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University of Glasgow

An analysis of the immune and vascular systems in untreated hypertension

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Submitted in fulfilment of the requirements for the Degree of Doctor of Medicine (MD)

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Abstract

Background: Blood pressure regulation leads to hypertension through complex environmental and genetic interactions, mediated by cardiac, vascular, endocrine, and renal systems. The immune system interacts with all of these, and may have a role in hypertension and associated organ damage.

Methods and Results: The Inflammatension study comprehensively assessed vascular function (endothelial function, arterial stiffness, intima-media thickness, and cardiovascular variability), the immune cell 'signature' (including B and T cell subsets, monocyte and dendritic cells, and intracellular stimulation studies), and circulating protein biomarkers, in an untreated hypertensive group compared to normotensive controls, and in consideration of phenotypic groups, as follows.

Does cardiovascular function differ between incident hypertension versus healthy controls? Hypertensive disease progression involves early arterial stiffness. Carotid atherosclerosis and impairment in endothelial function were not detected. Measures of arterial stiffness strongly correlate with each other, with ambulatory and central BP, and with cardiovascular variability.

Are phenotypic subgroups apparent in hypertension? White coat hypertension patients demonstrated arterial stiffening in excess of sustained hypertension; masked hypertension patients vascular characteristics were akin to normotension. Machine learning techniques generated three phenotypic groups of hypertension, 'arterially stiffened', 'vaso-protected', and 'non-dipper'.

Identifying immune cell 'signature' in patients: Flow cytometry demonstrated lower CD4+ naïve cells (CD45RA+CCR7+CD45RO+CD62L+) in hypertension. CD4+ T central memory cells were expanded in hypertension, along with CD62- T effector memory cells in an adjusted model. Hypertensive group had proportionally fewer CD28+ lymphocytes and CD8+ TEMRA cells, and T cells polarised towards Th1/Tc1 and Th17.1/Tc17.1. Intermediate monocytes demonstrated a differing pattern of CCR2 and CCR5 chemokine receptor

expression, and alterations in STAT1 and STAT6 phosphorylation cascades. Increased NK cell CD56+Dim expression and reduced NKT and T lymphocytes CD122 expression was linked to hypertension. Nocturnal non-dipping was associated with similar immune cell signature changes as hypertension, and dendritic cell mannose receptor downregulation in addition.

The circulating protein biomarker 'signature' of untreated hypertension and hypertensive phenotypes: Cytokines and chemokines dominated the 34 biomarkers differing between normotension and hypertension, though failed to meet Bonferroni-adjusted thresholds. Inflammatory biomarkers correlated with BP and arterial stiffness, but not endothelial function. Associations were concordant across systolic and diastolic BP; TPP1, CCL7, CCL11, and CCL21 positively correlating; IL18R1, and KYNU negatively. These relationships were more pronounced in the hypertensive subgroup, especially CD molecules and cytokines. HGF, AGE, and CCL21 showed greatest between-group differences and correlations across arterial parameters. Systolic nocturnal dipping demonstrated negative correlation with immune cell interaction and cellular adhesion biomarkers (CTRC, EPHA1, LGALS4, SIT1, SMOC, IL-18 and TNFSF11). Sixteen of the 85 correlating biomarkers also differed between the 'arterially stiffened', 'vaso-protected', and 'non-dipper' phenotypic groups.

Conclusions: In untreated hypertension arterial stiffness is already detectable, and along with nocturnal dipping and estimates of central BP, categorise hypertensive phenotypes. The exploratory data support alterations of circulating immune biomarkers, and innate (monocytes) and adaptive (T cells) immune compartments. Nocturnal dipping and hypertension phenotypes especially demonstrate immune system variances.

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AUTHOR'S DECLARATION

I declare that the work presented in this thesis were the work of the author, Dr Eleanor C Murray, under the supervision of Professors Christian Delles and Tomasz Guzik, unless otherwise stated. This thesis has been prepared by myself and is a record of work performed by myself.

No part of this work has previously been submitted in support of application for another degree or qualification by this or any other university.

Dr Eleanor C Murray, December 2023

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Other publications and presentations arising from this thesis

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Therapeutic targeting of inflammation in hypertension: from novel mechanisms to translational perspective. Murray EC, Nosalski R, MacRitchie N, Tomaszewski M, Maffia P, Harrison DG, Guzik TJ. Cardiovasc Res. 2021 Oct 26:cvab330. doi: 10.1093/cvr/cvab330. PMID: 34698811.

A Systematic Review of the Association between Air Pollution and Cardiovascular Parameters: Blood Pressure, Arterial Stiffness, and Endothelial Function. Kelten Clements, Eleanor C. Murray. Hypertension Journal. Vol. 7:3; Jul-Sep 2021. doi: 10.15713/ins.johtn.0234

Haemodynamic assessment in hypertension: the soloists and the orchestra. Murray, E., Rossitto, G. and Delles, C. (2020) Haemodynamic assessment in hypertension: the soloists and the orchestra. Journal of Hypertension. 2021, 39:1109–1111. DOI:10.1097/HJH.0000000000002796.

Abbreviations

24hr - 24 hour
ABPM - ambulatory blood pressure monitor
AIx - augmentation index
AI@75 - augmentation index adjusted to 75 bpm
ANOVA - analysis of variance
APC - antigen presenting cell
BMI - body mass index
BP - blood pressure
CAD - coronary artery disease
CART - Classification and regression tree
CCL - C-C motif chemokine ligand
CD - cluster of differentiation
CIMT - carotid intima-media thickness
CVD - cardiovascular disease
CXC - C-X-C motif chemokine ligand
DBP - diastolic blood pressure
DCP - diastolic central pressure
FMD - flow-mediated dilatation
GWAS - genome wide association studies
HGF - hepatic growth factor
HMOD - hypertension mediated organ damage
HR - heart rate
HTN - hypertension
ICAM - intercellular adhesion molecule
IDQ - InterHeart diet questionnaire
IPAQ - International Physical Activity Questionnaire
LnRHI - log transformed reactive hyperaemia index
MET-min - metabolic equivalent of task ie amount of energy expended during a minute
MHN - masked hypertension
m/s - meters per second
NO - nitric oxide
NTN - normotension
PAMPs - pathogen-associated molecular patterns
PAT - peripheral artery tonometry
PBMC - peripheral blood mononuclear cell
PWA - pulse-wave analysis
PWV - pulse-wave velocity
RAAS - renin-angiotensin-aldosterone system
ROS - reactive oxygen species
SBP - systolic blood pressure
SCP - systolic central pressure
SD - standard deviation
TNF - tumour necrosis factor
TLR - Toll-like receptor
UMAP - Uniform Manifold Approximation and Projection
WCH - white coat hypertension

Chapter 1. Introduction to hypertension

1.1 General introduction

Although blood pressure (BP) is a continuous parameter, elevated BP above consensus limits is a disease state known as hypertension. This is a common diagnosis across the UK and internationally and one with rising prevalence. [1,2] Hypertension is also the major risk factor for cardiovascular disease (CVD) such as coronary artery disease and myocardial infarction, stroke, and peripheral vascular disease. Hypertension arises from complex interactions of genetic and environmental factors. This chapter explores the regulatory systems involved in BP control, the definition and epidemiology of hypertension, and mechanisms by which hypertension leads to organ damage and disease.

1.1.1 Blood pressure regulation

1.1.2 Cardiovascular system

The cardiovascular system comprises the heart and vessels, commonly considered as arteries, capillaries and veins. Vessels share common features of a tunica intima including endothelial cell lining, tunica media with varying amounts of elastic lamina and smooth muscle, and tunica adventitia. However, the vascular system is more complex than a closed-circuit distribution system, beyond the force and volume of the cardiac output generated in a pulsatile fashion by the left ventricle, arterial pressure is also determined by heterogeneous mechanisms across the different vessel types, as follows.

Firstly, the dominant factor influencing the large distribution arteries is their elasticity which determines recoil, increasing flow efficiency and helping to convert pulsatile ventricular expulsion of blood into constant laminar flow by the time it reaches the capillaries. Secondly, arterial pressure is also determined by resistance arteries controlling perfusion of tissues. Resistance is regulated by the tone of these smaller arteries and arterioles with capacity for vaso-constriction and vaso-dilatation. The endothelium of resistance

vessels and micro-circulation actively controls vascular tone and thus regional blood flow via vasoactive mediators such as nitric oxide and prostaglandins, explored further below. Finally, blood returns to the heart through the venous system, with distensibility of the veins providing capacitance i.e. volume-induced stretch. Increasing age can impair BP regulation, as vascular compliance and elasticity are reduced and the vessel stiffens.

1.1.3 Endothelial function

Vascular tone, as alluded to above, is regulated by both paracrine and autocrine systems. The endothelium detects mechanical stimuli including pressure, stretch, and shear stress, increases in which induce vasodilatation via upregulated endothelial nitric oxide synthase (NOS), and increased release of vasodilatory nitric oxide (NO). NO diffuses from endothelial cells to adjacent vascular smooth muscle cells, triggering a cellular cascade that result in antagonism of calcium-mediated vasoconstriction. Endothelial cells also have receptors for catecholamines, endothelin, angiotensin II and others. Endothelium-dependent vasodilators that also act as NO agonists include bradykinin, acetylcholine, prostacyclin, and serotonin; endothelin and thromboxane meanwhile have a vaso-constrictive effect.[3-5]

In addition, the endothelium (and indeed many of the same vasoactive mediators, such as NO) regulates extravasation of solutes, fluids, macromolecules and cells; moderates coagulation and fibrinolytic systems, platelet aggregation, inflammation and local immune system activation, and angiogenesis.[6] The endothelium therefore should be considered as protective against hypertension; dysfunction in many of these roles being associated with hypertension and with organ damage. [7]

1.1.4 Other regulatory systems and genetics of hypertension

However, this cardiovascular-centric model of BP regulation, balancing cardiac output against endothelially-regulated systemic vascular resistance, does a disservice to the complexity of hypertension, which as a ‘syndrome’ is influenced by cardiac function, the vascular system, the kidneys, endocrine, and neurological systems, themselves each a product of genetic and

environmental factors. The term 'Mosaic Theory'[8] was coined by Dr Page over 60 years ago to describe this multifaceted approach (Figure 1.0), and can be exemplified by the heterogeneity of underlying pathology in the rare cases of secondary hypertension, such a hyperaldosteronism (endocrine aberration), WNK1 and WNK4 mutations in psuedohypoaldosteronism (monogenic cause), or renal artery stenosis (anatomical). In most cases of hypertension, no such underlying cause is identified, and the patient is assumed to have 'essential' or 'primary' hypertension relating to some combination of lifestyle and genetic factors, mediated through renal, neurological and endocrine systems, further discussed below.

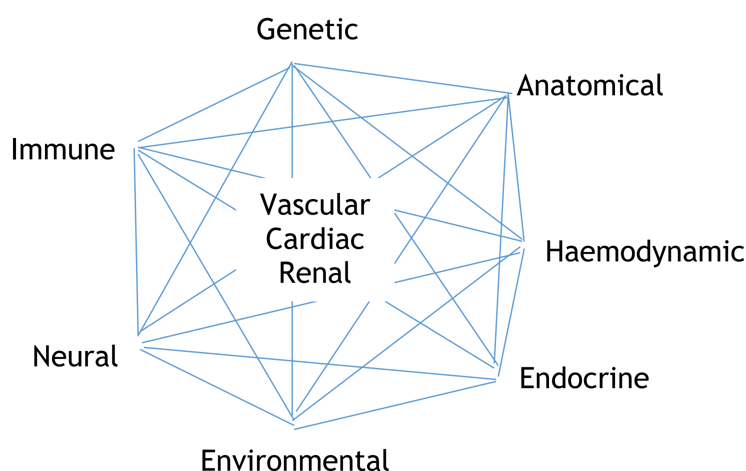


Figure 1.0 The complexity of BP regulation, highlighting the interplay of different systems leading to the final common endpoint of hypertension.

The kidneys maintain fluid and salt homeostasis through the renin-angiotensin-aldosterone system (RAAS) and through pressure-natriuresis, the process by which renal artery perfusion regulates sodium excretion. Both reduced nephron mass and dysfunctional tubule handling of sodium and chloride may elevate BP, even with a glomerular filtration rate in the normal range; and rare monogenic causes of hypertension commonly reflect mutations in tubular salt handling, as outlined below. Hypertension is an accepted cause of chronic kidney disease and a risk factor for CKD progression; at the same time, advanced chronic kidney disease causes hypertension through upregulated RAAS and impaired normal fluid and salt homeostatic functions.[9,10]

Neural influence on the cardiac, kidney and vascular systems occurs through baro-receptor sensing in the carotid sinus and aortic arch, in turn influencing the balance of sympathetic and parasympathetic (vagal) activity, with chronotropic and inotropic effects. Neuro-humoral control of salt and water homeostasis is also mediated by catecholamine-induced release of natriuretic peptides from the myocardium and renin release from the kidneys, as well as anti-diuretic hormone secreted by the hypothalamus. Detailed review is beyond the scope of this introduction and available elsewhere.[11]

Similarly, lifestyle and behavioural factors may facilitate BP regulation, or impair homeostatic mechanisms. The stress response stimulates the sympathetic nervous response outlined above, elevating blood pressure. Environmental factors such as diet (particularly salt, fibre content, and microbiome effects), physical activity, smoking, air pollution, and physiological effects such as obstructive sleep apnoea in patients with obesity, all influence BP regulatory systems and can contribute to hypertension. For example, dietary interventions have generally focussed on CVD reduction, though BP lowering has also been demonstrated in both normotensive and hypertensive cohorts. [12,13] Dietary salt in particular drives hypertension primarily through activation of RAAS. Decreasing salt intake by 4.4 g/d lowers SBP by 4 mmHg (95% CI -5 to -3) and DBP by 2 mmHg (95% CI: -3 to -1).[14] Evidence supports that such lifestyle factors are at least as strongly associated as genetic factors, if not more.[15]

Familial and twin genetic studies estimate that between 22 and 65% of BP is heritable. [16-18] The wide estimates reflect that BP is a complex trait with minor additive effects of multiple genes. These genes encode for proteins, ion channels, receptors, and enzymes involved in the endocrine, cardiac, renal, vascular, and neural regulatory systems. Figure 1.1 illustrating gene polymorphisms associated with vascular health and pathology, grouped by function. Complexity is illustrated by the heterogeneity of pathology in the rare monogenic causes of hypertension, other genes require genome wide association studies (GWAS) for identification. Examples of each follow to illustrate the diversity of genetic influences and regulatory systems.

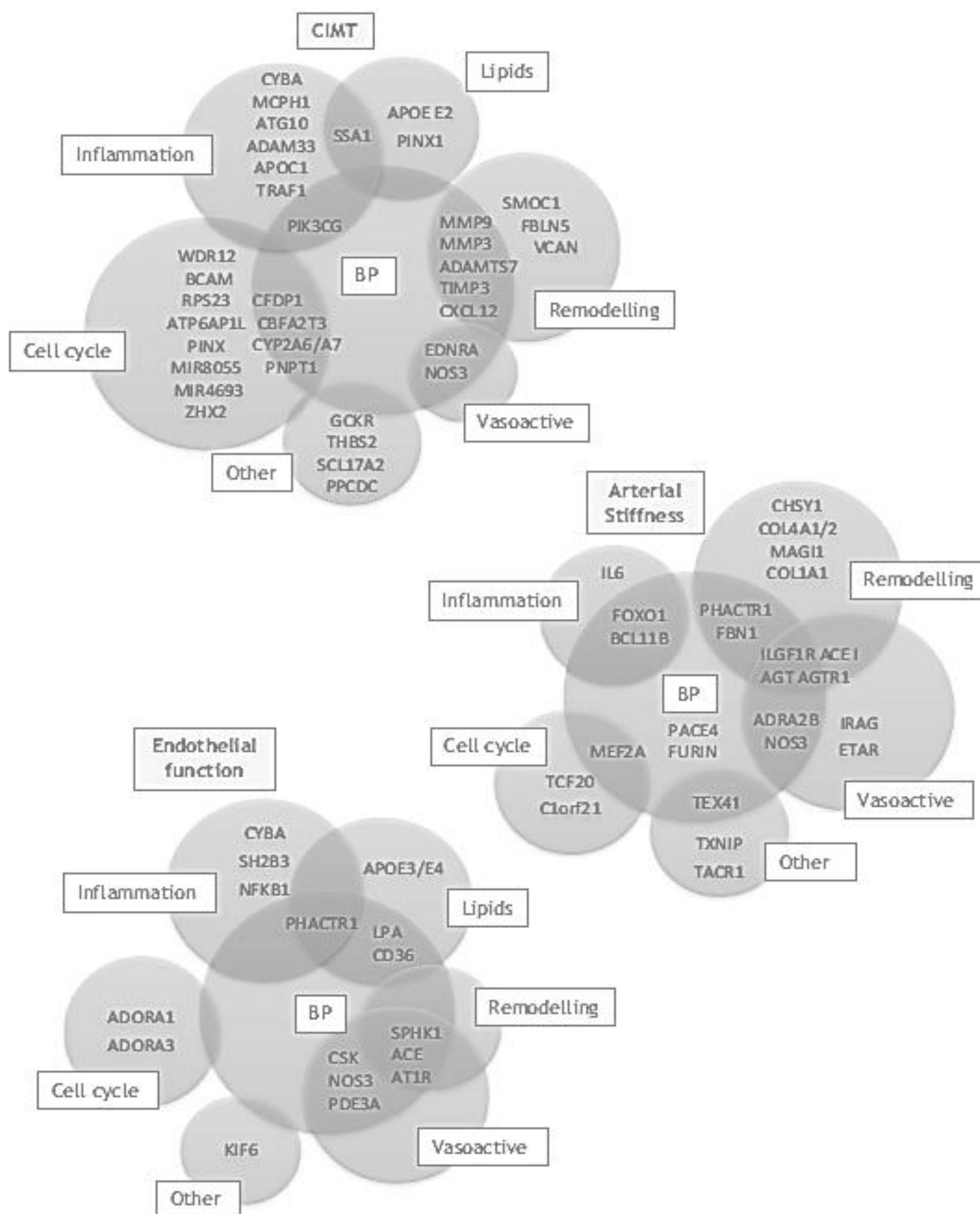


Figure 1.1. Gene polymorphisms relating to vascular health and pathology, genes grouped based on function. Reproduced with permission from: A Review of Vascular Traits and Assessment Techniques, and Their Heritability. Craig et al. Artery Research (2022) 28:61–78.

Children with homocystinuria develop premature atherosclerosis and early endothelial dysfunction;[19] in AD glucocorticoid-remediable aldosteronism, chimeric genes encoding steroid 11ß-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) result in aldosterone under ACTH regulation rather than angiotensin II,[20] salt and water retention, and elevation in BP.[21] GWAS meanwhile have identified multitudes of genetic loci associated with BP, for

example ATP2B1 encoding PMCA1, a vascular endothelial plasma membrane ATPase involved in pumping calcium from the cytosol to the extracellular compartment. Many further examples and covered in-depth elsewhere.[22]

1.1.5 Hypertension definitions and measurement

BP comprises of systolic and diastolic values, both parameters independently influencing cardiovascular outcomes [23] with most guidelines using 140 and 90 mmHg respectively as the thresholds for arterial hypertension (hence forth referred to simply as hypertension). For that reason, international guidelines advise the following:

"All adults should have their BP recorded in their medical record and be aware of their BP, and further screening should be undertaken at regular intervals". [24]

To define hypertension, one must first be able to accurately measure BP. Historical data is based on auscultatory methods with mercury sphygmomanometers, though more accurate oscillometric devices are now standard in both clinical practice and in research. Measurement can be as simple as a single numerical set of values; however, despite this technique remaining in common usage, it lacks sufficient accuracy. Consequently, valid and reliable BP measurement is not always achieved in a clinical setting;[25] despite being critical when assessing cardiovascular risk or in research when informing an evidence base. Furthermore, guidelines differ even in this basic practice of office BP measurements; NICE (National Institute of Clinical Excellence) guidelines suggest:

"If blood pressure measured in the clinic is 140/90 mmHg or higher, a second measurement should be taken during the consultation. If the second measurement is substantially different from the first, take a third measurement. The lower of the last two measurements should be recorded as the clinic blood pressure. Everyone with a clinic blood pressure of 140/90 mmHg or higher should have ABPM to make a diagnosis of hypertension." [26]

However, ESC (European Society of Cardiology)/ESH (European society of hypertension) guidelines suggest:

"Three BP measurements should be recorded, 1–2 min apart, and additional measurements only if the first two readings differ by >10 mmHg. BP is recorded as the average of the last two BP readings." [24]

Additional hypertension phenotypes are well described and briefly defined here. White coat hypertension is demonstration of elevated BP in the hypertensive range (>140 and/or 90 mmHg) in a clinical setting, but normal range values on ambulatory or home monitoring. Masked hypertension is conversely characterised by office BP <140/90 mmHg but ambulatory or home values above the threshold for hypertension. BP also tends to be lower during sleep, such that nocturnal dipping of BP on ambulatory monitoring is a normal phenomenon. Loss of this dip, defined as less than 10% reduction from daytime to night-time average, has been associated with increased CVD risk.[27]

To capture these additional data, guidelines suggest that ambulatory blood pressure monitoring (ABPM) is considered the gold standard measurement technique;[28,29] as ABPM offers attenuation of the 'white coat' effect, stronger associations with cardiovascular outcomes,[30] and data on prognostic phenotypes such as 'non-dippers'. Hypertension is defined on ABPM by the ESC/ESH as a 24-hour mean above 130 and/or 80 mmHg, or a daytime mean above 135 and/or 85 mmHg.[24]

1.1.5.1 Hypertension prevalence and consequences

Hypertension is usually asymptomatic. Incidence and prevalence are therefore challenging to measure, and diagnosis relies on screening or incidental findings. Data pooled from 844 studies worldwide suggests that throughout the last four decades mean BP has remained constant overall, though with lowering of mean BP in higher income countries and rising mean BP in lower income regions.[31] However, other large composite studies suggest increasing prevalence, for example in 2010 the global, age-standardised prevalence of hypertension was estimated as 31% based on data from almost 1 million participants across 90 countries, with a 5.2% increase in prevalence between 2000 and 2010.[2] WHO Global Health Observatory identify their African Region as having highest hypertension prevalence at 27%, with the Americas lowest at

18%, and similarly show rates rising from 594 million in 1975 to 1.13 billion in 2015, primarily in countries in the low- and middle income brackets. Reducing hypertension by 25% from 2010 to 2025 is one of the global targets for non-communicable diseases.[32] There is evidence that the UK is lagging behind other high-income countries in adequately controlling hypertension.[1]

CVD encompasses conditions relating to the various vascular beds – cerebral, cardiac, renal, and peripheral. Prevalence of hypertension translates into CVD morbidity and mortality, and thus is important at the individual and service provider level. The scale of the issue is considerable, in 2015 all-cause deaths associated with systolic BP ≥140 mmHg was 7.8 million (14.0% of all deaths), primarily ischaemic heart disease and stroke, associated with approximately 40% of deaths from these conditions. [33]

International screening events have endeavoured to increase awareness and identify this often 'hidden' population at increased risk – for example May Measurement Month screened over 1.2 Million individuals, reporting that 17.3% of those with hypertensive range BPs were not receiving treatment, and 46.3% of those who were on medication did not have their BP controlled, demonstrating the scale[1,2] of unmet need at a population level, and the need for additional therapeutic options targeting a broader range of contributory pathophysiological processes.[34]

1.1.5.2 Hypertension mediated organ damage (HMOD) and vascular mechanisms

BP is a continuous variable, and the relationship between BP and morbidity and mortality is approximately linear between 115/70 and 170/100 mmHg.[35] In section 1.4, diagnostic thresholds were described that dichotomise patients into normotensive and hypertensive. This reductionist approach is intended to highlight those at risk of the disease consequences of hypertension, though as a screening test does not include any direct evidence of target organ damage.

