.

Adjusted for: sex, socio-economic status, waist circumference, total cholesterol, glucose, gamma glutamyltransferase, cotinine, estimated glomerular filtration rate, activity energy expenditure.

Discussion

Here we report distinct pro- and anti-inflammatory mediator patterns in young and healthy white and black adults, independent of sex, hypertension or socio-economic status. Black individuals consistently showed higher pro-to-anti-inflammatory ratios when compared to their white counterparts. This suggests that the pro- and anti-inflammatory mediator profiles in this young healthy cohort are likely to be ethnic-specific and independent of blood pressure, sex or socio-economic status. With respect to blood pressure profiles, we found no independent relationships between individual inflammatory mediators or inflammatory mediator patterns with blood pressure in either of the young ethnic groups.

Ethnic Profiling

When examining the pro-to-anti-inflammatory mediator ratios, black adults consistently displayed higher ratios, even though they were leaner, had better lipid and glycaemic profiles, as well as lower immune cell counts. These results for mediator ratios were found, despite the black group having lower levels for six of the fifteen pro-inflammatory mediators compared to whites, and higher levels of only three of the fifteen mediators. It is the anti-inflammatory mediators where the black group displayed lower levels for three of the four mediators, which resulted in overall higher pro-to-anti-inflammatory ratios.

Literature regarding ethnic differences in cytokine concentrations remains inconsistent. Elkand *et al*.[26] and Schutte *et al*.[23,24] found CRP levels to be higher in black than in white populations, which is in line with our findings. However, Ford *et al.*[42] found no difference in CRP levels between African-American and white children and young adults.

Many studies and systematic reviews focussed on only a few inflammatory mediators at a time, and usually the same mediators, such as CRP, IL-6, TNF-α and IL-1β.[23, 24, 29, 43-46] This simplifies comparison between studies. In our study, we made use of a large range of mediators which, while complex, provides a better overall inflammatory profile.

One possible explanation for the ethnic-specific mediator profiles may be the differences in gene polymorphisms distribution across different ethnic groups.[47] It has been shown that differences in the inheritance of IL-6 and IL-10 genotypes in black populations result in higher expression when compared to white populations.[47] Differences in genotypes between black and white populations have also been seen where IL-2 is concerned.[48]

Fifty-nine percent of the black population (versus 20% in whites) falls within a low socio-economic category. It has recently been shown that childhood environment may play a role in the development of inflammatory phenotypes suggesting that the overall low socio-economic status of this black population may play a role.[49]

The effect of potential confounders on the difference in mediator concentrations should be taken into consideration. In our comparisons (**Figure 1**) we adjusted for age, sex and abdominal obesity. Yet tobacco use,[50] alcohol consumption,[51] and obesity,[52] which are often linked to socio-economic status,[53-55] may play a role in increasing pro-inflammatory mediator levels, as studies have found that those with a low socio-economic status show elevated systemic inflammation.[56, 57] However, when counteracting this by comparing black and white groups with similar mid-level socio-economic status, the original profiles remained robust, suggesting heritability.

Relationship with blood pressure

Our motivation for this ethnic comparative study was to identify the involvement of specific inflammatory mediators as a possible explanation for the high prevalence of hypertension in black populations.[58] Our analyses yielded no clear findings, with no individual mediator or pattern associating with a range of clinic, central and ambulatory blood pressure measures. Since at this early stage in life there are multiple factors that may dominate physiological variances in blood pressure– such as differences in renin-angiotensin-aldosterone profiles,[58] arterial stiffness,[59] and physical activity[60] – blood pressure may not be a measure sensitive enough to show small scale, early inflammatory changes in young otherwise healthy

individuals. It may thus be useful to focus on more sensitive markers of early cardiovascular changes such as arterial stiffness, the microvasculature,[61] and endothelial function.[62]

In addition to the relationship seen with blood pressure, inflammatory mediators are also associated with other disease states. It has been shown that IL-1β, IL-6, IL-17A and TNF-α are implicated in the development of atherosclerosis,[63, 64] while IL-10 is inversely associated.[65] More research is required regarding detailed inflammatory mediators in the development of CVD in black and white populations.

Our study results should be interpreted within the framework of its strengths and limitations. Participants were screened prior to participation using strict exclusion criteria for conditions that may affect the results. The absence of pre-existing chronic diseases afforded us the opportunity to investigate the underlying physiology in adults without influence from pathology. A further strength of our study is the inclusion of a wide range of pro- and anti-inflammatory mediators as well as our large sample size. Mediator concentrations were established using a high-sensitivity kit which allowed for detection even at low levels. In terms of limitations, the black group had a greater proportion of individuals with low socio-economic status, allowing for potential socio-economic bias. However, sensitivity analyses were performed to address this and yielded no changes in our main results. Additionally, this study uses cross-sectional data.

In conclusion, the black and white ethnic groups each consistently presented with unique inflammatory mediator patterns regardless of blood pressure, sex or socio-economic status. Despite a higher pro-to-anti-inflammatory status of the young black adults, there was no association with blood pressure in the black or white groups. Whether these ethnic specific patterns will relate to future disease development, needs to be established.

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Disclosures

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

No conflict of Interest.

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Supplementary Information

Table I. Interactions of sex and ethnicity on cytokines.

	cSBP		**24 hr SBP**	
	Ethnicity	Sex	Ethnicity	Sex
	p	*p*	*p*	*p*
CRP	**0.004**	**0.004**	0.089	**0.015**
Fractalkine	**0.008**	0.54	0.25	0.51
INF-γ	0.83	0.15	0.25	0.90
IL-1β	0.60	0.32	0.25	0.14
IL-2	0.56	**0.032**	0.59	0.17
IL-7	0.28	0.46	0.52	0.45
IL-8	0.16	0.072	0.17	0.18
IL-12	0.75	0.076	0.43	0.32
IL-17 A	0.52	**0.010**	0.53	0.11
IL-23	0.59	0.18	0.59	0.96
ITAC	0.89	0.54	0.76	0.43
MIP-1α	0.57	0.52	0.30	0.48
MIP-1β	0.94	0.080	0.63	0.92
MIP-3α	0.73	0.15	0.21	0.22
TNF-α	0.94	0.11	0.17	0.28
IL-4	0.99	0.90	**0.030**	0.36
IL-5	0.94	0.29	0.43	0.51
IL-10	0.91	0.24	0.39	0.31
IL-13	0.86	0.89	0.71	0.70
IL-6	0.84	0.44	0.17	0.73
IL-21	0.98	0.27	0.18	0.63
GM-CSF	0.80	0.82	**0.022**	0.48

Table II. A comparison of cytokine concentrations between normotensive black and white individuals, adjusted for age, sex and waist circumference.

	Black (n=536)	**White (n=507)**	*p*
Inflammatory Markers			
Pro-Inflammatory			
CRP (mg/L)	1.12 (1.01; 1.25)	0.65 (0.58; 0.72)	**<0.001**
Fractalkine (pg/mL)	28.1 (26.7; 29.7)	29.92 (28.3;31.6)	0.77
INF-γ (pg/mL)	6.76 (6.30; 7.24)	7.94 (7.40; 8.55)	**0.002**
IL-1β (pg/mL)	0.97 (0.91; 1.05)	1.08 (1.00; 1.17)	**0.047**
IL-2 (pg/mL)	0.79 (0.72; 0.87)	0.86 (0.78; 0.94)	0.23
IL-7 (pg/mL)	5.66 (5.26; 6.08)	5.68 (5.27; 6.11)	0.96
IL-8 (pg/mL)	1.76 (1.64;1.89)	1.94 (1.80; 2.08)	0.066
IL-12 (pg/mL)	1.77 (1.65; 1.91)	1.99 (1.84; 2.15)	**0.032**
IL-17 A (pg/mL)	3.21 (2.96; 3.48)	3.62 (3.33; 3.94)	**0.042**
IL-23 (pg/mL)	129 (107; 132)	136 (122; 151)	0.079
ITAC (pg/mL)	4.70 (4.41; 5.00)	3.68 (3.45; 3.93)	**<0.001**
MIP-1α (pg/mL)	9.59 (8.99; 10.2)	10.5 (9.86; 11.2)	**0.046**
MIP-1β (pg/mL)	7.05 (6.73; 7.40)	7.40 (7.05; 7.76)	0.17
MIP-3α (pg/mL)	2.21 (2.06; 2.38)	1.91 (1.77; 2.06)	**0.006**
TNF-α (pg/mL)	1.62 (1.51; 1.73)	1.82 (1.70; 1.95)	**0.018**
Anti-Inflammatory			
IL-4 (pg/mL)	42.8 (39.5; 46.5)	44.3 (40.6; 48.1)	0.58
IL-5 (pg/mL)	0.91 (0.85; 0.99)	1.05 (0.97; 1.13)	**0.018**
IL-10 (pg/mL)	4.36 (4.01; 4.2)	5.48 (5.05; 5.9)	**<0.001**
IL-13 (pg/mL)	3.90 (3.54; 4.30)	5.24 (4.27; 5.74)	**<0.001**
Pro- and Anti-Inflammatory			
IL-6 (pg/mL)	1.85 (1.68; 2.04)	2.42 (2.19; 2.67)	**<0.001**
IL-21 (pg/mL)	1.33 (122; 1.46)	1.53 (1.40; 1.67)	**0.029**
GM-CSF (pg/mL)	7.21 (7.59; 7.91)	8.59 (7.82; 9.44)	**0.010**
Pro-to-Anti Inflammatory Ratios			
IL-6 to IL-10	0.42 (0.39; 0.46)	0.44 (0.41; 0.47)	0.56
IL-1β to IL-10	0.22 (0.21; 0.23)	0.19 (0.18; 0.20)	**0.002**
TNF-α to IL-10	0.38 (0.36; 0.40)	0.33 (0.32; 0.35)	**0.001**
CRP to IL-10	0.26 (0.22; 0.30)	0.12 (0.10; 0.14)	**<0.001**
MIP-*1α* to IL-10	2.14 (2.00; 2.30)	1.84 (1.72; 1.97)	**0.002**
ITAC to IL-4	0.11 (0.10; 0.12)	0.08 (0.08; 0.09)	**<0.001**
ITAC to IL- 5	5.21 (4.81; 5.66)	3.52 (3.56; 3.83)	**<0.001**
ITAC to IL-10	1.09 (1.01; 1.18)	0.67 (0.62; 0.73)	**<0.001**
ITAC to IL-13	1.22 (1.10; 1.35)	0.71 (0.64; 0.79)	**<0.001**

Bold values indicate P<0.05.
Normotensive status determined using 24-hour ambulatory blood pressure.

Table III. A comparison of cytokine concentrations between hypertensive black and white individuals, adjusted for age, sex and waist circumference.

	Black (n=50)	**White (n=78)**	*p*
Inflammatory Markers			
Pro-Inflammatory			
CRP (mg/L)	1.44 (1.02; 2.04)	1.07 (0.82; 1.41)	0.21
Fractalkine (pg/mL)	27.5 (22.5; 33.6)	26.6 (22.9; 31.1)	0.82
INF-γ (pg/mL)	6.64 (5.22; 8.3)	7.64 (6.35; 9.20)	0.38
IL-1β (pg/mL)	0.97 (0.78; 1.22)	1.12 (0.94; 1.34)	0.34
IL-2 (pg/mL)	0.66 (0.49; 0.95)	0.86 (0.66; 1.13)	0.27
IL-7 (pg/mL)	5.38 (4.21; 6.86)	5.60 (4.62; 6.78)	0.81
IL-8 (pg/mL)	1.38 (1.09; 1.75)	1.88 (1.57; 2.26)	0.051
IL-12 (pg/mL)	1.57 (1.23; 2.01)	1.89 (1.56; 2.28)	0.27
IL-17 A (pg/mL)	3.10 (2.38; 4.06)	3.44 (2.80; 4.24)	0.57
IL-23 (pg/mL)	110 (77.1; 156)	127 (96.8; 167)	0.53
ITAC (pg/mL)	5.41 (4.19; 6.98)	3.50 (2.87; 4.27)	**0.012**
MIP-1α (pg/mL)	10.1 (8.00;12.7)	8.91 (7.46; 10.7)	0.43
MIP-1β (pg/mL)	7.38 (6.25; 8.71)	6.65 (5.85; 7.57)	0.35
MIP-3α (pg/mL)	1.80 (1.36; 2.38)	1.95 (1.57; 2.43)	0.67
TNF-α (pg/mL)	1.48 (1.19; 1.85)	1.68 (1.41; 2.01)	0.40
Anti-Inflammatory			
IL-4 (pg/mL)	40.9 (31.1; 54.1)	43.3 (34.9; 53.7)	0.77
IL-5 (pg/mL)	0.69 (0.52; 0.90)	0.93 (0.76; 1.15)	0.093
IL-10 (pg/mL)	4.29 (3.28; 5.61)	5.37 (4.44; 6.73)	0.18
IL-13 (pg/mL)	3.21 (2.19; 4.69)	4.71 (3.51; 6.32)	0.13
Pro- and Anti-Inflammatory			
IL-6 (pg/mL)	1.31 (0.91; 1.89)	2.22 (1.67; 2.94)	**0.033**
IL-21 (pg/mL)	1.29 (0.92; 1.79)	1.34 (1.03; 1.73)	0.86
GM-CSF (pg/mL)	6.85 (5.07; 9.27)	8.28 (6.55; 10.5)	0.35
Pro-to-Anti Inflammatory Ratios			
IL-6 to IL-10	0.31 (0.23; 0.40)	0.41 (0.34; 0.51)	0.094
IL-1β to IL-10	0.21 (0.17; 0.26)	0.20 (0.17; 0.23)	0.62
TNF-α to IL-10	0.35 (0.30; 0.40)	0.32 (0.28; 0.36)	0.41
CRP to IL-10	0.34 (0.22;0.53)	0.19 (0.14; 0.27)	**0.048**
MIP-*1α* to IL-10	2.28 (1.84; 2.82)	1.57 (1.33; 1.85)	**0.011**
ITAC to IL-4	0.13 (0.10; 0.18)	0.08 (0.06; 0.10)	**0.018**
ITAC to IL- 5	7.67 (5.78; 10.2)	3.78 (3.03; 4.69)	**<0.001**
ITAC to IL-10	1.26 (0.97; 1.63)	0.65 (0.53; 0.80)	**<0.001**
ITAC to IL-13	1.69 (1.14; 2.49)	0.74 (0.55; 1.01)	**0.002**

Bold values indicate P<0.05.
Hypertensive status determined using 24-hour ambulatory blood pressure.

Table IV. A comparison of cytokine concentrations between black and white men, adjusted for age, sex and waist circumference.

	Black (n=307)	**White (n=311)**	*p*
Inflammatory Markers			
Pro-Inflammatory			
CRP (mg/L)	0.81 (0.70; 0.95)	0.43 (0.37; 0.51)	**<0.001**
Fractalkine (pg/mL)	27.6 (25.5; 29.9)	30.3 (27.9; 32.9)	0.14
INF-γ (pg/mL)	6.76 (6.12; 7.48)	7.89 (7.11; 8.75)	0.054
IL-1β (pg/mL)	1.00 (0.91; 1.10)	1.08 (1.02; 1.19)	0.31
IL-2 (pg/mL)	0.81 (0.71; 0.93)	0.81 (0.71; 0.92)	0.97
IL-7 (pg/mL)	5.57 (5.04; 6.17)	5.77 (5.20; 6.41)	0.66
IL-8 (pg/mL)	1.74 (1.57;1.92)	1.94 (1.75; 2.15)	0.16
IL-12 (pg/mL)	1.74 1.57; 1.93)	1.97 (1.77; 2.19)	0.14
IL-17 A (pg/mL)	3.22 (2.87; 3.61)	3.56 (3.16; 4.01)	0.27
IL-23 (pg/mL)	118 (103; 136)	136 (188; 158)	0.20
ITAC (pg/mL)	4.75 (4.32; 5.25)	3.64 (3.29; 4.03)	**0.001**
MIP-1α (pg/mL)	9.95 (9.12; 10.9)	10.5 (9.59; 11.5)	0.45
MIP-1β (pg/mL)	7.38 (6.92; 7.89)	7.43 (6.95; 7.96)	0.89
MIP-3α (pg/mL)	2.17 (1.95; 2.42)	1.99 (1.79; 2.22)	0.29
TNF-α (pg/mL)	1.64 (1.49; 1.79)	1.88 (1.71; 2.07)	0.056
Anti-Inflammatory			
IL-4 (pg/mL)	42.0 (37.4; 47.0)	46.3 (41.1; 52.1)	0.28
IL-5 (pg/mL)	0.85 (0.65; 0.95)	1.01 (0.91; 1.14)	**0.047**
IL-10 (pg/mL)	4.37 (3.89; 4.90)	5.55 (4.93; 6.24)	**0.008**
IL-13 (pg/mL)	3.82 (3.30; 4.42)	5.05 (4.35; 5.86)	**0.016**
Pro- and Anti-Inflammatory			
IL-6 (pg/mL)	1.72 (1.50; 1.97)	2.43 (2.11; 2.80)	**0.002**
IL-21 (pg/mL)	1.29 (1.15; 1.45)	1.54 (1.37; 1.74)	0.056
GM-CSF (pg/mL)	7.06 (6.19; 8.04)	8.83 (7.73; 10.1)	**0.029**
Pro-to-Anti Inflammatory Ratios			
IL-6 to IL-10	0.39 (0.35; 0.44)	0.44 (0.40; 0.50)	0.18
IL-1β to IL-10	0.22 (0.20; 0.24)	0.19 (0.18; 0.21)	**0.047**
TNF-α to IL-10	0.38 (0.35; 0.41)	0.34 (0.32; 0.37)	0.073
CRP to IL-10	0.19 (0.16; 0.23)	0.08 (0.07; 0.10)	**<0.001**
MIP-*1α* to IL-10	2.20 (2.00; 2.42)	1.85 (1.68; 2.04)	**0.020**
ITAC to IL-4	0.11 (0.10; 0.13)	0.08 (0.07; 0.09)	**<0.001**
ITAC to IL- 5	5.68 (5.04; 6.41)	3.61 (3.18; 4.08)	**<0.001**
ITAC to IL-10	1.11 (0.99; 1.24)	0.65 (0.58; 0.73)	**<0.001**
ITAC to IL-13	1.26 (1.08; 1.48)	0.72 (0.61; 0.84)	**<0.001**

Bold values indicate P<0.05.
Hypertensive status determined using 24-hour ambulatory blood pressure.

Table V. A comparison of cytokine concentrations between black and white women, adjusted for age, sex and waist circumference.

	Black (n=307)	**White (n=311)**	*p*
Inflammatory Markers			
Pro-Inflammatory			
CRP (mg/L)	1.64 (1.42; 1.89)	1.00 (0.86; 1.15)	**<0.001**
Fractalkine (pg/mL)	28.1 (26.1; 30.2)	28.8 (26.9; 31.1)	0.58
INF-γ (pg/mL)	6.73 (6.11; 7.40)	7.82 (7.11; 8.59)	**0.029**
IL-1β (pg/mL)	0.96 (0.87; 1.06)	1.07 (0.97; 1.19)	0.13
IL-2 (pg/mL)	0.76 (0.67; 0.86)	0.87 (0.77; 0.99)	0.13
IL-7 (pg/mL)	5.69 (5.15; 6.28)	5.56 (5.04; 6.12)	0.74
IL-8 (pg/mL)	1.72 (1.56; 1.89)	1.90 (1.73; 2.09)	0.14
IL-12 (pg/mL)	1.77 (1.60; 1.96)	1.96 (1.77; 2.17)	0.17
IL-17 A (pg/mL)	3.18 (2.84; 3.55)	3.56 (3.18; 3.96)	0.16
IL-23 (pg/mL)	115 (103; 137)	132 (114; 151)	0.33
ITAC (pg/mL)	4.66 (4.29; 5.07)	3.69 (3.40; 4.00)	**<0.001**
MIP-1α (pg/mL)	9.91 (8.47; 10.1)	10.1 (9.25; 11.0)	0.17
MIP-1β (pg/mL)	6.84 (6.41; 7.29)	7.06 (6.62; 7.53)	0.50
MIP-3α (pg/mL)	2.17 (1.97; 2.40)	1.82 (1.65; 2.00)	**0.010**
TNF-α (pg/mL)	1.58 (1.44; 1.73)	1.70 (1.56; 1.87)	0.26
Anti-Inflammatory			
IL-4 (pg/mL)	42.9 (38.4; 479)	42.6 (38.1; 47.4)	0.92
IL-5 (pg/mL)	0.91 (0.82; 1.02)	1.05 (0.95; 1.16)	0.063
IL-10 (pg/mL)	4.32 (3.87; 4.82)	5.37 (4.83; 5.97)	**0.005**
IL-13 (pg/mL)	3.81 (3.35; 4.35)	5.19 (4.57; 5.90)	0.001
Pro- and Anti-Inflammatory			
IL-6 (pg/mL)	1.80 (1.58;2.07)	2.41 (2.11; 2.75)	**0.003**
IL-21 (pg/mL)	1.37 (1.21; 1.55)	1.45 (1.29; 1.63)	0.53
GM-CSF (pg/mL)	7.38 (6.53; 8.36)	8.20 97.26; 9.27)	0.24
Pro-to-Anti Inflammatory Ratios			
IL-6 to IL-10	0.42 (0.38; 0.46)	0.44 (0.40; 0.49)	0.41
IL-1β to IL-10	0.22 (0.20;0.24)	0.19 (0.18; 0.21)	**0.020**
TNF-α to IL-10	0.37 (0.35; 0.40)	0.32 (0.30; 0.34)	**0.001**
CRP to IL-10	0.38 (0.32; 0.45)	0.19 (0.16; 0.22)	**<0.001**
MIP-*1α* to IL-10	2.08 (1.91; 2.28)	1.73 (1.63; 1.94)	**0.013**
ITAC to IL-4	0.11 (0.10; 0.12)	0.09 (0.08; 0.10)	**0.006**
ITAC to IL- 5	5.15 (4.62; 5.75)	3.50 (3.15; 3.89)	**<0.001**
ITAC to IL-10	1.08 (0.98; 1.20)	0.69 (0.62; 0.76)	**<0.001**
ITAC to IL-13	1.22 (1.07; 1.40)	0.71 (0.63; 0.81)	**<0.001**

Bold values indicate P<0.05.
Hypertensive status determined using 24-hour ambulatory blood pressure.

Table VI. A comparison of cytokine concentrations between black and white individuals in the middle socio-economic group, adjusted for age, sex and waist circumference.

	Black (n=162)	**White (n=180)**	*p*
Inflammatory Markers			
Pro-Inflammatory			
CRP (mg/L)	1.22 (1.00; 1.49)	0.63 0.52; 0.77)	**<0.001**
Fractalkine (pg/mL)	30.5 (25.7; 33.7)	29.0 (26.4; 32.0)	0.51
INF-γ (pg/mL)	7.41 (6.55; 8.39)	7.78 (6.92; 8.77)	0.59
IL-1β (pg/mL)	1.04 (0.91; 1.18)	1.05 (0.93; 1.19)	0.85
IL-2 (pg/mL)	0.86 (0.73; 1.02)	0.81 (0.69; 0.94)	0.61
IL-7 (pg/mL)	5.70 (4.98; 6.53)	5.37 (4.72; 5.75)	0.55
IL-8 (pg/mL)	1.79 (1.56; 2.05)	1.89 (1.66; 2.16)	0.56
IL-12 (pg/mL)	1.85 (1.59; 2.14)	1.93 (1.67; 2.22)	0.67
IL-17 A (pg/mL)	3.37 (2.90; 3.91)	3.48 (3.02; 4.01)	0.76
IL-23 (pg/mL)	133 (110; 161)	130 (108; 155)	0.84
ITAC (pg/mL)	4.50 (4.00; 5.06)	3.61 (3.22; 4.04)	**0.011**
MIP-1α (pg/mL)	10.0 (8.89; 11.3)	10.1 (8.99; 10.3)	0.96
MIP-1β (pg/mL)	7.14 (6.53; 7.82)	7.29 (6.70; 7.94)	0.75
MIP-3α (pg/mL)	2.13 (1.87; 2.42)	1.91 (1.69; 2.16)	0.26
TNF-α (pg/mL)	1.68 (1.48; 1.91)	1.73 (1.53; 1.95)	0.77
Anti-Inflammatory			
IL-4 (pg/mL)	47.8 (41.2; 55.3)	42.5 (36.9; 48.9)	0.27
IL-5 (pg/mL)	0.92 (0.79; 1.07)	0.97 (0.85; 1.12)	0.62
IL-10 (pg/mL)	4.52 (3.88; 5.26)	5.24 (4.54; 6.04)	0.19
IL-13 (pg/mL)	4.22 (3.50; 5.08)	4.85 (4.06; 5.79)	0.31
Pro- and Anti-Inflammatory			
IL-6 (pg/mL)	1.93 (1.60; 2.32)	2.32 (1.94; 2.77)	0.18
IL-21 (pg/mL)	1.47 (1.25; 1.73)	1.42 (1.22; 1.66)	0.78
GM-CSF (pg/mL)	7.87 (6.65; 9.31)	8.43 (7.19; 9.91)	0.57
Pro-to-Anti Inflammatory Ratios			
IL-6 to IL-10	0.43 (0.37; 0.50)	0.44 (0.38; 0.50)	0.92
IL-1β to IL-10	0.24 (0.21; 0.26)	0.20 (0.18; 0.22)	0.017
TNF-α to IL-10	0.38 (0.35; 0.42)	0.33 (0.30; 0.36)	0.025
CRP to IL-10	0.27 (0.21; 0.35)	0.12 (0.10; 0.16)	**<0.001**
MIP-*1α* to IL-10	2.15 (1.91; 2.42)	1.84 (1.64; 2.06)	0.074
ITAC to IL-4	0.09 (0.08; 0.11)	0.09 (0.07; 0.10)	0.39
ITAC to IL- 5	4.88 (4.22; 5.64)	3.72 (3.25; 4.27)	**0.011**
ITAC to IL-10	1.00 (0.87; 1.15)	0.69 (0.61; 0.79)	**<0.001**
ITAC to IL-13	1.06 (0.88; 1.28)	0.74 (0.62; 0.88)	**0.007**

Bold values indicate P<0.05.
Hypertensive status determined using 24-hour ambulatory blood pressure.

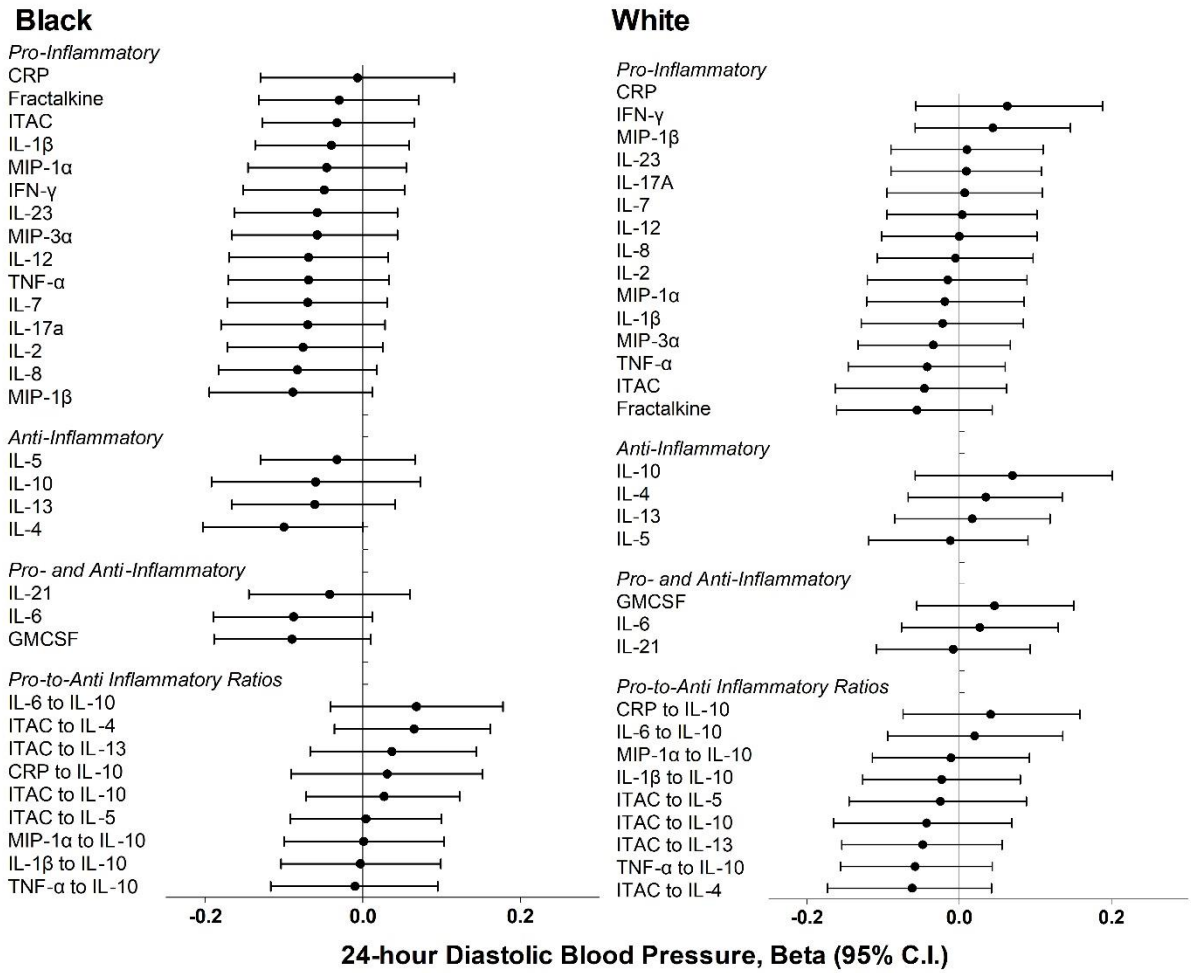

Figure I. Multiple regression analyses showing the relationship between cytokine concentrations and diastolic blood pressure in black and white adults.

Adjusted for: sex, socio-economic status, waist circumference, total cholesterol, glucose, gamma glutamyltransferase, cotinine, estimated glomerular filtration rate, activity energy expenditure.

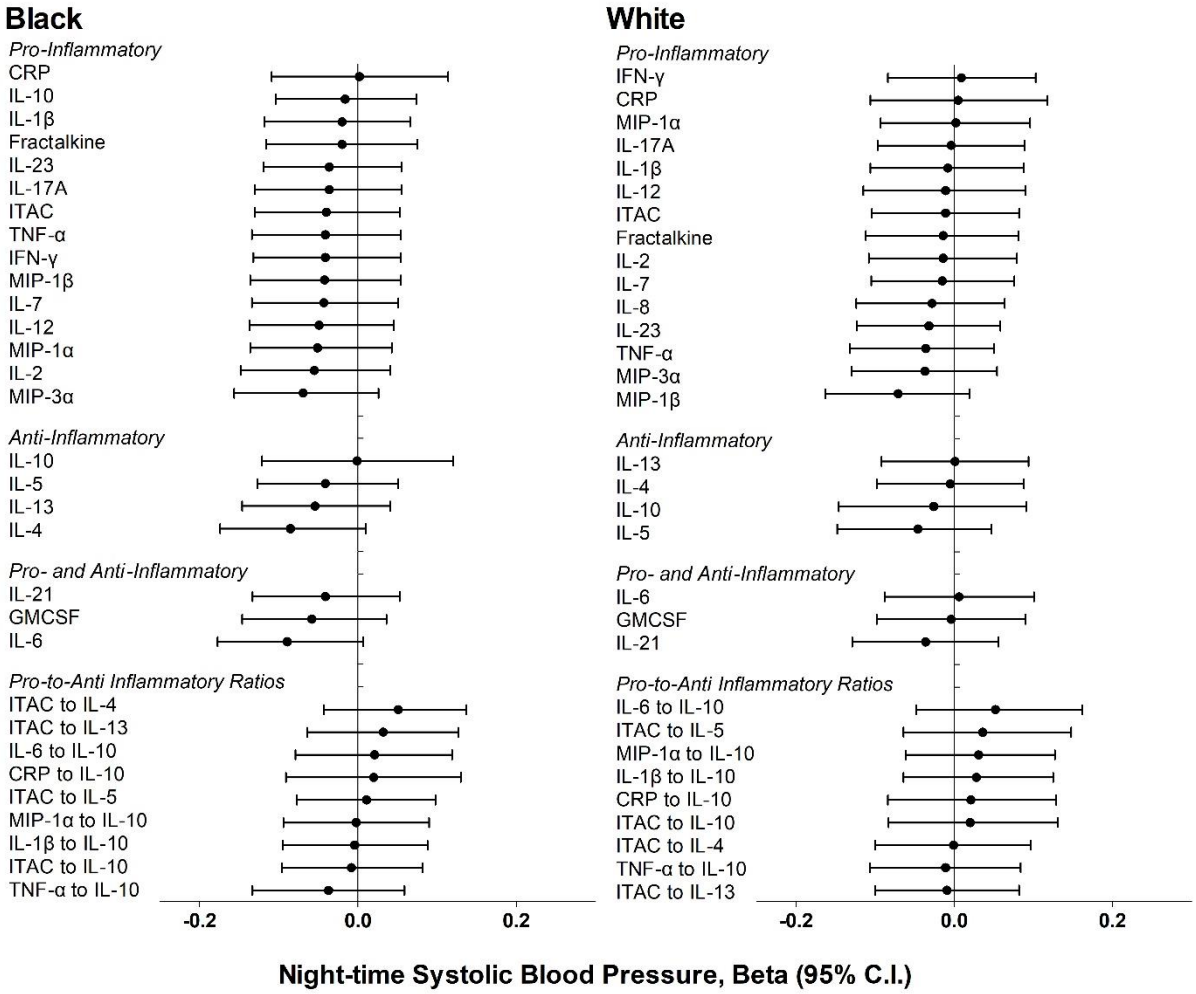

Figure II. Multiple regression analyses showing the relationship between cytokine concentrations and night-time systolic blood pressure in black and white adults.

Adjusted for: sex, socio-economic status, waist circumference, total cholesterol, glucose, gamma glutamyltransferase, cotinine, estimated glomerular filtration rate, activity energy expenditure.

Table VII. Inflammatory mediator factor scores in the total population.

	Factor 1	**Factor 2**	**Factor 3**	**Factor 4**
CRP				0.91
Fractalkine	0.65			
INF-γ	0.83			
IL-1β		0.59		
IL-2		0.80		
IL-7	0.71			
IL-8			0.77	
IL-12	0.72			
IL-17 A	0.76			
IL-23	0.68			
ITAC				0.43
MIP-1α	0.61			
MIP-1β	0.73			
MIP-3α		0.77		
TNF-α	0.72			
IL-4	0.83			
IL-5		0.62		
IL-10				
IL-13			0.86	
IL-6			0.82	
IL-21		0.55		
GM-CSF	0.82			
Eigenvalue	8.57	3.44	2.62	1.07
Cumulative %	71.4	68.8	87.4	53.3

Table VIII. Inflammatory mediator factor scores in the black population.

	Factor 1	**Factor 2**	**Factor 3**	**Factor 4**
CRP				0.81
Fractalkine	0.68			
INF-γ	0.84			
IL-1β	0.59			
IL-2		0.84		
IL-7	0.66			
IL-8			0.77	
IL-12	0.72			
IL-17 A	0.73			
IL-23	0.63			
ITAC				0.59
MIP-1α	0.57			
MIP-1β	0.65			
MIP-3α		0.73		
TNF-α	0.70			
IL-4	0.83			
IL-5		0.64		
IL-10	0.67			
IL-13			0.83	
IL-6			0.78	
IL-21		0.57		
GM-CSF	0.81			
Eigenvalue	9.16	2.82	2.60	1.08
Cumulative %	70.5	70.4	86.7	54.1

Table IX. Inflammatory mediator factor scores in the white population.

	Factor 1	**Factor 2**	**Factor 3**	**Factor 4**
CRP				0.99
Fractalkine	0.60			
INF-γ	0.82			
IL-1β		0.63		
IL-2		0.76		
IL-7	0.74			
IL-8			0.80	
IL-12	0.71			
IL-17 A	0.76			
IL-23	0.70			
ITAC		0.50		
MIP-1α	0.63	0.50		
MIP-1β	0.74			
MIP-3α		0.79		
TNF-α	0.69			
IL-4	0.84			
IL-5		0.61		
IL-10	0.61			
IL-13			0.87	
IL-6			0.84	
IL-21		0.56		
GM-CSF	0.81			
Eigenvalue	8.59	3.77	2.64	-
Cumulative %	71.6	62.8	88.0	-

Table X. Multiple regression analyses showing the relationship between inflammatory mediator factors and measures of blood pressure.

		24-hour SBP	24-hour DBP	Night-time SBP	Central SBP
Total					
	R^2	0.41	0.17	0.32	0.22
Factor 1	**β ± SE**	-0.03 ± 0.03	-0.05 ± 0.04	-0.05 ± 0.03	-0.02 ± 0.04
	p	0.32	0.22	0.15	0.65
	R^2	0.42	0.17	0.32	0.22
Factor 2	**β ± SE**	-0.04 ± 0.03	-0.05 ±0.04	-0.06 ± 0.03	-0.02 ± 0.04
	p	0.20	0.14	0.07	0.68
	R^2	0.42	0.17	0.31	0.22
Factor 3	**β ± SE**	-0.02 ± 0.03	-0.04 ± 0.04	-0.04 ± 0.03	-0.06 ± 0.04
	p	0.45	0.24	0.29	0.12
	R^2	0.42	0.17	0.31	0.22
Factor 4	**β ± SE**	-0.02 ± 0.03	-0.01 ± 0.04	-0.02 ± 0.04	0.03 ±0.04
	p	0.52	0.90	0.50	0.42
Black					
	R^2	0.33	0.16	0.23	0.13
Factor 1	**β ± SE**	-0.07 ± 0.04	-0.08 ± 0.05	-0.07 ± 0.05	-0.00 ± 0.05
	p	0.10	0.14	0.14	0.96
	R^2	0.33	0.15	0.23	0.13
Factor 2	**β ± SE**	-0.07 ± 0.04	-0.06 ± 0.05	-0.07 ± 0.05	-0.01 ± 0.05
	p	0.14	0.26	0.14	0.86
	R^2	0.33	0.16	0.23	0.14
Factor 3	**β ± SE**	-0.07 ± 0.04	-0.08 ± 0.05	-0.08 ± 0.05	-0.07 ± 0.05
	p	0.13	0.11	0.12	0.17
	R^2	0.32	0.15	0.23	0.13
Factor 4	**β ± SE**	-0.04 ± 0.05	-0.03 ± 0.05	-0.03 ± 0.05	-0.02 ± 0.05
	p	0.44	0.59	0.63	0.78
White					
	R^2	0.50	0.20	0.39	0.33
Factor 1	**β ± SE**	0.00 ± 0.04	-0.02 ± 0.05	-0.03 ± 0.05	0.01 ± 0.05
	p	0.92	0.77	0.56	0.91
	R^2	0.50	0.20	0.39	0.33
Factor 2	**β ± SE**	-0.01 ± 0.04	-0.05 ± 0.05	-0.05 ± 0.05	-0.01 ± 0.05
	p	0.76	0.34	0.26	0.81
	R^2	0.50	0.20	0.39	0.33
Factor 3	**β ± SE**	0.01 ± 0.04	0.01 ± 0.05	-0.01 ± 0.05	-0.01 ±0.05
	p	0.88	0.92	0.81	0.85

Adjusted for: sex, socio-economic status, waist circumference, total cholesterol, glucose, gamma glutamyltransferase, cotinine, estimated glomerular filtration rate, activity energy expenditure.

Chapter 4

Inflammation and salt in young adults:
The African-PREDICT study

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Keywords	4-6 keywords.
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Acknowledgements	Include acknowledgements.
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Ethical considerations	Manuscripts must contain a statement, in a separate section before the reference list., to the effect that all human and animal studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. It should also be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study.
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Inflammation and salt in young adults: The African-PREDICT study

Running title: Inflammation, salt and potassium in young adults.

Key Words: Sodium, cytokine, ethnicity, race, African, black

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Abstract

Purpose: Low-grade inflammation and a diet high in salt are both established risk factors for cardiovascular disease. High potassium (K^+) intake was found to counter increases in blood pressure due to high salt intake and may potentially also have protective anti-inflammatory effects. To better understand these interactions under normal physiological conditions, we investigated the relationships between 22 inflammatory mediators with 24-hour urinary K^+ in young healthy adults stratified by low, mid and high salt intake (salt tertiles). We stratified by ethnicity due to potential salt-sensitivity in black populations. **Methods:** In 991 healthy black (n=457) and white (n=534) adults, aged 20-30 years, with complete data for 24-hour urinary sodium and K^+, we analysed blood samples for 22 inflammatory mediators. **Results:** We found no differences in inflammatory mediators between low, mid and high sodium tertiles in either the black or white groups. In multivariable-adjusted regression analyses in white adults, we found only in the lowest salt tertile that K^+ associated negatively with pro-inflammatory mediators, namely interferon gamma, interleukin (IL) 7, IL-12, IL-17A, IL-23 and tumour necrosis factor alpha (all $p \leq 0.046$). In the black population, we found no independent associations between K^+ and any inflammatory mediator. **Conclusion:** In healthy white adults 24-hour urinary K^+ associated independently and negatively with specific pro-inflammatory mediators, but only in those with a daily salt intake less than 6.31 grams – suggesting K^+ to play a protective, anti-inflammatory role in a low-sodium environment. No similar associations were found in young healthy black adults.

Abbreviations

Na^+	Sodium
K^+	Potassium
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IFN-γ	Interferon gamma
IL-1 β	Interleukin 1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-17A	Interleukin 17A
IL-21	Interleukin 21
IL-23	Interleukin 23
ITAC	Interferon-inducible T-cell alpha chemoattractant
MIP-1α	Macrophage inflammatory protein 1-alpha
MIP-1β	Macrophage inflammatory protein 1-beta
MIP-3α	Macrophage inflammatory protein 3-alpha
NF-κB	Nuclear factor *kappa B*
TNFα	Tumour necrosis factor alpha

Introduction

Inflammation is involved in the development of cardiovascular disease [1-3]. Additionally, a diet high in salt (Na^+) is another well-known risk factor for cardiovascular diseases, including hypertension [4]. It was recently reported that Na^+ intake modulates the release of pro-inflammatory mediators [5-7]. These two cardiovascular risk factors may therefore be mechanistically involved. Interstitial Na^+ rapidly achieves equilibrium with plasma and excess Na^+ is then excreted by the kidneys [8]. However, osmotically inactive Na^+ can also be stored in tissues, such as the skin, which, in turn, leads to changes in immune cell function and increased inflammation [9].

A diet high in potassium (K^+) intake was shown to counter the usual increases in blood pressure in response to high salt intake [10,11]. This finding suggests that a high K^+ intake may have protective cardiovascular effects [12,13]. As inflammation and a diet high in Na^+ and low in K^+ may be additive risk factors for the development of cardiovascular disease, a better understanding is required to establish the potential impact of K^+ on cardiovascular health. As high K^+ intake has a beneficial effect on blood pressure [14], as well as cardiovascular events and mortality [13], an additional mechanism of K^+ may be its anti-inflammatory properties [15]. This notion is supported by a study indicating that K^+ supplementation inhibited interleukin (IL)17A production in human T lymphocytes that were induced by a salt load [5]. However, there is limited evidence on the role of K^+ in the regulation of other inflammatory mediators such as C-reactive protein (CRP), IL-6, and IL-23.

When examining Na^+ and K^+ handling, an essential factor to account for is black ethnicity. Black individuals have higher levels of sodium retention than their white counterparts [16]. Previous studies also reported a greater proportion of salt sensitivity in black populations[16]. The cardiovascular risk in black populations may be further increased based on a more pro-inflammatory profile when compared to white adults [17].

To better understand these potential mechanisms involved in the development of cardiovascular disease, we performed hypothesis generating work by investigating whether a detailed range of 22 pro- and anti-inflammatory mediators are associated with 24-hour urinary K^+ in young black and white adults. We specifically focussed on those with low, mid and high salt intake.

Methodology

Study population

This study forms part of the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT) [18]. We recruited young black and white, men and women, between the ages of 20-30 years. African-PREDICT included apparently healthy individuals who were HIV uninfected; had a screening office brachial blood pressure of <140 mmHg systolic and <90 mmHg diastolic; had no self-reported previous diagnosis or used any medication for a chronic disease; and, if female, were not currently pregnant or lactating. We analysed data of participants who were included in the baseline phase of the African-PREDICT study (n=1202). This study is a sub-cohort of a previously published larger cohort.[17] Participants on anti-inflammatory medication and with missing biochemical data (Na^+, K^+, and multiple inflammatory mediators) were additionally excluded resulting in a total of 991 participants. The exclusion of individuals with missing urine data (Na^+ and K^+) allowed for investigation of a more specific research question.

Questionnaires, anthropometry and physical activity measurements

Self-reported data with regards to demographic and lifestyle information were collected using a questionnaire. A 24-hour dietary recall questionnaire was administered by a trained dietitian or nutritionist on the study day and on two subsequent days. The average daily energy intake was then calculated. Socio-economic status was calculated using a point system that was adapted from Kuppuswamy's Socio-economic Status Scale [19] for a South African environment. Height, weight and waist circumference were measured using standard methods

[18]. Body mass index (BMI) was calculated using weight (kg) / height(m)^2. A compact, chest-worn accelerometric device (Actiheart4 CamNtech Ltd and CamNtech Inc, UK) was used to objectively measure physical activity over a maximum period of 7 days.

Ambulatory blood pressure

Participants were also fitted with a validated 24-hour brachial ambulatory blood pressure monitor (Card(X)plore® CE120, Meditech, Budapest, Hungary). The apparatus was programmed to record every 30 minutes during the day (06h00 to 22h00) and every hour during the night (22h00 to 06h00) [20]. Participants had a mean successful recording rate of 88%.

24-hour Urine Collection

Participants were instructed to collect a 24-hour urine sample on a day that was convenient for them and the date was noted. The first urine of the day was to be discarded and all urine passed thereafter was collected in the provided container, including the first urine of the following morning (day two). The start and finish times were recorded. The protocol for 24-hour urine collection followed that of the Pan American Health Organisation/World Health Organisation (PAHO/WHO) protocol for population level Na^+ determination in 24-hour urine samples [21]. Incomplete urine collections were defined as a volume <300 mL per 24 hours and/or a 24-hour creatinine excretion of <4 mmol or >25 mmol in women and <6 mmol or >30 mmol in men [22].

Biological sampling and biochemical analyses

Participants fasted overnight for at least eight hours prior to attending the day of research measurements. Blood samples were collected from the median cubital vein. The samples were prepared according to the standardised protocol of the African-PREDICT study and stored at -80°C until analysis [18].

Urinary Na^+, K^+ and chloride were measured by means of ion-selective electrode potentiometry on the Cobas Integra® 400 plus (Roche, Basel, Switzerland) and creatinine

concentrations were measured using the Creatinine Jaffé Gen.2 reagent (Roche, Basel, Switzerland). Daily urinary Na^+ and K^+ excretion (mmol/d) were calculated by multiplying the Na^+, K^+ and creatinine concentrations (mmol/l) of the 24-hour urine by the total 24-hour volume of urine (in litres). Daily salt intake was estimated from 24-hour urinary Na^+ excretion by converting Na^+ in mmol to mg: Na^+ (mmol) x 23= Na^+ (mg) [23] and then applying the conversion: 1g salt (NaCl) = 390 mg Na^+ [23].

A MILLIPEX Map Human High Sensitivity T Cell Magnetic Bead Panel (EMD Millipore, Merck, Missouri, USA) was used to analyse 21 cytokines. This multiplex panel was analysed using Luminex xMAP technology on the Luminex 200™ analyser.

Serum samples were analysed for high-sensitivity CRP, total cholesterol, low- and high-density lipoprotein cholesterol, glucose and γ-glutamyltransferase (GGT) (Cobas Integra® 400plus, Roche, Basel, Switzerland). Serum creatinine concentrations were measured using the Creatinine Jaffé Gen.2 reagent (Roche, Basel, Switzerland). Estimated creatinine clearance was determined using the Cockroft Gault formula (Men [(140-age) * weight in kg * 1.23]/serum creatinine or Women [(140-age) * weight in kg * 1.04]/serum creatinine). Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology (CKD-EPI) formula, without race in the equation, as correction for race is not suggested for a South African population [24,25]. Serum cotinine was analysed using a chemiluminescence method on the Immulite (Siemens, Erlangen, Germany) apparatus.

Statistical analyses

IBM®, SPSS® version 24 (IBM Corporation, Armonk, New York) was used for data analysis. GraphPad Prism 5.03 (GraphPad Software, San Diego) was used to develop all graphs. Continuous variables were inspected for normality using QQ plots as well as inspection of skewness and kurtosis. Variables with non-Gaussian distributions were logarithmically transformed. To substantiate analyses by ethnicity, we investigated the interactions of ethnicity on the relationship between Na^+, K^+ and the full range of pro- and anti-inflammatory mediators. Based on the interactions, we divided our groups by ethnicity (*Online Resource*

Table S1). Pro- to anti-inflammatory ratios were calculated based on the literature [26,27], and new ratios were suggested based on instances where pro-inflammatory mediators were higher and anti-inflammatory mediators were lower in the black and white groups. T-tests and Chi-square tests were used to compare the profiles of black and white participants. We further divided our groups by Na^+ tertiles, reflecting low, mid and high salt intake. Partial correlations and backward stepwise multiple regression were used to determine the relationship between K^+ and pro- and anti-inflammatory mediators. Partial correlations were adjusted for age, sex and waist circumference. Variables included in backward stepwise multiple regression models were: K^+, age, socio-economic status, AEE, waist circumference, total cholesterol, eGFR, cotinine, GGT, glucose and sex. In sensitivity analyses we also determined whether components of the renin-angiotensin-aldosterone system contribute to the model. Multiple regression analyses displayed the last model in which potassium remained.

Results

The general characteristics of the participants (n=991) are shown in *Table 1.* The black and white groups were similar in age (24.5 years; p=0.92) with an equal distribution in sex (p=0.71). When viewing the detailed inflammatory mediator profile of the two groups, the black group had higher pro-to-anti-inflammatory ratios than their white counterparts ($p \leq 0.021$) as was seen in a previous study in this population [17].

There were no ethnic differences for Na^+ excretion (p=0.47), but black participants had lower urine levels of K^+, with 94% black and 88% white participants having K^+ levels below recommended levels [28]. Black participants had higher Na^+/ K^+ ratios ($p<0.001$) than the white group.