Just as the heart, vasculature, and kidneys generate and regulate BP, they are also the organs most damaged when BP is persistently elevated. The Joint British Societies guideline on the prevention of cardiovascular disease defines

target organ damage as any of the following: heart failure, established coronary heart disease, stroke or transient ischaemic attack, peripheral arterial disease, abnormal renal function (elevated serum creatinine or proteinuria), retinopathy, or left ventricular hypertrophy.[36]

Multiple mechanisms underlie HMOD. As pressure within the artery increases, so does stretch and sheer stress, and compensatory processes occur resulting in endothelial dysfunction, remodelling of the vessel wall, increased stiffness of the vessel, and arteriosclerosis, each outlined in brief. Endothelial dysfunction (Section 1.2.2) refers to a pro-inflammatory, pro-thrombotic state of increased permeability and cytokine / chemokine production, leucocyte adhesion, and platelet aggregation, and reduced vasodilatation further impairing regulatory capacity. Impaired redox signalling and a pro-oxidant environment also contribute, reactive oxygen species inducing cellular injury and contributing to inflammation, cellular proliferation, angiogenesis, and fibrosis. A more detailed review of the immune system in cardiovascular disease follows in Section 1.3.

Arterial stiffness increases pulsatile load within the microvascular beds, promoting inward remodelling of small arteries with vascular smooth muscle cell proliferation and fibrosis, in turn increasing resistance and BP creating a positive feedback loop. The pulsatile load of stiffened vessels also increases left ventricular systolic load in the heart, placing greater demand on coronary artery perfusion. Inflammatory cells are involved in these processes; stimulated by damage associated molecular patterns the activated or pro-inflammatory state increases cell-adhesion molecule expression, cytokine and metalloproteinase production leading to degradation of elastic and collagen. Combined, the endothelial dysfunction and stiffening cause ischaemic damage in organ tissues, particularly those with high metabolic and oxygen demands such as the kidney.

Atherosclerotic coronary and cerebrovascular disease are meanwhile initiated when dendritic cells and other antigen presenting cells as well as endothelial cells displaying scavenger receptors take up modified LDL (low density lipoprotein) particles. As lipids and cholesterol crystals accumulate

intracellularly, they can activate NLRP3 inflammasome, leading to IL-1ß secretion. Modified LDL can also ligate and Toll-like receptors (TLRs), the subsequent intracellular signalling cascade involving NF-κB, IRF and AP-1 transcription factors increases expression of pro-inflammatory genes, upregulates production of cytokines, chemokines, reactive oxygen species (ROS), costimulatory molecules, and proteases. [37,38]

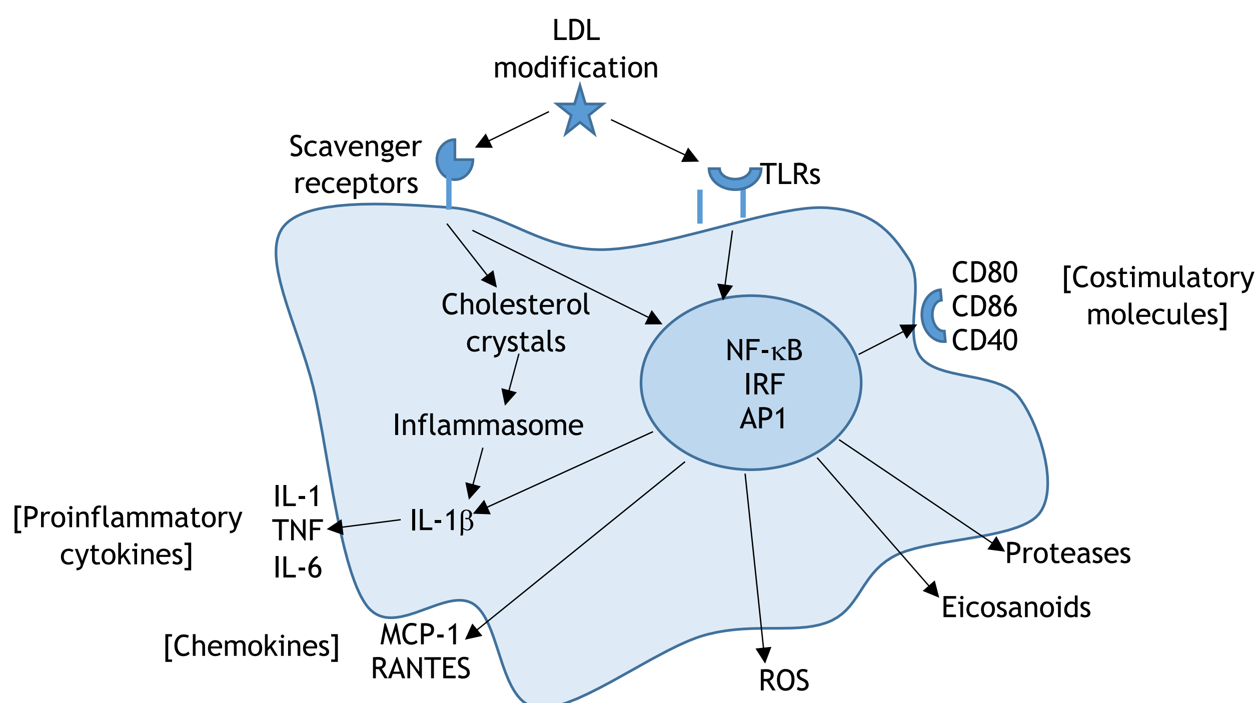


Figure 1.2 Activation of innate immune responses in the atheroma.
PRRs, pattern recognition receptors; LDL, low density lipoprotein; TLR, Toll-like receptor; IL, interleukin; NF-κB, nuclear factor κB; IRF, interferon regulatory factor; CD, cluster of differentiation. Adapted from Hansson, G., Hermansson, A. The immune system in atherosclerosis. Nat Immunol 12, 204–212 (2011). [38]

1.1.6 Informing the Inflammatension study

Consideration of these positive feedback cycles that exist in hypertension, and the knowledge gaps around early vascular dysfunction, were key factors in design of the Inflammatension study in which we aim to detect early traits relating to endothelial dysfunction, arterial stiffening and atherosclerosis. Similarly, I have outlined in this chapter the sensitivity of BP to environmental factors, reflected in our strenuous attempts to control such factors across participants. I have also illustrated diverse pathologies that can override BP homeostatic mechanisms; such health conditions were included when composing the study exclusion criteria.

Given the high prevalence of asymptomatic and even undiagnosed hypertension outlined above; one positive consequence arising from the Inflammatension Study is BP screening similar to protocols described for May Measurement Month.[34] Identification of hypertensive range BP can then prompt further investigation, changes in health-related behaviours, and commencement of antihypertensive medication.

Although inflammation did not feature in the original Mosaic theory, it has been mentioned multiple times in this chapter in relation to impaired BP regulation and HMOD. Indeed, hypertension guidelines now include inflammatory conditions as a cardiovascular risk factor.[24] The immune system is introduced in detail in Section 1.3.

I furthermore outline HMOD, as those at greatest cardiovascular risk (not necessarily the highest BP) stand to derive greatest benefit from hypertension treatment. The concept of individualised driving mechanisms of hypertension is central to the Inflammatension study, as subsequent chapters will report.

1.2 Introduction to the vascular system

1.2.1 Vascular dysfunction and hypertension

Chapter 1.1 outlined the clinical disease of hypertension, and the role of the vascular system in BP regulation and HMOD. This chapter further explores aspects of vascular dysfunction related to hypertension and relevant to the Inflammatension study.

1.2.1.1 Endothelial function

The anatomy of the vascular system has already been outlined in Chapter 1; notably it has an endothelial lining - a semi-permeable single-cell layer. Endothelial function refers to the ability of these endothelial cells to maintain appropriate vascular tone and permeability/barrier function, to manage oxidative and inflammatory stress, and deter haemostasis and platelet aggregation until required. To do so, the endothelium must detect biochemical signals and shear stress, and respond through expression of surface molecules and production of vasoactive and inflammatory mediators. Endothelial dysfunction thus refers to failures to maintain these homeostatic roles, including impairment of vasodilatation, excess permeability, damaging levels of oxidative and inflammatory stress, and dysregulated angiogenesis and haemostasis (Figure 1.3). Inflammation and elements of the immune system are integral to many of these processes. Endothelial dysfunction is thought to precede structural micro-circulatory changes such as rarefaction (the anatomical or functional loss of micro-vasculature), arterial stiffening, and atherosclerosis. Thus, hypertension can be both a cause and consequence of endothelial and microcirculatory dysfunction.

1.2.1.2 Vascular tone regulation

BP is partly determined by peripheral vascular resistance; vascular tone in turn is regulated by the sympathetic nervous system, endocrine system, and local autoregulation, each with polygenic influences, see Figure 1.0.[39] The primary vasodilatory molecule produced by the endothelium is nitric oxide (NO), generated via endothelial NO synthase (eNOS) and diffusing to local vascular smooth muscle cells (VSMC) to cause cGMP- and calcium-mediated

relaxation. Background NO release maintains a vasodilatory state, but shear stress can also induce eNOS.

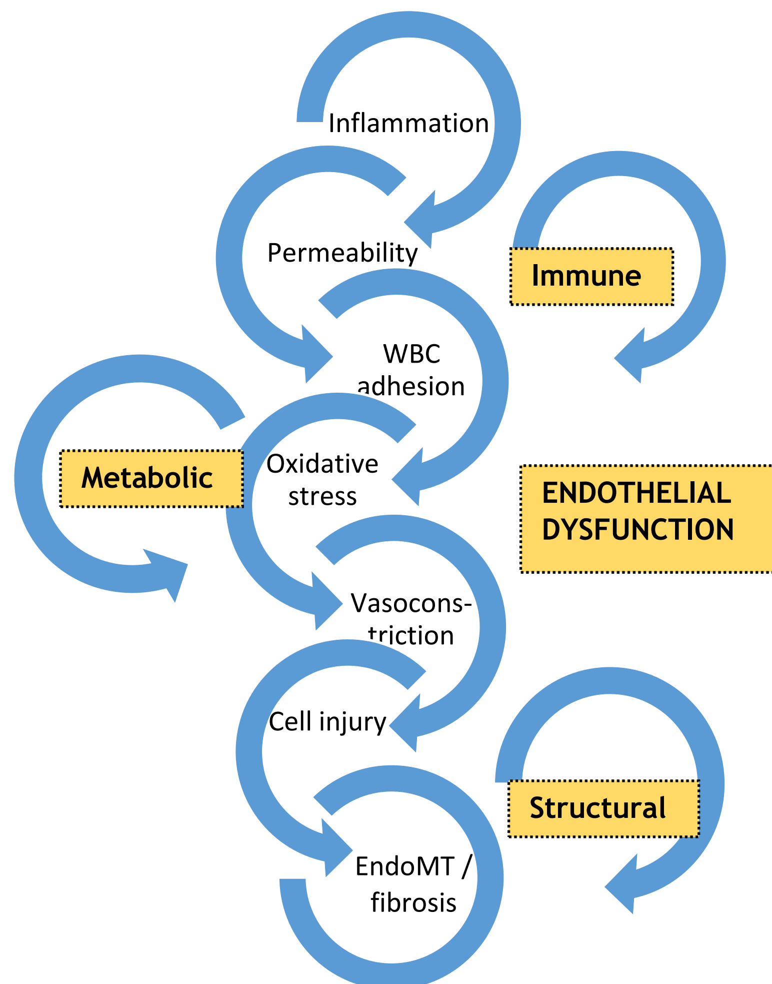


Figure 1.3 Pathophysiological features of human endothelial dysfunction and vascular disease. EndoMT, Endothelial to mesenchymal transition; WBC, white blood cell.

Other vasodilatory substances or NO agonists include prostacyclin, hydrogen sulphide, bradykinin, and naturetic peptides. Receptors for catecholamines, Angiotensin-II (Ang II), thromboxane A2, endothelin-1 (ET-1) and others meanwhile induce vasoconstriction, but also have a role in platelet aggregation and VSMC proliferation. Upregulation or overproduction of vasoconstrictive molecules will increase vascular tone, and hence peripheral resistance, and BP will rise. What remains less clear is the sequence of events and direction of causality. Methods chapter discusses the techniques employed in the study of vasodilatory capacity; Figure 1.4 demonstrates assessment tools available to study arterial function and structure, and what vascular bed they relate to.

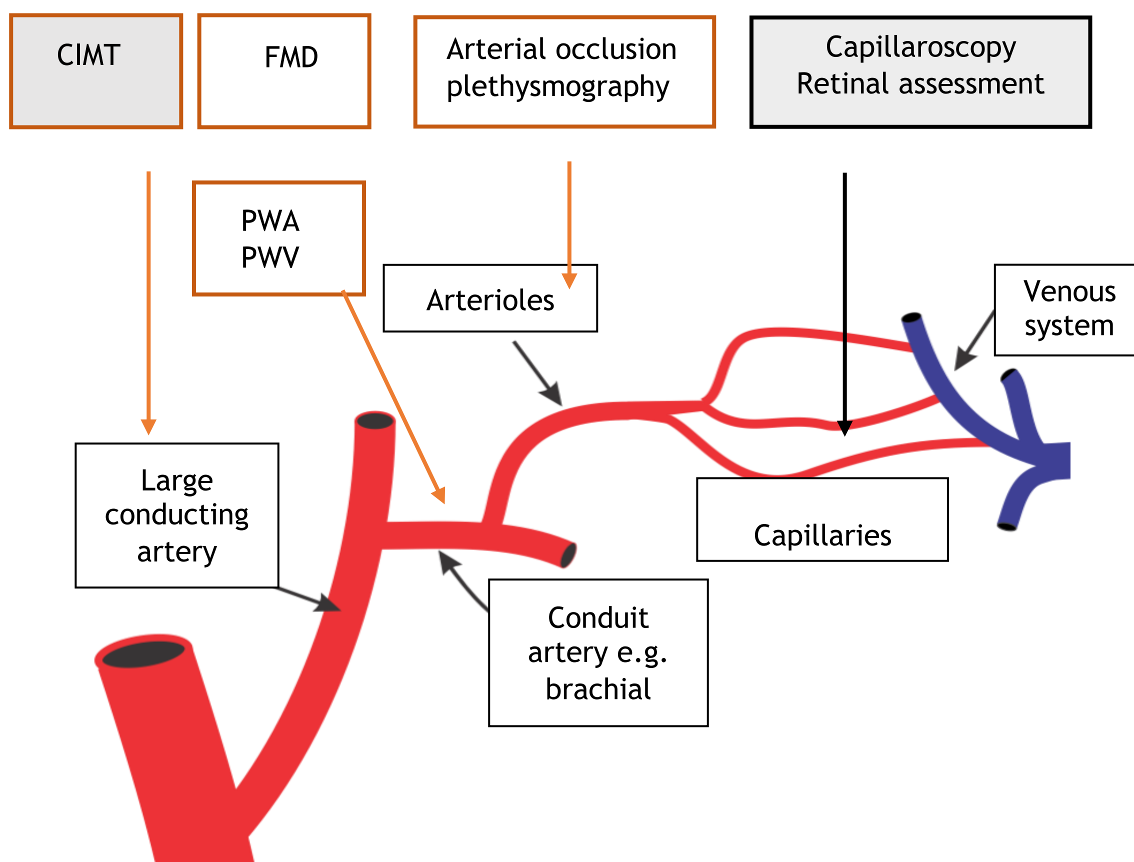


Figure1.4 Assessments of vascular function and structure across different vascular beds. Red box indicates those utilised in the Inflammatension study, grey shaing indicates structural assessment, while indicated functional. Adapted from The Endothelium and Its Role in Regulating Vascular Tone, Sandoo et al 2010. The Open Cardiovascular Medicine Journal 4(1):302-12

1.2.1.3 Arterial stiffening

Tissues require constant oxygen delivery and waste removal despite pulsatile cardiac output. This is achieved through augmentation of flow during diastole due to arterial compliance and recoil. Proximal arteries comprise more distensible elastin, than distal resistance vessels; thus, the pulse wave amplitude increases as it traverses the arterial tree. During the prodrome of hypertension, as well as with ageing, changes can be seen within the arterial system that include inward remodelling, with increased collagen and glycosaminoglycan formation thickening the vessel wall and degradation of elastin causing dilatation of the lumen. Hence the artery ‘stiffens’. This is described as arteriosclerosis when medial smooth-muscle hypertrophy, duplication of elastic laminae, intimal cellular hypertrophy and collagen

deposition give a histological appearance 'onion skin'. Arteriolosclerosis in smaller arterioles may limit blood flow, cause ischaemic damage, and ultimately obliterate the lumen – a cause of microcirculation rarefaction.

Arterial stiffening effects flow dynamics so that pulse waveforms are transmitted at greater velocity through the arteries.[40] As velocity rises the pulse-wave is reflected back more rapidly and the augmentation can arrive late in systole rather than in diastole, [41] augmenting forward pressure. Pulsatile pressure hence transmits further down the arterial tree to cause arteriosclerosis and rarefaction of the microcirculation of end organ tissues. [42] Arterial stiffening is an independent and reliable predictor of hypertension, and associated with risk of cardiovascular disease.[43]

1.2.1.4 Rarefaction and angiogenesis

Whilst the proximal arterial tree stiffens with age, structure of the arterioles and capillaries structure depend on endothelial cell proliferation, migration, and differentiation. This turnover and remodelling, as well as angiogenesis are mediated by a number of factors including vascular endothelial growth factor (VEGF), MMPs, and NO. Dysregulation of these can lead to reversible functional impairment or anatomical reduced density of the small vessels (rarefaction) through damage, inadequate repair, or lack of angiogenesis. Rarefaction consequently increases vascular resistance and blood pressure.[44] Techniques to assess microvascular rarefaction include finger nail-bed capillaroscopy and retinal assessment.[45,46]

1.2.1.5 Barrier and activator functions of the endothelium

The physical barrier function of the vascular endothelium protects the underlying tissue parenchyma, and regulates permeability to compartmentalise intravascular and interstitial space. Considering the autocrine and paracrine functions; endothelial secretion of biologically active molecules is balanced against degradation or uptake to control oxidative, contractile, pro-coagulant and inflammatory molecules. As an example of a local mediator, Sphingosine-1-phosphate (S1P) is a biologically active lipid that regulates vascular stability, permeability, and angiogenesis, as well as

lymphocyte trafficking. It maintains stability by inhibiting the degradation of the glycocalyx, thus promoting endothelial barrier function.[47,48]

Vascular inflammation leads to a state of increased endothelial permeability through the effects of molecules such as IL-1β, TNF-α (tumour-necrosis factor-alpha), histamine, and thrombin on tight junctions between cells. Shear stress mentioned above has additionally been linked to endothelial permeability through alterations in cellular glycocalyx components such as heparan sulfate.[49]

The active regulatory role of the endothelium extends to a requirement to remain inert to factors of the coagulation cascade and avoid activating platelets except in damage situations requiring thrombosis and haemostasis.[50] Similarly, inflammation destabilises endothelial intercellular junctions, the associated increased permeability facilitating immune cell trafficking. Complement activation and adhesion and infiltration of leucocytes must however remain regulated by the endothelium with appropriate downregulation and reversal if damage is to be avoided. In hypertension, dysregulation of endothelial barrier and paracrine functions can thus lead to a state favouring inflammation, vaso-constriction, coagulation, and oxidation.

1.2.1.6 Atherosclerosis

Two forces exert themselves on the vasculature; circumferential stretch from pressure generated by cardiac output and elastic recoil, but also frictional forces that whilst minimal in laminar blood flow, increase in areas of turbulence as energy of forward motion becomes pressure and friction against the endothelium. [4] The predisposition for atherosclerotic plaques to form at sites of turbulent blood flow illustrates firstly the ability of the endothelium to sense these frictional forces of turbulent flow, to ‘transduce’ mechanical forces into intracellular signalling pathways, and to alter cellular behaviour accordingly. Secondly, it highlights the importance of the endothelium’s regulatory role in lipid metabolism and inflammatory cell infiltrate, processes integral to atherosclerosis development in the subendothelium.

A range of vascular insults may initiate atherosclerosis, including oxidized LDL, cholesterol crystals, an inflammatory state, or mechanical stress. Once activated, endothelial cells express various adhesion molecules such as intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, monocyte chemoattractant protein (MCP)-1, and E-selectin. These induce leukocyte rolling, and migration into the subendothelial space. Inflammatory mediators such as cytokines TNF-α and IL-1β then amplify the response through further upregulating adhesion molecules. Monocytes, having migrated into the vessel wall, then differentiate into macrophages and phagocytose oxidised LDL to become foam cells.[51] Lymphocytes and dendritic cells also infiltrate and contribute to the cycle of inflammation, damage, and atherogenesis.[38] Ultimately, atherosclerosis in the vessel wall contributes to endothelial dysfunction and the activated state increases platelet interactions with risk of thrombosis and plaque rupture.

1.2.1.7 Vascular endothelium and inflammation

Vascular inflammation is cause and consequence of damage, and can initiate and progress atherosclerosis, arteriosclerosis, and calcification. I have already outlined how endothelial cells respond to injury, becoming activated to produce cytokines, chemokines, adhesion molecules, growth factors, and other inflammatory molecules. In turn this attracts leucocyte migration and activation, amplifying inflammation and potentially leading to damage if not ‘switched off’ appropriately. In a healthy state, resolution is achieved through peripheral tolerance-promoting T regulatory cells and anti-inflammatory cytokines such as IL-10, which reduce reactive oxygen species (ROS) production and inhibit endothelial activation.[52] ROS that give rise to oxidative stress in endothelial cells are derived from NADPH oxidases, xanthine oxidase, uncoupled eNOS, and dysfunctional mitochondria. [53]. Excess ROS can oxidise lipids, proteins, and nucleic acids, though antioxidant systems should prevent this, antioxidants including catalase, superoxide dismutase, glutathione peroxidase, and others.[53]

Chronic low grade inflammation (as well as oxidative stress) can be induced by air pollution, hyperglycaemia, inflammatory conditions including

periodontitis, or oxidised LDL (among others). Chronic inflammation has been hypothesised to lead to a perpetual state of activation of the endothelium, with recruitment of immune cells, up or down regulating of signalling pathways, accelerated endothelial senescence and dysfunction, and associated hypertension and vascular disease. [54] Additionally, all of these endothelial functions outlined above have complex interplay, for example vasoconstriction and arterial stiffening will both increase flow velocity and increase risk of shear stress; inflammation increases permeability, oxidative stress, cell adhesion molecule and damage-associated molecular pattern expression.

1.2.2 Informing the Inflammatension study

These processes outlined above link hypertension to vascular dysfunction: increased arterial stiffness, dysregulated vasoconstriction and vascular tone, oxidative stress, and inflammation. The Inflammatension study uses a range of techniques assessing vascular structure (carotid intima-media thickness), stiffness (pulse wave velocity and pulse wave analysis), endothelial function based on both vasodilatory capacity (flow-mediated dilatation) and peripheral artery tonometry, outlined in figure 1.4 and in detail in the Methods section.

1.3 Introduction: Inflammation and the immune system, in relation to arterial function and hypertension

1.3.1 Brief overview of adaptive and innate immune system

Human immunity is a complex system of cellular and biologically active protein defences against pathogens and tissue damage. Classically considered as innate and adaptive responses based on evolutionary development and functional traits. Both regulate inflammation and cell cycle, and dysregulation of either can lead to auto-immune, chronic inflammatory, malignant, or infective diseases.

1.3.2 Innate immune system

A much older defence system in terms of evolution, innate immunity is preserved across species and includes physical barriers such as skin, in addition to cellular mechanisms such as phagocytosis, and targeted protein defences such as the complement system. The cellular elements of the innate immune system relevant to hypertension and the Inflammatension study follow.