We determined differences in inflammatory mediator concentrations according to Na^+ tertiles (*Online Resource Table S2*). For all inflammatory mediators there were generally no differences.

To establish whether a relationship exists between Na^+ or K^+ with inflammatory mediators, we performed partially adjusted regression analyses in the total group as well as black and white groups separately (adjusted for age, sex and waist circumference as well as ethnicity in the total group) (*Online Resource Table S3*). These analyses yielded minimal correlations mostly with K^+ as indicated in detail in *Online Resource Table S3.*

Due to previous reports indicating the importance of Na^+/K^+ balance [29], we then performed partial correlations between K^+ and inflammatory mediators in groups stratified by Na^+ tertiles. In whites we found several prominent results in the lowest Na^+ tertile (T1). These include positive correlations between K^+ and both interferon-inducible T-cell alpha chemoattractant (ITAC)/IL-5 and ITAC/IL-10. In T1 we also found negative correlations between K^+ and interferon gamma (IFN-γ), IL-1β, IL-5, IL-6, IL-7, IL-8, IL-12, IL-17A, IL-21, IL-23, macrophage inflammatory protein 3-alpha (MIP-3α) and tumour necrosis factor alpha (TNF-α). Additionally, in the middle tertile (T2), K^+ correlated inversely with IL-4 (*Online Resource Fig. S1*). In fully adjusted regression analyses (*Fig. 1*), these findings were confirmed where K^+ associated negatively with the pro-inflammatory mediators IFN-γ, IL-7, IL-12, IL-17A, IL-23 and TNF-α, but only in the lowest Na^+ tertile T1 (all $p \leq 0.046$).

In the black population, with partial correlations we found in the highest Na^+ tertile (T3), positive correlations between K^+ and both ITAC and IL-5, and negative correlations in lowest tertile (T1) with ITAC/IL-4 and ITAC/IL-5 (all $p \leq 0.046$) (*Online Resource Fig. S2*). However, these results lost significance in fully adjusted regression analyses (*Fig. 2*). We examined renin, angiotensin II and aldosterone's impact on the model, all of which exhibited no effect (results not shown). We additionally examined the relationship between Na^+ and inflammatory mediators, stratified by Na^+, but found no significant correlations.

Table 1. Characteristics of young black and white adults.

	Black (n=457)	**White (n=534)**	***P***
Age, years	24.5 ± 3.12	24.5 ± 3.04	0.94
Male, n (%)	227 (49.7)	259 (48.5)	0.71
Socio-economic Status			
Low, n (%)	264 (57.8)	109 (20.4)	**<0.001**
Middle, n (%)	123 (26.9)	163 (30.5)	
High, n (%)	70 (15.3)	262 (49.1)	
Body Composition			
Body mass index (kg/m^2)	24.2 (17.8; 36.2)	25.0 (18.9; 35.1)	**0.014**
Waist circumference (cm)	77.6 (63.5; 98.5)	81.5 (64.9; 107)	**<0.001**
24-hour Urine Analysis			
Na^+ (mmol/day)	134 (44.5; 353)	130 (45.5; 294)	0.47
Salt (NaCl g/day)	7.88 (2.62; 20.8)	7.67 (2.68; 17.3)	0.47
Above 5g salt/day, n (%)	364 (79.6)	431 (80.7)	0.68
K^+ (mmol/day)	34.5 (12.7; 98.6)	49.7 (22.3; 107)	**<0.001**
Below 90 mmol/day K^+, n (%)	441 (94.3)	460 (88.3)	**0.001**
Na^+/K^+	3.94 (1.93; 7.85)	2.59 (1.09; 5.37)	**<0.001**
Inflammatory Markers			
Pro-Inflammatory			
CRP (mg/L)	1.02 (0.10; 12.0)	0.75 (0.08; 7.13)	**0.001**
Fractalkine (pg/mL)	28.1 (10.3; 74.4)	29.7 (10.8; 74.3)	0.15
IFN-γ (pg/mL)	6.84 (1.65; 22.0)	7.83 (1.61; 22.2)	**0.012**
IL-1β (pg/mL)	0.98 (021; 3.72)	1.10 (0.27; 3.70)	**0.031**
IL-2 (pg/mL)	0.76 (0.13; 3.88)	0.84 (0.16; 3.95)	0.16
IL-7 (pg/mL)	5.71 (1.37; 19.3)	5.63 (1.16; 18.6)	0.80
IL-8 (pg/mL)	1.75 (0.44; 6.91)	1.88 (0.47; 8.11)	0.16
IL-12 (pg/mL)	1.74 (0.36; 6.51)	1.97 (0.45; 6.72)	**0.027**
IL-17 A (pg/mL)	3.18 (0.64; 14.2)	3.53 (0.64; 14.1)	0.088
IL-23 (pg/mL)	118 (14.6; 609)	134 (12.9; 668)	0.10
ITAC (pg/mL)	4.77 (1.50; 18.0)	3.64 (1.40; 11.5)	**<0.001**
MIP-1α (pg/mL)	9.84 (2.98; 28.4)	10.3 (2.86; 27.4)	0.34
MIP-1β (pg/mL)	7.21 (2.87; 15.7)	7.28 (2.93; 16.4)	0.76
MIP-3α (pg/mL)	2.13 (0.56; 7.68)	1.87 (0.48; 5.77)	**0.015**
TNF-α (pg/mL)	1.60 (0.42; 5.29)	1.79 (0.49; 5.76)	**0.024**
Anti-Inflammatory			
LL-4 (pg/mL)	44.2 (7.97; 166)	44.6 (8.29; 154)	0.88
IL-5 (pg/mL)	0.89 (0.22; 3.90)	1.01 (0.26; 4.03)	**0.025**
IL-10 (pg/mL)	4.37 (0.94; 20.2)	5.38 (1.13; 21.2)	**<0.001**
IL-13 (pg/mL)	3.89 (0.58; 23.3)	4.98 (0.67; 31.4)	**0.001**
Pro- and Anti-Inflammatory			
IL-6 (pg/mL)	1.87 (0.25; 10.3)	2.34 (0.31; 13.2)	**0.002**
IL-21 (pg/mL)	1.31 (0.21; 6.05)	1.47 (0.26; 6.47)	0.088
GM-CSF (pg/mL)	7.34 (1.19; 32.6)	8.59 (1.23; 38.0)	**0.020**
Pro-to-Anti Inflammatory Ratios			
IL-6/IL-10	0.29 (0.04; 2.62)	0.16 (0.03; 1.22)	**<0.001**
IL-1β/IL-10	0.22 (0.08; 0.73)	0.20 (0.07; 0.52)	**0.021**
TNF-α/IL-10	0.37 (0.16; 0.97)	0.34 (0.15; 1.01)	**0.005**

	Black (n=457)	White (n=534)	*P*
CRP/IL-10	0.23 (0.01; 4.63)	0.14 (0.01; 2.36)	**<0.001**
MIP-*1α*/IL-10	2.20 (0.72; 7.04)	1.84 (0.61; 5.90)	**<0.001**
ITAC/IL-4	0.11 (0.02; 0.82)	0.08 (0.02; 0.67)	**<0.001**
ITAC/IL- 5	5.42 (1.16; 29.4)	3.61 (0.78; 18.1)	**<0.001**
ITAC/IL-10	1.10 (0.29; 6.78)	0.68 (0.22; 3.05)	**<0.001**
ITAC/IL-13	1.24 (0.18; 9.64)	0.73 (0.10; 5.90)	**<0.001**
Biochemical Markers			
Total Cholesterol (mmol/L)	3.49 ± 0.98	3.98 ± 1.31	**<0.001**
HDL-C (mmol/L)	1.14 ± 0.38	1.16 ± 0.45	0.58
LDL-C (mmol/L)	2.09 (1.01; 3.82)	2.42 (1.18; 4.39)	**<0.001**
Triglycerides (mmol/L)	0.63 (0.31; 1.35)	0.79 (0.33; 2.05)	**<0.001**
Glucose (mmol/L)	3.91 ± 1.05	4.23 ± 1.11	**<0.001**
eGFR (ml/min/1.73m^2)	123 ± 16.8	117 ± 20.3	**<0.001**
Estimated creatinine clearance (mL/min)	138 (87.0; 235)	147 (88.7; 262)	**0.001**
Creatinine Clearance (mL/min)	123 (56.4; 281)	128 (52.8; 311)	**0.28**
Plasma renin activity surrogate	63.2 (11.3; 267)	127 (38.8; 346)	**<0.001**
Angiotensin II (pg/mL)	47.6 (8.51; 197)	94.2 (29.3; 257)	**<0.001**
Aldosterone (pg/mL)	24.7 (5.00; 96.5)	52.3 (10.1; 223)	**<0.001**
Ambulatory BP (mmHg)			
24h SBP	116 ± 8.86	118 ± 9.94	**<0.001**
24h DBP	68.7 ± 5.73	68.5 ± 5.85	0.62
Health Behaviours			
Serum cotinine (ng/ml)	3.66 (1.00; 349)	3.13 (1.00; 306)	**0.27**
Self-Reported Tobacco use n (%)	110 (24.1)	116 (21.7)	0.37
γ-glutamyltransferase (U/L)	22.0 (8.62; 66.4)	14.7 (5.40; 48.3)	**<0.001**
Self-Reported Alcohol use, n (%)	236 (52.4)	292 (54.8)	0.46
Hormonal Contraceptive Use, n (% of women)	105 (46.5)	112 (41.0)	0.22
Energy Expenditure			
TEE (kcal/day)	2218 ± 394	2355 ± 497	**<0.001**
AEE (kcal/day)	430 ± 219	406 ± 204	0.12
Reported Energy Intake			
Energy intake (kCal/day)	2097 ± 806	2083 ± 710	0.82

Abbreviations: SBP, Systolic blood pressure; DBP, Diastolic blood pressure; BMI, Body mass index; HDL-C High density lipoprotein cholesterol; LDL-C, Low density lipoprotein cholesterol. Bold values indicate P<0.05. Data presented as mean ± SD; or geometric mean 95 C.I. Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interferon gamma (IFN-γ), Interleukin 1 beta (IL-1β), Interleukin 2 (IL-2), Interleukin 4 (IL-4), Interleukin 5 (IL-5), Interleukin 6 (IL-6), Interleukin 7 (IL-7), Interleukin 8 (IL-8), Interleukin 10 (IL-10), Interleukin 12 (IL-12), Interleukin 13 (IL-13), Interleukin 17A (IL-17A), Interleukin 21 (IL-21), Interleukin 23 (IL-23), Interferon-inducible T-cell alpha chemoattractant (ITAC), Macrophage inflammatory protein *1-alpha* (MIP-1α), Macrophage inflammatory protein *1-beta* (MIP-1β), Macrophage inflammatory protein 3-*alpha* (MIP-3α) and Tumour Necrosis Factor Alpha (TNFα).

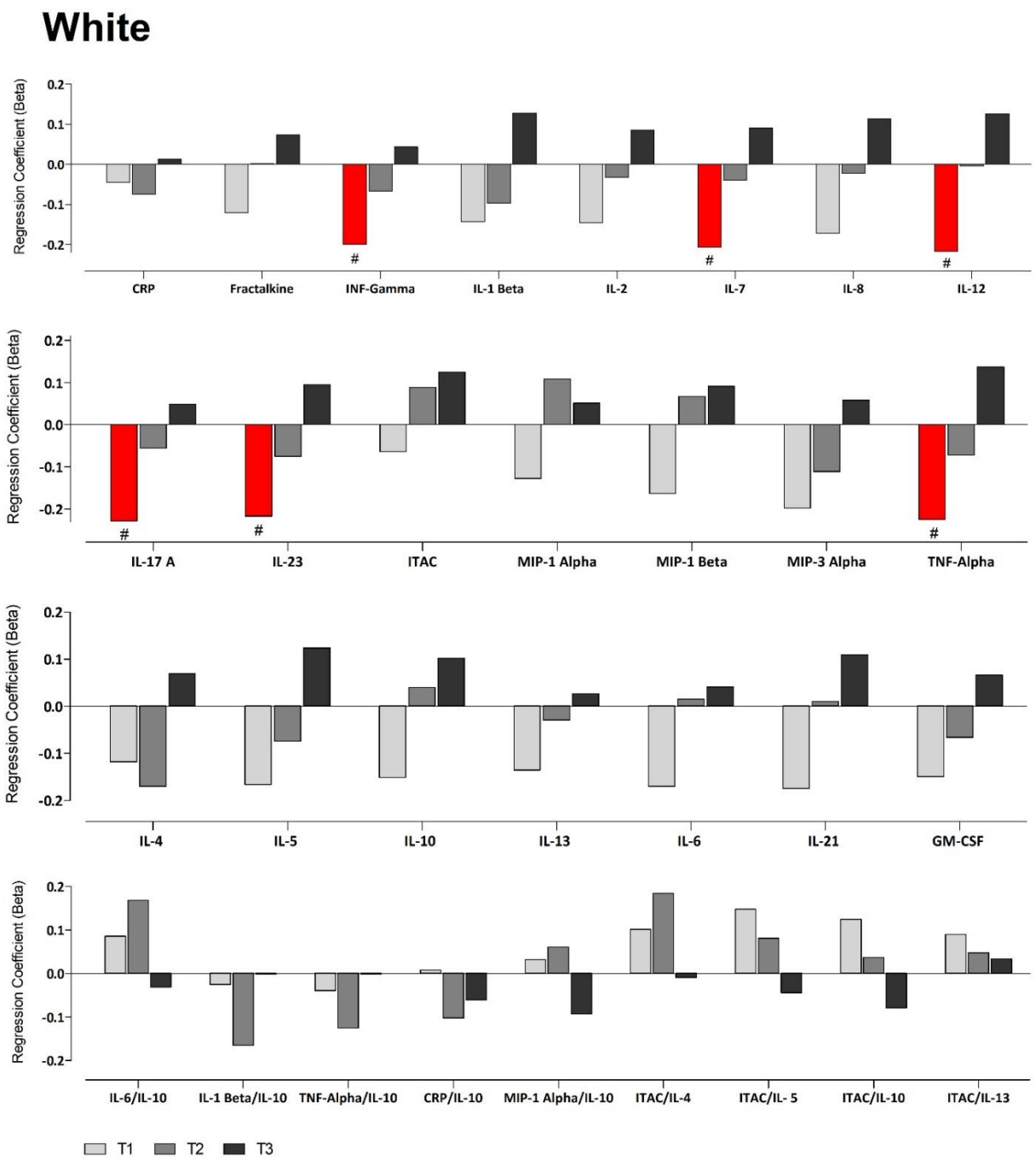

Fig. 1 Multi-variable adjusted regression analyses showing the relationship between inflammatory mediators and K^+ within each Na^+ tertile in white adults

Each model was adjusted for: age, sex, socio-economic status, waist circumference, total cholesterol, glucose, gamma glutamyltransferase, cotinine, estimated glomerular filtration rate, activity energy expenditure.
# indicates $p<0.05$ Red bars indicate $p<0.05$ in Na^+ Tertile 1, Blue bars indicate $p<0.05$ in Na^+ Tertile 2, Green bars indicate $p<0.05$ in Na^+ Tertile 3

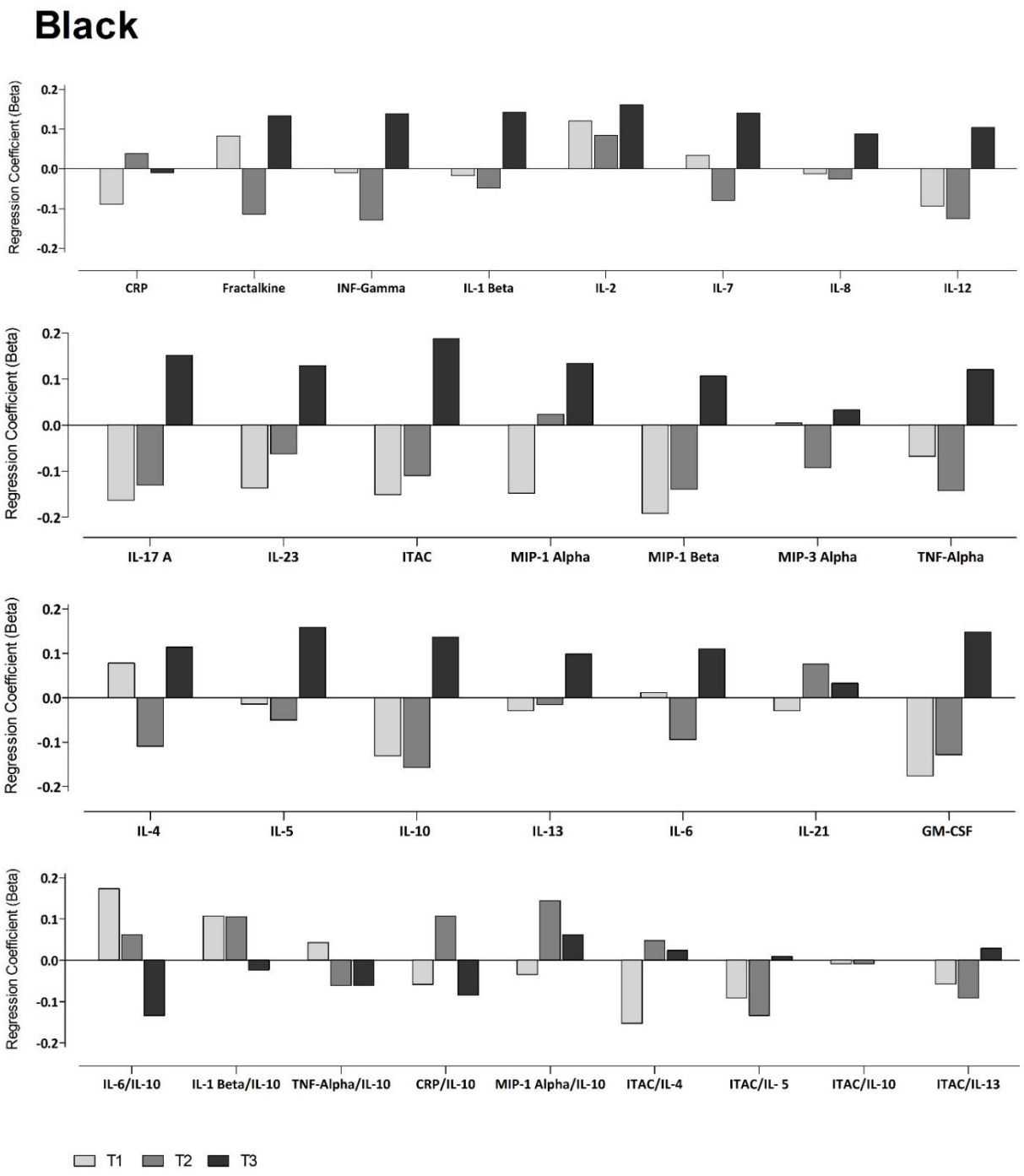

Fig. 2 Multi-variable adjusted regression analyses showing the relationship between inflammatory mediators and K^+ within each Na^+ tertile in black adults

Each model was adjusted for: age sex, socio-economic status, waist circumference, total cholesterol, glucose, gamma glutamyltransferase, cotinine, estimated glomerular filtration rate, activity energy expenditure.
# indicates $p<0.05$ Red bars indicate $p<0.05$ in Na^+ Tertile 1, Blue bars indicate $p<0.05$ in Na^+ Tertile 2, Green bars indicate $p<0.05$ in Na^+ Tertile 3

Discussion

Low-grade systemic inflammation and Na^+ are both risk factors for the development of cardiovascular disease [1-4]. It has been suggested that K^+ may provide a protective anti-inflammatory effect [15]. Therefore to better understand the possible mechanisms through which a high salt environment may predispose one to higher cardiovascular disease risk (potentially due to loss of the 'protective' anti-inflammatory role of K^+), we examined the relationships between a detailed range of inflammatory mediators and 24-hour urinary K^+, in those with low, mid and high salt intake. When we stratified 991 young healthy black and white participants by Na^+ excretion tertiles, we found negative independent relationships between urinary K^+ and six pro-inflammatory mediators; IFN-γ, IL-7, IL-12, IL-17A, IL-23 and TNF-α; but only in white adults and only in those within the lowest Na^+ tertile (with an equivalent of 4.21 [0.63-6.31] g salt intake/day). These findings suggest that K^+ may exert protective anti-inflammatory functions but only in individuals with a low salt intake as reflected by the 24h urinary Na^+ excretion.

Previous studies have shown that a diet high in Na^+ stimulates an inflammatory response [6,8,30]. In healthy human participants participating in the Mars520 study, Titze *et al.* found an increase in the pro-inflammatory mediators IL-6 and IL-23, as well as a decrease in the anti-inflammatory mediator IL-10 in those on a high salt diet [7].

In support of our findings of several negative relationships between pro-inflammatory mediators and urinary K^+, it was found that rats on a K^+ supplemented diet had suppressed renal inflammation [15]. This was evident by a decrease in macrophage infiltration and nuclear factor *kappa B* (NF-κB), as well as a lower expression of cytokines [15]. In addition, a study involving healthy humans found K^+ supplementation to have an inhibiting effect on the production of IL-17A by T-lymphocytes induced by salt loading [5]. One potential mechanism through which K^+ may suppress inflammation is via its anti-oxidant effect [5]. Increases in extracellular K^+ leads to elevated membrane Na^+ pump activity [31]. This, in turn, results in

hyperpolarization and ultimately a reduction in oxidase activity [31]. A second proposed mechanism is via K^+ inhibiting the effects of Na^+ on mitogen activated protein kinase *p38* which, when activated, leads to an immune response [5]. It has also been suggested that K^+ may suppress the activation of NF-κB, which is involved in regulating genes relating to inflammation in the kidneys [15,32,33].

When we examined the relationship between K^+ and inflammation, inverse relationships were seen with pro-inflammatory mediators, but not with anti-inflammatory mediators. This suggests a potential role of K^+ in pro-inflammatory processes. What is of particular interest is that this protective association is only seen in the lowest Na^+ tertile, with an average salt intake of 4.21 (0.63-6.31) g/day (or 10.7-107 mmol Na^+/day). The mean intake for the second and third Na^+ tertiles in the white group were 8.13 (6.31-10.0) g salt/day and 13.9 (10.0-50.1) g salt/day, respectively. These findings suggest that once Na^+ intake exceeds the levels of the first Na^+ tertile, or when the Na^+/K^+ equilibrium becomes significantly imbalanced, the protective effect of K^+ may be lost. This may imply that while it is important to maintain an acceptable Na^+/K^+ ratio, it is also of importance to do so at the recommended levels. Our findings thus suggest a loss of mediation of pro-inflammatory mediators by K^+ in individuals with increased Na^+ intake.

As previously mentioned, it is also important to consider the role of ethnicity on the relationship between inflammatory mediators and K^+ and Na^+. While numerous studies have examined differences in inflammation between ethnic groups, global findings remain contradictory [34]. However, multiple studies performed in South African populations have found that black individuals display higher levels of pro-inflammatory markers and an overall more pro-inflammatory profile [17, 35-37]. When examining Na^+, previous studies found that black adults have a predisposition for higher Na^+ retention [16]. Based on previous reports looking at salt-sensitivity, black populations also have a greater response in blood pressure to Na^+ [38]. Regardless, research into the role of Na^+ and K^+ in inflammation in any populations, but particularly black populations, is limited. While some studies have, to a limited extent, examined the role of K^+ in inflammation [5,15], to the best of our knowledge, none have

examined this relationship stratified by ethnicity. This is of importance as studies have found ethnic differences in K^+ excretion, with black populations being found to excrete less K^+ than their white counterparts even when intake is matched [39].

Our findings were only present in the white group. Although a previous study found that K^+ supplementation protects against an increase in blood pressure in black populations in response to a salt load [10]. In our study with the focus on inflammation, this potentially protective effect on blood pressure was not seen in terms of potential anti-inflammatory effects. It is unknown whether this lack of association in the black group may be due to the effects of salt-sensitivity. It should however be taken into account that the black group had particularly low urinary K^+ levels. Only 6% of the black population had a K^+ intake above the recommended minimum of 90 mmol/day [28], which may be a reason for the lack of association in this group. While protective associations are seen in the white adults, their mean K^+ intake was also below the recommended daily K^+ intake, albeit to a lesser extent than the black population. It would certainly be worth investigating whether an increase in K^+ intake in both groups would result in greater anti-inflammatory responses. However, it is important to note that an increase in K^+ levels should not be achieved by increasing calorie intake, but rather through the consumption of foods high in K^+ such as fruits and vegetables [40].

A strength of our study is the absence of pre-existing chronic diseases, which gave us the opportunity to test our hypotheses in adults without influence from pathology. Additionally, our study included a large panel of pro- and anti-inflammatory mediators which was analysed with a high-sensitivity kit. Although we included the renin-angiotensin-aldosterone system components in regression models, which yielded no contributory findings, the renin-angiotensin-aldosterone system is likely to be very important perhaps in those who have developed hypertension. In terms of limitations, the use of a single collection of 24-hour urine does not account for day-to-day variations in Na^+ and K^+ excretion.

In conclusion, in young apparently healthy white adults we found significant negative relationships between 24h urinary K^+ and specific pro-inflammatory mediators, but only in

those with a daily salt intake of less than 6.31 grams. Our results suggest that K^+ may play a protective, anti-inflammatory role in a low-sodium environment.

Access to data

The study methodology has been published[18], whereas the data dictionary, statistical analysis, protocol and deidentified individual participant data will be made available upon reasonable request to the corresponding author in agreement with all co-authors.