1.3.2.1 Phagocytic cells

Pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) released by necrotic cells bind to pattern-recognition receptors (PRRs) such as the archetype TLRs expressed on innate immune cells, activating them. Once activated, they are capable of phagocytosis, the ability to internalise and destroy microbial pathogens, and process them for antigen presentation. Monocytes are circulating phagocytic cells recruited to sites of inflammation. Once recruited, monocytes differentiate into dendritic cells (DCs) or macrophages, determined by the dominant cytokine milieu[55], see Figure 1.5. Monocytes are characterised by cell markers $CD14^{++}CD16^{-}CCR2^{high}CX_3CR1^{low}$ in classical phenotype, and $CD14^{+}CD16^{++}CX_3CR1^{high}CCR2^{low}$ in non-classical, with $CD14^{++}CD16^{+}CX_3CR1^{high}CCR2^{low}$ classed as intermediate monocytes.[56,57]

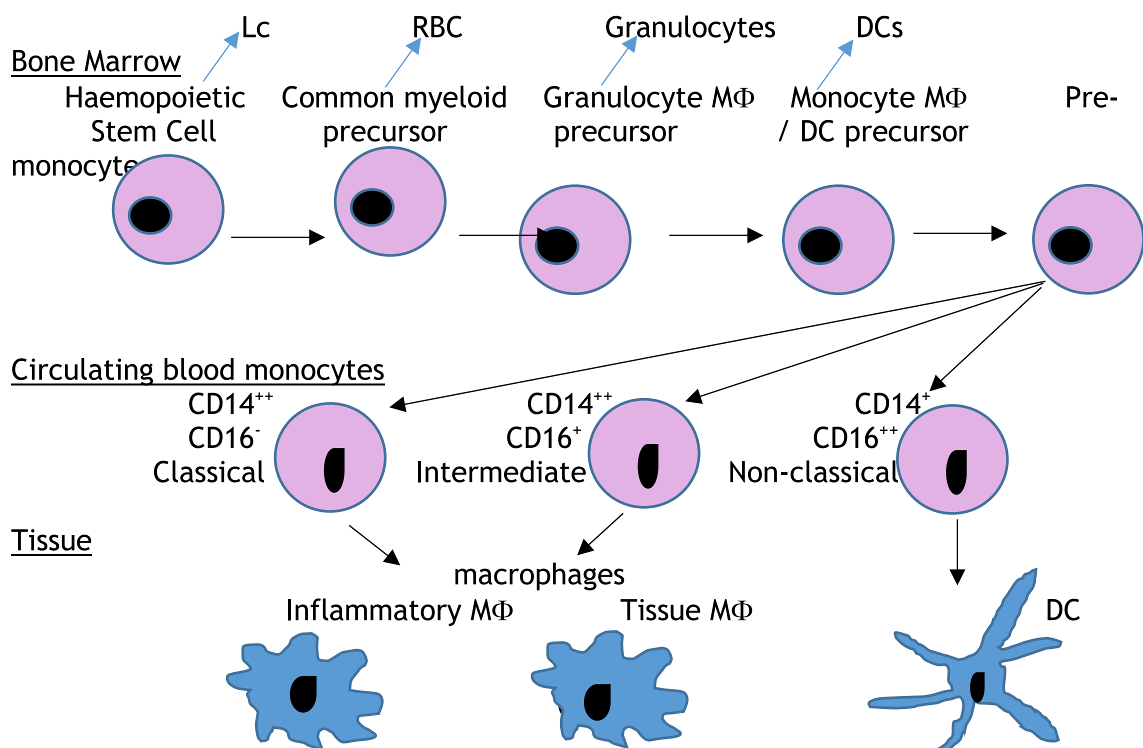


Figure 1.5 Monocyte progeny and maturation. Mφ, macrophage; DC, dendritic cell, Lc, lymphocyte; RBC, red blood cell.[58]

DCs also phagocytose, but in addition are key antigen presenting cells (APCs), linking the innate and adaptive immune systems through presentation of antigens to T cells and cytokine production. Heterogeneous subtypes of DCs exist, characterised by expression of distinct cell markers including CD4, CD8, CD11b and CD80, in addition to the shared CD11c marker.[59] DCs influence adaptive immune responses including memory, tolerance, and polarisation of helper T cells through antigen presentation, expression of co-stimulatory molecules and chemokine receptors, and secretion of cytokines.

1.3.2.2 Natural killer (NK) cells

NK cells are generated from innate lymphoid progenitor cells before migrating from the bone marrow to become tissue resident cytotoxic cells capable of amplifying inflammatory responses. They are defined by CD3^{-}CD56^{+} marker expression, sub-classified by CD56 levels; peripheral blood NK cells predominantly CD56^{dim} CD16^{++} with high perforin expression; CD56^{bright} CD16^{-} NK cells in contrast express inhibitory receptor CD94 and are enriched in secondary lymphoid tissues. [60] CD56^{dim} CD16^{++} cytotoxic NK cells can induce

lysis of infected cells through their secretion of apoptosis-inducing granzymes and perforin. NK cells in their activated state also produce cytokines that link them to the adaptive immune response, specifically IFN-γ, TNF-α, IL1β, IL-6 and IL-10.[61] NK cells have been linked to both vascular function and hypertension,[62,63] discussed further in Chapter 4.

1.3.3 Adaptive immune system

Antigen-specificity and immunological memory are the cardinal features of the adaptive immune response. Lymphocyte nomenclature is based on their tissue of origin, B lymphocytes from the bone marrow and T lymphocytes from the thymus.

1.3.3.1 B lymphocytes

The adaptive humoral immune response refers to B cells and their production of antigen-specific immunoglobulins (Ig), commonly known as antibodies. B cells circulate between secondary lymphoid organs, surveying for opsonised antigen. If encountered by multiple B-cell receptors/co-stimulatory receptors, the B cell becomes activated independent of T cells. Lower valency interactions require T helper signals to induce activation. B cells then differentiate into short-lived IgM-secreting plasma cells. Alternatively, germinal centres can develop in which B cell proliferation, maturation of affinity through somatic hypermutation, and class-switching occur.[64] This generates long-lived antibody-producing plasma cells, which migrate to the bone marrow where they can remain indefinitely, and memory B cells that continue to circulate.

Immunoglobulin isotypes IgA, IgD, IgE, IgG, and IgM have heterogeneous structures and functional capacities. Naïve B cells express surface IgM and IgD, isotypes that can also be secreted, with class-switch to predominantly IgG upon activation. IgA are enriched in mucosal tissues as a component of barrier immune defences. IgG is the most abundant antibody isotype. Although the key role of B lymphocytes is humoral immunity, a subset of regulatory B cells (Bregs) producing IL-10, TGFβ and other inhibitory

mediators also exit. [65] Consensus has yet to be reached regarding an identifying set of Breg surface phenotypic markers.

1.3.3.2 T lymphocytes

Naive T lymphocytes migrate through secondary lymphoid organs in search of antigen presented by APCs. All T cells express CD3 and can express the co-stimulatory CD4 or CD8 molecule. For the T cell receptor (TCR) to be activated, it requires antigen to be presented complexed with an MHC molecule. $CD4^+$ T helper cells recognise antigenic peptides presented on MHC class II molecules of APCs, their role is predominantly one of instigating and shaping the immune response.

T helper cells polarise their phenotype, Th1 driven by predominantly by IL-12 and IFN-γ, and Th2 by IL-4.[66] Th1 cells are critical for responses directed against intracellular pathogens, with production of IL-2 and IFN-γ. Th2 classically drive the immune response against extracellular pathogens including helminths, through production of IL-4, IL5 and support of B cells and eosinophils. The pro-inflammatory Th17 cell subgroup are driven by IL-23 and produce IL-17, with associations made to chronic inflammatory joint and bowel conditions.[67]
CD8+ cytotoxic T cells recognise MHC class I-presented peptides, so can be stimulated by any nucleated cell. They are a key component of the response against intracellular pathogens through their production of TNF-α and IFN-γ, secretion of cytotoxic granules causing direct cell death, or apoptosis induced via Fas/Fas-ligand interactions. Failure to regulate appropriately leads to tissue damage.

When naïve T cells are activated, they proliferate to generate effector cells that migrate to inflamed tissues. A proportion of these are maintained as circulating memory cells with enhanced responses upon repeat antigen encounter. Both CD4 and CD8 naïve and effector cells can be differentiated by their surface marker expression; naïve CD45RO−CD45RA+CCR7+, effector memory regress from lymphoid organs and migrate to damaged tissues expressing CD45RO+CCR7−CD62L-CD54RA−, central memory found in lymphoid

organs are CD45RO+ CCR7+ CD62L+ CD54RA−, and effector cells express CD45RO− CCR7− CD54RA+.[68] T central and effector memory with distinct proliferative and migratory capabilities reflected in their distinct expression profile.

Additional smaller subsets of T lymphocytes exist; Gamma-delta (γδ) T cells are a smaller population characterised by expression of heterodimeric γ and δ chain TCRs and undergo MHC-independent activation. They have diverse behaviours including release of cytokines and chemokines, interactions with other immune and epithelial cells, and cytotoxic effects. Regulatory T cells (Tregs) are essential for peripheral tolerance and for down-regulating immune responses through production of TGF-ß, IL-10 and adenosine. They are characterised by cell markers CD4+ CD25+ Foxp3+.[69] Similar to CD4 and CD8 T lymphocytes described above, activated effector Tregs express CD45RO, whilst naïve Tregs express CD45RA, CTLA4 (CD152), and glucocorticoid-induced TNF receptor (GITR). CTLA4 produced by naïve Tregs binds to CD80 and CD86 to block co-stimulation via CD28, sending a negative signal to TCR/CD3 and inhibiting T cell activation.[70]

1.3.4 Cytokines and chemokines

1.3.4.1 Cytokines

These glycoproteins allow communication between cells of the immune system, predominantly released by macrophages and helper T cells, though almost all nucleated cells can secrete cytokines. Binding of a cytokine to its receptor induces signalling cascades, modifying gene expression and differentiation and/or proliferation of the cell. Effects can be pro- or anti-inflammatory, broad classes including interleukins, interferons, tumour necrosis factors, and transforming growth factors.[71] Complexity of the immune response to cytokine stimulation arises due to redundancy, synergy, antagonism, amplification, and pleiotropism.[72] Such interactions allow orchestrated immune system activation, but also create a challenge for scientific study. Cytokines are produced transiently in response to inflammatory stimuli and have short half-lives, so when studying chronic states such as hypertension it is critical to avoid blood sampling during active

infection or inflammation, hence the stringent exclusion criteria of the Inflammatension study, discussed in Chapter 2.

1.3.4.2 Chemokines

Also described as chemoattractant cytokines, chemokines control immune cell migration, inducing cell movement along a chemokine gradient. They link innate and adaptive systems through regulation of T cell differentiation. Functionally, chemokines can be expressed constitutively with a role in homeostasis, or are inflammatory and induce rapid recruitment of immune cells. [73] Selectivity occurs as cells only respond to the chemokines to which they express G-protein coupled receptors. Although chemokines commonly bind to more than one receptor, and similarly receptors to multiple ligands, to create a complex system in a similar fashion to cytokines. Binding induces an intracellular signalling cascade, resulting in changes to cellular adhesion molecule expression, integrin affinity, and cell activation.

For example, CXCL13 chemokine guides B cell migration via CXCR5 receptor to lymphoid tissue follicles to survey for antigens, where BAFF and other B cell survival factors are also secreted.[74] If no antigen in encountered, sphingosine-1-phosphate (S1P) induces B lymphocyte egress from the lymphoid tissue back to the circulation.[75]

1.3.5 Immunology relevant to the cardiovascular system and blood pressure regulation

1.3.5.1 Animal models

Animal studies, predominantly angiotensin-II (ang-II) or DOCA-salt models, support a role for inflammation in hypertension. Knockout models with transgenic deficiency of an inflammatory mediator also provide evidence that inflammatory cytokines and receptors may contribute to the hypertensive phenotype. Caveats to germline knockout studies are the potential alterations in basal immune state, or compensatory increases in signalling through functional redundancy. Pharmacological targeting of immune

mediators to determine the effect of selective blockade on hypertension is an alternative approach. Detailed literature reviews have already been published for further perusal.[76] Summarising the findings, the data reveals a broad range of both innate (e.g. IL-1, TLR4) and adaptive (IL-17, CD80/86) immune targets with modulating effects on hypertensive phenotype.

As to the antigen(s) stimulating activation of the immune system, HSP70 is a potential candidate recently reviewed elsewhere [77], and isoketal-modified proteins are also possible auto-antigens, with the isoketal scavenger, 2-hydroxybenzylamine (2-HOBA) able to reduce dendritic cell activation and hypertension. [78] However, some studies have found little or no effect on BP of blocking TNF-α, IL-17 or TLR-4.[76] These discordant results may relate to treatment protocol differences (antibody clone, dosing regime, changes in immune function related to elevated immunoglobulins etc), or the model itself (ang-II dose/duration, presence of nephrectomy, different cytokine isoforms or ligand versus receptor target differences).

Hypertensive animal models report elevated levels of various chemokines and chemokine receptors including CCR2 and CCR5 [79-81] CXCL16 is a further example, induced in renal tubular epithelial cells in the setting of ang-II, there was no difference in BP rise between wildtype and CXCL16 knockout, but knockout mice demonstrated reduced T cell and macrophage infiltration and were protected from renal fibrosis and proteinuria. [82] This offers an example of how the immune system may be the variable factor explaining the heterogeneity in organ damage between hypertensive patients.

1.3.5.2 Clinical evidence

The wealth of animal model evidence has not been translated into human pharmacological BP trials, though cellular, cytokine and chemokine associations are supported by epidemiological and observational data. The third National Health and Nutrition Examination Survey (NHANES III) data for example, reveals a higher number of circulating leukocytes in hypertensive participants, correlating leukocyte count with systolic BP.[83] Evidence from UK Biobank data and Mendelian randomisation, also supports the causative role of lymphocytes.[84] Increased neutrophil to lymphocyte ratio (NLR) as a marker of

systemic inflammation also demonstrates an association with hypertension in several studies. [85–87] Finally, immunosenescent CD28null CD8+ T cells, with a highly cytotoxic phenotype are increased in the peripheral blood and target organs of patients with hypertension.[88] Inflammatory cytokines have also been linked to blood pressure and to the risk of developing hypertension;[89,90] these cytokines and their potential role as biomarkers is discussed in detail in Section 1.4. Chemotactic Monocyte chemoattractant protein-1 (MCP-1 / CCL2) levels are elevated in newly-diagnosed hypertension,[91] with evidence for CXCL9, CXCL10, CXCL11, and CXCR3 also circulating at higher levels in hypertensive patients. [88,92]

1.3.5.3 Genetic evidence

The heritability of BP has been estimated to lie between 33 and 57% based on epidemiological studies from Framingham Heart participant data, familial and twin studies.[18,93–95] However, the parallel genetic evolution of BP and immune regulatory systems since the Palaeozoic era has resulted in substantial overlap between these two complex traits. [96] Both GWAS studies and gene expression signatures of hypertension support the role of the immune system in hypertension. Extreme phenotypes can also offer particular genetic insights, in children with hypertension for example, genes of CD14 and of the RAAS demonstrate altered mRNA expression in leucocytes. Non-pharmacological treatment of BP resulted in downregulation of some genes, suggesting that the effects of genotype and phenotype may be bi-directional.[97]

SH2B3/LNK is one of the most extensively studied genes, with a role in cell signalling and proliferation of haematopoietic and endothelial cells, and has repeatedly been linked to both immune system activation and hypertension.[98,99] LNK gene encodes a modulator of T cell activation and has also been linked to diverse auto-immune diseases including coeliac disease, multiple sclerosis, and type 1 diabetes.[100] LNK knockout mice show increased sensitivity to Ang II, demonstrating hypertension and endothelial and renal dysfunction, including infiltration of inflammatory cells, oxidative stress, and reduced NO levels and vasorelaxation.[99,101] GWAS identifies the LNK/SH2B3 single nucleotide polymorphism (SNP) rs3184504 in hypertension.[98] Meta-

analysis gene expression profiles from individuals not on anti-hypertensive medication also supports the importance of the rs3184504 SNP as a transcription regulator of BP associated genes. Notably, six of the 34 signature genes associated with hypertension were co-expressed by neutrophils, all driven by the same BP-associated loci.[102]

To provide the context in which the Inflammatension study sits, and to explore why animal model, clinical, and genetic evidence has not translated into therapeutic approaches, I hereby review published data regarding BP effects of immuno-modulatory drugs; the effects of anti-hypertensive medications on the immune system, and non-pharmacological approaches targeting inflammation with BP outcomes.

1.3.5.4 Therapeutic targeting of the immune system in hypertension

The effects of immunomodulatory pharmaco-therapies on BP have predominantly been observational studies in transplantation and rheumatology, where immunosuppression is standard of care. Validity and replicability have been limited by polypharmacy, and in transplantation isolating the BP effects of medication from the physiological changes determined by the transplanted organ is an additional challenge. Despite these challenges, TNF-α inhibitors in particular suggest an association with BP, meta-analysis providing a combined estimate of 3.5 mmHg reduction in systolic BP (SBP) (95% CI -5.7, -1.3), P=0.001. Effects may however only be apparent in hypertension, as Figure 1.6 demonstrates. This has been covered in detail elsewhere, but supports the association of immune dysfunction with hypertension. [76]

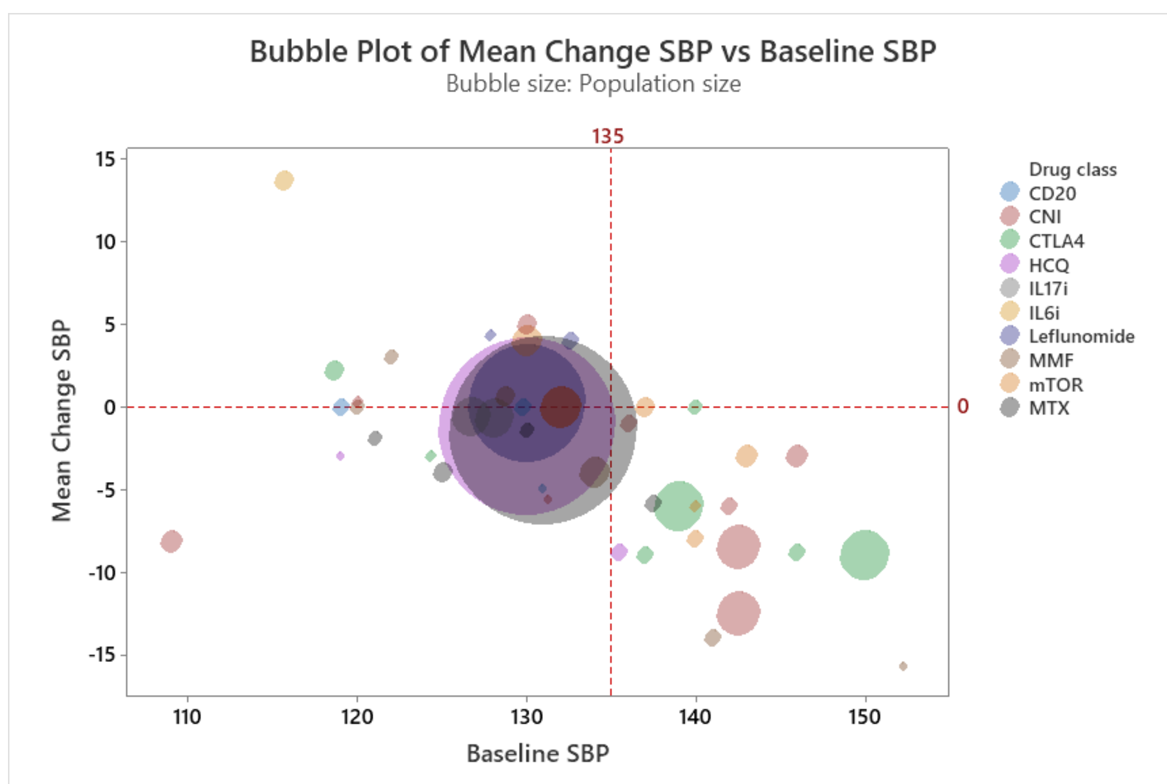


Figure 1.6. Bubble plot of immunomodulatory agents and BP effects; with bubble area representing cohort size, baseline systolic BP on the y-axis, and change in systolic BP on the x-axis (both in mmHg). R2 = 31% for average change in SBP by average baseline SBP (not shown). CNI, calcineurin inhibitor; CTLA4-Ig, cytotoxic T-lymphocyte-associated protein 4 immunoglobulin; HCQ, hydroxychloroquine; IL, interleukin; MMF, mycophenolate mofetil; mTOR: mammalian target of rapamycin; MTX: methotrexate; SBP, systolic blood pressure. Reproduced from Murray et al with permission. Therapeutic targeting of inflammation in hypertension. CVR; 17 (13): 2589-2609; 2021.

1.3.5.5 Impacts on the immune system of anti-hypertensive medications

Many BP medications modulate cells of the immune system, hence the design of Inflammatension to recruit only participants not receiving any antihypertensive pharmacological treatment. Beta-blockers and angiotensin-converting enzyme inhibitors (ACEi) are used as illustrative examples, evidencing the relationship between immune system activation and hypertension. Many cells of the innate immune system express α- and β-adrenoreceptors, and lymphocytes express β_2-adrenoreceptors. Immunotherapeutic potential of beta-blockers has been the subject of large observational studies of cancer patients, though initial claims of survival benefit [103] have not been subsequently been confirmed.[104-106] Initiating propranolol increased circulating T lymphocytes in 14 healthy individuals,

with concomitant increase in IL-2 secretion and receptor expression, NK cell activity in contrast was impeded following propranolol.[107] Furthermore, immunophenotyping of peripheral blood monocytes associated beta-blocker use with lower TLR4 expression in comparison to other anti-hypertensive agents,[108] and with reduced CCR2 expression and migratory capacity compared to drug-naïve patients. [109]

Angiotensin II, in addition to salt and water homeostasis and vasoconstriction, also mediates elements of the immune system, with sufficient elements of the RAAS within lymphoid organs to generate angiotensin II, and receptors allowing autocrine amplification.[110,111] AT1 receptors are present on activated T lymphocytes, macrophages, DCs, and NK cells.[112] Binding retards polarisation to inflammatory phenotypes, protecting organs from hypertensive injury, independent of BP lowering. [113–115] The following have been demonstrated in human studies of ACEi: reductions in IL-6, sCD40L, circulating vascular adhesion molecule-1 (sVCAM-1), and hsCRP levels associated with ramipril;[116,117] enalapril and irbesartan both increase circulating IL-10 and concomitantly reduce MMP-9; irbesartan also appears to reduce hsCRP, IL-6, and platelet aggregation;[118] whilst candesartan has been associated with lower plasma levels of CCL2 and TNF-α,[119] as well as soluble intercellular adhesion molecule-1 (sICAM-1), IL-6 and hs-CRP;[120] telmisartan with rosuvastatin demonstrates reduced Th17/Treg ratio, IL-1ß, IL-2, IFN-γ, hsCRP, and MCP-1, whilst elevating Treg-characteristic cytokines, and Foxp3 mRNA expression.[121]

However there are caveats: firstly, autocrine and tissue-specific immune effects may be masked by systemic impacts of 'global' RAAS blockade; secondly, the direction of causality question still remains, as BP reduction will reduce haemodynamic strain on organs known to release immunological mediators, and variation may exist in the BP 'threshold' at which these mediators are released. One solution in determining direct immune cell influence versus systemic BP-related effect is head to head comparisons across different antihypertensive classes, for example in acute stroke, ramipril-treated patients demonstrated lower CRP levels than those on other antihypertensive agents. [122]

1.3.5.6 Evidence from non-pharmacological interventions

In addition to medication effects, various non-pharmacological hypertension treatments have shown reductions in inflammation and better patient outcomes. For example, physical activity has both immediate elevating and longer-term lowering effects on BP regulation, but also has immune consequences, [123] including improved response to influenza vaccination.[124] Salt is another dominant driver of hypertension, primarily through activation of RAAS. A Cochrane review demonstrated decreasing salt intake lowered SBP by 4 mmHg (95% CI -5 to -3) and DBP by 2 mmHg (95% CI: -3 to -1).[14] Salt at higher concentrations favours pro-inflammatory phenotype T lymphocytes, with vessel and end-organ infiltration and induce microvascular dysfunction.[125,126] Correspondingly, dietary potassium less than 1.5 g/day has been associated with higher BP and risk of stroke; theorised to be mediated primarily by renal tubular sodium retention; potassium supplementation intriguingly also improves endothelial function, and may lower BP in hypertension, with a dose-response apparent. [127–131]

Dietary interventions beyond salt have focussed on CVD reduction, though BP lowering has also been demonstrated, e.g. Dietary Approaches to Stop Hypertension (DASH) employed strict dietary control SBP at 8 weeks 5.5 mmHg lower (95% CI 7.4-3.7), and DBP 3 mmHg (4.3-1.6).[132] Dietary immune effects are partially mediated through the microbiome; plant based dietary protein promoting bacterial species associated with anti-inflammatory effects. [133] Separating the proportionality of effect due to diet, antibiotics, and from the direct effect of gut flora is challenging.

Evidence for a link between periodontitis, inflammation, and hypertension spans animal studies, GWAS [134], and observational human data. [135] Recent controlled trial data demonstrates that intense treatment of periodontitis can improve endothelial function [136], lower inflammatory markers, and lower BP by as much as 7.5 mmHg.[137] A final example is renal nerve ablation, linked to reduced T cell activation and pro-inflammatory cytokine production (IL-1α, IL-1β, and IL-6) by DCs in Ang-II dependent hypertension. [138,139] While the beneficial effect of removing renal sympathetic nerves was clear in animal

models of hypertension, clinical trials in humans brought contrary or inconsistent results regarding BP lowering [140-143] and regarding circulating levels of pro-inflammatory markers. [144,145]

1.3.6 Informing the Inflammatension study.

The immune effects on BP regulatory systems in hypertension have been outlined, with evidence that hypertensive stimuli, such as Ang II, promotes a pro-inflammatory immune state, with immune cell infiltration of vasculature and organs. This inflammatory milieu exacerbates the hypertensive response, and contributes to endothelial dysfunction and organ damage. Examples of immune cell subsets with receptors for BP mediators (e.g. AT1, ACE2, adrenoreceptors), and non-pharmacological evidence of the relationship between hypertension and the immune system have provided both the clinical context and justification for the study design.

1.4 Biomarker background

Biomarkers are characteristics of biological or pathogenic processes that can be objectively measured and evaluated and relate to a clinical phenotype. The relationship may be causal or indirect, and may relate to pathogenesis of the disease state, to diagnosis, progression, or complications. An ideal biomarker would be acceptable to patients, non-invasive, sensitive to identify cases, specific to prevent false positives, and correlate with disease severity and with treatment.[146,147]

1.4.1 Existing blood biomarkers in hypertension

Measures of BP, be it office or ambulatory, offers only values and patterns. They do not suggest underlying pathology, nor determine risk or incidence of HMOD to permit individualised management of hypertension. Many circulating biomarkers are used to provide such information, both in clinical practice, and experimentally. These include cytokines, interleukins, soluble cell-adhesion molecules, leukocyte subgroups, components of the clotting cascade and micro-RNAs; however, no single biomarker can fully characterise the

function or pathology of the cardiovascular system. A selection relevant to the Inflammatension study are explored below.

1.4.2 Arterial function biomarkers in hypertension

The measures of vascular function already discussed are essentially biomarkers of primary hypertension, both causal and consequential.[146] They are not currently widely available in the office setting however, do require additional consultation time, and trained operatives and environmental controls of limited acceptability to patients (such as fasting and avoidance of nicotine, caffeine, and exercise). Additional circulating protein biomarkers have been associated with these functional cardiovascular traits; they are briefly outlined here, as a detailed review is beyond the remit of Inflammatension and can be found elsewhere. [146,148,149]

1.4.2.1 Endothelial function in hypertension

Examples of the diverse range of biomarkers studied include vascular endothelial growth factor (VEGF), submicron membrane fragments known as Endothelial microparticles (EMPs), and regulators of the renin-angiotensin and NO systems; an illustration of each follows. Plasma VEGF can induce cell proliferation and angiogenesis, and may also be a biomarker for endothelial dysfunction and vascular damage, VEGF levels inversely correlating with FMD ($r=-0.35$, $P=0.03$), and higher VEGF associated with hypertensive retinopathy.[150] EMPs meanwhile are expelled by damaged or stressed cells, with altered levels seen in many cardiovascular diseases. Hypertension demonstrates increased CD144+ and possibly CD31+/CD41- EMPs, the association supported by anti-hypertensive medication-related changes. Validating evidence is limited by challenges in specimen processing, restricting the potential usefulness of EMPs as routine biomarkers. [151,152]

Angiotensin A is a derivative of angiotensin II (Ang II) with dose-dependent vasoconstrictive actions mediated in the kidney seemingly via AT1 receptor. [153] Vasoconstriction inhibiting factor (VIF) is another regulatory protein, modulating vasoactive Ang II via AT2 receptors. VIF is a biomarker for cardio-renal organ damage, with the elevated levels theorised as protective as

countering Ang II. [154] The NO system similarly regulates vascular tone, and asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthesis, similarly has evidence of elevation in primary hypertension.[155]

1.4.2.2 Arterial stiffness in hypertension

Examples of arterial stiffness biomarkers have been again been selected to illustrate the heterogeneity of reported biomarkers, a full review being beyond the scope of this thesis. Homocysteine is an amino acid derived from methionine via trans-methylation; perturbation in metabolism of homocysteine causes elevated concentrations. Raised homocysteine levels have demonstrated both an increased risk of incident hypertension (OR 1.66),[156] and increased arterial stiffness as measured by brachial-ankle PWV (baPWV), suggesting possible predictive clinical utility.[157] Adrenomedullin meanwhile is a vasoactive peptide predominantly secreted by vascular endothelial cells. Its' precursor mid-regional pro-adrenomedullin (MR-proADM) offers longer circulating half-life, with evidence of higher area under the curve than high-sensitivity C-reactive protein (hsCRP) with regard to baPWV.[158] Finally, MicroRNAs have also demonstrated vascular function biomarker potential; for example, microRNA-21 demonstrates association with PWV in hypertensive patients, independent of BP.[159]

1.4.2.3 Atherosclerosis in hypertension

Elevated homocysteine levels demonstrate an association with increased IMT and CRP,[160] whilst serum cystatin-C concentration independently correlates with cIMT as well as glomerular filtration rate in hypertensive patients.[161] Adiponectin, involved in various metabolic processes, also has direct effects on endothelial cell VCAM-1 expression, and macrophage TNF-α generation and scavenger receptor expression.[162] Low serum adiponectin levels have been independently associated with incident hypertension,[163] with CIMT in both healthy and diabetic patients,[164,165] and with coronary artery disease (CAD) as determined by angiography.[166] MMP-9, an extracellular matrix-degrading enzyme, also has evidence of a biological link to atherosclerosis, particularly studied in CAD and summarised by Packard and Libby.[162]

1.4.3 Immune and inflammatory biomarkers in hypertension

The bidirectional relationship between vascular function and hypertension appears to be mediated in part by inflammation, with many circulating biomarkers studied across epidemiological, RCT, and GWAS studies (Figure 1.7). Key examples include CRP, an acute phase reactant associated with BP elevation in observational and clinical trial data. [167,168] For example, normotensive women in a nested case-control study demonstrated elevated risk of incident hypertension with higher quartiles of CRP and interleukin-6 (IL-6).[89] Though interestingly, this was not replicated in a similar study in males once association between CRP and BMI was adjusted for, nor in the CANTOS trial.[169,170] Soluble-ICAM-1, MCP-1, IL-10, and adiponectin levels also appear to be increased in hypertension and may confer risk of developing the disease,[91,171] whilst CRP, TNF, and IL-6, IL-1β, IL-8, IL-17, IL-18, Sphingosine-1-phosphate (S1P, regulates immune cell trafficking), among others, are higher in those with hypertension relative to normotensive controls.[48,172–178] Many of these cytokines are related and share common regulators, though not all markers changed in parallel within studies.[173] This may reflect a discrepancy between local cytokine concentration with paracrine effects, and circulating levels; or it may be methodological with variation between assays and between labs.

Multiple biomarkers in combination (or their relative concentrations) have also been considered to improve accuracy. For example evaluation of 1456 Framingham study participants identified CRP, plasminogen activator inhibitor-1 (PAI-I), and urine albumin/creatinine ratio (UACR) as the biomarkers most strongly associated with hypertension risk. Serum aldosterone lost significance after adjusting for these three, supporting interactions between aldosterone and inflammation.[179] Mechanistically, inflammatory cytokines promote cell infiltration, affect renal sodium transport, and alter vascular function and structure, ultimately leading to sodium and volume retention, increased systemic vascular resistance, and hypertension. Subgroups of circulating leukocytes also lend themselves as biomarkers of inflammation and hypertension, as were explored in Section 1.3.

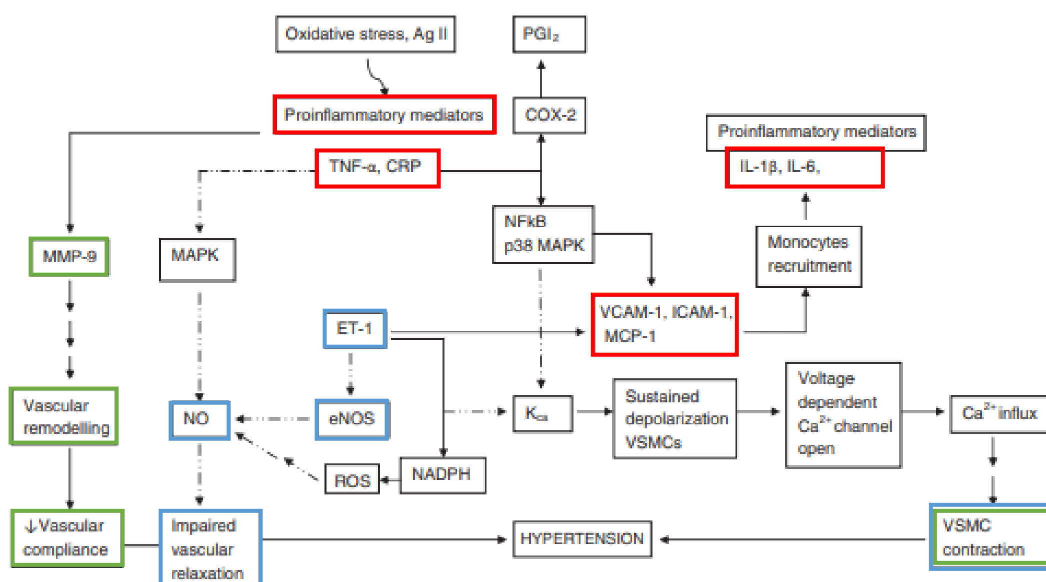


Figure 1.7 Illustration of markers of endothelial dysfunction, inflammation, and oxidative stress that favour vasoconstriction and contribute to hypertension. Solid arrow, induce; broken arrows, impair. Inflammatory biomarkers in red boxes, arterial stiffness in green, and endothelial function in blue. Adapted from: The role of oxidative stress, antioxidants and vascular inflammation in cardiovascular disease. H. N. Siti, Y. Kamisah, J. Kamsiah. Vascular Pharmacology; 2015; 71: 40-56. doi.org/10.1016/j.vph.2015.03.005.

Biomarkers can also have utility in considering the risk for developing hypertension, risk of progression, predicting response to treatment, and as markers of complications of hypertension; Figure 1.8.[148] Some such as CRP overlap these areas of utility, but most are specific e.g. cystatin-C as a marker of kidney damage, and thus consideration must be given to which biomarkers to investigate to ensure relevance to the research question.

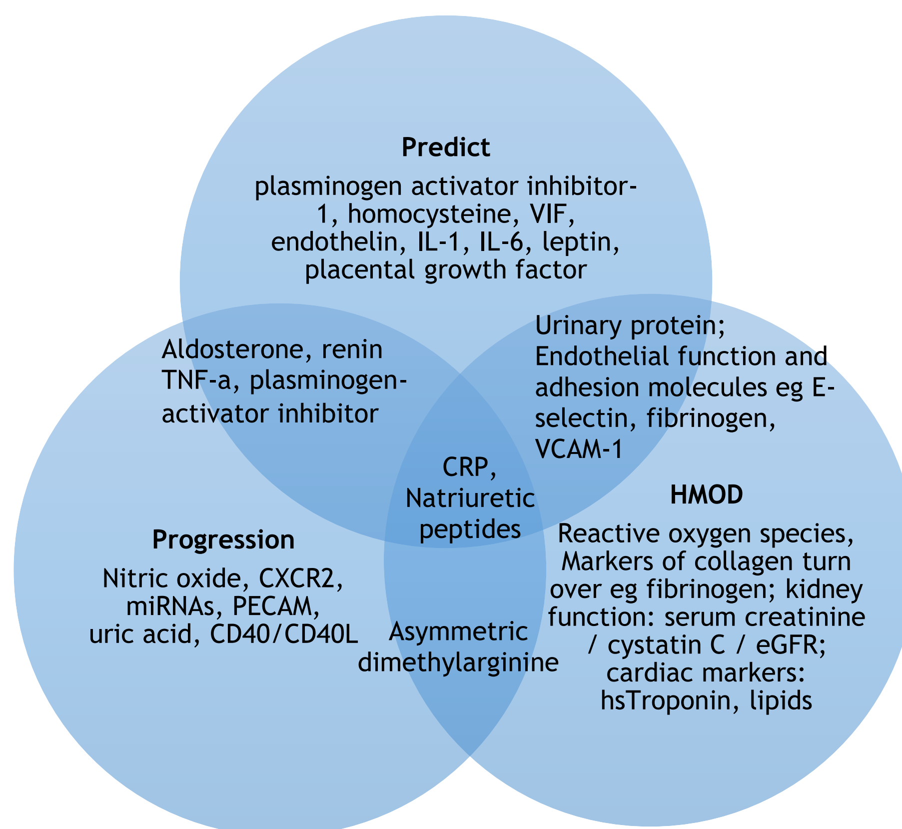


Figure 1.8 Circulating biomarkers of primary hypertension: predicting incidence, associated with progression, and with hypertension-mediated organ damage (HMOD). IL, interleukin; VIF, vasoconstriction inhibiting factor; VCAM, vascular cell adhesion molecule; PECAM, platelet endothelial cell adhesion molecule; TNF, tumour necrosis factor; eGFR, estimated glomerular filtration rate.

1.4.4 Immune and inflammatory biomarkers in arterial function

The measures of arterial function and damage (endothelial dysfunction, stiffening, atherosclerosis, and HMOD) have been linked to various biomarkers; examples are described below, with more detailed information available published elsewhere.[148,149]

1.4.4.1 Immune biomarkers of endothelial function

Arterial tone is regulated by vasoactive mediators endothelin-1 and nitric oxide (NO) released from endothelial cells. They demonstrate a bidirectional modulating relationship with cytokines such as TNF-α, IL-1 and IL-6.[5,180] In the context of CAD, endothelin-1 but not IL-6 demonstrated an association with FMD.[16] As a non-specific marker of inflammation, elevated CRP levels in patient populations with diabetes or peripheral arterial disease have also been linked to endothelial dysfunction as measured by FMD, unrelated to

traditional risk factors for CVD.[181,182] In rheumatological disease, pharmacologic treatment lowers CRP levels, with improved FMD values also observed, though multivariate analyses were not reported. [183-186]

A study of a wide array of biomarkers and acetylcholine-induced forearm arterial vasodilation in untreated hypertension demonstrated higher levels of e-selectin, p-selectin, MCP-1, tissue inhibitor of metalloproteinases type 1 (TIMP-1), and glutathione peroxidase and superoxide dismutase (enzymes involved in clearance of oxidative products) in the tertile with most impaired vasodilatation. IL-6, hsCRP, sICAM-1, sVCAM-1, homocysteine, and MMPs however did not demonstrate between-tertile differences.[187]

1.4.4.2 Immune biomarkers of arterial stiffness

Studies across diverse patient populations have demonstrated correlation between arterial stiffness (as well as consequent organ damage) and various inflammatory markers. These circulating inflammatory biomarkers include leukocyte count and ratio of neutrophils to lymphocytes, CRP, cell adhesion molecules, fibrinogen, cytokines, microRNAs, and cyclo-oxygenase-2. [149]

For example, in spondyloarthritis, PWV >8m/sec demonstrates increased levels of IL-6, TNF-α, and IL1β in multivariate analysis;[188] and a Framingham Heart Study applied multivariable analysis with backward elimination to conclude associations between IL-6 and osteoprotegerin with carotid-femoral PWV; and reflected pressure wave associated positively with CRP and inversely with lipoprotein-associated phospholipase-A2.[189] Studies of cell adhesion molecules report divergent results, some associating VCAM-1 with PWV in hypertension,[190] another (in patients referred for echocardiography) finding no correlation between VCAM-1, ICAM-1 and aortic distensibility.[191]

CRP is the most widely reported biomarker, linked to arterial stiffness in varied populations, and by different measurement techniques (PWV, PWA, AIx).[149,184,186,189] However, CRP does not always maintain independent significance in adjusted analyses,[192] and reductions in CRP do not always demonstrate improvement in AIx.[193,194] The link between inflammatory proteins and arterial stiffness is mediated via effects on cell expression,

vasoactive capacity, and proliferative and fibrotic effects on vascular smooth muscle and interstitial cells.[159]

1.4.4.3 Immune biomarkers in atherosclerosis

Inflammation has been linked to the formation, progression and destabilisation of atherosclerotic plaques. CD40, IL-18 and IL-6R in particular are associated with these processes and have been reported as an independent predictors of coronary artery events.[195-197] Mechanistically, IL-18 signalling induces atherogenic factors including VCAM-1, chemokines and cytokines (IL-6), and MMP-1, -9, and -13. IL-18 levels were associated with CIMT ($P<0.001$) and plaque prevalence ($P<0.001$), but lost significance after adjustment for traditional risk factors. [198] Further examples of inflammatory-atherosclerotic biomarkers comes from a study of HIV-infected and uninfected men, with elevated sTNF-αR2 levels positively associated with CIMT; fibrinogen with bifurcation-IMT and carotid plaque; and ICAM-1 with carotid plaque.[199]

1.4.5 Summary

Many circulating biomarkers relating to the immune system and inflammation have been associated with hypertension, with arterial function, and with HMOD, though so far few have made it into routine clinical practice. Those that have relate predominantly to advanced disease and HMOD, fewer to early onset hypertension and risk of incidence. Inflammatension therefore offers novel analysis of incident patients, combined with vascular and cellular immune markers.

1.5 Inflammatension study aims

The Inflammatension project studies hypertension in young, incident patients with no overt cardiovascular disease, encompassing three fields of data, cross examined by three key research questions, as illustrated in Figure 1.9. The data relate to measures of cardiovascular function, such as endothelial function, arterial stiffness, intima-media thickness, and cardiovascular variability; detailed analysis of immune cells, including B cell subsets, T cell

subsets, monocyte and dendritic cells, and intracellular stimulation studies; and circulating immune biomarkers. The three research questions applied to these data were comparisons of hypertensive patients against normotensive controls assessing for between group differences; clinically relevant associations between the variables measures, and analysis of any phenotypic subgroups identified.

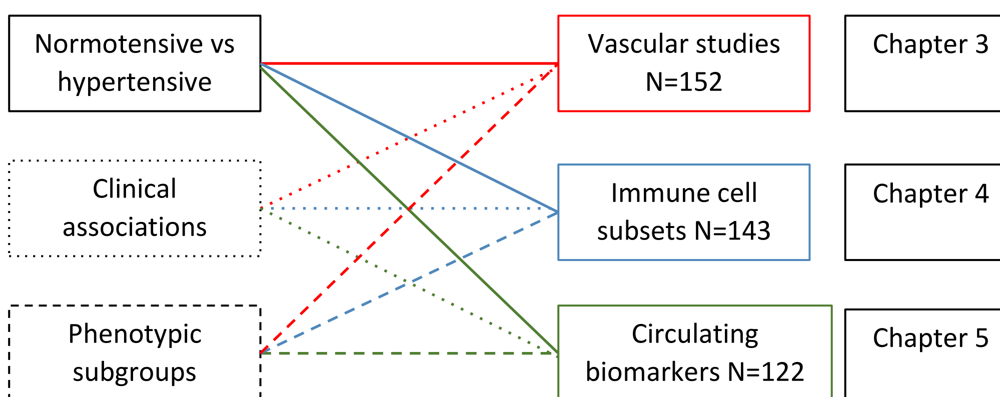


Figure 1.9 Research themes on left, will be applied to the generated data sets presented on the right. A more detailed exploration of the aims follows.

1.5.1 Vascular function in hypertension

The aim was to collate comprehensive data on arterial function to explore relationships between traits that could allow for early identification of hypertensive phenotypes, with the following research questions: 1) Do measures of cardiovascular function differ in young, incident patients with hypertension but no overt cardiovascular disease, versus healthy controls? 2) Do measures of cardiovascular function demonstrate clinically applicable associations? 3) Are phenotypic subgroups apparent in hypertension, and which cardiovascular parameters can discriminate these subgroups?

1.5.2 Immune cell subsets and arterial hypertension

Detailed analysis of immune cells, including B cell subsets, T cell subsets, monocyte and dendritic cells, and intracellular stimulation studies permitted consideration of the following research questions: 1) Do circulating immune cells differ in young, incident patients with hypertension but no overt cardiovascular disease, versus healthy controls? 2) Does nocturnal dipping status demonstrate leucocyte subset associations?

1.5.3 Protein biomarkers in hypertension

The aim was to obtain and analyse comprehensive biomarker data from blood samples of normotensive and hypertensive patients, to explore the following research questions: 1) Do circulating immune biomarkers differ between young, incident patients with hypertension but no overt cardiovascular disease, versus healthy controls? 2) Which immune system biomarkers are associated with demographic features, BP, hypertension, and arterial function? 3) Relating to the immune milieu, are phenotypic subgroups apparent in hypertension?

Chapter 2 General methods

The structure of this chapter recapitulates the proposed experimental aims and the sequence of subsequent chapters; aspects pertaining to the general study methodology and approach are detailed here, with specific methodological considerations presented in the relevant subsequent chapters.

2.1 Funding, ethics, participants

Funding was provided by the European Research Council (grant number ERC-2016-726318). Inflammatension was approved by the West of Scotland Research Ethics Service, reference number 17/WS/0115, as well as NHS Greater Glasgow and Clyde Research and Development (IRAS project ID: 224036). Patient data was anonymised with a unique study identification number given; this study number was used for blood samples, all electronic and paper data relating to vascular function and immune signature. The study ran in accordance with GDPR legislation regarding data. Biological samples collected from participants as part of this study were transported, stored, accessed and processed in accordance with 2004 Human Tissue Act and the 2006 Human Tissue (Scotland) Act. The study ran in accordance with the World Medical Association Declaration of Helsinki (1964) and its revisions (Tokyo [1975], Venice [1983], Hong Kong [1989], South Africa [1996] and Edinburgh [2000]). Participants were free to withdraw from the study at any point without giving reasons and without prejudicing his/her further treatment. In the case of a withdrawal of consent for lab analysis, all samples were destroyed, though any data already generated was retained if the participant was in agreement.

2.2 Recruiting strategies and challenges

2.2.1 Power calculations

Target recruitment was set at 120 hypertensive and 120 control participants, based on a power calculation derived from published and preliminary data of multiple cytokines and biomarkers, estimating a minimum of 116 subjects to allow detection of difference in IL-6 of 25% at a two-sided alpha of 0.1% and power of 90%, TNF-α would similarly require 58 subjects.[169,174] To detect a 25% difference in CD8+CD25+ T cell (α=0.1%, power - 90%), power calculations suggested 87 participants per group [200]. These analyses took into account adjustments required for multiple comparisons.

2.2.2 Recruitment Challenges and Strategies in the COVID-19 era

Study visits were undertaken 23/05/19 until 06/09/21. Participants were recruited from Greater Glasgow and Clyde, UK; patient information sheet is included in Appendix 1.

2.2.2.1 Recruitment challenges

Three dominant factors were anticipated to restrict recruitment to the Inflammatension study. Firstly, untreated hypertension is a 'hidden' diagnosis, with recognition rapidly followed by pharmaceutical therapy in the majority of individuals. This necessitates screening of apparently healthy individuals with unknown hypertension to identify cases, or identification at referral to secondary care. Whilst population estimates of hypertension are around 30% [2], our community events targeted younger populations with a higher likelihood of meeting the inclusion criteria, where a far lower percentage registered a BP in the hypertensive range, and of those who did go on to complete an ABPM, few were confirmed as hypertensive range, as Chapter 3.3 (Demographic results) reports.

Secondly, COVID-19 abolished our ability to recruit through community screening events and pre-operative assessment clinics, and even blood pressure clinics were mostly cancelled or deferred. Furthermore, primary care

also minimised face-to-face consultations, hence incidental diagnoses of hypertension fell. Additionally, approval for all non-COVID research was revoked so the study halted.

Thirdly, Inflammatension's restrictive recruitment criteria. Identifying a drug-naive, hypertensive cohort without concomitant health conditions is a particular challenge, but not unique; many studies require narrow inclusion and broad exclusion criteria in order to ensure a 'clean' study population, controlling as many potential confounding variables as possible. Within clinical research, recruitment is frequently the greatest challenge and the largest demand on staff resources, with delays in starting recruitment occurring in approximately 40% of trials.[201] Reviewing the relative merits of different recruitment strategies has been valuable in facilitating recruitment to Inflammatension, and so is reported herein.