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Disclosures

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

The authors declare that there is no conflict of interest.

Authorship

SHC, SBL and AES contributed to the conception or design. SHC, SBL, CD, LAG, AES contributed to the acquisition, analysis, or interpretation of data. SHC drafted the manuscript. SBL, CD, LAG and AES critically revised the manuscript. All gave final approval and agree to be accountable for all aspects of work ensuring integrity and accuracy.

Ethical Standards

The study was approved by the Health Research Ethics Committee (HREC) of the North-West University (NWU-00058-18-A1), adheres to the 1964 Declaration of Helsinki and its later amendments and all participants in the study provided written informed consent prior to participation.

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Supplementary Information

Table S1. Interactions of ethnicity on cytokines

	Na^+	**K^+**
	p	*p*
Fractalkine	0.43	0.29
CRP	0.71	0.16
GM-CSF	0.55	0.29
IFN *gamma*	0.55	0.15
IL-1 *beta*	0.90	0.16
IL-2	0.15	**0.022**
IL-4	0.36	0.26
IL-5	0.37	**0.019**
IL-6	0.98	0.16
IL-7	0.89	0.15
IL-8	0.96	0.40
IL-10	0.95	0.35
IL-12	0.76	0.25
IL-13	0.81	0.11
IL-17 A	0.31	0.20
IL-21	0.56	0.18
IL-23	0.61	0.34
ITAC	0.46	0.77
MIP *1-alpha*	0.31	0.54
MIP *1-beta*	0.77	0.84
MIP 3-*alpha*	0.58	0.62
TNF *alpha*	0.98	0.28

Table S2. Analysis of variance between Na^+ tertiles T1, T2 and T3 in black and white adults.

	Black				White			
	T1	**T2**	**T3**	**p**	**T1**	**T2**	**T3**	**p**
24-hour Urine Analysis								
Na^+ (mmol/day)	67.5[ab] (63.0; 72.2)	134[ac] (131; 137)	248[bc] (237; 259)	**<0.001**	71.4[ab] (67.7; 75.3)	138[ac] (135; 140)	236[bc] (226; 247)	**<0.001**
Salt (NaCl g/day)	3.98[ab] (3.71; 4.26)	7.92[ac] (7.74; 8.10)	14.6[bc] (14.0; 15.3)	**<0.001**	4.21[ab] (3.99; 4.44)	8.13[ac] (7.97; 8.29)	13.9[bc] (13.3; 14.6)	**<0.001**
K^+ (mmol/day)	21.1[ab] (19.4; 23.0)	33.5[ac] (31.4; 35.7)	53.6[bc] (49.6; 58.0)	**<0.001**	36.4[ab] (33.9; 39.1)	50.1[ac] (46.6; 53.9)	69.2[bc] (65.3; 73.4)	**<0.001**
Na^+/K^+	3.23[ab] (2.97; 3.52)	4.03[ac] (3.79; 4.28)	4.58[bc] (4.29; 4.89)	**<0.001**	1.97[ab] (1.82; 2.14)	2.66[ac] (2.50; 2.83)	3.41[bc] (3.23; 3.59)	**<0.001**
Biochemical Analyses								
Creatinine Clearance (mL/min)	82.5[ab] (76.7; 88.8))	117[ac] (109; 125)	175[bc] (164; 187)	**<0.001**	91.8[ab] (84.9; 99.2)	134[ac] (126; 143)	175[bc] (163;189)	**<0.001**
Plasma renin activity surrogate	72.8[a] (62.4; 84.9)	62.4 (53.8; 72.5)	54.7[a] (47.1; 63.5)	**0.029**	145[a] (131; 160)	125 (114; 137)	110[a] (99.0; 121)	**<0.001**
Angiotensin II (pg/mL)	54.6[a] (46.8; 63.8)	47.2 (40.5; 54.9)	41.2[a] (35.2; 48.1)	**0.036**	105[a] (95.2; 116)	93.3 (84.5; 103)	83.9[a] (75.7; 92.9)	**0.008**
Aldosterone (pg/mL)	30.2[ab] (26.3; 34.6)	22.2[a] (19.4; 25.4)	22.2[b] (19.7; 25.2)	**0.001**	63.9[a] (56.0; 73.0)	51.9 (45.0; 59.9)	43.2[a] (38.0; 49.1)	**<0.001**
Inflammatory Markers								
Pro-Inflammatory								
CRP (mg/L)	1.05 (0.81; 1.36)	1.02 (0.78; 1.32)	1.01 (0.80; 1.26)	0.97	0.82 (0.65; 1.03)	0.71 (0.59; 0.86)	0.72 (0.59; 0.88)	0.56
Fractalkine (pg/mL)	27.6 (25.0; 30.5)	28.7 (25.7; 32.0)	28.0 (25.4; 30.8)	0.87	29.8 (27.1; 32.8)	28.3 (25.9; 30.9)	31.3 (28.4; 34.5)	0.32
IFN-γ (pg/mL)	6.92 (6.07; 7.88)	7.30 (6.37; 8.37)	6.41 (5.63; 7.29)	0.38	7.94 (7.04; 8.95)	7.53 (6.63; 8.54)	8.05 (7.10; 9.13)	0.73
IL-1β (pg/mL)	0.92 (0.80; 1.06)	1.02 (0.89; 1.16)	1.00 (0.86; 1.16)	0.59	1.10 (0.97; 1.24)	1.05 (0.94; 1.18)	1.15 (1.03; 1.29)	0.56
IL-2 (pg/mL)	0.70 (0.59; 0.84)	0.79 (0.65; 0.95)	0.79 (0.65;0.96)	0.62	0.86 (0.74; 0.99)	0.82 (0.70; 0.94)	0.83 (0.70; 0.98)	0.89
IL-7 (pg/mL)	5.57 (4.93; 6.30)	5.85 (5.08; 6.74)	5.71 (5.03; 6.48)	0.88	5.47 (4.79; 6.24)	5.32 (4.70; 6.03)	6.21 (5.48; 7.03)	0.21
IL-8 (pg/mL)	1.77 (1.54; 2.05)	1.71 (1.50; 1.96)	1.75 (1.53; 2.00)	0.94	1.81 (1.61; 2.05)	1.93 (1.70; 2.19)	1.91 (1.69; 2.14)	0.75
IL-12 (pg/mL)	1.76 (1.53; 2.02)	1.76 (1.50; 2.04)	1.71 (1.53; 1.97)	0.95	1.97 (1.73; 2.24)	1.86 (1.62; 2.14)	2.10 (1.87; 2.37)	0.43
IL-17 A (pg/mL)	3.04 (2.60; 3.55)	3.37 (2.84; 3.95)	3.17 (2.74; 3.67)	0.66	3.53 (3.07; 4.07)	3.45 (3.00; 3.97)	3.63 (3.14; 4.20)	0.88
IL-23 (pg/mL)	121 (102; 144)	121 (99.5; 148)	113 (93.2; 136)	0.82	132 (109; 158)	131 (109; 157)	140 (116; 167)	0.87
ITAC (pg/mL)	4.85 (4.27; 5.50)	4.86 (4.23; 5.59)	4.62 (4.10; 5.20)	0.81	3.46 (3.13; 3.81)	3.79 (3.40; 4.22)	3.70 (3.35; 4.10)	0.42
MIP-1α (pg/mL)	9.66 (8.61; 10.8)	10.1 (8.97; 11.4)	9.78 (6.68; 11.0)	0.86	9.72 (8.70; 10.9)	10.8 (9.74; 12.0)	10.4 (9.26; 11.7)	0.39
MIP-1β (pg/mL)	7.25 (6.68; 7.87)	7.36 (6.75; 8.03)	7.04 (6.44;7.69)	0.75	7.03 (6.48; 7.63)	7.33 (6.75; 7.69)	7.51 (6.89; 8.19)	0.54
MIP-3α (pg/mL)	2.02 (1.76; 2.31)	2.32 (2.03; 2.65)	2.08 (1.85; 2.36)	0.31	1.70 (1.48; 1.95)	1.94 (1.74; 2.17)	1.99 (1.75; 2.27)	0.17
TNF-α (pg/mL)	1.57 (1.38; 1.78)	1.65 (1.45; 1.87)	1.60 (1.41; 1.82)	0.87	1.70 (1.51; 1.91)	1.76 (1.57; 1.98)	1.94 (1.74; 2.17)	0.25

	Black				White			
	T1	**T2**	**T3**	**p**	**T1**	**T2**	**T3**	**p**
Anti-Inflammatory								
IL-4 (pg/mL)	46.2 (40.3; 52.9)	44.3 (37.6; 52.2)	42.4 (36.5; 49.2)	0.72	46.5 (40.7; 53.4)	39.8 (34.4; 46.1)	48.4 (42.3; 55.4)	0.11
IL-5 (pg/mL)	0.88 (0.76; 1.01)	0.88 (0.74; 1.04)	0.91 (0.78; 1.05)	0.95	1.02 (0.90; 1.16)	0.99 (0.87; 1.13)	1.02 (0.90; 1.16)	0.92
IL-10 (pg/mL)	4.40 (3.81; 5.09)	4.56 (3.89; 5.35)	4.18 (3.58; 4.88)	0.72	5.39 (4.69; 6.19)	5.36 (4.70; 6.11)	5.41 (4.73; 6.20)	0.99
IL-13 (pg/mL)	3.69 (3.06; 4.44)	3.95 (3.27; 4.77)	4.02 (3.35; 4.82)	0.79	5.04 (4.27; 5.94)	5.03 (4.22; 5.99)	4.86 (4.06; 5.82)	0.95
Pro- and Anti-Inflammatory								
IL-6 (pg/mL)	1.86 (1.55; 2.24)	1.94 (1.61; 2.33)	1.82 (1.52; 2.18)	0.89	2.37 (2.02; 2.79)	2.32 (1.95; 2.76)	2.33 (1.96; 2.76)	0.98
IL-21 (pg/mL)	1.24 (1.06; 1.44)	1.35 (1.12; 1.64)	1.35 (1.15; 1.58)	0.70	1.41 (1.23; 1.63)	1.41 (1.21; 1.65)	1.60 (1.36; 1.88)	0.42
GM-CSF (pg/mL)	7.91 (6.78; 9.23)	7.38 (6.19; 8.80)	6.83 (5.69; 8.19)	0.48	8.37 (7.17; 9.77)	8.22 (7.03; 9.62)	9.29 (8.00; 10.8)	0.51
Pro-to-Anti Inflammatory Ratios								
IL-6/IL-10	0.28 (0.22; 0.34)	0.27 (0.21; 0.34)	0.32 (0.25: 0.40)	0.50	0.17 (0.13; 0.21)	0.16 (0.13; 0.19)	0.17 (0.13; 0.21)	0.92
IL-1β/IL-10	0.21 (0.19; 0.23)	0.22 (0.20; 0.25)	0.23 (0.20; 0.26)	0.50	0.20 (0.18;0.22)	0.18 (0.17; 0.20)	0.21 (0.19; 0.24)	0.14
TNF-α/IL-10	0.36 (0.33; 0.39)	0.37 (0.34; 0.41)	0.38 (0.35:0.42)	0.70	0.32 (0.29;0.35)	0.33 (0.30; 0.36)	0.36 (0.33; 0.39)	0.12
CRP/IL-10	0.24 (0.18; 0.33)	0.21 (0.16; 0.29)	0.24 (0.18; 0.32)	0.80	0.15 (0.12; 0.21)	0.13 (0.11; 0.17)	0.14 (0.10; 0.17)	0.67
MIP-*1α*/IL-10	2.21 (1.95; 2.51)	2.19 (1.96; 2.46)	2.21 (1.95; 2.50)	1.00	1.75 (1.56; 1.96)	1.88 (1.68; 2.10)	1.90 (1.70; 2.12)	0.54
ITAC/IL-4	0.11 (0.09; 0.12)	0.11 (0.09; 0.13)	0.11 (0.09; 0.13)	0.92	0.07 (0.07; 0.09)	0.10 (0.08; 0.11)	0.07 (0.07; 0.09)	0.024
ITAC/IL- 5	5.52 (4.73; 6.43)	5.64 (4.71; 6.77)	5.15 (4.42; 6.01)	0.71	3.41 (3.02; 3.85)	3.83 (3.34; 4.41)	3.59 (3.18; 4.04)	0.42
ITAC/IL-10	1.11 (0.94; 1.29)	1.09 (0.92; 1.28)	1.10 (0.95;1.29)	0.99	0.64 (0.57; 0.72)	0.71 (0.62; 0.80)	0.68 (0.61; 0.76)	0.52
ITAC/IL-13	1.31 (1.08; 1.59)	1.24 (1.02; 1.51)	1.17 (0.97; 1.41)	0.71	0.69 (0.58; 0.81)	0.75 (0.62; 0.90)	0.77 (0.63; 0.92)	0.67

Table S3. Partial correlations between Na^+ and K^+ and inflammatory mediators in total, black and white population.

	Total *		**Black**		**White**	
	Na^+ (mmol/day)	K^+ (mmol/day)	Na^+ (mmol/day)	K^+ (mmol/day)	Na^+ (mmol/day)	K^+ (mmol/day)
Pro-Inflammatory						
CRP (mg/L)	-	r= -0.083 p= 0.010	r= -0.22 p= 0.031	-	-	r= -0.104 p= 0.018
Fractalkine (pg/mL)	-	-	-	-	-	-
IFN-γ (pg/mL)	-	-	-	-	-	-
IL-1β (pg/mL)	-	-	-	-	-	-
IL-2 (pg/mL)	-	-	-	r= 0.129 p= 0.013	-	-
IL-7 (pg/mL)	-	-	-	-	-	-
IL-8 (pg/mL)	-	-	-	-	-	-
IL-12 (pg/mL)	-	-	-	-	-	-
IL-17 A (pg/mL)	-	-	-	-	-	-
IL-23 (pg/mL)	-	-	-	-	-	-
ITAC (pg/mL)	-	-	-	-	-	-
MIP-1α (pg/mL)	-	-	-	-	-	-
MIP-1β (pg/mL)	-	-	-	-	-	-
MIP-3α (pg/mL)	-	-	-	-	-	-
TNF-α (pg/mL)	-	-	-	-	-	-
Anti-Inflammatory					-	-
IL-4 (pg/mL)	-	-	-	-	-	r= -0.092 p= 0.038
IL-5 (pg/mL)	-	-	-	-	-	-
IL-10 (pg/mL)	-	-	-	-	-	-
IL-13 (pg/mL)	-	-	-	-	-	-
IL-6 (pg/mL)	-	-	-	-	-	-
IL-21 (pg/mL)	-	-	-	-	-	-
GM-CSF (pg/mL)	-	-	-	-	-	-
Pro-to-Anti Inflammatory Ratios						
IL-6/IL-10	-	-	-	-	-	-
IL-1β/IL-10	-	-	-	-	-	-
TNF-α/IL-10	-	-	-	-	-	-
CRP/IL-10	-	r= -0.067 p= 0.041	-	-	-	-
MIP-*1α*/IL-10	-	-	-	-	-	-

	Total *		**Black**		**White**	
Na^+ (mmol/day)	K^+ (mmol/day)	Na^+ (mmol/day)	K^+ (mmol/day)	Na^+ (mmol/day)	K^+ (mmol/day)	Na^+ (mmol/day)
ITAC/IL-4	-	-	-	-	-	r= 0.115 p= 0.009
ITAC/IL- 5	-	-	-	-	-	-
ITAC/IL-10	-	-	-	-	-	-
ITAC/IL-13	-	-	-	-	-	-

Adjusted for: Age, sex and waist circumference. * Additionally, adjusted for ethnicity.

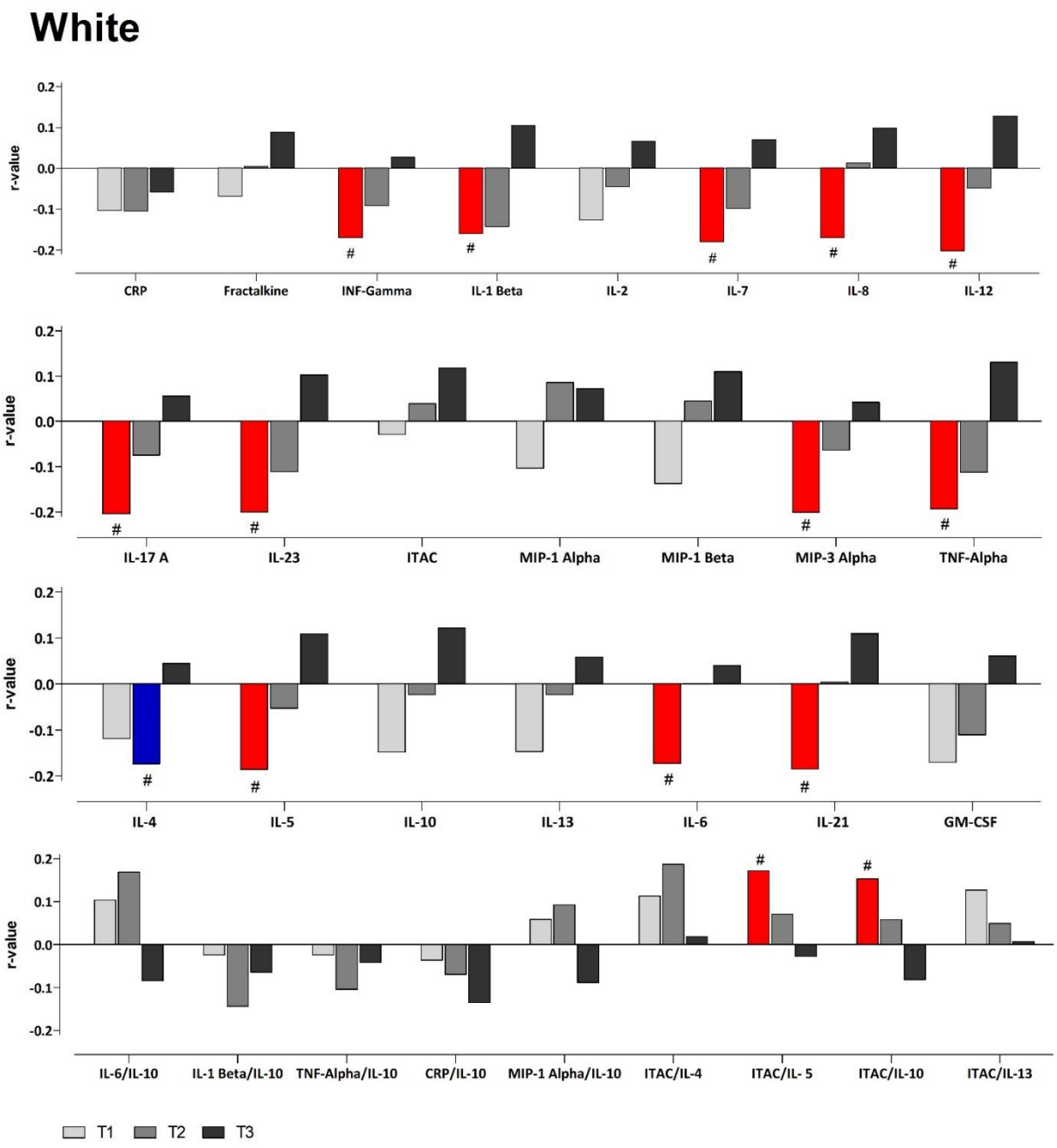

Fig. S1 Partial correlations between 24 hr K^+ and inflammatory mediators in white individuals, within 24h Na^+ tertiles.

Each model was adjusted for: age, sex and waist circumference.
# indicates correlation with $p<0.05$ Red bars indicate $p<0.05$ in Na^+ Tertile 1, Blue bars indicate $p<0.05$ in Na^+ Tertile 2, Green bars indicate $p<0.05$ in Na^+ Tertile 3

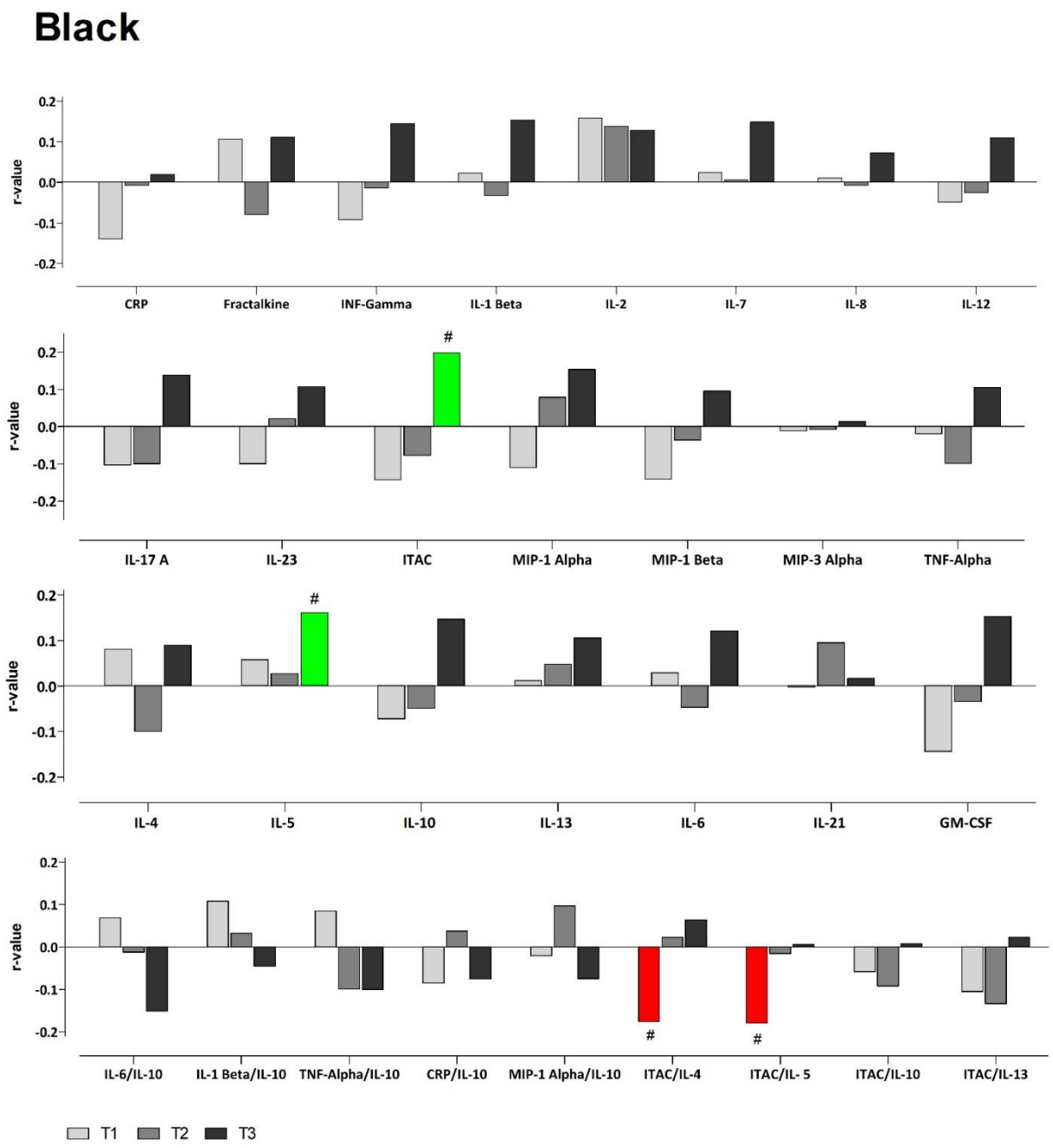

Fig. S2 Partial correlations between 24 hr K^+ and inflammatory mediators in black individuals, within 24h Na^+ tertiles.

Each model was adjusted for: age, sex and waist circumference.
# indicates correlation with p<0.05. Red bars indicate p<0.05 in Na^+ Tertile 1, Blue bars indicate p<0.05 in Na^+ Tertile 2, Green bars indicate p<0.05 in Na^+ Tertile 3

Chapter 5

Inflammation and hypertension development:
a longitudinal analysis of the African-PREDICT study

Journal of Hypertension			
Impact factor	4.209		
Publisher	Wolters Kluwer Health		
Citation	None 		
Aims & Scope	The *Journal of Hypertension* publishes papers reporting original clinical and experimental research which are of a high standard and which contribute to the advancement of knowledge in the field of hypertension.		
Author Instructions			
Language	Not specified.	**Font**	Not specified.
Spacing	Double.	**Margins**	3 cm.
Word count	Not specified.	**Tables& figures**	Not specified.
References	Not specified.	**Alignment**	Not justified.
Manuscript	The manuscript should include the following sections, each starting on a separate page: title page, abstract and keywords, text, acknowledgements, references, individual tables and captions.		
Title page	The title page should carry: • Full title (20 words) • Running title (40 characters, including spaces) • Full first name, middle initial(s) and last (family name- in capital letters) name of each author should appear • Author affiliations • Affiliations of all the authors connected using a,b,c • Sources of funding • Conflicts of interest statement		

	• Corresponding author information • Word count: including references, but not tables and legends • Number of tables • Number of figures • Number of supplementary digital content files
Abstract	Structured abstract of no more than 250 words
Keywords	3-10 keywords
Text	Introduction, Methods, Results & Discussion
Acknowledgements	Include acknowledgements
Conflict of interest	Include statement on title page
Funding	Include funding on title page
Ethical considerations	All work must be conducted in accordance with the Declaration of Helsinki. Papers describing experimental work on human participants which carries a risk of harm must include (1) a statement that the experiments were conducted with the understanding and the consent of each participant, and (2) a statement that the responsible ethical committee has approved the experiments.
References	• References should be numbered consecutively • Assigned Arabic numerals, which should be given in brackets, e.g. [17]. • References should include the names of all authors and any Study Group named in the primary author list when six or fewer; when seven or more, list only the first six names and add et al. • Journal names should be abbreviated as MEDLINE *Articles in journals:* Zhou M-S, Schulman IH, Raij L. Vascular inflammation, insulin resistance, and endothelial dysfunction in salt-sensitive hypertension: role of nuclear factor kappa B activation. J Hypertens 2010; 28:527–535
Tables	• Each table should be typed on a separate page in double spacing. • Each table should be assigned an Arabic numeral,(Table 3) and a brief title. • Vertical rules should not be used. • Place explanatory matter in footnotes, not in the heading. • Be sure that each table is cited in the text.
Figures	• Cite figures consecutively in your manuscript. • Number figures in the figure legend in the order in which they are discussed.
Legends for illustrations	• Captions should be typed in double spacing, beginning on a separate page.