2.2.2.2 Recruiting strategies

Attempts have previously been made to associate trial features and recruitment strategies with success rates, but the relationship is complex [201]. Inflammatension employed traditional approaches to recruitment with careful consideration of ethical and data protection issues. Approvals from Ethics, R&D, and the Medical Director included initial screening at routine outpatient appointments for the blood pressure (BP) clinics. For example, if NHS staff identified possible participants through their regular duties, such referrals were acted on only if the patient had given their consent to be contacted by the research team. This contact was also restricted to sharing of the patient-information leaflet (PIL), with an 'opt in' policy. Two hypertension-based research studies performed locally were also screened for potential participants, after confirming written participant consent regarding willingness to be approached about further research trials.

BP clinic screening involved weekly review of the clinic list via the NHS GGC Trackcare software system for attendees who fit the inclusion and exclusion criteria, with postal or email PIL. Similarly, those awaiting ABPM were on occasion flagged as potentially suitable for the study, and a PIL could be sent to them.

An amendment was added (V 1.1, 18/12/18, approved 10/05/19) to include Secondary care more generally, including the 'Clinical Decision-Making unit' as the site to which hypertensive urgencies are commonly referred to from primary care; and pre-operative assessment clinics, with the rationale being that those fit for day case orthopaedic procedures would likely be a population more aligned with the inclusion and exclusion criteria, and also a population otherwise less likely to be obtaining BP screening routinely. However, medical staff rotate through the Clinical Decision-Making unit, limiting referrals to the research team; Renal live-donor assessment team did not identify anyone suitable, and only one orthopaedic pre-op assessment site was agreeable and engaged with identifying potential participants. A later amendment expanded opportunistic medical encounters as a source of referrals through the addition of the Emergency Department. However, the approval process carried significant delay that this option was not available until almost completion date.

Inflammatension initially managed to recruit predominantly male Caucasian hypertensive (9/30) and healthy control female participants (12/30) with lower numbers of normotensive males (n=6) and hypertensive females (n=3). Recruitment success has been studied previously, with reported variation in rates and cost of recruitment by pharmacies, paid media, word or mouth, and unpaid media, but also demonstrating differences in participant quality of life, educational attainment, and income.[202] None of the strategies was singularly effective.[203] This supported the use of multiple recruitment strategies to increase equitable access to participation and improve generalisability of the data to an unselected population.

A further comparison of 19 virtual versus traditional recruitment strategies concluded that the former recruited younger and more female and ethnic minority participants, suggesting that expanding our electronic recruitment strategies could benefit our sex imbalance; virtual studies also achieved higher numbers of participants per month, and had shorter duration to target recruitment.[204] Studies often include electronic or 'virtual' components, with potential for all aspects of recruitment, consent, data collection, and storage being online or via electronic applications. Inflammatension embraced

electronic methods of disseminating recruitment information about the opportunity to take part in research, with a listing on the Volunteer Glasgow website and use of social media. The main benefit of such being the diverse populations of local individuals who may be interested. Examples included Facebook volunteer and community pages, University of Glasgow Yammer message board, and Nextdoor local community message board. This was not a prolific source of participants and was more successful at identifying healthy control rather than hypertensive individuals (Figure 2.0), but provided a small numbers of volunteers when other means were restricted. Rates of expressed interest to study completion were low, hypothesised as due to the ease and spontaneity with which electronic adverts allow potential participants to express interest, with subsequent waning of commitment such that the potential participant never follows up on offered study visit dates, or cancels at short notice. Broader benefits include financial and environmental sustainability considerations, with electronic systems avoiding need for travel of staff or potential participants, and electronic rather than paper PIL – avoiding paper, printing, envelope, and sorting/delivery.[204]

Community-based screening events were undertaken prior to the COVID-19 pandemic, with interested individuals being offered a BP check and brief lifestyle advice at workplaces, community events and places of significant footfall, such as large stores and hospital entrances. Where hypertensive individuals were identified they were advised to attend their GP for further investigation and management, and individuals fitting the inclusion criteria were provided with an Inflammatension PIL to contact me if they wished to opt in. Additionally, in collaboration with other active hypertension studies, the Primary care research group affiliated with the University of Glasgow invited Primary care sites to permit screening of their registered patients on our behalf, with opt-in letters sent to those meeting criteria. Only three practices agreed, and only one volunteer completed the Inflammatension study via this route.

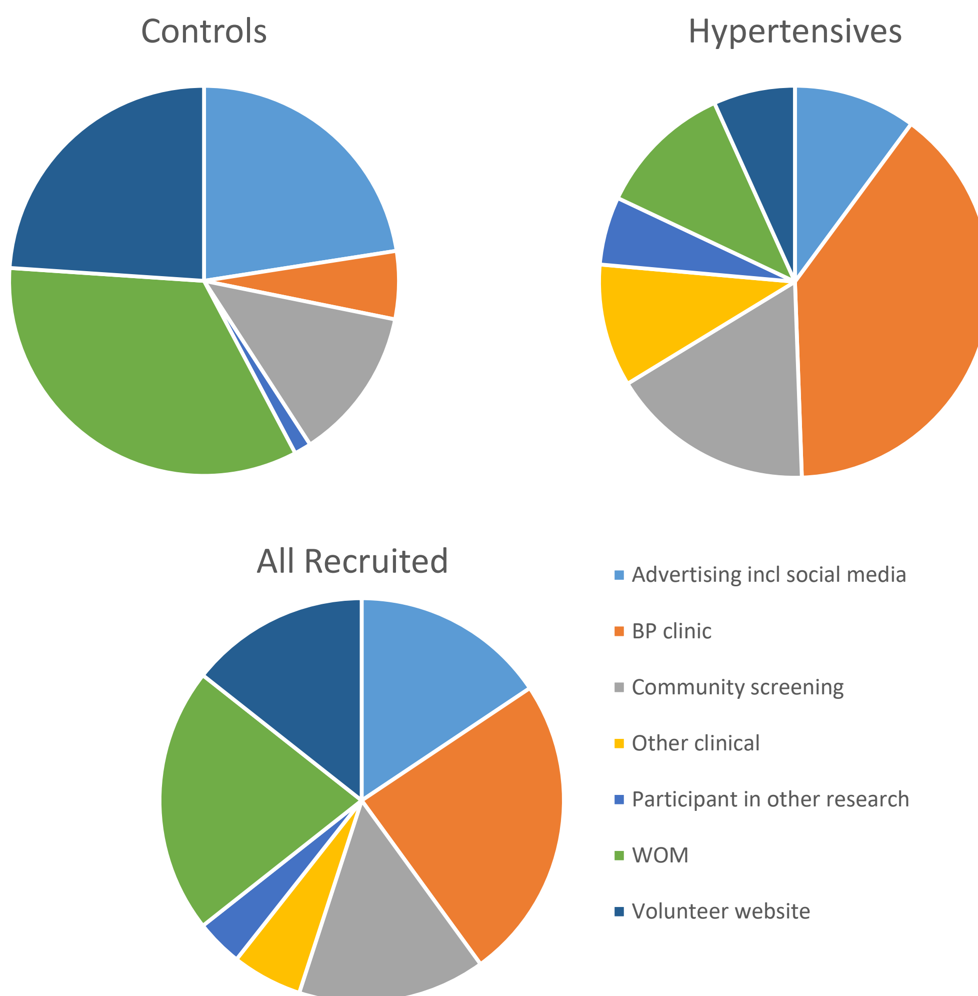


Figure 2.0 The recruitment strategies grouped by theme, and highlights that the most successful approaches differed between control and hypertensive participants. BP, blood pressure; Other clinical, includes Immediate Assessment Unit, Medical Receiving Unit, Pre-Operation Assessment; WOM, word of mouth.

Diverse additional recruitment strategies trialled with Inflammatension: posters; email distribution around colleagues; paid media strategies included radio (Sunny Govan) and print (the Herald), both trialled but without great success; SHARE (Scottish health research register) was considered, but there was limited optimism regarding its' ability to identify the correct individuals due to Inflammatension's broad exclusion criteria.

Limiting the damage of COVID-19 on recruitment focused on developing recruitment strategies whilst awaiting approval to restart study visits. I also contacted the volunteer participants who had booked in for study visits but

were cancelled when R&D approval was suspended, to establish if still keen to volunteer. Alternative study design options were considered, given that the suspension was for an unknown period of time.

In summary, a 'hidden' population for recruitment, compounded by ineffectual R&D approval processes, significantly limited recruitment, the statistical power, and the timeline of the project. These are not specific to my project but rather a threat to any similar research, hence the inclusion of this chapter so that others may design their studies cognisant of the challenges and possible mitigating strategies.

2.3 Study design: study protocol

2.3.1 Participant inclusion criteria

Inclusion criteria specified age between 18 and 50 years; with hypertensive cases demonstrating office blood pressure (BP) greater than either 140 mmHg systolic, and/or 90 mmHg diastolic; controls required office BP under 140 and 90 mmHg and were age, sex, and body mass index-matched to cases.

Potential participants were screened and excluded for any potentially confounding factors known to influence the immune system, including acute and chronic infections and inflammatory disorders, or vaccines within the last three weeks. Or were in place to ensure recruitment remained ethical, specifically inability to provide valid consent. Finally, targeting recruitment to participants with primary hypertension, with exclusion of anti-hypertensive medications, known secondary hypertension, and BMI above 35. See Appendix 2 for full protocol including exclusion criteria. An amendment was requested Sept 2019 and approved Sept 2020 to raise the age limit to 55 years, with the intention of improving recruitment, particularly of the female hypertensive group. Participants were empowered to withdraw from the Inflammatension study at any point; one chose to do so following ABPM but prior to any other investigations, their ABPM data was not included in reporting of the study.

2.3.2 Design

Inflammatension was designed as a cross-sectional clinical and laboratory study of primary hypertensive and age-, sex- and BMI-matched controls. The workbook for the study visit, as well as protocols covering vascular studies and laboratory techniques are included as Appendices 2 to 4. Chapter 1.2 included justification for collection of all data. Outlined here are the methodological details of the various vascular and laboratory studies, and of data analysis in the order of the Table of Contents. Further technical detail regarding individual techniques can be found in the relevant results chapters.

2.3.2.1 Clinical and demographic data

Factors known to have a role in determining BP and immune function were recorded, specifically age, sex, body-mass index (BMI), smoking status, menstruation and hormonal contraception (known to influence FMD), and ethnicity. Healthy controls were selected to match these demographics.

Height was measured (without shoes) to the nearest millimetre; weight was measured to the nearest 100g on Seca weighing scales which were calibrated regularly, and the same equipment used for all visits. BMI was calculated according to the standardised kg/m2. Medical history and use of prescribed and over the counter medication were recorded to confirm no basis for exclusion and to detect potentially confounding variables. Smoking was categorised as current cigarette smoker, current e-cigarette smoker, ex-smoker (no cigarettes for more than 3 months) or never smoked. Ethnicity was categorised as European-descent, African-descent, Asian-descent, Chinese/Japanese descent, or Middle-Eastern descent.

All study visits conformed to a start time between 8.30 and 10am to minimise circadian rhythm variability and in consideration of fasting. Patients attended fasted and avoiding caffeine from midnight, until after office BP measurements, EndoPAT-2000, and FMD were completed, as these components have been shown to be influenced by the post-prandial state.[205] The order of investigations was standardised to avoid any between-participant differences due to repeated measures effects of occlusive techniques. All

studies were conducted in a quiet, temperature-controlled room; participants were requested to avoid exercise that morning; vasoactive drugs were a contra-indication to inclusion in the study; stage of the menstrual cycle was noted where appropriate, and it was recorded if there were any deviations from these points. Two technicians did all study visits and data collection, the less experience receiving training until competent and confident and regular communication between the two ensured that study visits followed the same order (partially automated analysis of BP, FMD, EndoPAT-2000, PWA, PWV), and technical aspects were identical to minimise inter-rater reliability issues. Any subsequent software analysis (CIMT and FMD) were all completed by one assessor for the same reason. Guidance on precise methodologies were taken from the product literature for each technique, and additional published guidelines where available, further detail in Chapter 3 and below.

All Inflammatension participants underwent non-invasive assessment of the following cardiovascular parameters: blood pressure variables, endothelial function assessed via brachial artery dilatory capacity and digital pulse amplitude (peripheral artery tonometry) in response to hyperaemia; arterial stiffness quantified with pulse wave velocity and pulse wave analysis; and intima media thickness as a marker of atherosclerosis. Justification and underlying basis in pathophysiology have been discussed in Chapter 1.2, and more detailed protocols can be found in the relevant results chapters.

2.3.2.2 Blood pressure (BP) measurement

Office BP accuracy was optimised by consistent technique and following standardised methods.[206] BP was measured during the study visit in the sitting position after five minutes of rest, with an appropriately sized cuff, using an automated calibrated sphygmomanometer; systolic and diastolic were recorded in triplicate and summarised in data reporting as the mean of these three sets of values. ABPM was commenced on the day of the study visit, though if already completed within the last three months to the same protocol, and no other lifestyle or demographic parameters had changed, then these results were accepted.

In line with NICE recommendations,[26] Ambulatory blood pressure monitoring (ABPM) duration was 24 hours, with twice hourly BP measurements through the day and hourly overnight (22.00 until 7am) using a Spacelabs Healthcare monitor and associated Spacelabs Healthcare software to upload and analyse. A failed recording resulted in one repeat attempt. Data are presented as heart rate range derived from minimum and maximum heart rate, average 24-hour systolic and diastolic BP, average daytime and night-time values, and percentage nocturnal reduction with 'dipping' status defined as a nocturnal decrease of 10% or more from daytime average.

For data analysis, cases and controls were designated based on their ABPM result, with the threshold for hypertension defined according to ESC/ESH guidelines [207] i.e. a 24-hour mean above 130 and/or 80 mmHg, or a daytime mean above 135 and/or 85 mmHg. If ABPM was declined by the participant or failed, values were estimated from the average of three office BP measurements to permit categorisation as case or control. Estimation involved adjustment of the office BP value by the average difference between office and ambulatory BP values (-7.3 mmHg systolic and -6.3 mmHg diastolic).

2.3.2.3 Pulse wave analysis (PWA)

As outlined in Chapter 1.2, arterial stiffening reflects structural changes in the arterial wall, with degradation of elastin and increases in collagen and fibrin, causing increases in the thickness of the artery wall and in the size of the lumen. Such changes occur as part of the aging process, but also in hypertension, and are also associated with chronic inflammation and oxidative stress.

This stiffening has a predictable and measurable effect on flow dynamics, demonstrable in the pulse waveform and increased pulse pressure. Hence, variation in the pulse waveform can occur even with similar systolic and diastolic BP values, representing a distinct parameter relating to both cardiac output and arterial stiffness. PWA using SphygmoCor XCEL (Atcor medical, West Ryde, Australia) captures these characteristics. The generated report includes central systolic, diastolic, and augmentation pressures (AP) - the ancillary peak superimposed on the systolic waveform and determined by

the pressure reflected from the arterial wall i.e. the compliance of the vessel. Technical details and quality control specifics are provided in Chapter 3.

2.3.2.4 Pulse wave velocity (PWV)

PWV is a non-invasive measure of arterial stiffness, with numerous technical variations published. Carotid-femoral PWV is the best validated,[208] with subtraction technique to determine distance (supra-sternal notch to the thigh cuff, the palpable femoral artery at the groin to the thigh cuff, and the carotid artery to the supra-sternal notch). The Inflammatension study employed SphygmoCor XCEL to measure PWV (Atcor medical, West Ryde, Australia). The femoral BP cuff and carotid probe both capture the pulse wave based on the systolic upstroke, as determined by integral software. These data permit automated PWV calculation and quality control analysis. See Chapter 3 for technical details and figures.

2.3.2.5 Flow mediated dilatation (FMD)

The endothelium is another key determinant of vascular function, responsible for the release of vasodilatory substances including nitric oxide (NO). FMD is a validated, non-invasive technique to estimate arterial endothelium-dependent dilatation following an ischaemic stimulus.[209,210] Methodological variation exists, imaging of the brachial artery using high-resolution ultrasound being the most accepted approach.[209,211] UNEX semi-automated device and proprietary software (UNEX EF, Japan) were employed to measure brachial FMD performed according to guidelines [209] and manufacturer's instructions, with detailed methodology, figures, and protocol provided in Chapter 3.

2.3.2.6 Peripheral arterial tonometry (PAT)

PAT is a measure of endothelial function, as described in Chapter 1.2, quantified with the EndoPAT™ 2000 Device (Itamar, Israel), a plethysmographic tool with fingertip probes equipped with pressure sensors to detect arterial tone. A baseline measure precedes occlusion of the brachial artery; following release of the occlusion, the amplitude of the PAT signal generates a ‘hyperaemic response’ value indicative of endothelial-related

dilatation. PAT ratio pre- and post-occlusion generates the 'reactive hyperaemia index' (RHI). Simultaneous analysis of the contra-lateral arm offers a control for vascular tone. LnRHI is a natural log transformation of RHI to normalise distribution of the data. Standardised procedure is detailed in Chapter 3.2 and included a controlled environment as described above.

EndoPAT™ 2000 technique also reports Augmentation Index (AIx), a composite measure of arterial stiffness determined by reflected wave amplitude, velocity, and site of reflection. EndoPAT™ 2000 generates AIx through automated analysis of multiple pulse waveforms captured during baseline measurements. AI@75 is this figure adjusted to 75 beats/min to counter heart-rate dependent confounding.

2.3.2.7 Intima media thickness (IMT)

Thickness of the intima and media layers of the common carotid artery were measured with B-mode ultrasonography performed on Acuson Sequoia c512 (Siemens AG, Germany). The vessel wall was visualised at both 90 and 135 degrees and both static images and video clips saved in reference to the R wave of the ECG, corresponding to end-diastole. Off-line bath analysis was performed blind to hypertensive status, with Carotid Studio (QUIPU, Version 3.6.0, 2019, Italy) software, with multiple data points along the artery wall recorded and an average measurement generated. Detailed methodology, figures, and protocol are provided in Chapter 3. Unfortunately, the COVID-19 pandemic restricted access to the CIMT equipment and not all Inflammatension participants underwent this assessment.

2.3.3 Questionnaires

Physical activity questionnaire: Frequency and intensity of physical activity was self reported using the validated open-access "International Physical Activity Questionnaire (IPAQ; short version)".[212] Combined with demographic data, this provided a metabolic equivalent of task (MET)-min per week value. Appendix 3 contains the questionnaire.

Cardiovascular risk: This was assessed and quantified with the InterHeart Risk Score Questionnaire, a scoring system validated to predict acute myocardial infarction events.[213]

2.4 Blood and urine sampling for immune studies

2.4.1 Obtainment of samples

Phlebotomy was performed in participants fasted and without caffeine intake for the prior 12 hours to avoid post-prandial changes; any deviations to this were recorded. A vacutainer system was used and standard venepuncture technique employed in the interval between PAT and FMD measurements. All sampling was performed between the hours of 9.00 and 10.30 am to abrogate potential effects of circadian rhythm.[214] All blood samples (Table 2.0) were gently inverted four times, other than Tempus RNA tube, which was vigorously shaken for ten seconds. Urine sample was a mid-stream sample collected during the study visit, with urinalysis assessment for protein, blood, and glucose, then sample then processed as below. In addition, renal function was determined with serum creatinine (mMol/L) and estimated glomerular filtration rate (eGFR, ml/min, MDRD equation) if the participant had not had this measured in the last 6 months.

Sample	Purpose	Volume
Plasma (EDTA)	Protein Arrays/ELISA	6ml
Serum (clot-activating SST)	Cytokines	5ml
PBMC (EDTA)	FACS & *ex vivo* studies	20ml
PBMC (Sodium citrate)	Phospho-FACS	4ml
RNA (Tempus tube)	Transcriptional studies	3ml
Urine (universal container)	For proteomics/ metabolomics	5-10ml

Table 2.0 Biological samples obtained during the study visit.

2.4.1.1 Transport

Risk analysis was undertaken prior to project commencement to anticipate any potential problems. Urine and the 6ml EDTA blood sample were transported on a cool pack, the remainder of the samples were at room

temperate; maximal time from venepucture to arrival at the laboratory was one hour. Biological samples were transported in accordance with national legislation relating to the Dangerous Goods Act (ADR: European Agreement concerning the International Carriage of Dangerous Goods by Road 2019).

2.4.2 Sample preparation

For full laboratory protocols please see Chapters 4.2 and 5.2 and Appendix 4, but in summary, samples were processed same day for Flow cytometry analysis, the remainder were stored in -80 degrees Celsius initially, then moved into liquid nitrogen for long-term storage at the University of Glasgow in a secure location, identified only by the participant identification or laboratory number. Biological samples were stored, accessed and processed in accordance with national legislation relating to the 2004 Human Tissue Act, and the 2006 Human Tissue (Scotland) Act.

2.4.2.1 Flow cytometry

Sample preparation, cell staining, and flow cytometry methodology and analysis are described in detail in Chapter 4.

2.4.2.2 Biomarker studies

Protein biomarkers were analysed with Olink® Explore Inflammation panel, described in full in Chapter 5.

2.5 Data analysis and statistical considerations

2.5.1 Data preparation

2.5.1.1 Exclusions

Two participants were excluded from all analyses. One was on an injectable biological agent not disclosed during the study visit and not apparent on screening of community-prescribed medications; subsequent to the study visit, another participant was found to have fibromuscular dysplasia of the renal artery underlying their hypertension, so was also excluded.

2.5.1.2 Missing data points

Missing demographic or vascular data points remained blank, the exception being missing ABPM data (n=6) as a defining characteristic of the groups. Person correlation supported mean office BP as an estimate of daytime average BP (r=0.78 [95%CI 0.70,0.83] for systolic, and 0.79 [95% CI 0.72, 0.84] for diastolic). For these six participants the missing ABPM parameter was substituted with office-corrected BP values. Correction involved adjustment by the mean difference between office and ABPM values across the group. For example, missing 24 hour average SBP values were estimated from office SBP minus 7.34 mmHg (the mean difference between the two measures across the group).

2.5.1.3 Outliers

All data were assessed visually for outliers using box plots. Instances were cross-referenced with the primary data source to identify computational or data entry errors, which were corrected or removed; the remaining outliers were considered individually and only removed if they skewed the data significantly or appeared to reflect failure of measurement technique.

2.5.1.4 Distribution

Continuous parameters were assessed via histograms and Anderson-Darling test of normality. If skewed, data were normalised with logarithmic or square root transformation. In parameters with positive and negative integers requiring normalisation, these were first transformed to a positive distribution in a linear fashion. i.e. if the lowest value was –65, then each value had 65 added to ensure the smallest value was positive. Continuous parameters were dichotomised where appropriate for stratified analyses.

2.5.1.5 Confounders

Confounding, also known as effect measure modification, is the association of a third variable with the main exposure and outcome of interest. Demographic and environmental factors were considered as potential confounders,

including age, sex, BMI, medications not requiring patient exclusion, stage of menstrual cycle, and smoking behaviour. Confounding was assessed for through stratified histograms and summary statistics, looking for overlap of confidence intervals around each stratum-specific measure. The results of these analyses are discussed in the respective Vascular and Immune chapters (3.2.9 and 4.2.6).

2.5.2 Software

2.5.2.1 Demographic and Vascular data

Software specific to measures of vascular function are discussed in the relevant results sections. Once retrieved from Castor EDC, demographic and vascular data were analysed in Microsoft Excel (2013), Minitab (Version 19), and Rstudio (Version 1.4.1717).

2.5.2.2 Immune data

Flow cytometry data analysis was performed on FlowJo™ v10.8 Software (BD Life Sciences). Statistical analysis was then performed with Minitab as above. Additional detail for these software techniques is reported in Chapter 4.2 and 5.2.

2.5.2.3 Analysis methods

Study Statistician and bio-informatician (Dr John McLure) was involved in the study design from conception. Descriptive statistics were employed to summarise the data. Between group comparisons of hypertension versus normotension employed statistical testing according to the nature of the data. Predominantly, T-test for continuous data normally distributed, Mann-Whitney if non-parametric; or Mood's median test if non-parametric and distributions differed. Categorical data were analysed with Chi^2, and multiple groups with ANOVA. Correlation and multiple regression were employed to assess the strength of association and relative importance of multiple predictors to the outcome of interest. Statistical significance was assumed for $P<0.05$ across all

analyses. Bonferroni approach offered adjusted statistical significance thresholds for multiple comparisons. This was calculated as as alpha ($P<0.05$) / n (number of comparisons). Mathematical methodology details specific to the respective Vascular and Immune chapters are described in Sections 3.2.9 and 4.2.9.

Exploratory unsupervised approaches (Machine learning) such as Principle Component Analysis and hierarchical clustering 'heatmap' analysis were employed to identify signature patterns or phenotypes. Inflammatension took guidance on this from bio-mathematicians Pawel Renc and Patryk Orzechowski. Detail regarding these statistical techniques, including classification methods, are covered in the specific results chapters.

2.5.2.4 Data storage and accessibility

An electronic case report form (e-CRF) was the primary source and storage facility for study data, and was provided by CASTOR EDC (https://castoredc.com). The structure of the data entry form followed the structure of the study visits to minimise data entry errors. Data was validated at the point of entry into the e-CRF, automated alerts flagged data or review at the point of entry if beyond the anticipated range of values. CASTOR EDC recorded any data changes in order to maintain a complete audit trail. Access to the e-CRF was restricted to authorised, site-specific personnel running study visits. A study specific data management plan was followed, and all members of the delegation log undertook GDPR and Good Clinical Practice training.

Pseudo-anonymised participant identification numbers were used for eCRF, for paper workbooks, for ABPMs and for blood and urine samples; this ensured blinding of immune/laboratory analyses towards hypertension status. Full data sets will be retained within the University of Glasgow for a minimum of 10 years, including sufficient information to link records to participants, and all original signed consent forms kept secure at the Clinical Research

Facility, in accordance with GCP. Data is accessible by request from the University's Institutional Data Repository Enlighten: Research Data.

2.6 Sustainability/environ impact assessment

Inflammatension funders (European Research Council) and host organizations (University of Glasgow and NHS Greater Glasgow and Clyde) have all made commitments to improving environmental sustainability. [215,216] Methodology of the Inflammatension project included efforts to minimise environmental impact and resource use. Biological samples were transported by cycle courier rather than motorized vehicle, participants were also encouraged to use public transport (prior to COVID-19 restrictions), reducing fossil fuel reliance and air pollution. Rechargeable batteries powered ABPM machines, and sample bags were reused where no contamination or damage had occurred for transporting samples.

Chapter 3 Demographic and Vascular Results

3.1 Background and aims

Despite well-established demographic and lifestyle risk factors for primary hypertension, the early phenotypic features remain undefined. Where ABPM data is available, loss of nocturnal dipping status, masked hypertension, and white coat hypertension are subgroups with prognostic value,[217] as discussed in Chapter 1.1.5. Otherwise, identifying early on which individuals will develop HMOD continues to evade us. To overcome this, surrogate markers such as endothelial dysfunction, microvascular abnormalities, IMT and stiffness of the large arteries are employed. These early functional traits reflect different aspects of vascular health and are known predictors of more advanced pathophysiological changes and cardiovascular disease. However, the natural progression of arterial dysfunction, hypertension, and cardiovascular disease is neither linear nor uniform: hypertension can be both cause and consequence of microcirculatory dysfunction and arterial stiffness,

and cardiovascular disease accelerates this cycle. Heterogeneous inciting elements and regulatory systems account for the variation across different vascular parameters and in their progression, leading to the concept of hypertensive 'phenotypes'. Understanding the discriminatory capacity of these characteristics could allow for early identification of disease phenotypes and tailoring of management. The techniques measuring these functional traits (FMD, PAT, PWA, PWV, IMT) have been discussed in Chapters 1.2 and 2.0 and additional methodological details follow in Section 3.2.

The aim was therefore to collate comprehensive data on arterial function to explore relationships between traits that could allow for early identification of hypertensive phenotypes, with the following research questions: 1) Do measures of cardiovascular function differ between young, incident patients with hypertension but no overt cardiovascular disease, versus healthy controls? 2) Do measures of cardiovascular function demonstrate clinically applicable associations? 3) Are phenotypic subgroups apparent in hypertension, and which cardiovascular parameters can discriminate these subgroups?

3.2 Study methods specific to this chapter

3.2.1 Blood pressure (BP) measurement

Office and ambulatory 24 hour BP measurements were performed as outlined in the Methods section (Chapter 2.0). BP variability was represented by standard deviation (SD) in an individual's BP readings in mmHg. Heart rate range was also recorded as a measure of cardiovascular variability.

3.2.2 Pulse wave analysis (PWA)

PWA using SphygmoCor XCEL (ATCOR Medical, West Ryde, Australia) captures characteristics of the pulse waveform relating to arterial stiffness with a non-invasive technique. Testing was performed supine after 10 minutes of rest. An appropriately sized cuff was placed on the upper arm and
the SphygmoCor automatically acquired two brachial BP readings with a one-

minute interval. The cuff then partially inflated to capture the brachial artery waveform for 10 seconds based on volumetric assessment of air displaced by the arterial pulse; automated analysis generated a PWA report, avoiding inter-rater influences. The report included estimates of central systolic, central diastolic, and augmentation pressures (AP) in mmHg, as well as Augmentation index (AIx) adjusted to 75 beats/min (AIx@75). Augmentation pressure is the ancillary peak superimposed on the systolic waveform and determined by the pressure reflected from the arterial wall i.e. the compliance of the vessel. AIx is influenced by heart rate, correcting to a HR of 75 bpm is therefore standard practice. Two measurements were performed, and integrated quality control analysis judged if measurements met quality control criteria. Both measures were recorded, and the mean was used in data analysis. If the duplicate measure failed on concordance, a 3rd measure was undertaken and the mean of the two closest formed the final value.

3.2.3 Pulse wave velocity (PWV)

An additional non-invasive surrogate marker for arterial stiffness; carotid-femoral pulse wave velocity was determined using the SphygmoCor XCEL (ATCOR Medical, West Ryde, Australia). With the participant supine and rested, a BP cuff of appropriate size was applied to the right thigh as close to the femoral artery as possible, detecting volumetric displacement of air related to arterial pulse volume. Three distance measurements were entered into the software: supra-sternal notch to the thigh cuff, the palpable femoral artery at the groin to the thigh cuff, and the carotid artery to the supra-sternal notch; a validated measurement technique.[208] The pressure probe was placed over the right common carotid artery at the measured distance to identify the pulse wave. Once a sufficient quality and duration (10 seconds) of pulse waveforms were detected at the carotid artery, the leg cuff automatically inflated. Both pressure waves were captured simultaneously based on the systolic upstroke, referred to as the 'foot' of the pressure wave, as determined by integral software. These data formed the basis for automated PWV calculation, reported in meters per second (m/s). A quality control analysis generated by the software was also recorded, indicating pulse-to-pulse variability. PWV was repeated to generate two values, assessed

for concordance; if results differed by >1m/s or quality control was not met then PWV was repeated and those data with confirmed quality control or greatest concordance were recorded. PWV is influenced by BP, this was dealt with in multiple regression models through inclusion of BP parameters.

3.2.4 Flow mediated dilatation (FMD)

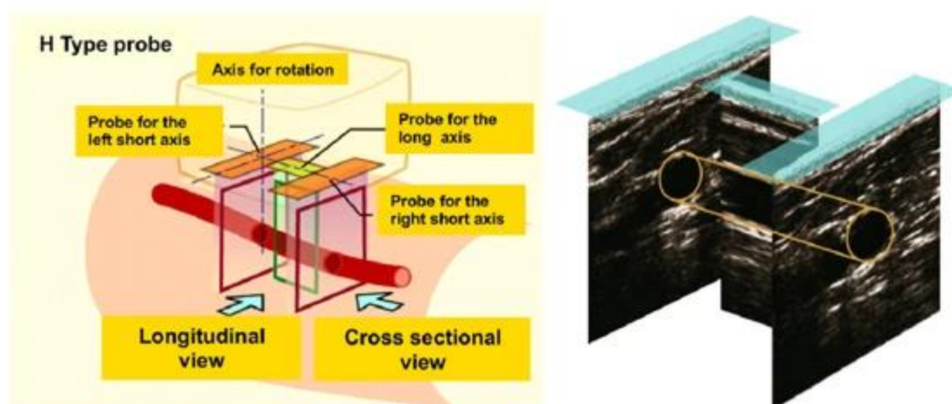


Figure 3.0. UNEX H-shaped probe displays longitudinal and cross-sectional ultrasound images simultaneously. Reproduced from Iguchi, T., Takemoto, Y., Shimada, K. et al. Simultaneous assessment of endothelial function and morphology in the brachial artery using a new semiautomatic ultrasound system. Hypertens Res, 2013; 36, 691–697.

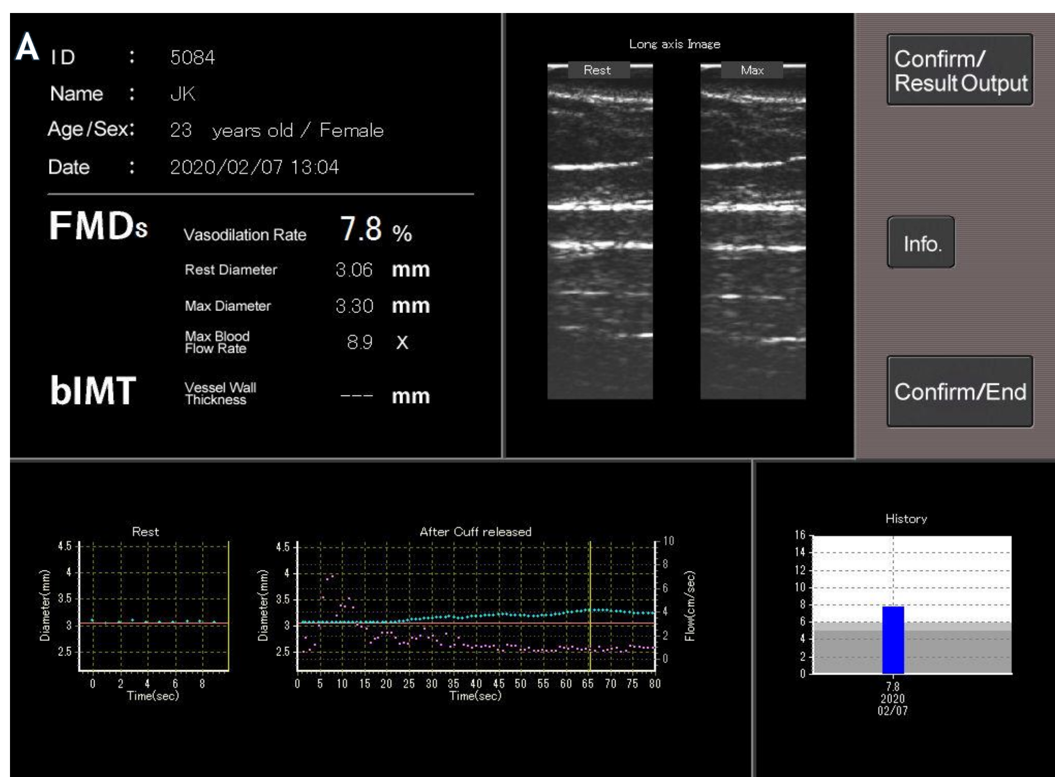
UNEXEF; Unex, Nagoya, Japan) provides one longitudinal and two short-axis images simultaneously using a 10 MHz H-type probe (Figure 3.0). The ultrasound probe was held in place by a positioning arm with capacity for automated adjustments to optimise image quality. Participant lay supine after a minimum 15 minutes rest and minimum 10 minutes since any BP measurements or cuff occlusions, with the dominant arm outstretched and supported at the wrist and humerus. A rapid-inflator/-deflator blood pressure cuff was placed on the forearm distal to the ultrasound probe. The brachial artery was identified in the short axis: the automated tracking system then optimised the probe position. A baseline BP was recorded on the contralateral arm, and pulse detection clasps were placed on both wrists. A baseline image of the brachial artery was obtained for 3min, and flow velocity recorded. The cuff was inflated to 50 mm Hg above the systolic pressure, maximum 200 mmHg for 5min. The cuff was subsequently deflated rapidly, and a further minute of measuring commenced. Sources of variation such as duration of occlusion and position of cuff and ultrasound probe were minimised through

adherence to published guidelines and manufacturers' instructions (UNEX Corporation, Nagoya, Japan), where additional methodological detail is available.[209]

Images were reviewed throughout and if automated analysis failed to accurately detect the vessel wall, potentially giving an inaccurate result, the clip was analysed separately, and manual adjustments made to identify the intima and permit accurate measurements. Analysis was performed with reference to both A mode (wave pattern) and B mode (image), Figure 3.1. Baseline diameter and absolute change in diameter were also recorded, and percent FMD was automatically generated from integrated software as:

% FMD = (peak diameter – baseline diameter) / baseline diameter.

Automated measurement of baseline diameter was calculated during end diastole (identified by the R wave on the ECG), averaged over 10 cardiac cycles.



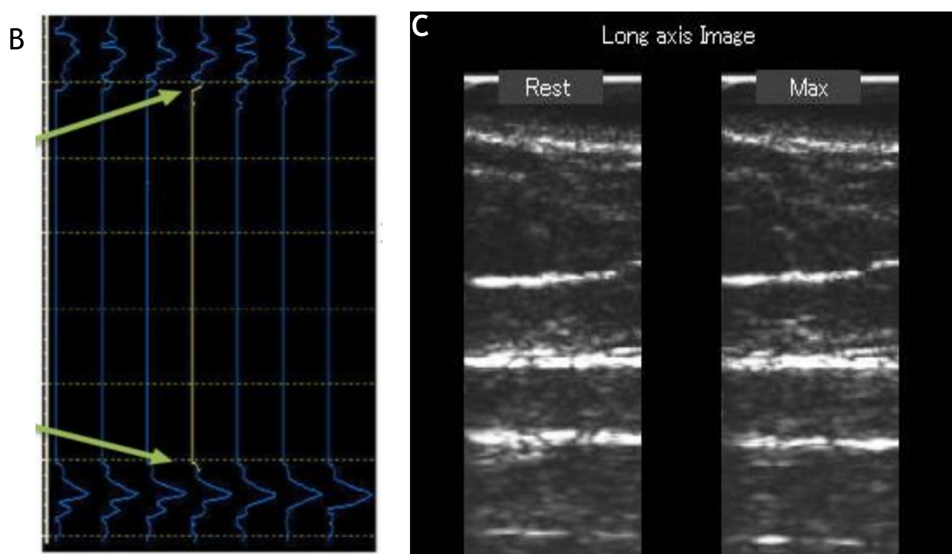
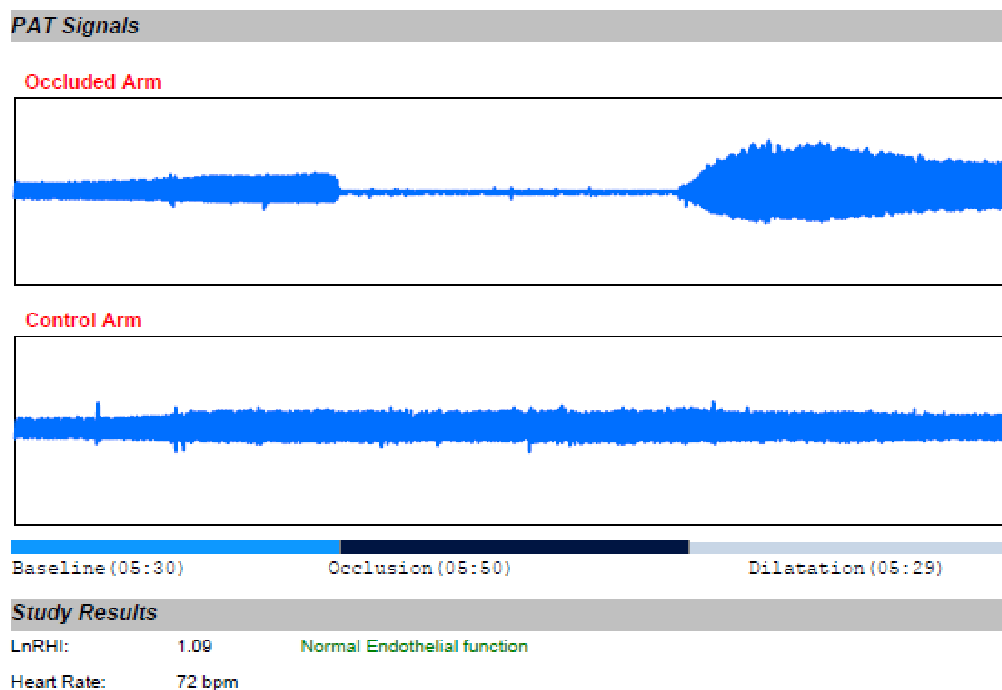


Figure 3.1. Flow mediated dilatation (FMD) by UNEX. Panel A is an example image of results screen. Panel B: A mode illustrating measurement by wave pattern. Panel C: is B mode with an ultrasound image of the artery.

3.2.5 Peripheral arterial tonometry (PAT)

The EndoPAT^{TM} 2000 Device (Itamar, Israel) was used to quantify PAT as a measure of endothelial function. Standardised procedure involved a controlled environment as described in Methods (Chapter 2.4). A BP cuff was on the participant's non-dominant upper arm and plethysmography probes connected to the device on both index fingers while the participant lies supine. Probe-holders ensured correct finger position during probe inflation, following which arms were placed on arm rests. False nails occasionally led to failure of the technique. Baseline recordings of PAT were taken for 5 minutes whilst remaining supine, then for precisely 5 minutes with the blood pressure cuff inflated to supra-systolic pressure (50 mmHg above office SBP recorded earlier in the study visit), and finally for 5 minutes upon release of the blood pressure cuff occlusion. Analysis of the post-ischemic vascular responsiveness i.e. the 'reactive hyperaemia index' (RHI) was based on the amplitude of the PAT signal and the ratio of the signal pre- and post-occlusion as illustrated in Figure 3.2, using integrated software analysis. Confounding (non-endothelial) influences on vascular tone were controlled for by use of the contra-lateral arm. RHI was log transformed to LnRHI to generate a more normal data distribution. LnRHI > 0.51 was considered normal, see Figure 3.2. Mean pulse rate captured during the technique was recorded. Augmentation Index (AIx) was based on automated analysis of multiple pulse waveforms obtained during

the baseline measurement period, see Figure 3.3; AIx was adjusted to heart rate of 75 beats per minute (AI@75) to avoid rate-related confounding. Participants with Raynaud's' disease did not undertake EndoPAT due to concerns of validity and accuracy in individuals with this condition.



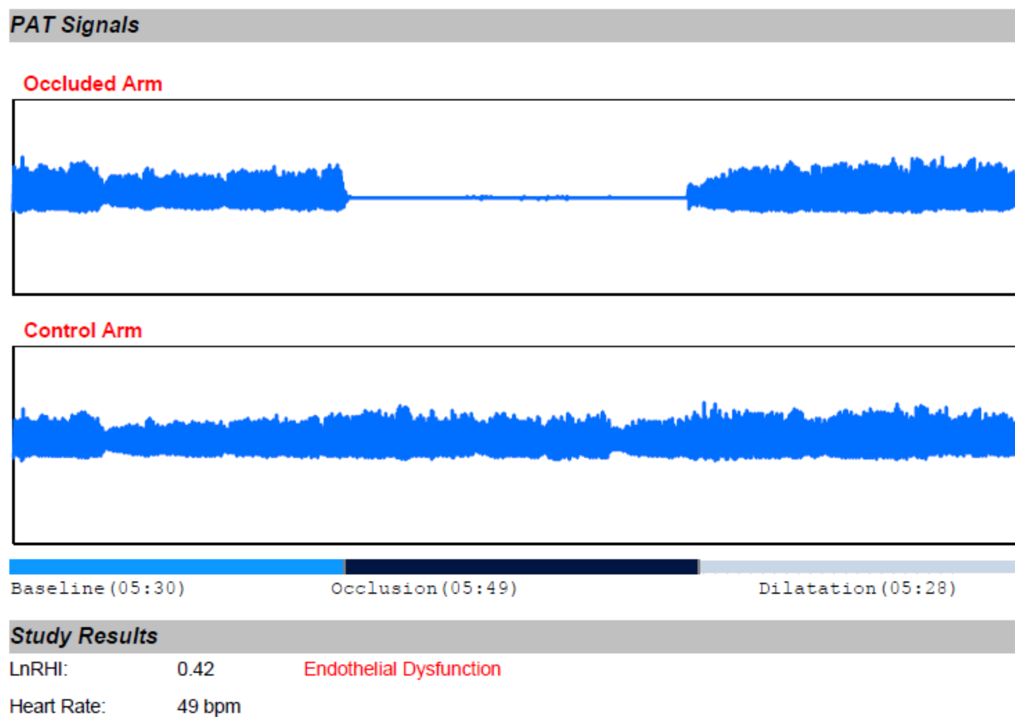


Figure 3.2. EndoPAT-2000 result sheet for LnRHI, with control and occluded arm indicated, periods of baseline, occlusion and dilatation quantified, and LnRHI >0.51 indicating normal endothelial function (Panel A) and <0.51 indicating endothelial dysfunction (Panel B).

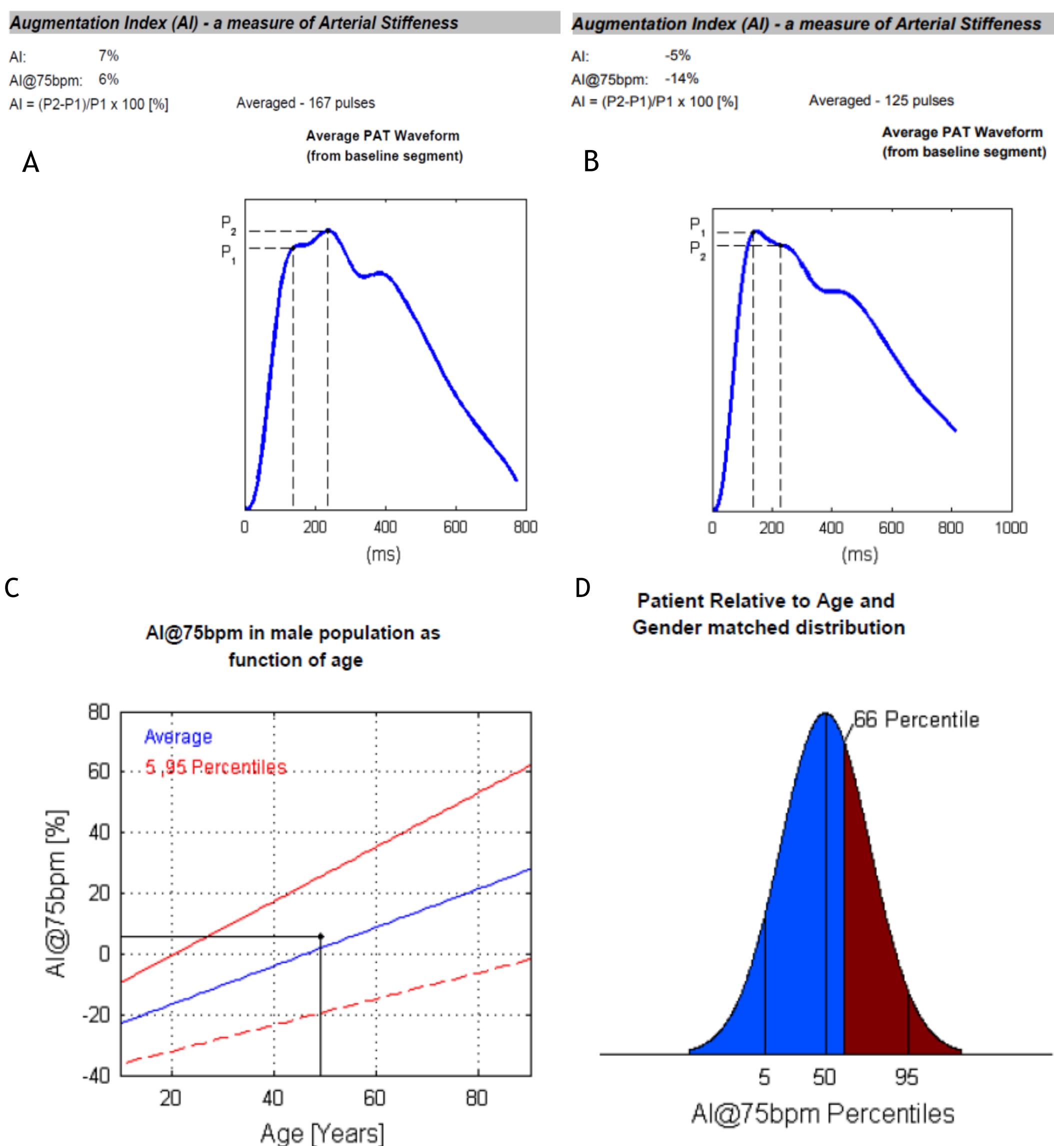


Figure 3.3. EndoPAT-2000 result sheet for AI@75 (Augmentation index adjusted to 75 beats per minute) based on pulse waveform and mean heart rate. Panel A an example within normal range, Panel B an example of abnormal. AI@75 value relative to the age-appropriate normal range (Panel C), and age and gender adjusted percentiles (Panel D).