	• Each one should have an Arabic numeral corresponding to the illustration to which it refers.
Supplemental Digital Content	• Cited consecutively in the text of the submitted manuscript. • Should include the type of material submitted (Audio, Figure, Table, etc.), be clearly labelled as "Supplemental Digital Content," include the sequential list number, and provide a description of the supplemental content.

**Formatting changes were made to maintain uniformity throughout this thesis, including text font, line spacing, margins, page numbers, tables and figures.*

Inflammation and hypertension development: a longitudinal analysis of the African-PREDICT study

Running title: Inflammation, blood pressure and ethnicity

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Abstract

Objective: The role of inflammation in the development of hypertension remains incompletely understood. While single inflammatory mediators have been shown to associate with changes in blood pressure (ΔBP), the role of clusters of inflammatory mediators has been less comprehensively explored. We therefore determined whether individual or clusters of inflammatory mediators from a large biomarker panel were associated with ΔBP over 4.5 years, in young healthy adults. **Methods:** We included 358 adults (white, n=156; black n=202) with detailed information on ambulatory blood pressure (BP) at baseline and follow-up. Baseline blood samples were analysed for 22 inflammatory mediators using multiplexing technology. Principal component analysis was used to study associations between clusters of inflammatory mediators and ΔBP. **Results:** In the total cohort in multivariable-adjusted regression analyses, percentage change in 24-hour systolic BP associated positively with Factors 1 (Interferon-gamma, interleukin (IL)-4, IL-7, IL-10, IL-12, IL-17A, IL-21, IL-23, macrophage inflammatory protein (MIP)-1α, MIP-1β, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF)) and 2 (IL-5, IL-6, IL-8, IL-13). Change in daytime systolic BP associated positively with Factors 1, 2 and 3 (C-Reactive protein, IL-1β, IL-2, MIP-3α). Subgroup analysis found these findings were limited to white study participants. Numerous associations were present between individual inflammatory mediators (Interferon-gamma, GM-CSF, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, IL-21, IL-23, MIP-1α and MIP-1β) and ΔBP in the white but not black subgroups. **Conclusion:** We found independent relationships between numerous inflammatory mediators (individual and clusters) and ΔBP over 4.5 years. The relationship between inflammatory markers and ΔBP was only found in white participants.

Key Words: Hypertension, cytokine, ethnicity, African, black

Introduction

Hypertension is the most prominent risk factor for the development of cardiovascular disease.[1] The Global Burden of Disease study found raised systolic blood pressure (BP) to account for 10.4 million deaths per year.[2] Hypertension is a multi-factorial trait that develops as a result of both environmental and genetic factors.[3] One important factor found to contribute to BP elevation is low-grade inflammation.[4]

The infiltration of innate and adaptive immune cells, along with other inflammatory processes such as expression of adhesion molecules, cytokines and reactive oxygen species by the brain, kidneys and the vasculature are consistently found in individuals with hypertension.[5-8] C-reactive protein (CRP), interleukin (IL) 6, and tumour necrosis factor alpha (TNF-α) relate positively to hypertension,[9] and risk prediction models that include CRP have been developed.[10,11] However, the inflammatory pathways involved in early vascular ageing and hypertension development may be more complex and cannot be fully represented by single markers.[12] While single inflammatory mediators have been found to be associated with raised BP,[13] the role of clusters of inflammatory mediators has been less comprehensively explored. The activation of pro- and anti-inflammatory pathways does not occur in isolation and numerous interactions between inflammatory mediators exist, making investigations into the role of inflammation in the development of hypertension challenging.[14]

We have shown, using data from several studies including a cross-sectional analysis in 1189 participants from the African-PREDICT study,[15] that the profiles of inflammatory mediators differ in populations from African descent when compared to populations of European descent.[16] Black populations are known to have a greater burden of hypertension than most other populations.[17] As such, we hypothesised that pro-inflammatory mediators would associate adversely with change in blood pressure (ΔBP). Therefore, we evaluated the role of inflammatory mediators in the early stages of development of hypertension in both young black and white adults.

Methodology

Study population

This study forms part of the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT), and detailed methods have been described before.[18] As shown in **Figure 1**, from 2013-2017 we recruited 1202 young black and white men and women, between the ages of 20-30 years. Participants were recruited from Potchefstroom and surrounding areas in the North West province, South Africa via community health workers, work place and public advertisement. Individuals from low, middle and high socio-economic status groups were specifically included. Although individuals with office brachial BP of ≥140 and/or ≥90 were excluded during baseline screening, there was an average two-week period between the screening and research phases. Some participants were classified as being hypertensive based on 24-hour ambulatory blood pressure during the research phase and were included in this study. This sub-study included data of the first 358 participants who were successfully followed up during 2018-2019. The study was approved by the Health Research Ethics Committee (HREC) of the North-West University (NWU-00058-18-A1), adheres to the guidelines as set out by the Declaration of Helsinki and all participants in the study provided written informed consent prior to participation.

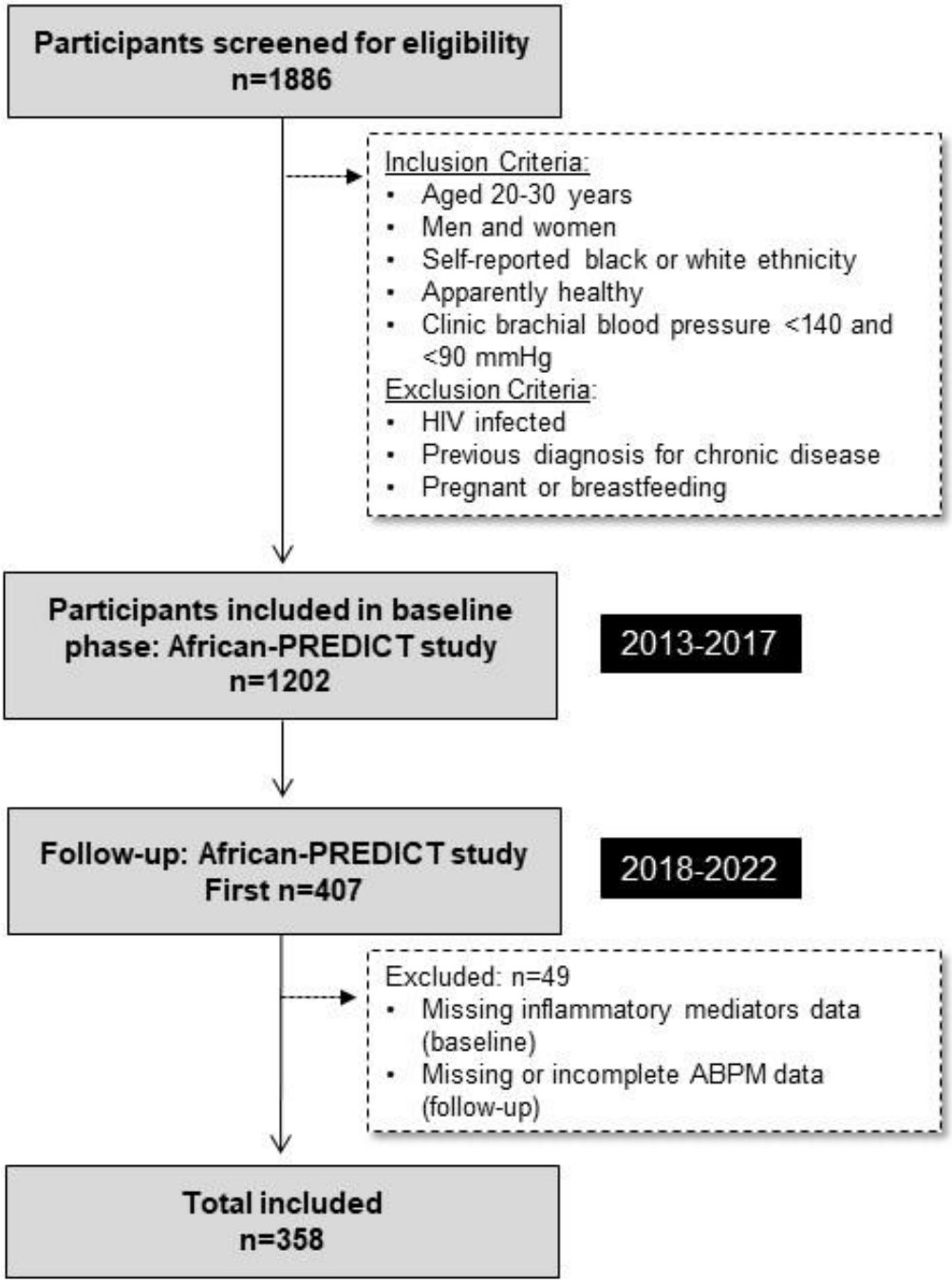

Figure 1. Layout of the study population.

General Measurements

Self-reported data with regards to demographic, lifestyle, socio-economic status and medication use were collected using questionnaires. Anthropometry was measured using standard methods.[18] Body mass index (BMI) was calculated as weight (kg) / height $(m)^2$.

Blood pressure

All BP measurements were performed by a trained researcher using an appropriately sized cuff for each participant. Cuffs were selected from adults' sizes small, medium and large and sizing was checked upon fitting.

Office blood pressure

After a 10-minute rest, duplicate brachial BP measurements were done on the left and right arms, with a 5-minute interval in-between, whilst participants were seated (Dinamap® Procare 200; GE Medical Systems, Milwaukee, WI, USA). BP were measured in a temperature-controlled room in the research clinic, with a single researcher present.

Ambulatory blood pressure

Participants non-dominant arm were fitted with a validated 24-hour ambulatory BP (ABPM) monitor (Card(X)plore® CE120, Meditech, Budapest, Hungary) at approximately the same time every day (late morning). The monitor was programmed to record every 30 minutes during the day (06h00 to 22h00) and every hour during the night (22h00 to 06h00).[19] The monitor was fitted in a temperature-controlled room in the research clinic. Participants were instructed on how and when to remove the ABPM monitor the following day. Participants were provided a diary card to record all activity during monitoring. Data was checked for missed measurements or premature removal. In this study, participants had a mean successful inflation rate of 83%.

As ABPM were measured during baseline and follow-up, we calculated ΔBP over time, expressed at percentage change.

Biological sampling and biochemical analyses

Participants fasted overnight for at least eight hours prior to attending the day of research measurements. Blood samples were collected from the median cubital vein. The samples were prepared according to standardised protocols and stored at -80°C until the time of analysis.

Serum samples were analysed for high-sensitivity CRP, total cholesterol, glucose and γ-glutamyltransferase (GGT) (Cobas Integra® 400plus, Roche, Basel, Switzerland). Serum creatinine concentrations were measured using the Creatinine Jaffé Gen.2 reagent (Roche, Basel, Switzerland). Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology (CKD-EPI) formula, without race in the equation as this is not appropriate for South African populations.[20,21] Serum cotinine was analysed using a chemiluminescence method on the Immulite (Siemens, Erlangen, Germany) apparatus.

A MILLIPEX Map Human High Sensitivity T Cell Magnetic Bead Panel (EMD Millipore, Merck, Missouri, USA) was used to analyse 21 inflammatory mediators from baseline samples, using Luminex xMAP technology on the Luminex 200™ analyser.[15] These include fractalkine, Granulocyte-macrophage colony-stimulating factor, Interferon gamma, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, IL-21, IL-23, Interferon-inducible T-cell alpha chemoattractant, Macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-3α and TNF-α.

Statistical analyses

IBM®, SPSS® version 26 (IBM Corporation, Armonk, New York) was used for data analyses. GraphPad Prism 5.03 (GraphPad Software, San Diego) was used for all graphics. Continuous variables were inspected for normality using QQ plots as well as inspection of skewness and kurtosis. Variables with non-Gaussian distributions were logarithmically transformed. Pro- to anti-inflammatory ratios were calculated based on literature,[22,23] and new ratios were used based on instances where pro-inflammatory mediators were higher and anti-inflammatory mediators were lower in the black and white groups.[15] Dependent and independent T-tests, Chi-Square and McNemar tests were used to compare the profiles of black and white participants at baseline and follow-up. Factor analyses of the multiple inflammatory mediators were performed using the factor function of SPSS. Principal component analyses were used and factors with an eigenvalue of >1 were retained. Varimax rotation was used to obtain independent interpretable factors. A factor loading of ≥0.3 was used to interpret the factor patterns. Double loading was handled by placing the variable in the factor with the strongest

loading factor. Factor scores with a cumulative percentage of >50 was subsequently used for multiple regression analyses. This procedure was followed in the total group and each ethnic group individually.

We determined the relationships between ΔBP as the dependent variable, and pro- and anti-inflammatory mediators at baseline using multivariate forward stepwise regression analyses.

Sensitivity power analyses were performed using G*Power 3.1 statistical analysis program.[24] This study should be able to detect an effect size of 0.0220 with a power of 80% using 358 participants as the sample size and significance level set at 0.05 for a multiple linear regression with a maximum of 11 covariates. Should participants be stratified into groups according to ethnicity, the study should be able to detect an effect size of 0.0510 with a power of 80% given a sample size of 156 and significance level set at 0.05, for a multiple linear regression with a maximum of 11 covariates.

Results

The general characteristics of the participants (n=358) at baseline and follow-up are shown in **Table 1.** Participants had a median age increase of 4.45 years. When reviewing BP, black participants showed increases in all ambulatory BP measures (all p<0.001) during follow whereas the white participants showed an increase only in nighttime DBP (p=0.035) (**Table 1**). Consequently, percental ΔBP were higher in the black compared to the white group (**Figure 2**). The number of young black adults with hypertension increased from 7.4% to 20.3% over time (p<0.001), whereas these numbers remained stable in the white group (15.3%, p=0.59).

The levels of most inflammatory mediators were similar (p>0.05) between the ethnic groups at baseline (**Table S1**). Black participants had higher levels of ITAC and MIP-3α (both p≤0.006) and lower levels of IL-6, IL-10 and IL-13 (all p≤0.040), similar to what we previously reported in a larger sample.[15] Additionally, black participants had higher ratios of IL-1β/IL-10, TNF-α/IL-10, MIP-1α/IL-10, ITAC/IL-4, ITAC/IL-5, ITAC/IL-10 and ITAC/IL-13 (all p≤0.022).

We performed factor analyses with the pro- and anti-inflammatory mediator data to determine factor scores (**Table S2-S4**). Factor scores were subsequently used for multiple regression analyses to determine whether the pro- and anti-inflammatory mediator factors at baseline associate with the ΔBP. In the total population (**Table 2**), percentage change in 24-hour SBP and daytime SBP associated positively with Factors 1 and 2, and percentage change in daytime SBP also associated positively with Factor 3. Black ethnicity contributed significantly to ΔBP in all the models. When performing these analyses separately in the two ethnic groups, in white participants (**Table 3**), 24-hour SBP as well as 24-hour daytime SBP (both $p \leq 0.020$) were associated positively with Factor 1. Additionally, Factor 2 associated positively with 24-hour SBP, 24-hour DBP, 24-hour daytime SBP and 24-hour daytime DBP (all $p \leq 0.038$). No statistically significant associations were found in the black group (**Table 3**).

To dissect the potential contribution of individual inflammatory markers to BP changes, we also performed multiple regression analyses on individual inflammatory mediators. In white participants (**Table S5**), numerous associations were present between pro- and anti-inflammatory mediators (IFN-γ, GM-CSF, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, IL-21, IL-23, MIP-1α and MIP-1β) and ΔBP after adjusting for multiple covariates ($p<0.05$). No associations were found for black participants (**Table S5**).

We calculated hazard ratios (**Table S6**) to determine whether inflammatory mediator factors predict the development of hypertension. Black ethnicity significantly contributed to risk prediction for all models. No significant prediction was found for any of the inflammatory factors.

Table 1. Characteristics at baseline and follow-up.

	Black (n=202)			White (n=156)		
	Baseline	Follow-Up	p	Baseline	Follow-Up	p
Age, years	24.5 ± 3.26	28.9 ± 3.39	**<0.001**	26.2 ± 2.75	30.9 ± 2.70	**<0.001**
Men, n (%)	82 (40.6)	-		73 (46.8)	-	
Socio-economic Status						
Low, n (%)	129 (63.9)	74 (45.1)	**<0.001**	10 (6.4)	7 (5.4)	0.69
Middle, n (%)	51 (25.2)	53 (32.3)		29 (18.6)	30 (23.1)	
High, n (%)	22 (10.9)	37 (22.6)		117 (75.0)	93 (71.5)	
Body Composition						
Body mass index (kg/m^2)	24.5 ± 5.45	26.8 ± 6.70	**<0.001**	26.4 ± 5.32	27.2 ± 5.66	**<0.001**
Waist circumference (cm)	76.9 ± 11.3	79.1 ± 13.6	**0.001**	84.1 ± 14.7	84.8 ± 15.4	0.31
Office BP (mmHg)						
SBP	118 ± 12.4	117 ± 12.6	0.36	118 ± 12.3	114 ± 12.4	**<0.001**
DBP	79.7 ± 8.90	80.8 ± 9.82	0.078	78.4 ± 8.40	77.9 ± 8.41	0.39
Ambulatory BP (mmHg)						
24h SBP	115 ± 9.01	118 ± 10.3	**<0.001**	119 ± 10.1	118 ± 10.8	0.27
24h DBP	68.4 ± 5.86	72.1 ± 7.18	**<0.001**	69.7 ± 6.26	70.5 ± 6.84	0.054
Daytime SBP	119 ± 9.43	122 ± 10.6	**<0.001**	124 ± 10.7	123 ± 11.4	0.76
Daytime DBP	72.8 ± 6.44	76.3 ± 7.61	**<0.001**	74.7 ± 10.7	75.5 ± 7.21	0.079
Nighttime SBP	107 ± 10.3	110 ± 11.5	**<0.001**	109 ± 10.6	108 ± 10.7	0.072
Nighttime DBP	60.1 ± 6.94	63.5 ± 8.00	**<0.001**	59.8 ± 6.54	60.8 ± 7.22	**0.035**
Hypertensive, n (%)	16 (7.4)	41 (20.3)	**<0.001**	27 (15.3)	27 (15.3)	0.59

Findings presented as mean ± SD.

Abbreviations: SBP Systolic blood pressure; DBP Diastolic blood pressure.

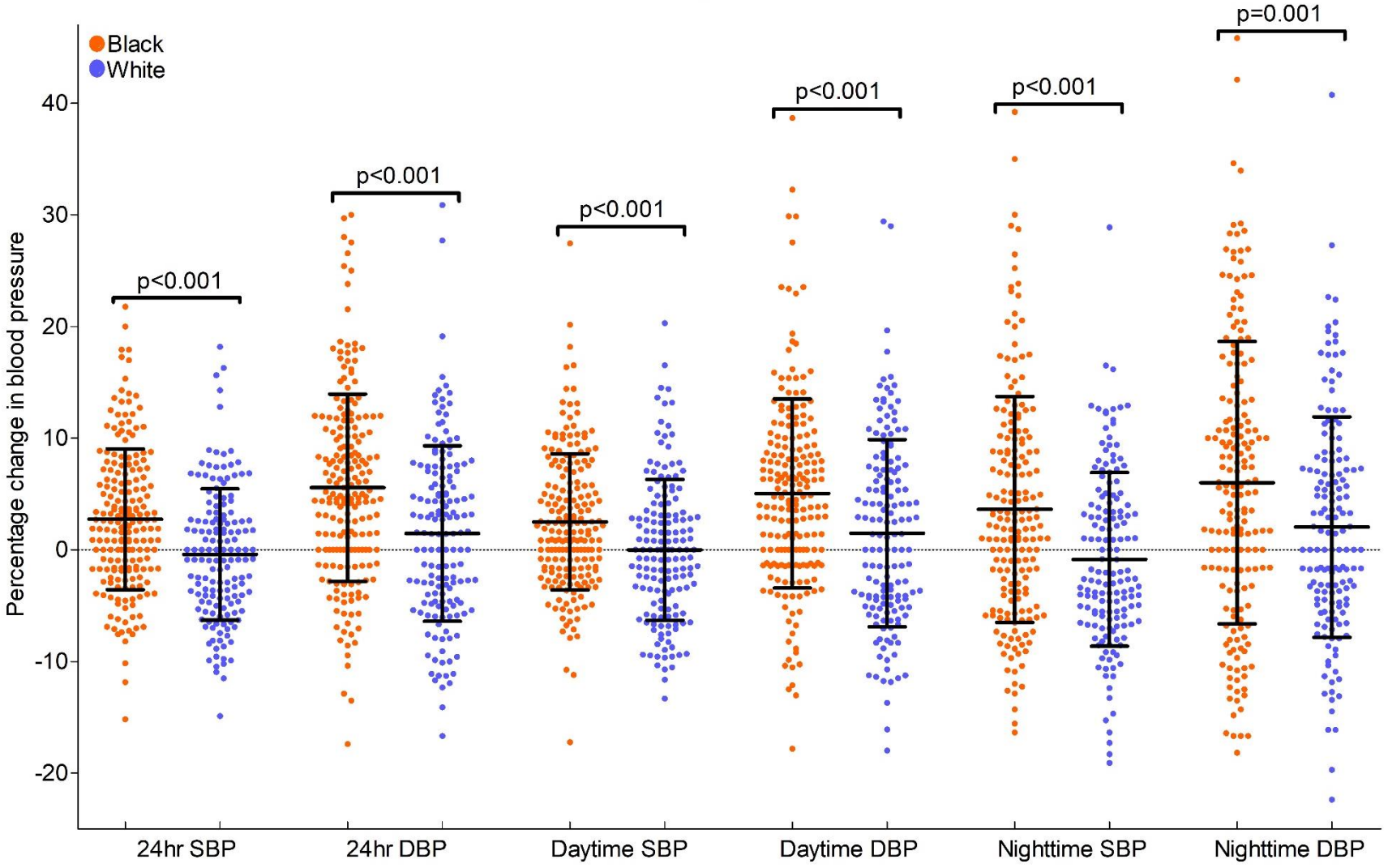

Figure 1. Percentage change in ambulatory blood pressure over 4.5 years in young black and white adults.
Horizontal line and whiskers: Mean ± SD
Abbreviations: SBP Systolic blood pressure; DBP Diastolic blood pressure.

Table 2. Multivariable adjusted forward stepwise regression analyses in the total group to show the relationship between percentage change in blood pressure and clusters of inflammatory mediators.

	Percentage change in					
	24-hour SBP (n=354)	**24-hour DBP (n=356)**	**Daytime SBP (n=354)**	**Daytime DBP (n=355)**	**Nighttime SBP (n=340)**	**Nighttime DBP (n=341)**
Factor 1	R^2=0.083 β=0.120 (0.008; 0.232) P=0.035	R^2=0.073 ---	R^2=0.052 β=0.142 (0.028; 0.255) P=0.015	R^2=0.039 ---	R^2=0.053 ---	R^2=0.025 ---
Ethnicity (0, black / 1, white)	β=-0.182 (-0.311; -0.055) P=0.005	β=-0.206 (-0.324; -0.091) P=0.001	β=-0.197 (-0.313; -0.084) P=0.001	β=-0.205 (-0.322; -0.092) P<0.001	β=-0.238 (-0.357; -0.123) P<0.001	β=-0.170 (-0.291; -0.052) P=0.005
Total Cholesterol (mmol/L)	β=-0.140 (-0.289; -0.014) P=0.035	---	---	---	---	---
Age (years)	---	β=-0.146 (-0.256; -0.029) P=0.014	---	---	---	---
Factor 2	R^2=0.088 β=0.140 (0.027; 0.252) P=0.035	R^2=0.073 ---	R^2=0.058 β=0.160 (0.046; 0.275) P=0.006	R^2=0.039 ---	R^2=0.053 ---	R^2=0.025 ---
Ethnicity (0, black / 1, white)	β=-0.202 (-0.332; -0.074) P=0.002	β=-0.206 (-0.324; -0.091) P=0.001	β=-0.218 (-0.334; -0.104) P<0.001	β=-0.205 (-0.322; -0.092) P<0.001	β=-0.238 (-0.357; -0.123) P<0.001	β=-0.170 (-0.291; -0.052) P=0.005

	Percentage change in					
	24-hour SBP (n=354)	**24-hour DBP (n=356)**	**Daytime SBP (n=354)**	**Daytime DBP (n=355)**	**Nighttime SBP (n=340)**	**Nighttime DBP (n=341)**
Total Cholesterol (mmol/L)	β=-0.136 (-0.284; -0.010) P=0.002	---	---	---	---	---
Age (years)	---	β=-0.146 (-0.256; -0.029) P=0.014	---	---	---	---
	R^2=0.072	R^2=0.072	R^2=0.046	R^2=0.039	R^2=0.053	R^2=0.025
Factor 3	---	---	β=0.118 (0.002; 0.34) P=0.046	---	---	---
Ethnicity (0, black / 1, white)	β=-0.180 (-0.311; -0.050) P=0.007	β=-0.206 (-0.326; -0.088) P=0.001	β=-0.193 (-0.311; -0.078) P=0.001	β=-0.205 (-0.324; -0.090) P=0.001	β=-0.238(-0.357; -0.123) P<0.001	β=-0.170 (-0.291; -0.052) P=0.005
Total Cholesterol (mmol/L)	β=-0.146 (-0.297; -0.017) P=0.028	---	---	---	---	---
Age (years)	---	β=-0.146 (-0.258; -0.027) P=0.014	---	---	---	---

Factor 1: Fractalkine, IFN-γ, IL-4, IL-7, IL-10, IL-12, IL-17A, IL-23, ITAC, MIP-1α, MIP-1β, TNF-α, GM-CSF
Factor 2: IL-6, IL-8, IL-13
Factor 3: IL-1β, IL-2, IL-5, IL-21, MIP-3α
Findings presented as β (95%CI).
Adjusted for: age, sex, ethnicity, socio-economic status, waist circumference, total cholesterol, glucose, gamma glutamyltransferase, cotinine, estimated glomerular filtration rate and activity energy expenditure.
Abbreviations: SBP Systolic blood pressure; DBP Diastolic blood pressure.