3.2.6 Intima media thickness (IMT)

B-mode ultrasonography of the right carotid artery was performed with Acuson Sequoia c512 (Siemens AG, Germany). Detailed procedural guidelines have been published elsewhere, [218] but in summary, the patient was positioned supine, head extended and rotated slightly to the left. On a longitudinal image of the common carotid artery, the vessel wall was visualised at both 90 and 135 degrees with the carotid bifurcation (the ‘bulb’) included for reference, and the images saved in reference to the R wave of

the ECG corresponding to end-diastole (static images and video clips). IMT was measured on high resolution 1.2 x 1.2 cm image, as the distance between lumen-intima interface and the media-adventitia border, as illustrated in Figure 3.4. Analysis was performed off-line in batches, by a single (blinded) assessor, to minimise variation in analysis technique, utilising Carotid Studio (QUIPU, Version 3.6.0, 2019, Italy) software designed specifically for this purpose with multiple data points along the wall of the vessel captured and an average measurement generated.

Inter-observer variability was assessed by both sonographers measuring one in 10 CIMTs, blinded to the result of the other operator. The duplicate measures demonstrated sufficient consistency for assurance of quality control (r=0.90).

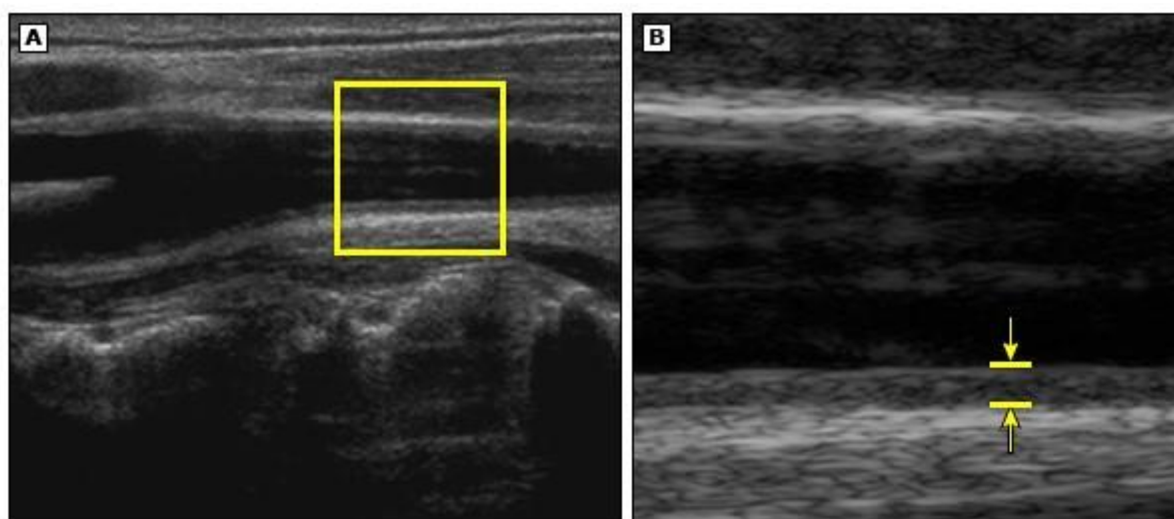


Figure 3.4. Carotid artery intima-media thickness assessment. Panel A demonstrates the site of measurement of the common carotid artery, proximal to the bifurcation of the internal and external carotid arteries. Panel B illustrates the intima-media thickness measurement, performed in triplicate.

3.2.7 Physical activity questionnaire

Self-reported physical activity was assessed using the validated open access "International Physical Activity Questionnaire (IPAQ; short version)".[212] Automatic calculation of data from IPAQ (available freely from the website [212]) was used to reduce human error risk. Appendix 3 contains the questionnaire. The final score was expressed as MET-min per week:

[MET level x minutes of activity x events per week]

3.2.8 Cardiovascular risk

The InterHeart Risk Score Questionnaire was employed, as described in the InterHeart study, as a scoring system to predict cardiovascular events.[213] Participants self-reported responses were entered directly into the online calculator with electronic summation of accrued points. The output is a score from 0 to 48 for future risk of myocardial infarction. Appendix 3 contains the questionnaire and scoring.

3.2.9 Statistical approaches

General data analysis approaches have been outlined in Section 2.6. For multivariable regression, missing ABPM data (n=6) were imputed using office BP values adjusted for the mean difference between office and ABPM values. Confounding variables included in adjusted models included sex, age, BMI and physical activity score (IPAQ). Analysis of variables' discriminatory value in identifying categorical subgroups of white-coat and masked hypertension employed Minitab Classification and regression tree (CART) to create a decision tree. This unsupervised approach generates a model to explain classification of cases in the form of "decision tree", hence determines thresholds by which subgroups are best classified.

Within the hypertensive group, distinct phenotypes were identified using Uniform Manifold Approximation and Projection (UMAP) [219] as a dimensionality reduction technique, combined with Spectral Biclustering. To permit these techniques, variables that were highly correlated or with a substantial number of values missing were removed, giving 19 features in total. Missing data points were imputed from within-sex group median values, and data scaled to a columnar mean of 0 and standard deviation of 1 (z-score). Taking each in turn, UMAP learns the manifold structure of data by graphing neighbouring samples, where edge weights reflect similarities (or distances) between nodes according to a given metric (e.g. Euclidean distance). The embedded data is then projected to lower-dimensional space while preserving its topological structure with k-means algorithm. This partitions data into k groups, aggregating the most similar samples. Data centroids are determined iteratively, first initialising centroids randomly, assigning each data point to

the nearest one, and then correcting their positions to minimise within-cluster variances.

The optimal number of clusters to capture distinct groups was defined by Silhouette score.[220] This measure assesses the quality of data clustering when ground-truth labels are unavailable. The value ranges from -1 to 1, with higher scores when clusters are well-separated and dense. Shapley (SHAP) values reveal the gains and costs to measure the contribution of features to assignment to a particular class, allowing further interpretation of a decision made by a model. These were computed to better interpret discriminating features for each of the UMAP clusters. [221]

Spectral biclustering approach [222] captures homogenous patterns appearing in the data. This technique assumes the data has a checkerboard structure, with the number of partitions in both dimensions as input. Each row is thus assigned to the same number of biclusters as the number of column partitions, and vice-versa. While classical clustering focuses on detecting 'global' similarities based on all features, biclustering reveals patterns containing 'local' subsets of features and subgroups of patients. Differences between groups identified in biclustering analysis were assessed with box-plots and ANOVA.

To interpret the model, the optimal settings were identified using a grid of different parameters, including different UMAP metrics (Euclidean, Manhattan, Canberra, Correlation), the number of sample neighbours for UMAP (4, 5, 6, 7, 8, 9, 10), numbers of dimensions in reduced space for UMAP (2, 3, 4, 5, 7, 10), and the number of clusters for k-means (3, 4, 5). Min_dist parameter of UMAP was set to 0 in order to improve clustering. The quality of clusterings for particular settings was assessed using Silhouette Score. Using dimensionality reduction technique (UMAP) was aimed at improving separation of the clusters. The compromise between the interpretability of the model and training accuracy is maintained by using a single tree with depth up to 6. This low complexity model obtained nearly 100% accuracy and reflected underlying structures in the data.

3.2.10 Data checks

Prior to undertaking data analysis, assessment was made for effect measure modification of variables (also known as confounding). Histograms and stratified statistical analyses included male versus female sex, dichotomised age, dichotomised BMI, and dichotomised physical activity (Table 3.0). Variables where data were not normally distributed, as determined by histograms and Anderson-Darling test for normality, were logarithmically or square-root transformed, see Table 3.1. These findings determined the use of most appropriate statistical test for data analysis i.e. T-test for normally distributed data and Mann-Whitney or Moods' test otherwise.

Age and sex appear to have a modifying effect on measures of augmentation index. Age also influenced cIMT, PWV and SCP, all $P<0.05$, whilst sex influenced LnRHI, IPAQ, and SBP SD, all $P<0.05$. BMI demonstrated an association with various BP parameters (Table 3.0), for example office BP 129/82mmHg in low BMI subgroup versus 140/89mmHg in high BMI subgroup, $p<0.001$; % FMD, cIMT, and PWV also demonstrated association with BMI (all $P<0.05$), see Table 3.0.

Physical activity only reached statistical significance for office SBP and measures of augmentation index. Factors demonstrated as having effect measure modification were included in multiple regression analyses. Consideration was given to other potential confounders; firstly, the effect of medications not specified as exclusions but with potential to influence results. Inhaled steroids (n=3) all demonstrated negative values for AIx/AI@75, this was likely due to chance as studies report no association.[223] Other medications in use by participants included inhaled salbutamol (a beta agonist) and anti-depressants; neither showed any obvious pattern with vascular parameters. Secondly, stage of the menstrual cycle has been

Variable	Sex	Age	BMI	Physical activity	Anderson-Darling P value	Transformation of data
BMI	NS	NS	N/A	0.047	NS	
Age	NS	N/A	NS	NS	0.018	Unchanged (log/square root transformation not effective)
Office SBP	NS	NS	<0.001	0.04	0.14	
Office DBP	NS	NS	0.001	NS	NS	
% FMD	NS	NS	0.014	NS	<0.005	Corrected by log transformation
LnRHI	0.048	NS	NS	NS	NS	Value already logarithmic
AI %	NS	<0.0001	NS	NS	<0.005	Linear increase converts to positive integers, then log transformation
Mean HR	NS	NS	NS	NS	NS	
AI@75 %	<0.001	<0.001	NS	0.05	<0.005 (0.047 F, 0.005 M)	Linear increase to convert to positive integers, then log transformation
cIMT	NS	0.014	0.026	NS	NS	
PWV	NS	0.008	0.002	NS	<0.005	Corrected when 2 outliers removed
AIx	<0.001	<0.001	NS	0.001	0.022 (<0.005 F, 0.78 M)	Linear increase to convert to positive integers; transformation not effective
SCP	NS	0.02	<0.001	NS	NS	
DCP	NS	NS	0.009	NS	NS	
IDQ	NS	NS	<0.001	NS	<0.005	Unchanged (log/square root transformation not effective)
IPAQ	0.042	NS	NS	N/A	<0.005	Corrected by log transformation
SBP24	NS	NS	<0.0001	NS	NS	
DBP24	NS	NS	0.003	NS	NS	
SBP SD	0.044	NS	NS	NS	<0.005	Corrected by log transformation
DBP SD	NS	NS	0.020	NS	<0.005	Corrected by log transformation
dip SBP %	NS	NS	0.003	NS	NS	
dip DBP %	NS	NS	0.017	NS	NS	
HR range day	NS	NS		NS	<0.005	Corrected by log transformation
HR range night	NS	NS	0.05	NS	<0.005	Corrected by log transformation

Table 3.0. Stratified analyses to assess for effect measure modification and distribution of data, with any transformation of the data undertaken to correct. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; cIMT, carotid IMT; LnRHI, natural logarithmic scaled reactive hyperaemia index; AIx, augmentation index; AI@75%, AIx adjusted for heart rate; SD, standard deviation; HR, heart rate; PWV, pulse wave velocity; PWA, pules wave analysis; SCP systolic central pressure; DCP, diastolic central pressure; IDQ, interheart diet score; IPAQ, International Physical Activity Questionnaire; SBP24, 24 hour average SBP; DBP24, 24 hour average DBP; SBP SD, standard deviation of SBP readings i.e. BP variability; DBP SD, standard deviation of DBP readings; HR, heart rate; M, male; F, female.

hypothesised to influence measures of arterial function including LnRHI, AI@75%, and FMD, and was thus assessed for potential confounding.[224] Mean and SD did not differ by stage of the menstrual cycle for LnRHI (p=0.75) or FMD (p=0.17). AI@75% did demonstrate differences in mean values (day 1-7 17.9% ±20.7, day 8-14 -0.33±17.8, day 15-28 14.1±14.6), though demonstrated borderline statistical significance (p=0.05). This is in line with meta-analysis concluding that the menstrual cycle has a small effect on macrovascular but not on microvascular endothelial function.[225]

Finally, smoking prevalence was low (Table 3.1), further analysis of 'ever-' versus 'never-smoked' also demonstrated no between group differences (p=0.891); nor was smoking associated with any of the following variables through regression modelling with hypertension status: % FMD, LnRHI, AIx@75, PWV, AIx by PWA, cIMT or central BP (all P<0.05). These factors are not therefore included in analyses that follow.

3.3 Results

3.3.1 Participant demographics and blood pressure parameters

Inflammatension patient cohort, illustrated in a flow chart

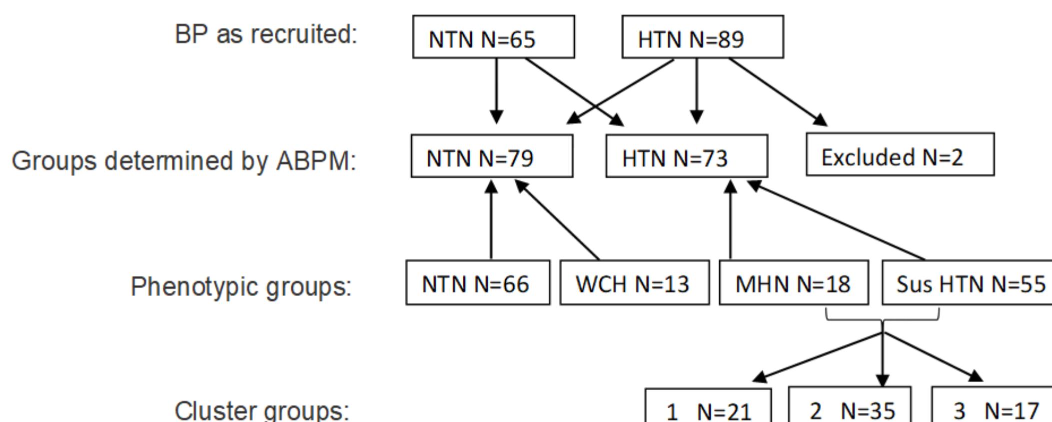


Figure 3.5. Flow chart of participant numbers as recruited, and as assigned to subgroups based on ambulatory blood pressure values (ABPM) for vascular analyses. Participant numbers for flow cytometry and biomarkers are reported in Chapters 4 and 5 respectively. NTN, normotension; HTN, hypertension; N, number

of participants; WCH, White-coat hypertension; MHN, masked hypertension; Sus HTN, sustained hypertension.

Figure 3.5 demonstrates participants as recruited, based on reported or screening office BP, exclusions, and final subgroupings. It illustrates that despite efforts to recruit discreet hypertensive and normotensive groups, office BP values at screening were often not confirmed on the later ABPM. As a result, the distribution of participant BP is continuous, as Figure 3.6 illustrates.

To answer the research question: "Do measures of cardiovascular function differ between young, incident patients with hypertension but no overt cardiovascular disease, versus healthy controls?" participants were dichotomised into normotensive and hypertensive groups (24 hour ABPM above 130 and/or 80 mmHg or daytime ABPM above 135 and/or 85 mmHg). Comparisons of the BP data of these groups follows.

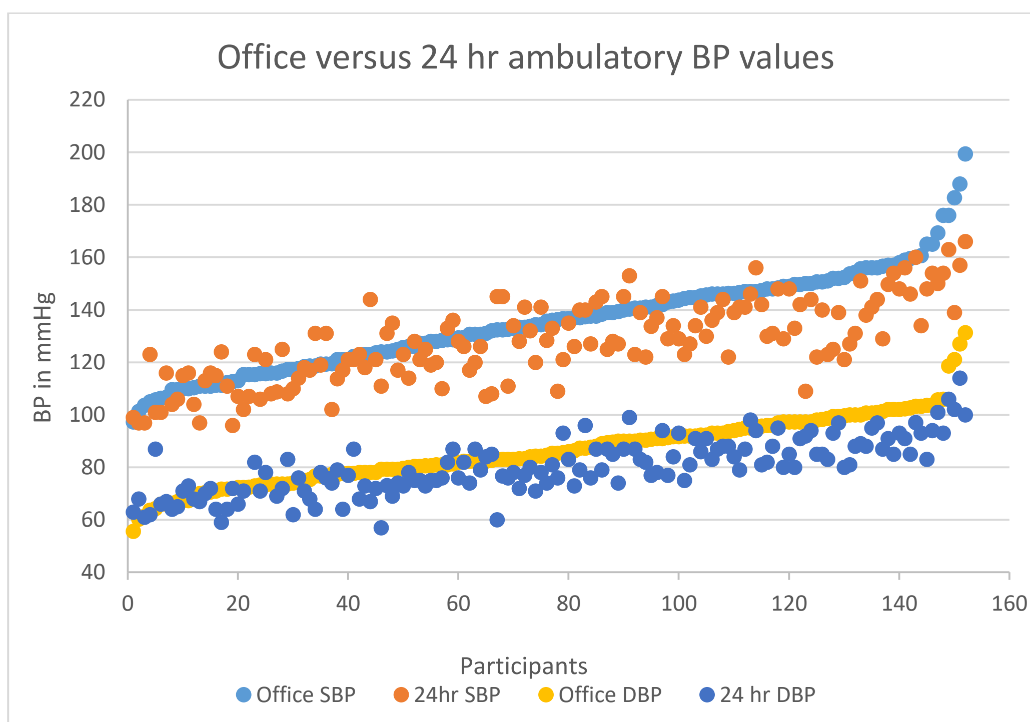


Figure 3.6. Scatter plot of office BP values, and subsequent 24 hour average ABPM values. Participant numbers ordered by office blood pressure values (X axis), and BP (blood pressure) in mmHg (Y axis). SBP, systolic BP; DBP, diastolic BP; 24hr, 24 hour average.

	NTN =79	HTN =73	P value
Male sex (%)	41 (52)	41 (56)	0.598
BMI, kg/m2, mean (SD)	26.3 (4.8)	28.8 (4.1)	0.001
Age, years, median (IQR)	39 (12)	39 (14)	0.83
SBP, mmHg, Mean (SD)	124.6 (13.9)	146.8 (15.3)	<0.0001*
DBP, mmHg, mean (SD)	80.0 (10.5)	93.0 (11.8)	<0.0001*
BAME Ethnicity N (%)	8 (11.0)	11 (15.1)	0.50
Smoking N (%)	6 (8.2)	1 (1.4)	n/a
SBP24, mmHg, mean (SD)	115.7 (8.9)	141.1 (9.0)	<0.0001*
DBP24, mmHg, mean (SD)	72.7 (7.0)	88.1 (7.9)	<0.0001*
SBP SD, mmHg, median (IQR)	9.6 (2.7)	11.4 (3.0)	<0.0001*
DBP SD, mmHg, median (IQR)	8.0 (4.1)	9.0 (3.6)	0.07
SBP day, mmHg, mean (SD)	119.7 (9.5)	145.4 (8.7)	<0.0001*
DBP day, mmHg, mean (SD)	76.4 (7.4)	91.5 (7.7)	<0.0001*
SBP night, mmHg, mean (SD)	102.9 (10.5)	124.7 (13.3)	<0.0001*
DBP night, mmHg, mean (SD)	61.9 (7.9)	74.2 (11.1)	<0.0001*
% dip SBP mean (SD)	11.7 (5.3)	12.0 (6.3)	0.75
% dip DBP mean (SD	15.5 (6.2)	12.0 (6.3)	0.66
% group with nocturnal dip	43 (58.9)	44 (60.3)	0.57
HR range day, median (IQR)	40 (26.5)	34 (13.3)	0.18
HR range night, median (IQR)	22 (15)	19 (11.5)	0.79

Table 3.1 Demographic results by blood pressure group. HTN, hypertension based on 24-hour systolic blood pressure (SBP) above 130 mmHg or daytime SBP above 135 mmHg; NTN, normotension with BP values below these thresholds; DBP, diastolic blood pressure; BAME, Black Asian and minority ethnic; SD, standard deviation; HR, heart rate. Two-tailed students T-test or Mann-Whitney test if non-parametric data. * Indicates statistical significance between the groups was maintained following adjustment for BMI as the only demographic variable to differ between groups (regression analysis with BP as a categorical response).

Table 3.1 demonstrates the clear separation of groups with regard to BP parameters. Measures of cardiovascular variability were discordant, with SBP standard deviation (SD) higher in hypertensive individuals, nocturnal BP dipping and HR range showing no between-group differences. Table 3.1 also demonstrates that groups were well matched for age and sex. BMI did differ between the groups by 3.5 kg/m^2 and was included in adjusted analyses of between group differences, as were age and/or sex and/or physical activity where analyses presented in Table 3.0 had demonstrated potential for confounding. Statistical significance in BP separation between the groups was maintained following this adjustment (indicated by an asterix on Table 3.1); other variables remained not significant despite adjustment.

3.3.2 Vascular function: hypertension versus normotension

Having confirmed the groups separated on BP variables, comparisons of vascular measures were undertaken to answer the first research question: “Do measures of cardiovascular function differ between young, incident patients with hypertension but no overt cardiovascular disease, versus healthy controls?”

	NTN =79		HTN =73		P value	
CIMT mm mean (SD)	0.55	(0.12)	0.54	(0.12)	0.58	T-test
FMD % mean (SD)	4.9	(4.25)	6.1	(4.05)	0.40	MW test
Rest diameter mean (SD)	3.94	(0.74)	4.2	(0.77)	0.07	T-test
Max diameter mean (SD)	4.26	(0.83)	4.5	(0.76)	0.04	T-test
LnRHI mean (SD)	0.69	(0.28)	0.78	(0.31)	0.06*	T-test
AI% median (IQR)	6	(18)	14	(31)	0.06	Mood's test
HR mean (SD)	63.1	(10.2)	67.1	(10.6)	0.02*	T-test
AI@75% median (IQR)	-1.00	(24.2)	9.00	(28.5)	0.01*	Mood's test
PWV m/sec mean (SD)	6.57	(1.29)	7.50	(1.7)	<0.0001*	T-test
PWA AIx mean (SD)	9	(28.0)	11.75	(27.3)	0.05*	MW test
SCP mmHg mean (SD)	113.1	(12.5)	133.0	(14.0)	<0.0001*	T-test
DCP mmHg mean (SD)	77.9	(9.5)	93.0	(10.6)	<0.0001*	T-test
IDQ median (IQR)	7.0	(8.0)	12.0	(6)	<0.0001	MW test
IPAQ median (IQR)	2706	(2629)	1896	(1402)	0.01	MW test

Table 3.2. Vascular results by blood pressure groups. HTN, hypertension based on 24 hour systolic blood pressure (SBP) above 130 mmHg or daytime SBP above 135 mmHg; NTN, normotension with BP values below these thresholds; MW test, Mann-Whitney U test; DBP, diastolic blood pressure; cIMT, carotid IMT; Rhi, reactive hyperaemia index; AIx, augmentation index; AI@75%, AIx adjusted for heart rate; SD, standard deviation; HR, heart rate; PWV, pulse wave velocity; PWA, pules wave analysis; SCP systolic central pressure; DCP, diastolic central pressure; IDQ, interheart diet score; IPAQ, International *Physical Activity* Questionnaire. * Indicates statistical significance was maintained or attained following adjustment: regression analysis with BP as a categorical response and including confounding variables (age and/or sex and/or BMI [body mass index]) as described in Table 3.1.

Arterial stiffness was greater in the hypertensive group (Table 3.2), both in measures performed on SphygmoCor (PWV, PWA-AIx), and

AI@75 derived from EndoPAT-2000. Arterial stiffness measures remained statistically different following adjustment for confounding variables (age and/or sex and/or BMI) as described in Table 3.0, as did estimates of central arterial pressure (SCP and DCP). Measures of endothelial function (LnRHI and % FMD) and CIMT did not differ between normotensive and hypertensive groups. Following adjustment, LnRHI did reach statistical significance for between group differences (P=0.016), but with higher values (better microvascular function) suggested in the hypertensive group. FMD and CIMT remained non-significant.