Table 3. Multivariable adjusted forward stepwise regression analyses in black and white groups to show the relationship between percentage change in blood pressure and clusters of inflammatory mediators.

	Percentage change in					
White	**24-hour SBP (n=156)**	**24-hour DBP (n=156)**	**Daytime SBP (n=156)**	**Daytime DBP (n=155)**	**Nighttime SBP (n=154)**	**Nighttime DBP (n=154)**
Factor 1	R^2=0.032 β=0.198 (0.029; 0.340) P=0.020	---	R^2=0.039 β=0.215 (0.049; 0.381) P=0.012	---	---	---
Factor 2	R^2=0.033 β=0.200 (0.031; 0.341) P=0.019	R^2=0.024 β=0.178 (0.009; 0.323) P=0.038	R^2=0.036 β=0.207 (0.040; 0.373) P=0.015	R^2=0.026 β=0.183 (0.015; 0.342) P=0.033	---	---
Factor 3	---	---	---	---	---	---
Factor 4	---	---	---	---	---	---
Black	**24-hour SBP (n=198)**	**24-hour DBP (n=200)**	**Daytime SBP (n=198)**	**Daytime DBP (n=200)**	**Nighttime SBP (n=186)**	**Nighttime DBP (n=187)**
Factor 1	---	---	---	---	---	---
Factor 2	---	---	---	---	---	---
Factor 3	---	---	---	---	---	---

White

Factor 1: IFN-γ, IL-4, IL-7, IL-10, IL-12, IL-17A, IL-21, IL-23, MIP-1α, MIP-1β, TNF-α, GM-CSF

Factor 2: IL-5, IL-6, IL-8, IL-13

Factor 3: CRP, IL-1β, IL-2, MIP-3α

Factor 4: Fractalkine, ITAC

Black

Factor 1: Fractalkine, IFN-γ, IL-4, IL-7, IL-10, IL-12, IL-17A, IL-23, ITAC, MIP-1α, MIP-1β, TNF-α, GM-CSF

Factor 2: IL-6, IL-8, IL-13

Factor 3: IL-1β, IL-2, IL-5, IL-21, MIP-3α

Findings presented as β (95%CI).

Adjusted for: age, sex, socio-economic status, waist circumference, total cholesterol, glucose, gamma glutamyltransferase, cotinine, estimated glomerular filtration rate and activity energy expenditure.

Abbreviations: SBP Systolic blood pressure; DBP Diastolic blood pressure.

Discussion

Inflammation has been implicated in the development of cardiovascular disease, including hypertension.[25,26] There is, however, a very limited understanding of whether complex inflammatory processes are already involved in the early phases of cardiovascular disease development in humans. In this study we evaluated a detailed panel of pro- and anti-inflammatory mediators in young, apparently healthy black and white adults and determined whether these mediators predict ΔBP over 4.5 years. We found independent, positive associations between clusters of inflammatory markers and ΔBP in the total group. Although black ethnicity also associated with ΔBP, associations between individual and clusters of inflammatory mediators with ΔBP were evident in white individuals only.

Other studies conducted in 6112 children aged 8-17 years,[27] 193 obese children with a mean age of 13,[28] and 281 obese children aged 6-18 years,[29] all found positive relationships between BP and the well-known inflammatory mediators CRP, IL-6 and IL-1β. We, however, found no associations between CRP or IL-1β and any measure of BP, but did find that IL-6 associates with ΔBP. The limited studies available examining these relationships in young individuals focussed on a restricted number of inflammatory mediators, as indicated above.[27-29] Our findings allowed us to show that relationships with BP are in fact present across a wide range of inflammatory mediators, further emphasising the link between inflammation and BP.

Non-steroidal anti-inflammatory drugs used to treat inflammation have been shown to raise BP as opposed to lowering it [30] – thereby demonstrating that the relationship between inflammation and BP is complex. In this study we used factor analyses to investigate the relationship between BP and clusters of inflammatory mediators. This approach may help to provide clarity on the mediators or inflammatory pathways involved. Factor 1 consisted largely of pro-inflammatory mediators, including IL-12,[31] IL-17A[6,32] and TNF-α,[32] which have previously been found to associate with increased BP in rat and human models. Factor 1 also

included IL-7, IL-23, MIP-1α and MIP-1β which associated positively with ΔBP. Although a study did find IL-7 to be higher in participants with hypertension than in the control group,[33] research into the relationship of these mediators with BP remains limited.

Factor 2 comprised mostly of anti-inflammatory mediators such as IL-13, which despite positively correlating with BP in our study, was previously suggested to elicit a protective cardiovascular role.[34] Factor 2 additionally included IL-6 which has been associated with increased BP in previous studies,[13,32] and IL-5 for which no previous reports on a direct association with BP could be found. Both Factors 1 and 2 showed robust positive relationships with ΔBP – most prominently with measures of systolic BP, in the white group. This finding supports the notion that the complex interplay between inflammatory mediators plays a role in BP regulation and not only pro-inflammatory mediators.[35] The independent associations between ΔBP and inflammatory mediators in young healthy adults suggest that inflammatory mediators may be early indicators of cardiovascular change, reflected by increasing BP.

Hypertension and other cardiovascular diseases are prominent features in populations of African descent. Previous South African studies have shown that black participants display higher levels of pro-inflammatory mediators than their white counterparts.[36,37] In the larger young African-PREDICT baseline cohort (n=1189), we also previously showed that black participants have an overall more pro-inflammatory profile than their white counterparts.[15] It is therefore surprising that the significant relationship between inflammatory mediators and ΔBP was only present in the white participants of our study. The prominent increase in BP over 4-5 years seen only in the young black population (Figure 2) may result from factors other than their overall pro-inflammatory profile. Another study also reported an accelerated progression from prehypertension to hypertension in black compared to white counterparts.[38] Some potential driving factors include genetic polymorphisms in renal sodium handling resulting in salt-sensitivity[39] and low plasma renin levels.[40] Additionally, even at younger ages black populations have shown to have increased measures of arterial stiffness,[41] vascular resistance,[42,43] and left ventricular mass[44] all of which may drive

increases in BP.[45] In the white group, subtle changes in inflammation appear to be associated with more subtle ΔBP and it is unclear how much inflammation will contribute to hypertension in the longer term.

It is well established that biological age and chronological age are not necessarily comparable.[46] A study found that the progression of vascular ageing is accelerated in the presence of cardiovascular risk factors.[47] Exposure to risk factors in early life plays a prominent role in the deterioration of vascular structure and function.[47] It was suggested that childhood environment could have an effect on the development of inflammatory phenotypes,[48] suggesting that the effects of the inflammatory mediator profile on the cardiovascular system may start at a very early age. Studies into the mechanisms involved have shown that both innate and adaptive immune responses contribute to the pathophysiology of hypertension via inflammatory changes in the kidney, blood vessels and the brain.[6,49] Inflammatory mediators aid in the development of hypertension through contribution to increased vascular permeability, release of cytokines, reactive oxygen species, nitric oxide and metalloproteinases.[50] Cytokine release leads to decreased lumen diameter of resistance vessels, increased vascular resistance and stiffness,[50] angiotensinogen and angiotensin II production, as well as sodium and volume retention.[6]

A strength of this study is the large panel of pro- and anti-inflammatory mediators which was analysed with a high-sensitivity analysis kit. The use of factor analyses allowed to identify clusters of multiple inflammatory mediators. We included young adults, which allowed us to examine the relationship between inflammation and BP in the absence of pre-existing health conditions. Furthermore, the presence of individuals with early hypertension ensures relationships are not confounded by target organ damage. However, due to the young healthy status of the cohort, it is unlikely that inflammatory mediators would predict clinical hypertension at this point which may only become evident with continued follow-up. Additionally, incidence of masked hypertension may differ between black and white populations.[51]

In conclusion, when evaluating a detailed range of inflammatory mediators (individually or in clusters) in young healthy adults, we found independent relationships with ΔBP. Although black ethnicity strongly associated with ΔBP over time, associations between inflammatory mediators and ΔBP were evident in white adults only. These findings suggest that at this young age, the development of hypertension in black populations may not be driven by inflammation while, in the white population, subtle changes in inflammation may predict the early changes in BP.

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Disclosures

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

The authors report no conflict of Interest.

Access to Data

The study methodology has been published,[18] whereas the data dictionary, statistical analysis, protocol and deidentified individual participant data will be made available upon reasonable request to the corresponding author in agreement with all co-authors.

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Supplementary Information

Table S1. A comparison of cytokine concentrations between black and white individuals at baseline.

	Black (n=202)	**White (n=156)**	*p*
Inflammatory Markers			
Pro-Inflammatory			
CRP (pg/mL)	1.24 (0.15; 9.41)	1.02 (0.12; 10.6)	0.18
Fractalkine (pg/mL)	27.6 (8.28; 72.5)	27.0 (11.1; 73.5)	0.74
INF-γ (pg/mL)	6.62 (1.61; 19.7)	7.33 (1.44; 21.0)	0.24
IL-1β (pg/mL)	0.94 (0.15; 3.79)	1.06 (0.27; 4.02)	0.23
IL-2 (pg/mL)	0.80 (0.12; 3.88)	0.79 (0.15; 3.96)	0.92
IL-7 (pg/mL)	5.62 (1.51; 17.3)	5.41 (0.86; 19.2)	0.68
IL-8 (pg/mL)	1.66 (0.45; 5.53)	1.95 (0.50; 6.99)	0.060
IL-12 (pg/mL)	1.76 (0.32; 6.47)	1.84 (0.45; 5.89)	0.64
IL-17 A (pg/mL)	3.19 (0.63; 12.9)	3.27 (0.64; 12.1)	0.80
IL-23 (pg/mL)	111 (13.5; 575)	134 (12.9; 772)	0.13
ITAC (pg/mL)	4.63 (1.46; 16.9)	3.24 (1.40; 8.63)	**<0.001**
MIP-*1α* (pg/mL)	8.75 (2.60; 22.9)	10.5 (3.47; 26.4)	**0.019**
MIP-*1β* (pg/mL)	6.92 (2.30; 14.9)	6.92 (3.00; 15.8)	1.00
MIP-3α (pg/mL)	2.26 (0.56; 8.68)	1.76 (0.57; 4.85)	**0.005**
TNF-α (pg/mL)	1.62 (0.39; 4.94)	1.72 (0.55; 5.20)	0.43
Anti-Inflammatory			
IL-4 (pg/mL)	43.4 (8.99; 153)	43.1 (9.28; 144)	0.94
IL-5 (pg/mL)	0.89 (0.19; 3.71)	1.04 (0.27; 3.88)	0.10
IL-10 (pg/mL)	4.34 (0.81; 17.4)	5.45 (0.94; 22.1)	**0.023**
IL-13 (pg/mL)	3.60 (0.54; 20.7)	5.51 (0.78; 31.0)	**<0.001**
Pro- and Anti-Inflammatory			
GM-CSF (pg/mL)	6.92 (1.35; 31.0)	8.12 (1.27; 30.9)	0.15
IL-6 (pg/mL)	1.73 (0.23; 8.60)	2.42 (0.28; 12.0)	**0.005**
IL-21 (pg/mL)	1.39 (0.23; 5.69)	1.35 (0.27; 4.82)	0.79
Pro-to-Anti Inflammatory Ratios			
IL-6 to IL-10	0.25 (0.04; 1.65)	0.15 (0.02; 1.23)	**<0.001**
IL-1β to IL-10	0.22 (0.08; 0.85)	0.19 (0.05; 0.73)	0.11
TNF-α to IL-10	0.38 (0.17; 1.03)	0.32 (0.13; 1.14)	**0.002**
CRP to IL-10	0.29 (0.02; 3.00)	0.19 (0.01; 3.54)	**0.011**
MIP-*1α* to IL-10	2.04 (0.72; 6.48)	1.84 (0.57; 6.50)	0.21
ITAC to IL-4	0.11 (0.02; 0.85)	0.08 (0.02; 0.41)	**0.001**
ITAC to IL- 5	5.42 (1.11; 39.0)	3.12 (0.76; 10.1)	**<0.001**
ITAC to IL-10	1.09 (0.26; 8.86)	0.60 (0.17; 2.88)	**<0.001**
ITAC to IL-13	1.30 (0.22; 9.88)	0.59 (0.09; 4.19)	**<0.001**

Fractalkine, Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interferon gamma (IFN-γ), Interleukin 1 beta (IL-1β), Interleukin 2 (IL-2), Interleukin 4 (IL-4), Interleukin 5 (IL-5), Interleukin 6 (IL-6), Interleukin 7 (IL-7), Interleukin 8 (IL-8), Interleukin 10 (IL-10), Interleukin 12 (IL-12), Interleukin 13 (IL-13), Interleukin 17A (IL-17A), Interleukin 21 (IL-21), Interleukin 23 (IL-23), Interferon-inducible T-cell alpha chemoattractant (ITAC), Macrophage inflammatory protein *1-alpha* (MIP-1α), Macrophage inflammatory protein *1-beta* (MIP-1β), Macrophage inflammatory protein 3-*alpha* (MIP-3α) and Tumour Necrosis Factor Alpha (TNFα).

Table S2. Inflammatory mediator factor scores in the total population.

	Factor 1	**Factor 2**	**Factor 3**	**Factor 4**
CRP				-0.718
Fractalkine	0.636			
INF-γ	0.804			
IL-1β			0.609	
IL-2			0.819	
IL-7	0.698			
IL-8		0.716		
IL-12	0.698			
IL-17 A	0.776			
IL-23	0.629			
ITAC	0.510			
MIP-1α	0.571			
MIP-1β	0.687			
MIP-3α			0.759	
TNF-α	0.684			
IL-4	0.778			
IL-5			0.652	
IL-10	0.631			
IL-13		0.855		
IL-6		0.814		
IL-21			0.554	
GM-CSF	0.782			
Eigenvalue	8.35	2.61	3.36	-
Cumulative %	64.2	86.9	67.2	-

Table S3. Inflammatory mediator factor scores in the white population.

	Factor 1	**Factor 2**	**Factor 3**	**Factor 4**
CRP			0.347	
Fractalkine				0.711
INF-γ	0.831			
IL-1β			0.685	
IL-2			0.813	
IL-7	0.752			
IL-8		0.824		
IL-12	0.735			
IL-17 A	0.819			
IL-23	0.767			
ITAC				0.805
MIP-1α	0.548			
MIP-1β	0.754			
MIP-3α			0.691	
TNF-α	0.580			
IL-4	0.773			
IL-5		0.604		
IL-10	0.614			
IL-13		0.871		
IL-6		0.834		
IL-21	0.492			
GM-CSF	0.821			
Eigenvalue	8.37	3.23	2.23	1.46
Cumulative %	69.7	80.8	55.88	73.1

Table S4. Inflammatory mediator factor scores in the black population.

	Factor 1	**Factor 2**	**Factor 3**	**Factor 4**
CRP				-0.600
Fractalkine	0.636			
INF-γ	0.785			
IL-1β			0.609	
IL-2			0.834	
IL-7	0.617			
IL-8		0.691		
IL-12	0.633			
IL-17 A	0.709			
IL-23	0.596			
ITAC	0.590			
MIP-1α	0.575			
MIP-1β	0.573			
MIP-3α			0.725	
TNF-α	0.652			
IL-4	0.698			
IL-5			0.635	
IL-10	0.582			
IL-13		0.585		
IL-6		0.814		
IL-21			0.602	
GM-CSF	0.752			
Eigenvalue	8.41	2.56	3.39	---
Cumulative %	64.8	85.4	67.8	---

Table S5. Multivariable adjusted forward stepwise regression analyses in the black and white group to show the relationship between percentage change in blood pressure and inflammatory mediators.

	Percentage change in					
White	**24hr SBP (n=156)**	**24hr DBP (n=156)**	**Daytime SBP (n=156)**	**Daytime DBP (n=155)**	**Nighttime SBP (n=154)**	**Nighttime DBP (n=154)**
Pro-Inflammatory						
CRP (mg/L)	---	---	---	---	---	---
Fractalkine (pg/mL)	---	---	---	---	---	---
IFN-γ (pg/mL)	---	---	R^2=0.041 β=0.220 (0.054; 0.390) P=0.010	---	---	---
IL-1β (pg/mL)	---	---	---	---	---	---
IL-2 (pg/mL)	---	---	---	---	---	---
IL-7 (pg/mL)	R^2=0.045 β=0.229 (0.055; 0.345) P=0.007	R^2=0.029 β=0.191 (0.021; 0.315) P=0.025	R^2=0.064 β=0.266 (0.096; 0.405) P=0.002	R^2=0.032 β=0.197 (0.027; 0.335) P=0.021	---	---
IL-8 (pg/mL)	---	R^2=0.029 β=0.189 (0.021; 0.344) P=0.027	R^2=0.028 β=0.190 (0.024; 0.369) P=0.026	R^2=0.037 β=0.211 (0.044; 0.380) P=0.014	---	---
IL-12 (pg/mL)	R^2=0.040 β=0.217 (0.048; 0.363) P=0.011	---	R^2=0.055 β=0.250 (0.086; 0.422) P=0.003	R^2=0.031 β=0.194 (0.026; 0.359) P=0.024	---	---
IL-17 A (pg/mL)	R^2=0.023 β=0.174 (0.006; 0.325) P=0.042	---	R^2=0.052 β=0.243 (0.080; 0.418) P=0.004	$R^2$0.032 β=0.198 (0.030; 0.364) P=0.021	---	---
IL-23 (pg/mL)	R^2=0.052 β=0.244 (0.068; 0.353) P=0.004	R^2=0.029 β=0.172 (0.003; 0.294) P=0.045	R^2=0.054 β=0.247 (0.076; 0.381) P=0.004	R^2=0.023 β=0.174 (0.006; 0.309) P=0.042	---	---

	Percentage change in					
White	**24hr SBP (n=156)**	**24hr DBP (n=156)**	**Daytime SBP (n=156)**	**Daytime DBP (n=155)**	**Nighttime SBP (n=154)**	**Nighttime DBP (n=154)**
ITAC (pg/mL)	---	---	---	---	---	---
MIP-1α (pg/mL)	R^2=0.024 β=0.176 (0.008; 0.319) P=0.040	R^2=0.028 β=0.186 (0.018; 0.330) P=0.029	R^2=0.029 β=0.190 (0.022; 0.356) P=0.026	---	---	---
MIP-1β (pg/mL)	R^2=0.024 β=0.177 (0.008; 0.321) P=0.039	---	R^2=0.034 β=0.202 (0.036; 0.369) P=0.018	---	---	---
MIP-3α (pg/mL)	---	---	---	---	---	---
TNF-α (pg/mL)	---	---	---	---	---	---
Anti-Inflammatory						
IL-4 (pg/mL)	R^2=0.023 β=0.175 (0.007; 0.354) P=0.041	---	R^2=0.045 β=0.229 (0.070; 0.438) P=0.007	---	---	---
IL-5 (pg/mL)	---	---	---	---	---	---
IL-10 (pg/mL)	R^2=0.025 β=0.182 (0.014; 0.330) P=0.033	---	R^2=0.033 β=0.199 (0.033; 0.372) P=0.020	R^2=0.021 β=0.169 (0.000; 0.334) P=0.050	---	---
IL-13 (pg/mL)	R^2=0.044 β=0.226 (0.058; 0.377) P=0.008	R^2=0.042 β=0.222 (0.054; 0.374) P=0.009	R^2=0.055 β=0.249 (0.087; 0.427) P=0.003	R^2=0.044 β=0.226 (0.060; 0.395) P=0.008	---	R^2=0.027 β=0.185 (0.015; 0.310) P=0.031
Pro- and Anti- Inflammatory						
IL-6 (pg/mL)	R^2=0.031 β=0.196 (0.028; 0.348) P=0.022	R^2=0.031 β=0.195 (0.027; 0.349) P=0.022	R^2=0.046 β=0.230 (0.066; 0.408) P=0.007	R^2=0.035 β=0.206 (0.039; 0.375) P=0.016	---	---

	Percentage change in					
White	**24hr SBP (n=156)**	**24hr DBP (n=156)**	**Daytime SBP (n=156)**	**Daytime DBP (n=155)**	**Nighttime SBP (n=154)**	**Nighttime DBP (n=154)**
IL-21 (pg/mL)	R^2=0.041 β=0.220 (0.048; 0.343) P=0.010	---	R^2=0.063 β=0.265 (0.098; 0.410) P=0.002	R^2=0.026 β=0.182 (0.013; 0.326) P=0.034	---	---
GM-CSF (pg/mL)	R^2=0.043 β=0.223 (0.058; 0.391) P=0.009	---	R^2=0.052 β=0.242 (0.083; 0.439) P=0.004	R^2-0.026 β=0.183 (0.015; 0.369) P=0.033	---	---
Pro-to-Anti Inflammatory Ratios						---
IL-6/IL-10	---	---	---	---	---	---
IL-1β/IL-10	---	---	---	---	---	---
TNF-α/IL-10	---	---	---	---	---	---
CRP/IL-10	R^2=0.030 β=-0.192 (-0.371; -0.026) P=0.024	---	R^2=0.037 β=-0.209 (-0.417; -0.048) P=0.014	---	---	---
MIP-*1α*/IL-10	---	---	---	---	---	---
ITAC/IL-4	---	---	---	---	---	---
ITAC/IL- 5	---	---	---	---	---	---
ITAC/IL-10	---	---	---	---	---	---
ITAC/IL-13	---	---	R^2=0.026 β=-0.181 (-0.376; -0.015) P=0.034	---	---	---
	Percentage change in					
Black	**24hr SBP (n=198)**	**24hr DBP (n=200)**	**Daytime SBP (n=198)**	**Daytime DBP (n=200)**	**Nighttime SBP (n=186)**	**Nighttime DBP (n=187)**
All Pro-Inflammatory	---	---	---	---	---	---
All Anti-Inflammatory	---	---	---	---	---	---

	Percentage change in					
Black	**24hr SBP (n=198)**	**24hr DBP (n=200)**	**Daytime SBP (n=198)**	**Daytime DBP (n=200)**	**Nighttime SBP (n=186)**	**Nighttime DBP (n=187)**
All Pro- and Anti-Inflammatory	---	---	---	---	---	---
All Pro-to-Anti Inflammatory Ratios	---	---	---	---	---	---

Findings presented as β (95%CI).
Adjusted for: age, sex, socio-economic status, waist circumference, total cholesterol, glucose, gamma glutamyltransferase, cotinine, estimated glomerular filtration rate and activity energy expenditure.

Table S6. Hazard Ratio for the development of hypertension over 4.5 years

	β	95%(CI)	p
Factor 1	0.794	(0.572; 1.101)	0.17
Ethnicity (0, black / 1, white)	0.244	(0.094; 0.632)	**0.004**
Waist Circumference (cm)	1.724	(1.253; 2.373)	**0.001**
Glucose (mmol/L)	1.462	(0.917; 2.331)	0.11
Socio-economic Status	0.749	(0.483; 1.163)	0.20
Activity Energy Expenditure (kCal/day)	0.882	(0.619; 1.255)	0.48
Total Cholesterol (mmol/L)	0.963	(0.622; 1.492)	0.87
Estimated glomerular filtration rate (ml/min/1.73m^2)	1.072	(0.724; 1.586)	0.73
Cotinine (ng/ml)	1.231	(0.906; 1.674)	0.18
Gamma glutamyltransferase, (U/L)	1.025	(0.716; 1.467)	0.89
Sex (0, women / 1, men)	1.362	(0.715; 2.594)	0.35
Age (years)	1.136	(0.802; 1.608)	0.47
Factor 2	0.823	(0.593; 1.143)	0.25
Ethnicity (0, black / 1, white)	0.350	(0.151; 0.808)	**0.014**
Waist Circumference (cm)	1.508	(1.124; 2.024)	**0.006**
Glucose (mmol/L)	1.287	(0.814; 2.036)	0.28
Socio-economic Status	0.778	(0.509; 1.187)	0.24
Activity Energy Expenditure (kCal/day)	1.018	(0.739; 1.401)	0.92
Total Cholesterol (mmol/L)	0.998	(0.645; 1.546)	0.10
Estimated glomerular filtration rate (ml/min/1.73m^2)	1.062	(0.737; 1.529)	0.75
Cotinine (ng/ml)	1.093	(0.812; 1.471)	0.56
Gamma glutamyltransferase, (U/L)	1.208	(0.880; 1.656)	0.24
Sex (0, women / 1, men)	1.475	(0.794; 2.738)	0.22
Age (years)	1.081	(0.786; 1.489)	0.63
Factor 3	0.737	(0.529; 1.027)	0.071
Ethnicity (0, black / 1, white)	0.224	(0.084; 0.597)	**0.003**
Waist Circumference (cm)	1.744	(1.253; 2.427)	**0.001**
Glucose (mmol/L)	1.758	(1.048; 2.951)	**0.033**
Socio-economic Status	0.732	(0.458; 1.027)	0.19
Activity Energy Expenditure (kCal/day)	0.848	(0.584; 1.229)	0.38
Total Cholesterol (mmol/L)	1.033	(0.661; 1.616)	0.89
Estimated glomerular filtration rate (ml/min/1.73m^2)	1.031	(0.691; 1.537)	0.88
Cotinine (ng/ml)	1.214	(0.896; 1.644)	0.21
Gamma glutamyltransferase, (U/L)	1.080	(0.752; 1.546)	0.68
Sex (0, women / 1, men)	1.129	(0.580; 2.199)	0.72
Age (years)	1.187	(0.829; 1.700)	0.35

Factor 1: Fractalkine, IFN-γ, IL-4, IL-7, IL-10, IL-12, IL-17A, IL-23, ITAC, MIP-1α, MIP-1β, TNF-α, GM-CSF

Factor 2: IL-6, IL-8, IL-13

Factor 3: IL-1β, IL-2, IL-5, IL-21, MIP-3α

Chapter 6

Summary, Conclusion and Recommendations
Proposed for Future Studies

Introduction

This conclusive chapter addresses the aims, objectives and hypotheses laid out in Chapter 1, presents a summary of the main findings of the manuscripts and draws final conclusions. Additionally, recommendations for future studies to investigate the role of inflammatory mediators in the cardiovascular system are suggested.

Summary of Findings and Responses to Hypotheses

Despite the traditional understanding that inflammation protects during illness and injury,[1] there has been an increased interest in inflammatory mediators and their potential contribution to the development of cardiovascular disease (CVD). This contribution includes their role in changes to the structure and function of the cardiovascular system;[2-5] however, it remains a matter of debate precisely which inflammatory mediators and mechanisms are involved.[6-10] Therefore, the central aim of this study was to present a detailed inflammatory mediator profile and describe how it relates to sodium and potassium excretion and the cardiovascular profile of young, healthy South African adults. This was achieved using a 22-inflammatory mediator panel to describe a detailed inflammatory profile of young black and white adults and how this profile related to both dietary sodium and potassium intake and blood pressure (BP).

Manuscript 1: Distinct inflammatory mediator patterns in young black and white adults: The African-PREDICT study

This manuscript described and compared a detailed cytokine profile and investigated the relationships between BP and the pro- and anti-inflammatory mediator profiles of young black and white adults.

Hypothesis 1: The inflammatory mediator profile will differ between black and white groups, with black adults presenting with a more pro-inflammatory profile than the white group.

A number of studies investigating ethnic differences in inflammatory mediators focussed on only a few inflammatory mediators at a time, such as C-reactive protein (CRP), interleukin (IL)-6, tumour necrosis factor alpha (TNF-α) and IL-1β.[11-17] In addition, literature concerning ethnic differences in inflammatory mediator concentrations remains inconsistent.[11-13, 18, 19] The large panel of inflammatory mediators allowed us to evaluate the inflammatory profile of these black and white individuals as a whole. In the first manuscript, which is presented in Chapter 3, we demonstrated that each ethnic group presented with unique inflammatory mediator patterns regardless of BP, sex or socio-economic status. Black participants presented with a predominantly pro-inflammatory profile when compared to their white counterparts. These results were found despite the black group having lower levels of six and higher levels of only three of the fifteen pro-inflammatory mediators compared to the white group. The black group displayed lower levels of three of the four mediators with the anti-inflammatory mediators, resulting in overall higher pro-to-anti-inflammatory ratios. While the cause of these ethnic-specific differences remain unclear, one potential mechanism may be the differences in gene polymorphisms relating to genes coding for inflammation seen in different ethnic groups.[20]

Therefore, based on the results detailed above, I accept Hypothesis 1.

Hypothesis 2: Measures of BP will be adversely related to pro-inflammatory mediators and beneficially related to anti-inflammatory mediators in both black and white participants.

Inflammatory mediators have been implicated in the development of chronic diseases,[21-23] such as hypertension.[24] A number of pro-inflammatory mediators have been found to associate with increases in BP.[3, 25, 26] In contrast, some anti-inflammatory mediators have shown inverse associations with blood pressure.[3, 5] Despite the black individuals, in this large cross-sectional cohort, presenting with a predominantly pro-inflammatory profile when compared to their white counterparts, there were no associations with BP in the black or white groups for either single mediators or clusters of inflammatory mediators. This may suggest that at an early age, other physiological factors such as sympathetic activation, the renin-

angiotensin-aldosterone system,[27] arterial stiffness[28] and physical activity[29] may play a more prominent role in BP regulation than inflammation.

Based on these findings, I reject Hypothesis 2.

Manuscript 2: Inflammation and salt in young adults: The African-PREDICT study

In this manuscript we investigated the relationships between a detailed range of 22 pro- and anti-inflammatory mediators with 24-hour urinary sodium and potassium, respectively, in young black and white adults.

Hypothesis 1: Inflammatory mediators will associate adversely with sodium in both black and white adults.

It is well established that inflammation plays a role in the development of cardiovascular disease.[3, 24, 30] Furthermore, a diet high in salt is a risk factor for the development of hypertension. [31] It was recently reported that sodium intake may play a role in increases in pro-inflammatory mediators,[32-34] suggesting these two risk factors may be mechanistically involved. However, in this study no consistent associations between inflammatory mediators and 24-hour urinary sodium were found.

As such, I reject Hypothesis 1.

Hypothesis 2: Inflammatory mediators will be beneficially associated with potassium in both black and white adults.

Potassium intake has been shown to mitigate increases in BP as a result of salt intake, thus suggesting a protective role of potassium.[35-38] As potassium intake has a beneficial effect on BP, [39] it has been proposed that potassium may additionally possess anti-inflammatory properties.[40] One study found potassium supplementation in humans to inhibit interleukin-17A production induced by a salt load.[32] We demonstrated that in healthy white adults, 24-hour urinary potassium associated independently and negatively with several pro-inflammatory

mediators, but only in those with a daily salt intake of less than 6.31 grams (those in the lowest sodium tertile). No associations were seen in the black population. One possible explanation for these ethnic-specific results may be the differences in both sodium retention and potassium excretion observed between black and white populations.[41, 42] Black populations have shown increased sodium retention and decreased potassium excretion when compared to their white counterparts.[41, 42]

Since independent negative associations between potassium and several inflammatory mediators were found in white, but not in black participants, I partially accept Hypothesis 2.

Manuscript 3: Inflammation and hypertension development: a longitudinal analysis of the African-PREDICT study

In this manuscript we investigated the relationship between a detailed range of 22 pro- and anti-inflammatory mediators, both individual mediators and clusters and changes in BP over 4.5 years in young adults.

Hypothesis 1: Pro-inflammatory mediators will associate adversely with changes in BP in both black and white adults.

Hypothesis 2: Anti-inflammatory mediators will associate beneficially with changes in BP in both black and white adults.

The infiltration of innate and adaptive immune cells, along with other inflammatory processes have consistently been found in individuals with raised BP.[30, 43-45] A number of inflammatory mediators such as CRP,[26] IL-6,[3, 25, 26] IL-17A[3, 30] and TNF-α[3] have been found to positively associate with BP. In the third research article, which is presented in Chapter 5, we demonstrated that when evaluating a detailed range of inflammatory mediators (individually or in clusters) in young healthy adults, inflammatory mediators (both pro- and anti-) showed independent relationships with the change in BP over time. Although the black group presented with an increase in BP over time, independent associations between inflammatory

mediators and change in BP were evident only in white adults. This finding suggests that increases in BP over time, which were seen only in the young black population, may result from factors other than inflammation, such as genetic polymorphisms in renal sodium handling resulting in salt-sensitivity,[46, 47] low plasma renin levels or exposures to unhealthy behaviours related to socio-economic status.[48] Additional driving factors may be the increased arterial stiffness,[49] vascular resistance[50, 51] and left ventricular mass[52] seen in black populations, all of which may influence increases in BP.[53]

Based on the positive independent relationships seen between numerous pro-inflammatory mediators, factors (a mix of pro-and anti-inflammatory mediators) and change in BP in white participants, I partially accept Hypothesis 1. Based on the positive independent relationships between factors (which included both pro- and anti-inflammatory mediators) and change in BP, I reject Hypothesis 2.

Summary of the Main Findings

Below is a summary of the main findings from the three manuscripts of this thesis (**Figure 1**).

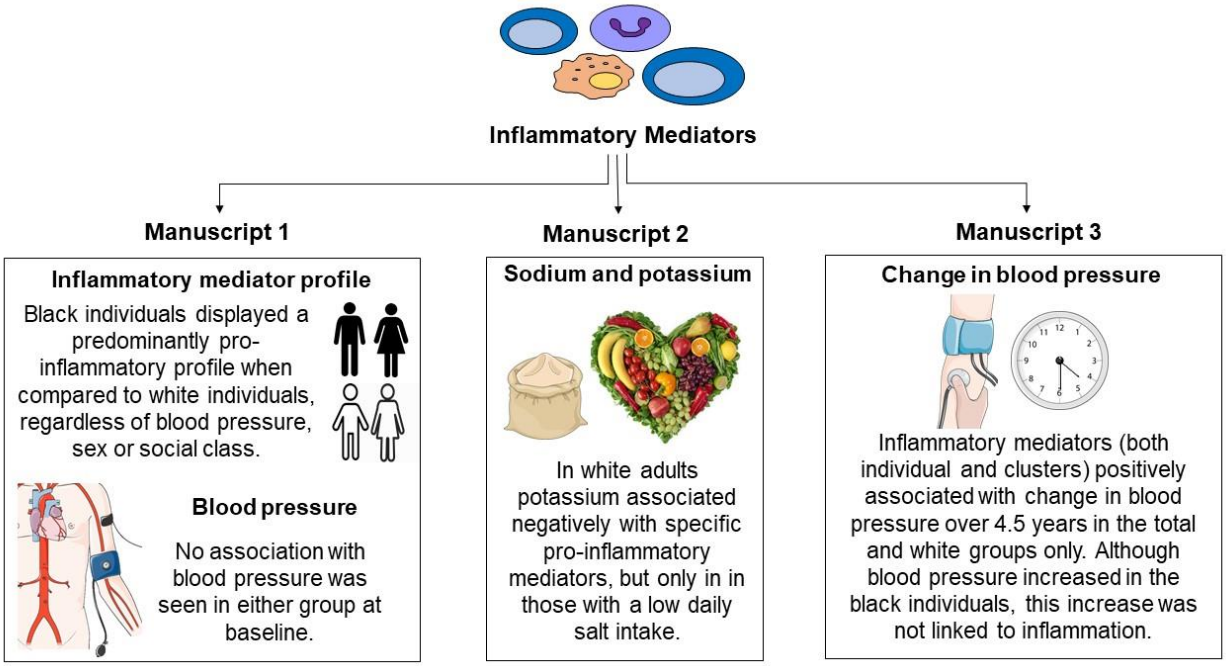

Figure 1. Summary of manuscript findings.

In a large cross-sectional analyses' of 1189 participants, with a mean age of 24.5 ± 3.12 years, we found no link between a unique range of inflammatory mediators and BP. This was unexpected, as literature reported previous associations between inflammation and BP. [3, 5, 30, 54, 55] What was found in this cross-sectional analysis was unique ethnic profiles; however, it remains to be determined whether those unique profiles predict future CVD or other disease outcomes. Although no associations with BP were seen in the cross-sectional analyses, when we followed a sub-sample of n=358 over 4.5 years (mean age 29.9 ± 3.05), independent associations between both single and clusters of inflammatory mediators at baseline and change in BP over time were found. This indicates that cross-sectional analyses in youth are unlikely to already show associations, particularly in healthy individuals (such as those in this study who were HIV uninfected, screening BP <140mmHg and <90 mmHg with no chronic diseases). However, with progression in age it is clear that specific inflammatory mediators in this study (IFN-γ, GM-CSF, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, IL-21, IL-23, MIP-1α and MIP-1β) can predict change in BP. Unexpectedly, these findings were seen in the white participants only, and may potentially have wider application to similar populations. It has been shown that individuals of black African descent may present with features contributing to a higher risk for the development of hypertension (such as salt-sensitivity and suppression of renin-angiotensin-aldosterone system).[56-58] Our study suggests that early inflammation may not be a dominant contributor to elevated BP in African populations. Based on our observations of this study in older populations it is likely that inflammation will play a more prominent role later in life.[59] To determine whether inflammation may associate with some behavioural risk factors of hypertension, we also evaluated sodium and potassium, and a potential protective role of potassium intake was identified. It is thus likely that additive to the role it plays in countering increases in BP as a result of a salt load,[35, 36] potassium also counters inflammation. It may therefore have wider implications in inflammatory diseases such as arthritis and ulcerative colitis.

Strengths and Limitations

This thesis included both observational cross-sectional and prospective study designs. Manuscripts 1 and 2 made use of baseline data from the African-PREDICT study. As a result of their cross-sectional design, these two studies are a reflection of associations and it is thus not possible to determine pathophysiological sequence or causation. While Manuscript 3 included longitudinal data allowing the observation of blood pressure changes over time, the results of this study were based on associations. Therefore, it is not possible to infer causality.

A significant strength of this study lies in the inclusion of a wide range of pro- and anti-inflammatory mediators which were analysed using a high-sensitivity multiplex kit. The African-PREDICT study recruited young, apparently healthy individuals, and as such, the absence of pre-existing chronic diseases thus allowed investigation of adults without influence from pathology. However, the young healthy status of this cohort does infer that associations may only become evident with continued follow-up appointments with the research participants.

The African-PREDICT study recruited participants from the North West province in South Africa and due to the design of study recruitment, our results may not reflect the true nature of the inflammatory and cardiovascular profiles of the South African population in its entirety. However, efforts were made to recruit participants with equal distribution of ethnicity, sex and socio-economic status to ensure each of these groups were equally represented. Regardless, this study provides some of the first findings profiling a large panel of inflammatory mediators and their relation with cardiovascular function in an African population. Therefore, these results contribute to the limited knowledge on the role of the inflammatory profile of Sub-Saharan Africans, particularly in the early development of cardiovascular disease.

An additional limitation was the calculation of estimated salt intake based on a single collection of 24-hour urine which does not take into account day-to-day variations in sodium excretion.[60] However, the African-PREDICT study protocol for 24-hour urine collection followed that of the Pan American Health Organisation/World Health Organisation protocol for population level

sodium determination in 24-hour urine samples.[61] The use of a single 24-hour urine sample when estimating salt intake in large populations is accepted by the World Health Organisation.[61, 62]

Confounders and Chance

It is important to take into consideration the possibility of chance and confounding factors. While all efforts were made to adjust for a number of carefully selected confounders in the multivariate regression models, we were unable to account for any other unknown interactions that may have affected our findings. On the contrary, numerous adjustments may result in under or overestimations of associations.

One confounder that could not be adjusted for in this study is the occurrence of masked hypertension. While individuals with office brachial BP of ≥140 and/or ≥90 were excluded during baseline screening, there was an average two-week period between the screening and research phases. This resulted in participants being classified as being hypertensive (masked hypertensive) based on 24-hour ambulatory blood pressure during the research phase. These participants were still included in this study. Differences in incidence of masked hypertension between black and white populations,[63] and the use of only office BP during screening may have resulted in uneven inclusion of hypertensive participants between ethnic groups, although the mean blood pressures of the groups were generally comparable.

Finally, statistically significant findings in this study were interpreted from a physiological standpoint, as such statistical significance was relied upon while also taking into consideration physiological significance.

Recommendations for Future Studies

Inflammation and its role in the development of cardiovascular disease remains an important topic, both globally and in Sub-Saharan African populations. Therefore, the following are

recommendations for future research into the relationship between inflammation and cardiovascular disease:

- Additional longitudinal studies are required to profile the changes in the inflammatory profile of individuals as they age, as well as evaluate the long-term changes in inflammation related to dietary sodium and potassium, taking into account sex and ethnicity. Furthermore, longitudinal studies may allow for better evaluation of the ability of inflammatory mediators to predict clinical hypertension that may only occur with increased age.
- While studies have shown associations between inflammation and BP,[3, 25, 26, 30] questions relating to causation remain. More clinical trials examining causal relationships, such as the CANTOS targeting IL-1β,[64] are required.
- As participants in the African-PREDICT study were recruited exclusively from the North West province, research in larger study populations from the different regions of Africa is encouraged. This will allow for the investigation of results in the wider African context.
- The lack of associations between inflammation and BP in black populations should be further investigated in longitudinal studies.
- While we included the renin-angiotensin-aldosterone system components in regression models, and it produced no contributory findings, the renin-angiotensin-aldosterone system is likely to be very important at a later stage in those who develop hypertension. Therefore, studies investigating the role of other potential contributors such as the renin-angiotensin-aldosterone system on the link between inflammation and cardiovascular disease are encouraged and may help shed light on these ethnic-specific findings.
- When examining potassium intake, statistically significant differences were present between black and white population groups. However, 94% of black and 88% of white participants reflected potassium levels below the recommended levels.[65] We therefore

suggest that research into the relationship between inflammation and potassium should be performed in a population with a potassium intake at or above the minimum recommended intake of 90 mmol/day.[65] This may allow for a better representation of the protective role that potassium plays.

- Similar to the efforts made to reduce sodium intake in the South African population through the national sodium reduction legislation implemented in 2016, similar efforts may be needed to encourage increased potassium intake.

Conclusion

This study aimed to present a detailed inflammatory mediator profile and describe how it relates to sodium and potassium and the BP profile of South African adults. Our study showed, for the first time in a large, young and healthy population, distinct differences in a detailed panel of inflammatory mediators between black and white adults. Although BP in black participants increased in longitudinal analyses, all findings relating to inflammation were seen only in the white groups. We found multiple associations between specific inflammatory mediators and change in BP, as well as a protective anti-inflammatory effect of potassium in those with a lower salt intake in the white population. Raised BP is a leading contributor to the development of CVD.[66, 67] Therefore, our findings highlight the potential role of inflammation in the development of CVD in humans. As such, the use of anti-inflammatory medications to target inflammatory mediators may prove effective.

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Appendix A

Publications

Crouch SH, Botha-Le Roux S, Delles C, Graham LA, Schutte AE. Distinct inflammatory mediator patterns in young black and white adults: The African-predict study. Cytokine. 2020;126:154894. https://doi.org/10.1016/j.cyto.2019.154894

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Distinct inflammatory mediator patterns in young black and white adults: The African-predict study

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ABSTRACT

Objective: Inflammatory mediators have been implicated in the early stages of cardiovascular disease development, including hypertension. Since global reports reflect a higher hypertension prevalence in black than white populations, we hypothesise the involvement of specific inflammatory mediators. We therefore compared a detailed range of 22 inflammatory mediators between young black and white adults, and determined the relationship with blood pressure.

Approach and results: We included 1197 adults (20–30 years; 50% black; 52% female) with detailed ambulatory blood pressures. Blood samples were analysed for 22 inflammatory mediators. For pro-inflammatory mediators, the black adults had higher C-reactive protein, interferon-inducible T-cell alpha chemoattractant, macrophage inflammatory protein 3 alpha (all $p \leq 0.008$), but lower interferon-gamma, interleukin (IL)-1β, IL-8, IL-12, IL-17A, and tumour necrosis factor alpha (all $p \leq 0.048$). For anti-inflammatory mediators the black group consistently had lower levels (IL-5, IL-10 and IL-13 (all $p \leq 0.012$)), resulting in generally higher pro-to-anti-inflammatory ratios in black than white adults ($p \leq 0.001$). In mediators with pro- and anti-inflammatory functions, the black group had lower granulocyte-macrophage colony-stimulating factor and IL-6 (both $p \leq 0.010$). These patterns were confirmed after adjustment for age, sex and waist circumference, or when stratifying by hypertensive status, sex and socio-economic status. Multi-variable adjusted regression analyses and factor analysis yielded no relationship between inflammatory mediators and blood pressure in this young healthy population.

Conclusions: Black and white ethnic groups each consistently presented with unique inflammatory mediator patterns regardless of blood pressure, sex or social class. No association with blood pressure was seen in either of the groups.

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ORIGINAL CONTRIBUTION

Inflammation and salt in young adults: the African-PREDICT study

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Abstract

Purpose Low-grade inflammation and a diet high in salt are both established risk factors for cardiovascular disease. High potassium (K^+) intake was found to counter increase in blood pressure due to high salt intake and may potentially also have protective anti-inflammatory effects. To better understand these interactions under normal physiological conditions, we investigated the relationships between 22 inflammatory mediators with 24-h urinary K^+ in young healthy adults stratified by low, medium and high salt intake (salt tertiles). We stratified by ethnicity due to potential salt sensitivity in black populations.

Methods In 991 healthy black ($N=457$) and white ($N=534$) adults, aged 20–30 years, with complete data for 24-h urinary sodium and K^+, we analysed blood samples for 22 inflammatory mediators.

Results We found no differences in inflammatory mediators between low-, mid- and high-sodium tertiles in either the black or white groups. In multivariable-adjusted regression analyses in white adults, we found only in the lowest salt tertile that K^+ associated negatively with pro-inflammatory mediators, namely interferon gamma, interleukin (IL) -7, IL-12, IL-17A, IL-23 and tumour necrosis factor alpha (all $p \leq 0.046$). In the black population, we found no independent associations between K^+ and any inflammatory mediator.

Conclusion In healthy white adults, 24-h urinary K^+ associated independently and negatively with specific pro-inflammatory mediators, but only in those with a daily salt intake less than 6.31 g, suggesting K^+ to play a protective, anti-inflammatory role in a low-sodium environment. No similar associations were found in young healthy black adults.

Keywords Sodium · Cytokine · Ethnicity · Race · African · Black

Appendix B

Health Research Ethics Committee approval of
the African-PREDICT Study

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT

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Ethics Committee
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Email Ethics@nwu.ac.za

Prof A Schutte

2012/07/31

ETHICS APPROVAL OF PROJECT

The North-West University Ethics Committee (NWU-EC) hereby approves your project as indicated below. This implies that the NWU-EC grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

Project title : African Prospective study for the Early Detection and Identification of Cardiovascular disease and hyperTension. (African-PREDICT study)

Project Leader: Prof A Schutte

Ethics number:

N	W	U	-	0	0	0	0	1	-	1	2	-	A	1
Institution				Project Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Approval date: 2012/04/12 **Expiry date:** 2017/04/11

Special conditions of the approval (if any): None

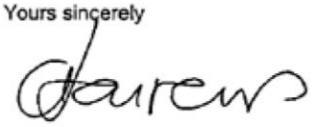
General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- *The project leader (principle investigator) must report in the prescribed format to the NWU-EC:*
  - *annually (or as otherwise requested) on the progress of the project,*
  - *without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.*
- *The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-EC. Would there be deviated from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-EC and new approval received before or on the expiry date.*
- *In the interest of ethical responsibility the NWU-EC retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the project;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the project are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the NWU-EC or that information has been false or misrepresented,*
    - *the required annual report and reporting of adverse events was not done timely and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

Yours sincerely



Prof Amanda Lourens
(chair NWU Ethics Committee)

Appendix C

Department of Health Approval of
the African-PREDICT Study

Appendix L

health

Department:
Health
REPUBLIC OF SOUTH AFRICA

Private Bag X828, PRETORIA, 0001
Civitas Building Corner Andries and Struben Street, PRETORIA, 0001
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North-West University
Private Bag X6001
Potchefstroom
2520

To whom it may concern

ENDORSEMENT OF THE HYPERTENSION RESEARCH AND TRAINING CLINIC OF THE NORTH-WEST UNIVERSITY

It has come to the attention of the Department of Health (Cluster: Non-communicable Diseases) that the Hypertension in Africa Research Team (HART) at the Potchefstroom Campus of the North-West University wishes to establish a Hypertension Research and Training Clinic on campus.

Based on detailed information provided by Professor Alta Schutte, I hereby wish to upport the concept of the Hypertension Clinic – with regards to the three main aspects that they wish to address. These components include:

(a) cardiovascular and HIV screening and preventive educational workshops in the general African population in the North West Province;

(b) a long-term research project that initially include young and older healthy normotensive African individuals. This research population will be tracked over time as pre-hypertension and ultimately hypertension develops to identify early markers of cardiovascular disease. These markers may be potential targets for future prevention and therapy;

(c) training of BSc Honours, Masters and PhD students within the activities of the proposed Clinic.

An important aspect of this work is the fact that hypertension in African populations is physiologically mechanistically different from hypertension in European populations. Results found in similar studies performed in Europe are therefore not necessarily applicable to the unique African populations of South Africa. If significant early markers are identified, the research could potentially lead to much more effective prevention and treatment programmes in Africa.

Yours sincerely

PROF MC FREEMAN
CLUSTER MANAGER: NON-COMMUNICABLE DISEASES
DATE: 15 | 9 | 2011

health
Department of
Health
North West Province
REPUBLIC OF SOUTH AFRICA

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POLICY, PLANNING, RESEARCH, MONITORING AND EVALUATION

To : Prof. A. Schutte

From : Policy, Planning, Research, Monitoring & Evaluation

Subject: Approval Letter: African prospective study on the early detection and identification of cardiovascular disease and hypertension.

Purpose

To inform Prof. A. Schutte that permission to undertake the above mentioned study has been granted by the North West Department of Health. The researcher is expected to issue this letter as prove that the Department has granted approval to the districts or health facilities that form part of the study.

Arrangements in advance with managers at district level or facilities shall be facilitated by the researcher and the department expects to receive the final research report upon completion.

Kindest regards

Moaisi 31/05/2013

Acting Director: Policy, Planning, Research, Monitoring & Evaluation Date
Mr L. Moaisi

DEPARTMENT OF HEALTH
PRIVATE BAG x2068
2013 -05- 31
SUPERINTENDENT GENERAL

Healthy Living for All

Appendix D

Health Research Ethics Committee approval of this PhD Study

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Health Sciences Ethics Office for Research, Training and Support

Health Research Ethics Committee (HREC)
Tel: 018-285 2291
Email: Wayne.Towers@nwu.ac.za

Prof AE Schutte
Physiology-HART

18 June 2018

Dear Prof Schutte

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

Ethics number: NWU-00058-18-S1

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

Study title: Cytokines and the cardiovascular profile of young South Africans: The African-PREDICT study

Study leader: Prof AE Schutte

Student: SH Crouch-27231569

Application type: Single study

Risk level: Minimal (monitoring report required annually)

Expiry date: 30 June 2019 (monitoring due end of June annually until completion)

You are kindly informed that after review by the HREC, Faculty of Health Sciences, North-West University, your ethics approval application has been successful and was determined to fulfil all requirements for approval. Your study is approved for a year and may commence from 18 June 2018. Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation. A monitoring report should be submitted two months prior to the reporting dates as indicated i.e. annually for minimal risk studies, six-monthly for medium risk studies and three-monthly for high risk studies, to ensure timely renewal of the study. A final report must be provided at completion of the study or the HREC, Faculty of Health Sciences must be notified if the study is temporarily suspended or terminated. The monitoring report template is obtainable from the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-HRECMonitoring@nwu.ac.za. Annually, a number of studies may be randomly selected for an internal audit.

The HREC, Faculty of Health Sciences requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the proposal or other associated documentation must be submitted to the HREC, Faculty of Health Sciences prior to implementing these changes. These requests should be submitted to Ethics-HRECApply@nwu.ac.za with a cover letter with a specific subject title indicating, "Amendment request: NWU-XXXXX-XX-XX". The letter should include the title of the approved study, the names of the researchers involved, the nature of the amendment/s being made (indicating what changes have been made as well as where they have been made), which documents have been attached and any further explanation to clarify the amendment request being submitted. The amendments made should be indicated in yellow highlight in the amended documents. The *e-mail*, to which you attach the documents that you send, should have a *specific subject line* indicating that it is an amendment request e.g. "Amendment request: NWU-XXXXX-XX-XX". This e-mail should indicate the nature of the amendment. This submission will be handled via the expedited process.

Appendix E

African-PREDICT Study informed consent form

INFORMED CONSENT FORM FOR THE African-PREDICT STUDY (RESEARCH PHASE):

TITLE OF THE RESEARCH PROJECT: African **PR**ospective study on the **E**arly **D**etection and **I**dentification of **C**ardiovascular disease and Hyper**T**ension (African-PREDICT)

ETHICS REFERENCE NUMBER: NWU-00001-12-A1

PRINCIPAL INVESTIGATOR: Prof. Alta Schutte (PhD Physiology)

Prof. Schutte and the research team have the expertise and interest in Cardiovascular Physiology, namely to understand the biological processes in humans when high blood pressure and heart disease develop.

ADDRESS: NORTH-WEST UNIVERSITY (Potchefstroom Campus); Hypertension in Africa Research Team (HART); Hypertension Research and Training Clinic Building F11, Office 101.

CONTACT NUMBERS: 018 299 2444 / 018 285 2466 / 018 299 2780

You are invited to take part in the African-PREDICT research study. Please take some time to read the information presented here, which will explain the details of this study. Please ask the researcher or person explaining the research to you any questions about any part of this study that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research is about and how you might be involved. Also, your participation is **entirely voluntary** and you are free to say no to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part now.

This study has been approved by the Health Research Ethics Committee of the Faculty of Health Sciences of the North-West University (NWU-00001-12-A1) and will be conducted according to the ethical guidelines and principles of Ethics in Health Research: Principles, Processes and Structures (DoH, 2015) and other international ethical guidelines applicable to this study. It might be necessary for the research ethics committee members or other relevant people to inspect the research records.

What is this research study all about?

You will know already from taking part in the screening phase of the study that heart disease and especially high blood pressure (or hypertension) is a big problem in South Africa. Also, many people are unaware of it, as it has no symptoms. High blood pressure is a very important risk factor which may result in heart disease, kidney disease and stroke. (*When blood stops flowing to the heart, this can cause a heart attack and part of the heart dies. A stroke is when there is a problem with the blood supply to the brain and a part of the brain is damaged.*) That is why many people in South Africa suffer from these diseases resulting in death.

Since heart disease is mostly seen in older people, the purpose of this study is to include and focus on young healthy people to understand how high blood pressure and heart disease develop. It is believed that our lifestyle (e.g. what we eat, drink, and do) may have an impact on whether we will develop high blood pressure and heart disease. Also, it is not well known whether there are perhaps certain measurements (e.g. in your blood or urine) that may predict whether you will develop heart disease when you are older.

The aim of this study is therefore to determine how high blood pressure and heart disease develop in a group of 1200 healthy young South Africans living in and around Potchefstroom, by tracking everyone over 5-20 years. It is therefore of great importance that we take detailed measurements of your lifestyle, and your current health (e.g. heart, blood vessels, eyes, blood and urine). These measurements will be made at the beginning of the study, but it will be most important to repeat these measurements in following visits every 5 years, to see how these health measurements have changed. We expect that some participants will remain healthy with normal blood pressures, and other will develop high blood pressure. Only by tracking the changes in blood pressures and other detailed measurements will be able to understand the influences of e.g. lifestyle on changes in blood pressure.

If your results show that a certain measurement predicts that high blood pressure will develop later in life, this information could help doctors and nurses to prevent more people in the local community having strokes and heart attacks in the future.

Why have you been invited to participate?

Your screening tests show that you are healthy and suitable to take part in this study. You are also in the most important age group of 20 to 30 years. As we would like to follow you over time it is ideal that you have indicated that you intend to stay in or around or visit Potchefstroom for the next 5 years at least.

It will also be very important for us to be able to keep in touch with you. We kindly ask that you **tell us immediately about any changes of your contact details** (address, telephone number, email address etc.).

Once we have performed all of the measurements as described below, we will have a much better understanding of your health status. If we are not able to obtain important measurements (such as 24-hour blood pressure measurements, or if we are unable to obtain a blood sample, or if we detect a serious health abnormality), you will most likely not be able to take further part in the research project. Once we have completed the measurements, we will discuss your results with you and the way forward.

What will be expected of you?

The research team will make an appointment with you, and if necessary, transport will be provided to bring you to the Hypertension Clinic (Building F12) on the Potchefstroom Campus of the North-West University. Such an appointment will be made for early in the morning, as the measurements will start at approximately 08:00, and in total will take about 5 hours to complete.

To make sure that your results are valid and useful, it is important to take note of the following:

1. The **evening before** – **Do not eat or drink anything except water after 10pm** or before you come to the clinic in the morning.
2. **On the study day, please wear comfortable clothing** such as trousers and a top that can be easily removed for the tests (please avoid wearing skirts, dresses or tights as we will need to access your bare foot and put a blood pressure cuff around your thigh over your trousers).
3. **Please bring with you**:
    - All medication you currently are taking
    - Your ID document & clinic card/book

- Some good quality sunglasses to protect your eyes after the measurements

4. Let us know if transport should be arranged for you.

If you are happy to participate, we will ask you to sign this consent form stating that you are volunteering to participate in this study and that you understand all the procedures that will be performed. You are free to contact us with any questions should there be any uncertainty about any of the information provided. Then we will take the measures listed in the table below. Tests will be done in the Hypertension Clinic and we will provide you with a meal during the day. You will not be able to bath or shower for 24 hours after your clinic appointment due to the equipment you will be wearing when you leave the clinic.

WHAT TESTS WILL BE DONE?

- **Body composition:** we will measure your height, weight, waist, hip and neck circumference in a private room, while you are wearing your underwear. In another room, while you are clothed and lying down on a bed, we will also measure your body fat percentage by using a device that connects with sensors on your hand and on your foot. This is a completely painless procedure. (the measurements should take about 20 minutes to complete)
- **Biological samples:** early in the morning while you are lying down on a bed, a research nurse will take a blood sample from a vein in your arm by using standard clinical procedures.(10-20 min) We will also ask you to provide a urine sample in the morning, in a private restroom. At the end of the day, we will kindly request that you collect your urine over the next 24 hours (we will give you the containers and detailed instructions for this). These urine and blood samples will be used to test for genetic and a detailed range of biochemical markers (biomarkers) related to high blood pressure, heart disease and diabetes, such as glucose, cholesterol and markers of inflammation. You are more likely to have high blood pressure if one of your parents or a close family member has high blood pressure. This is because high blood pressure can be caused by differences in our genes. Our genes are like a very complicated "manual" in each of our cells that tells the body how to work properly. When there are changes in the genes, it changes the "manual" and the body then does not work as well as it should for example causing high blood pressure. We share our genes with our family because half of the gene "manual" comes from your mother and half from your father. Therefore, if they have high blood pressure due to differences in their genes then it is likely that you will get the same changes in your genes and develop high blood pressure. We would like to find out what these differences are in order to better understand how they cause high blood pressure so that we can find ways to stop it happening.
  Take note that some of your samples may be stored for many years in freezers before we will analyse the samples. We may also need to ship some of your to other local or international expert laboratories for analyses.
- **Blood pressure:** while you are sitting down in a private room, we will measure blood pressure twice on both arms, by placing a cuff around your upper arm. (20 min) Another blood pressure measurement will also be done by placing a small blood pressure cuff around your finger, and upper arm, while you are lying on a bed. We will then test your blood pressure responses when you do a colour word reading test and when you place your hand in cold water for 1 minute. (30 min) At the end of the measurement day, we will fit a portable blood pressure monitor to you which will assess your blood pressure over the next 24 hours, thus over a day and when you are sleeping at night. It is important that the device is not removed during this time to ensure a reliable measurement.
- **Blood vessel & heart health:** in a private room we will again ask you to lie down comfortably on a bed. We will first test your blood pressure at your upper arm, with a device that will also measure the blood pressure at your heart. We will then test how stiff your blood vessels are by using a small pen-like device rested on your neck to register the pulse in your neck on a computer. At the same time another blood pressure cuff will be placed around your thigh. (15 min) Afterwards, in a

semi-dark room we will use a sonar device (usually used during pregnancy) to take some sonar pictures and video clips of the blood vessels in your neck and of your heart on the bare chest. We will provide a blanket or gown for cover. (20 min)

- **ECG** (Electrocardiography test) **for heart health:** while you are lying down on a bed in a private room, we will test the natural electrical activity of your heart by placing several stickers with sensors on your chest. We will take care to ensure your privacy. (10 min)
- **Eye Pressure:** a research nurse will put some eye drops in both eyes and then she will measure the pressure in your eyes with a device that rests lightly on your eye. (10 min) This test will inform us whether you have a condition called glaucoma, which means that the pressure within your eyes are quite high. If so, we will advise you and refer you for necessary treatment. If the pressure is normal, we will continue with the next eye test as described below.
- **Testing the small vessels of the eye:** a research nurse will put an eye drop in one eye, and a researcher will ask you to look into a special camera, named a fundoscope. This is the same device used by ophthalmologists (eye doctors). This camera will shine a light into your eye and we will take some pictures of the small blood vessels at the back of your eye (there will be a camera-like flash). We will also check how well your small blood vessels respond to light flickering, by doing a light-flicker test with this special camera. (20-30 min)
- **Physical activity:** at the end of the measurement day, a researcher will place a small monitor on your chest that will record your activity and movement levels for 7 days. No pain or discomfort is associated with this device, and you are kindly requested not to remove the device before the 7 day measurements were completed.
- **HIV test:** As this test was done during the screening phase, we will not test again for HIV. However, with each follow-up visit every 5 years, will would like to perform this test again.
- **Questionnaires:** during the course of the morning, you will be asked to complete several questionnaires with the help of a researcher. These include a general health questionnaires (with questions about your age, family history of disease, education, occupation, lifestyle habits, 15 min), Berlin sleep questionnaire (asking questions about how well you sleep, 5 min), physical activity questionnaire (to report on how active your lifestyle is, 5 min), dietary questionnaire (with the help of a dietician you will be asked what you ate during the past day (30 min). Within the next week the dietician will contact you again on two occasions to complete the questionnaire again. This should give us the best reflection on your eating habits). Finally, a trained psychologist will help you to complete a number of questionnaires on your personal well-being (including questions on stress and how well you cope with stress , 30-45 min).

Will you gain anything from taking part in this research?

- You will receive direct feedback during each advanced measurement on your health status. All of these advanced clinic tests are provided to you at no cost (worth ±R3 000).
- Should any abnormalities be detected, we will refer you to doctors, clinics or hospitals for further tests or treatment and the test results may assist your doctor in making decisions about further treatment.
- Apart from this personal benefit, your research data will help biomedical health researchers to gain a better understanding on how high blood pressure and heart disease develops, and may help us to develop better programmes to prevent or treat these diseases in our community and elsewhere. The data may also be used to advise the Ministry of Health on changes to the health system that may benefit the broader South Africa.

Are there risks involved in you taking part in this research and what will be done to prevent them?

To help you with a better understanding of the potential risks, and what we are doing to prevent these, please refer to the table below:

Risks	**Precautions**
• Taking a blood sample at a vein in the upper arm, may cause some pain and discomfort;	• A trained registered research nurse perform all blood sampling and regularly undergo training on clinical measurements.
• Applying an eye drop may cause a slight burning sensation; • Performing the eye pressure test is slightly uncomfortable; • Performing a light flicker test may also be slightly uncomfortable. • After the eye measurement some discomfort may be experienced (similar to a visit to an eye doctor) while waiting for the pupil to constrict.	• She also performs the eye pressure test and apply the eye drop. To ensure correct procedures and minimum participant discomfort she has undergone training at an eye doctor to ensure that she use the safest techniques to make the measurement quickly and correctly. The light flicker test may cause discomfort but the researcher is highly experienced and ensures that the measurement is done quickly and accurately. It does not cause any long term harm and is comparable to standard eye doctor measures. Afterwards, when the pupil is dilated, the eye is sensitive to light. Therefore an eye patch is provided and all lights of the clinic turned off when these assessments start (at the end of the day's measurements). You are also encouraged to bring sunglasses for when you leave the clinic. We also provide transport to you after we are finished as you are not encouraged to drive if your eye has not yet returned to normal.
• Placing the hand in an ice water bucket for 1 minute may cause some pain in your hand.	• Placing the hand in ice water causes some pain due to the very cold water. The time is only for 1 minute to reduce discomfort to a minimum, and a small electric blanket or hot water bottle is provided afterwards to heat up the hand and ensure comfort.
• You may experience some discomfort when having to undress for the body measurements or heart sonar measurements.	• All measurements are done in private temperature controlled rooms. For sensitive measurements a female scientist is trained to perform measurements to ensure especially comfort of female participants. All staff are also trained in these aspects to be highly professional and discreet and to ensure maximum comfort and to avoid any embarrassment. For heart sonars, an expert clinical technologist has vast experience in performing the sonars in a semi-dark room and also provides a blanket should you require this.
• When you complete the psychological questionnaires you may feel uncomfortable when giving personal information, such as feeling depressed or stressed. • All health measurements may cause some anxiety when you are worried about the results of the tests. • If a health abnormality is identified, others may become aware of this private information, e.g. diabetes.	• For psychological questionnaires a psychologist is well trained to complete the questionnaires in a private area. All necessary aspects are adhered to to make sure it is done in a professional and comfortable manner. If any abnormality is detected, the psychologist informs the research nurse, who will then privately discuss the results with you. • For other health measurements, such blood pressure, the results may be stressful. We will therefore provide you with the information privately and if we note something abnormal, we will ensure that you are referred appropriately for further tests or treatment. • If any health abnormalities was identified, you will meet individually with the research nurse in a private room for a feedback session. She will explain your results to you and provide you with a letter of

• As measurements take place during the working week you may suffer from a loss of income, or may get into trouble for not being at work due to time spent in the project.	referral for further testing or treatment. This will also be placed in a sealed envelope. • If you will lose wages due to your participation in the study, you need to inform the research nurse, who will make sure that communication is taken up with your employer. We will normally discuss your participation with your employer beforehand to make sure there won't be any loss in income. Once your employer agrees that you can attend the study during normal working hours without having to take leave or lose any wages, you can join the study.

There are more gains for you in joining this study than there are risks.

How will we protect your confidentiality and who will see your findings?

Anonymity of your findings will be protected by all of the researchers involved. A number, and not your name, will be assigned to your research results, and all scientists using your data will only note this number, and not your name. Your privacy will be respected by making sure that all the measurements are taken in private rooms and performed by well-trained scientists. Your results will be kept confidential by storing hard copies of your documentation in a locked cupboard within the Hypertension Clinic, and only the Principal Investigator, Head of the Hypertension Clinic and Data Manager having direct access. Electronic files with data are stored and handled by the Data Manager in a password protected online database using the University web-network (with firewall and security features), as well as some backup files on external password protected hard drives. Only the researchers, their postgraduate students and local and international collaborators will be able to look at your findings – however, all findings will be anonymised using your unique participant number. As this is a long term project, your data will be stored for 20 years or longer.

What will happen with the findings or samples?

As indicated above, your research results are safely stored on electronic files, with some results on hard copies, and in the form of blood or urine samples in biofreezers. We will store your data and your blood and urine samples for at least 30 years. Over time the research team will make sure that all of this information is analysed in the utmost detail to create new knowledge on how high blood pressure, heart disease, and related diseases develop over time. It is important to store the data and samples for a long period, as new scientific discoveries on markers of high blood pressure will be made by other scientists or ourselves in the future. It will then allow us to test if these markers are also useful in your (the South African) samples, and whether these can be used throughout South Africa in the future.

Some of your biological samples (from urine and blood) will be analysed immediately, but others will be stored for many years before analyses are performed. Please note that we will perform the biochemical analyses in our laboratories on the Potchefstroom Campus. But we may need to ship some of your samples to other laboratories in South Africa or internationally, when we do not have the funds, skills or the equipment to perform the analyses locally. Samples will be shipped using courier services approved for handling biological samples, to ensure the safekeeping and protection of the samples during transit. We will also ensure that the appropriate approvals from the South African Department of Health (export permit) and the Health Research Ethics Committee are obtained prior to shipping the samples.

Apart from your samples, your anonymised data may also be shared with other national or international collaborators. It is therefore possible that your anonymised results will be reported as stand alone data as part

of the African-PREDICT study, or your data may be pooled into other datasets from the province, country or globally in further research studies on high blood pressure and related health status. Your data will therefore be used to analyse your original state of blood pressure and health – in South Africa and in comparison to other local and international populations – and to analyse how your health status changes over time.

If we were to share your anonymised data or samples with external groups, the external groups will sign confidentiality and data or material transfer agreements with us. This process is overseen by the Legal Services of the North-West University. This will ensure that your information is adequately handled and protected, and that your data is only used for the intended purpose as described in the agreement.

It is also possible that your data may be useful for other purposes apart from the aim of the present study. When the data is to be used for such purposes, new applications will be submitted to the Health Research Ethics Committee, where the Committee will stand in on your behalf.

Findings from the study will be published in scientific journals, and discussed locally and internationally with scientific experts and the Department of Health.

How will you know about the results of this research?

During the course of the day you will receive direct feedback from each research station on your health status and findings. As described earlier, if any abnormalities are detected, a detailed report within a referral letter will be compiled by the research nurse and you will be directed to the appropriate healthcare provider. If at any stage (also after you have visited the clinic) you wish to know any of your research results, you are welcome to contact the researchers at the Hypertension Clinic.

The research team also intends to publish the research findings of the larger study in scientific literature, but also in local media, and perhaps also national media. This will not include you as an individual, but the collective findings of all the research participants. Furthermore, as this is a longitudinal study, the research team may provide you with further results of the study when you return to the clinic during follow-up measurements. As the research team will contact you annually to ensure that your contact details are still correct, we will inform you if any important research findings became apparent that you need to take note of.

Will you be paid to take part in this study and are there any costs for you?

No, you will not be paid to take part in the study, but the research team will provide you with a R300 gift voucher as a token of appreciation for your participation. We hope that the results of the measurements will be useful to you to understand your own health status.

We will provide transport to all participants, and a meal will be served during the course of the morning after you have given a blood sample.

There will thus be no costs involved for you, if you do take part in this study.

To cover all of the research expenses, this study is funded by several local and international funding bodies, including the Department of Science and Technology (National Research Foundation), Medical Research Council of South Africa and the Medical Research Council of the United Kingdom, as well as scientific grants from industry (GlaxoSmithKline, Pfizer, Boehringer-Ingelheim, Medi-Clinic Hospital Group).

Note* What happens after the study day?

At the end of the study, you may have one eye covered so it is advisable not to drive until you see that your eye has recovered, due to a possible loss of depth perception. You will know that the eye is fully recovered when the black part of the treated eye (pupil) has been reduced to a similar size as the pupil of the untreated

eye. In the week following the study day, we will make **three short appointments** with you to collect the blood pressure monitor, your urine collection and the activity monitor and to do two more short interviews (20-30 minutes) about your diet. We will give you a diary sheet so you can keep track of these appointments and they will be arranged to suit your schedule.

Is there anything else that you should know or do?

- You can contact Sr. Adele Burger (or Prof. Alta Schutte) at 018 285 2261/2446 if you have any further questions or have any problems.
- You can also contact the Health Research Ethics Committee via Mrs Carolien van Zyl at 018 299 1206 or carolien.vanzyl@nwu.ac.za if you have any concerns that were not answered about the research or if you have complaints about the research.
- You will receive a copy of this information and consent form for your own purposes.

Address: Building F11, Potchefstroom Campus, North-West University, Potchefstroom 2520
Tel: 018-285 2261 (Office hours Mon-Fri) Fax: 018-285 2260; Email:adele.burger@nwu.ac.za

Declaration by participant

By signing below, I …… agree to take part in the research study titled: The African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT).

I declare that:

- I have read this information/it was explained to me by a trusted person in a language with which I am fluent and comfortable.
- The research was clearly explained to me.
- I have had a chance to ask questions to both the person getting the consent from me, as well as the researcher and all my questions have been answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be handled in a negative way if I do so.
- I may be asked to leave the study before it has finished, if the researcher feels it is in the best interest, or if I do not follow the study plan, as agreed to.

I agree that my blood or urine samples may be sent to laboratories in South Africa or in other countries for analyses (with my personal details removed, and only identifiable by an anonymous number).	**Yes**	**No**

Signed at (*place*) …… on (*date*) …….. 20….

_____ _____

Signature of participant **Signature of witness**

Declaration by person obtaining consent

I *(name)* …… declare that:

- I clearly and in detail explained the information in this document to ……
- I did not use an interpreter.
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above.
- I gave him/her time to discuss it with others if he/she wished to do so.

Signed at (*place*) …… on (*date*) …….. 20….

_____ _____

Signature of person obtaining consent **Signature of witness**

Declaration by researcher

I, *Aletta E.Schutte*, declare that:

- I explained the information in this document to the Head of the Hypertension Clinic, Head of Screening, and research assistants.
- I did not use an interpreter.
- I encouraged them to ask questions and took adequate time to answer them.

And that I was available should they want to ask any further questions.

- The informed consent was obtained by an independent person.
- I am satisfied that she adequately understands all aspects of the research, as described above.
- I am satisfied that she had time to discuss it with others if she wished to do so.

Signed at (*place*) ..... on (*date*) ..... 20....

_____	_____
Signature of researcher	**Signature of witness**

Appendix F

Turn-it-in Report

27231569:Inflammatory_mediators_and_the_cardiovascular_pro. PREDICT_study_TII..docx

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Appendix G

Language Editing

Linguistique
LANGUAGE SERVICES
ENGLISH/FRENCH TUITION | EDITING | PROOFREADING | WRITING

CONFIRMATION OF LANGUAGE EDITING

Candidate:
S.H. Crouch

Research study for the Doctor of Philosophy in Science with Physiology thesis entitled:

INFLAMMATORY MEDIATORS AND THE CARDIOVASCULAR PROFILE OF YOUNG SOUTH AFRICANS: THE AFRICAN-PREDICT STUDY

This serves to confirm that I, Lisa Kirsten Gouws,
have provided language editing services to the candidate,
SIMONE CROUCH,
in preparation for the submission of the aforementioned research thesis.

Yours faithfully,

LKGouws

Lisa K. Gouws (Mrs)

Linguistique Language Services
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