However, the continuous nature of BP values has already been discussed, with Figure 3.6 illustrating that our data failed to achieve two distinct BP groups, but rather some participants recruited to either normotensive or hypertensive arms had ABPM results around the BP threshold. To confirm the validity of dichotomising groups based on continuous variables (SBP and DBP), summary statistics were generated to include a 'borderline BP' group, Table 3.3, with analysis of the more distinctly separated normotensive and hypertensive arms.

Table 3.3 demonstrates that removing the 'borderline' 24 hour BP subgroup, does not alter the between-group differences in vascular or demographic parameters. This validates the dichotomisation of data into normotensive and hypertensive. The analysis demonstrated intermediate values for PWV, AI@75 derived from Endo-PAT-2000 and PWA-determined AIx, estimates of central BP, and IDQ for the borderline group; supporting the assumption that these parameters follow a continuum from normotension to hypertension. Considering this continuum, and being cognizant that hypertension is a single diagnosis that can be made based on many different parameters (systolic, diastolic, daytime, night-time, or 24 hour average pressures), the relationship between each vascular variable and BP was analysed in more detail. Table 3.5 reports these correlations, illustrated in Figures 3.7 and 3.8.

	NTN N=64 (42%)	Borderline N=32 (21%)	HTN N=56 (37%)	P value	NTN vs HTN
BMI kg/m^2 mean (SD)	25.9 (4.6)	28.7 (4.3)	28.6 (4.1)	0.001	T-test
Age years median (IQR)	39 (12)	38 (13)	39 (16)	0.83	MW test
Male Sex (%)	31 (48%)	21 (66%)	30 (54%)	0.2	Chi^2
CIMT mmHg, mean (SD)	0.54 (0.11)	0.58 (0.12)	0.53 (0.12)	0.52	T-test
FMD % mean (SD)	4.8 (4.0)	7.25 (6.15)	5.9 (3.8)	0.7	MW test
LnRHI mean (SD)	0.69 (0.29)	0.6 (0.29)	0.79 (0.41)	0.13	T-test
HR Mean (SD)	61 (9.8)	64.6 (21.5)	64.0 (16.3)	0.32	T-test
AI@75% median (IQR)	-2.0 (24.5)	0.0 (23.0)	12.5 (25.0)	0.001	MW test
PWV m/sec, mean (SD)	6.4 (1.2)	7.6 (1.3)	7.5 (1.5)	<0.0001	T-test
PWA AIx, mean	5.8 (22.8)	12.5 (24.5)	16.5 (28.3)	0.015	MW test
SCP mmHg, mean (SD)	110.6 (11.8)	123.7 (14.7)	134.6 (14.7)	<0.0001	T-test
DCP mmHg, mean (SD)	75.4 (8.9)	88.9 (8.4)	93.0 (17.5)	<0.0001	T-test
IDQ median (IQR)	5 (7.0)	11.5 (8.8)	12 (7.0)	<0.0001	MW test
Adjusted IDQ, median (IQR)	5 (6.5)	9.5 (7.5)	11 (5.0)	0.02	MW test
IPAQ median (IQR)	3159 (2801)	1422 (1600)	1908 (1287)	0.01	MW test

Table 3.3. Data divided into tercile groups based on ambulatory blood pressure monitoring. NTN, normotensive (24 hour SBP [systolic blood pressure] <125mmHg); 'borderline' (125-134 mmHg); HTN, hypertensive (24 hour SBP >135 mmHg); MW test, Mann-Whitney U test; DBP, diastolic blood pressure; cIMT, carotid intima media thickness; LnRHI, logarithmic transformation of reactive hyperaemia index; AIx, augmentation index; AI@75%, AIx adjusted for heart rate; SD, standard deviation; HR, heart rate; PWV, pulse wave velocity; PWA, pules wave analysis; SCP systolic central pressure; DCP, diastolic central pressure; IDQ, interheart diet score; IPAQ, International Physical Activity Questionnaire.

3.3.3 Associations between vascular and BP parameters

Consistent with between group comparisons, CIMT showed no correlation with any BP variable. Of measures of endothelial function, % FMD did not correlate with BP variables, and LnRHI demonstrated

only weak correlation (strongest with daytime SBP, r = 0.24) and restricted to hypertensive patients (24hr SBP r = 0.41, 24hr DBP 0.23, both P<0.05), Figure 3.8. Measures of arterial stiffness demonstrated correlation that was stronger for diastolic than systolic BP, consistent across PWV, PWA-AIx as measured by SphygmoCor, and HR-adjusted AI@75 derived from EndoPAT-2000. The pattern was conserved across daytime, night-time and 24 hour average parameters, with strongest correlation for PWV and 24 hour DBP with r=0.46, P<0.0001, see Table 3.4. Figure 3.8 illustrates the key correlations discussed and Figure 3.8 a summary correlation matrix.

	LnRHI	AI@75	% FMD	PWV	PWA-AIx	SCP	DCP	CIMT
SBP24	0.22#	0.34*	0.035	0.40*	0.26#	0.73*	0.75*	0.008
DBP24	0.17#	0.38*	0.029	0.46*	0.37*	0.74*	0.84*	0.044
MAP	0.20#	0.35*	0.032	0.45*	0.34*	0.76*	0.82*	0.029
SBP day	0.24#	0.29#	0.025	0.38*	0.26#	0.73*	0.73*	0.004
DBP day	0.21#	0.38*	0.022	0.43*	0.35*	0.73*	0.82*	0.051
SBP noct	0.17#	0.15#	0.045	0.32*	0.20#	0.60*	0.65*	0.035
DBP noct	0.10	0.30#	0.021	0.42*	0.33*	0.59*	0.72*	0.06
dip SBP %	-0.005	0.18	-0.037	0.038	0.03	0.049	-0.03	-0.043
dip DBP %	0.051	0.04	0.017	-0.09	-0.05	-0.002	-0.08	-0.029

Table 3.4. Pearson correlation (r values) of measures of vascular function with blood pressure (BP) variables.* indicates P<0.001, # indicates P<0.05. SBP24, 24 hour average systolic BP; DBP24, 24 hour average diastolic BP; MAP, mean arterial pressure; SBP SD, standard deviation i.e. variability of systolic BP; DBP SD, standard deviation i.e. variability of diastolic BP; SD, standard deviation; day, daytime average BP; noct, nocturnal average BP; dip, percentage reduction from day to night BP; cIMT, carotid intima-media thickness; RHI, reactive hyperaemia index; AI@75%, EndoPAT-2000-derived augmentation index adjusted for heart rate; HR, heart rate; PWV, pulse wave velocity; PWA, pules wave analysis; SCP systolic central pressure; DCP, diastolic central pressure.

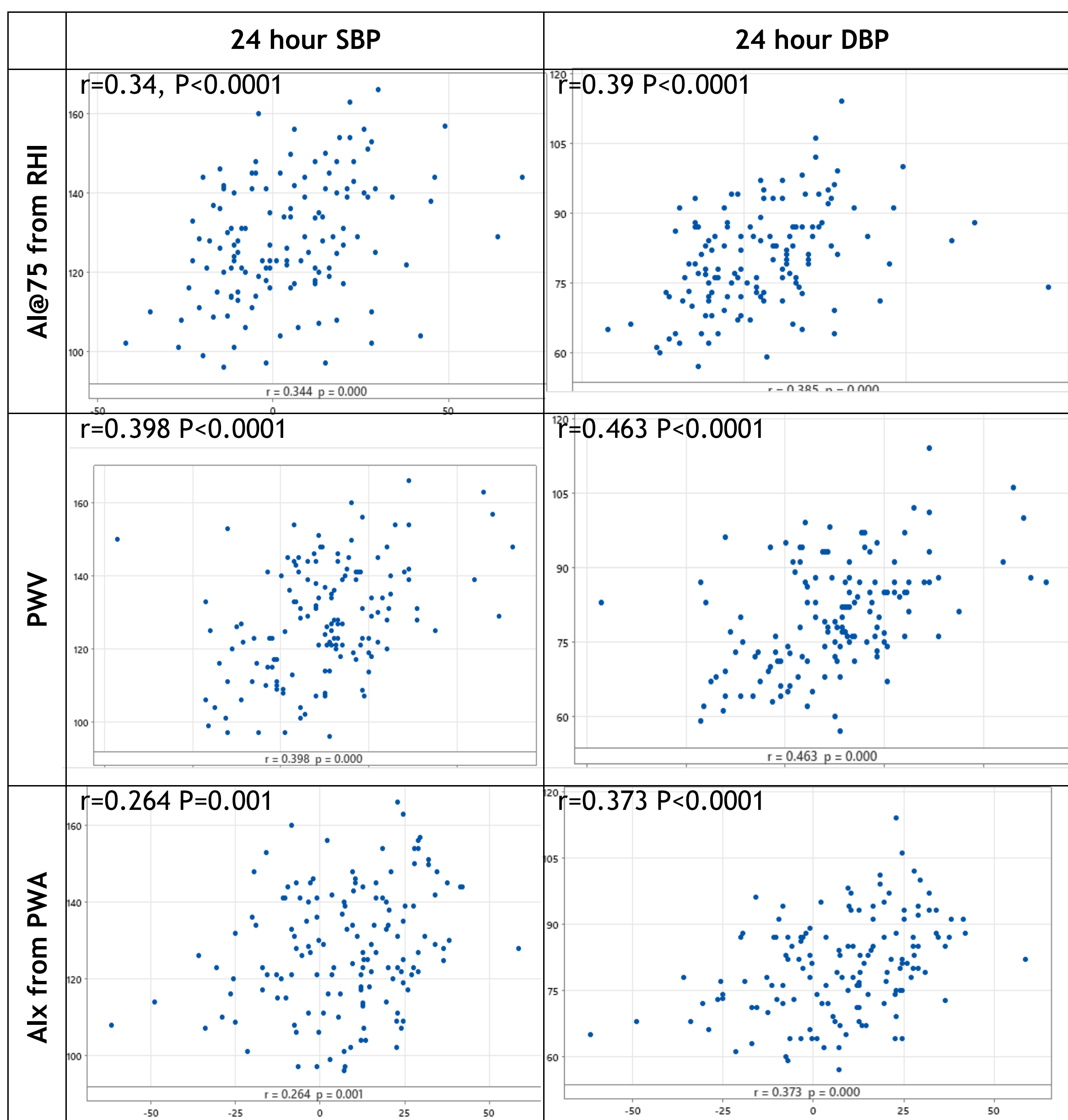
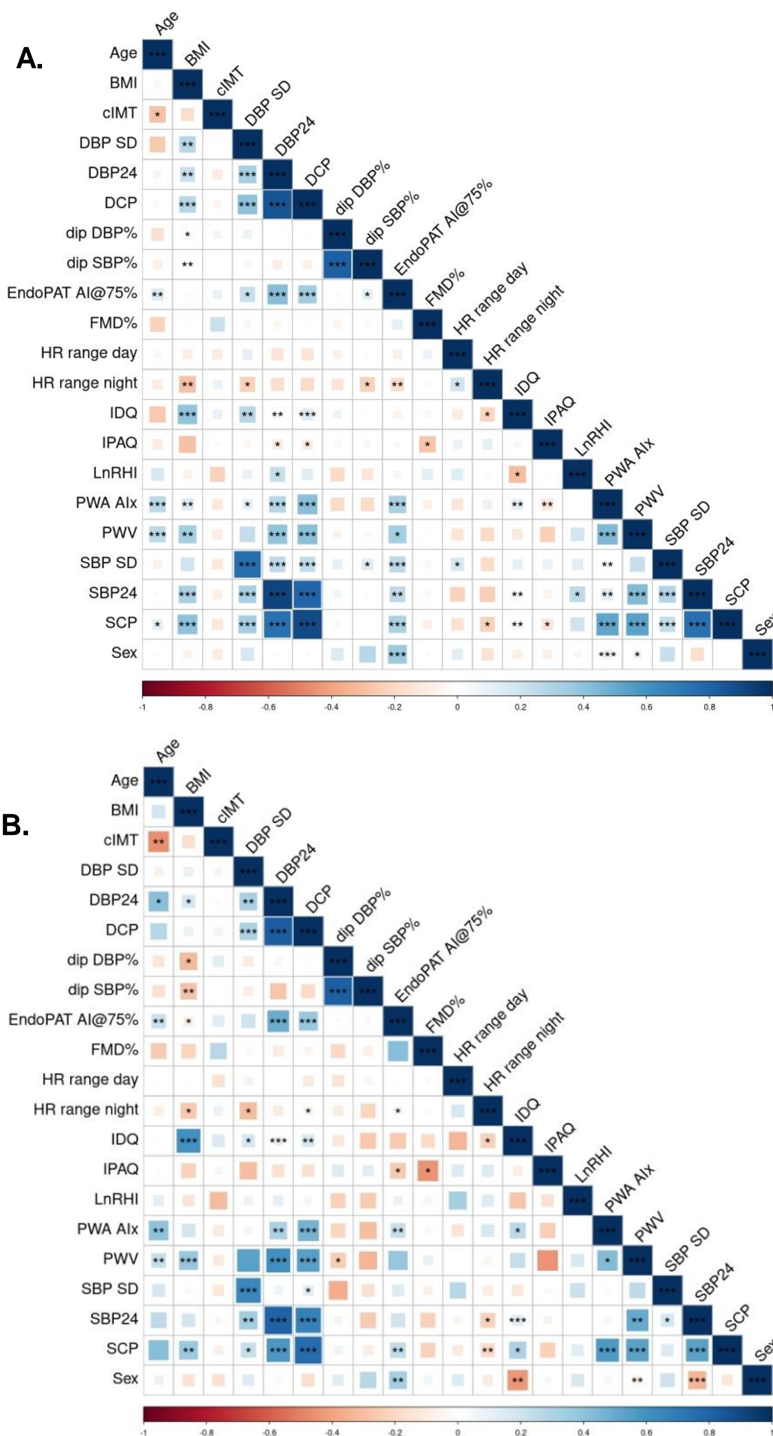


Figure 3.7. Correlation charts for significant findings between vascular indicators of arterial stiffness and blood pressure variables, corresponding to Table 3.4. 24 hour systolic blood pressure (SBP) and diastolic blood pressure (DBP) versus AIx adjusted to heart rate 75 (AI@75), pulsewave velocity (PWV), and AIx derived from pulsewave analysis (PWA).

Figure 3.8 illustrates that correlations were similar between hypertension and normotension for PWV and PWA-AIx, but diverged for AI@75, with 24hr SBP reaching significance only in the hypertensive population. The correlation matrix also highlights that SCP and DCP demonstrate broad association with both arterial stiffness and 24hr BP parameters, preserved across normotensive and hypertensive groups and all statistically significant.

3.3.3.1 Correlation between measures of vascular function

The cardiovascular parameters studied in Inflammatension fall broadly into the categories of BP variables, endothelial function measures, arterial stiffness indices, and atherosclerosis markers. Assessing these in relation to normotension and hypertension does not determine if the strength of the relationship is equal across the range of values e.g. if PWV predicts BP at 5 m/s as well as it does at 12 m/s, or only in hypertension disease state. I explored the associations *between* these variables, split by hypertension status, to answer the second research question: “Do measures of cardiovascular function demonstrate clinically applicable associations?”



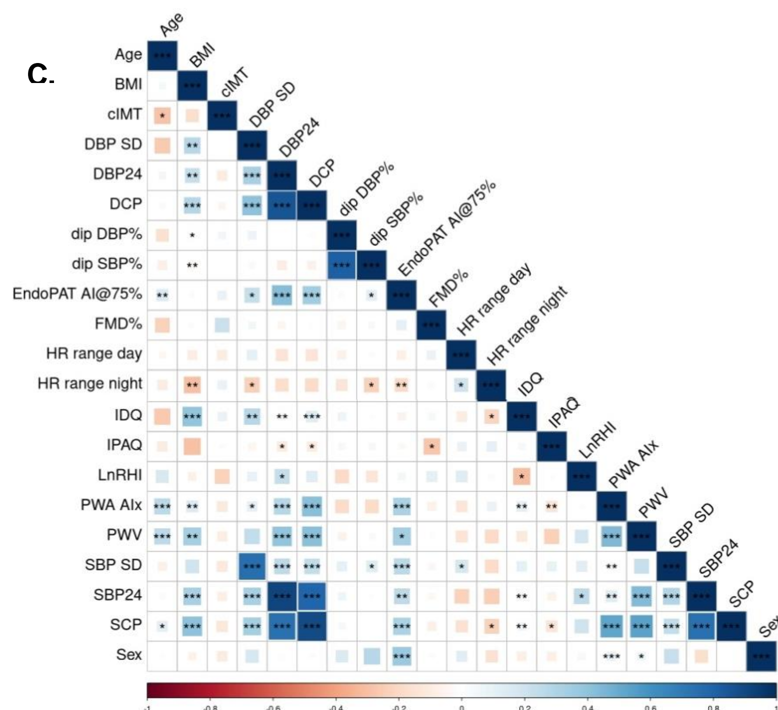


Figure 3.8. Correlation matrix of Cardiovascular and blood pressure parameters in all studied participants (Panel A) and separately by normotension and hypertension groups (Panel B and C respectively), colour indicating the strength and direction of correlation. Colour and colour intensity indicate r values i.e. direction and strength of correlation (red negative correlation, blue positive), as per X axis. *P<0.05 **P<0.01 ***P<0.001.
BMI, body mass index; CIMT, carotid intima media thickness; DBP, diastolic blood pressure; SD, standard deviation; DCP, diastolic central pressure; AI@75%, AIx adjusted for heart rate; FMD%, percent flow-mediated dilatation; HR, heart rate; IDQ, interheart diet score; IPAQ, International Physical Activity Questionnaire; LnRHI, logarithmic transformation of reactive hyperaemia index; PWV, pulse wave velocity; PWA, pulse wave analysis; AIx, augmentation index; SBP, systolic blood pressure; SCP systolic central pressure.

Collinearity was apparent among techniques measuring arterial stiffness, with correlation strongest between AI@75 derived from EndoPAT-2000, and AIx as measured by SphygmoCor (r=0.50, P<0.001). PWV demonstrated weaker association with both (Table 3.4 and Figure 3.8). Regarding measures of endothelial function, % FMD did not correlate with LnRHI across the whole cohort (r=0.05, P = 0.56). Though participants with 'abnormal' LnRHI results (<0.51) did demonstrate higher % FMD (7.5% vs 5.8%, P=0.03). Neither LnRHI nor % FMD correlated with measures of arterial stiffness or CIMT (Table 3.4 and Figure 3.8).

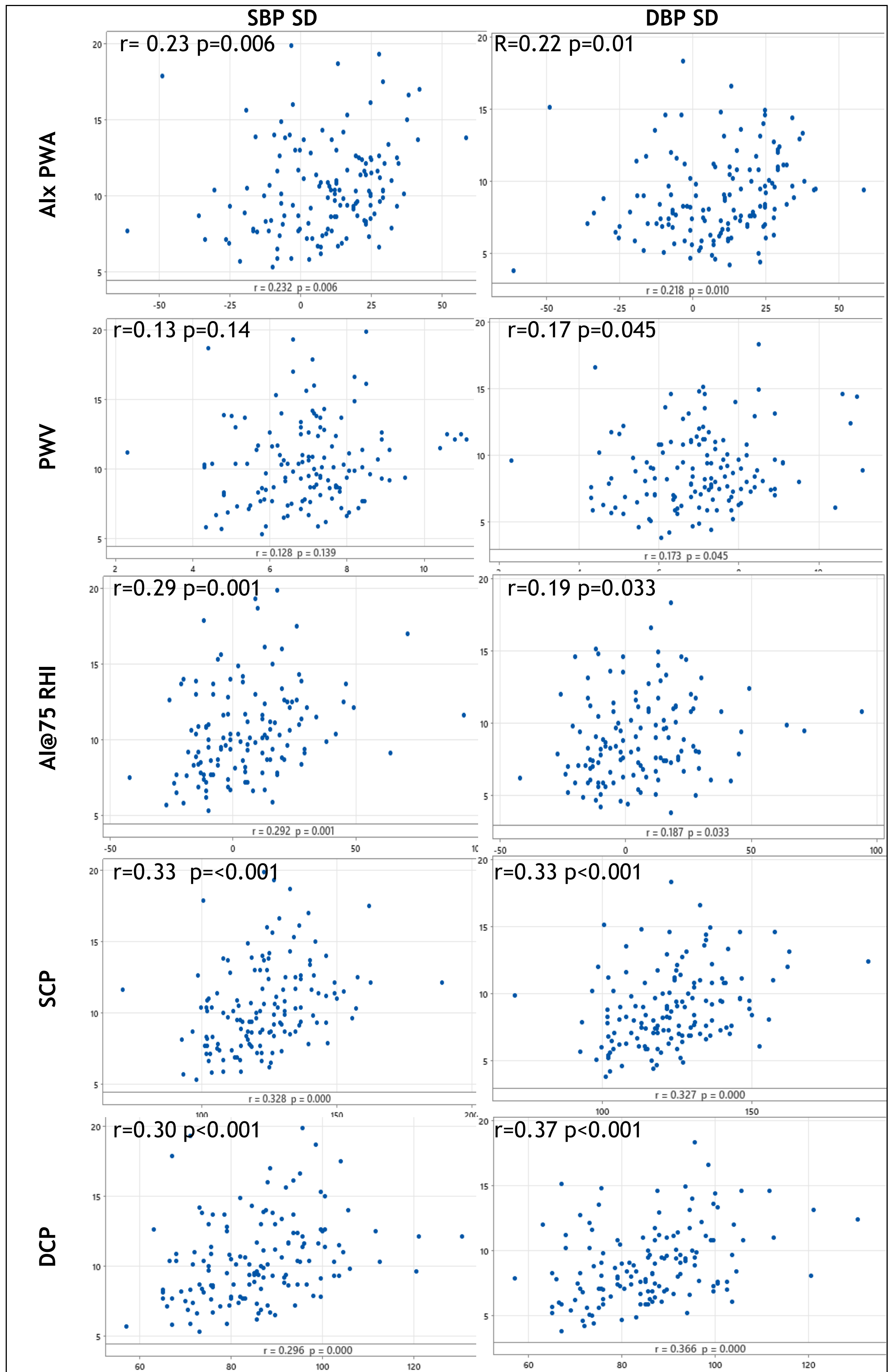

SBP SD
DBP SD
AIx PWA
PWV
AI@75 RHI
SCP
DCP
r= 0.23 p=0.006
R=0.22 p=0.01
r=0.13 p=0.14
r=0.17 p=0.045
r=0.29 p=0.001
r=0.19 p=0.033
r=0.33 p=<0.001
r=0.33 p<0.001
r=0.30 p<0.001
r=0.37 p<0.001
r = 0.232 p = 0.006
r = 0.218 p = 0.010
r = 0.128 p = 0.139
r = 0.173 p = 0.045
r = 0.292 p = 0.001
r = 0.187 p = 0.033
r = 0.328 p = 0.000
r = 0.327 p = 0.000
r = 0.296 p = 0.000
r = 0.366 p = 0.000

Figure 3.9. Systolic and diastolic blood pressure (SBP, DBP) variability as indicated by standard deviation (SD) correlated with measures of arterial stiffness. AIx PWA, augmentation index derived from pulse-wave analysis; PWV, pulse wave velocity; AI@75 RHI, augmentation index adjusted to 75 beats/minute derived from EndoPAT-2000; SCP, systolic central pressure; DCP diastolic central pressure. Strength of correlation (r values) and statistical significance (p values) from Pearson correlation.

3.3.3.2 Adjusted models corroborate early hypertension association with arterial stiffness

Expanding on research questions one and two, multivariable regression explored if the associations reported above remained significant after confounding variables age, sex, BMI and physical activity were entered into the model.

The sequence of events in endothelial dysfunction, arterial stiffening, and hypertension remains incompletely understood, with the possibility of bi-directional interactions. These parameters were therefore considered as either predictor or response in a multivariable regression. Consistent with analyses already reported, neither CIMT nor % FMD demonstrated ability to predict vascular or BP parameters in multivariable regression. Equally, none of the other variables explained the variance in CIMT or % FMD.

In these adjusted models, elevated systolic BP demonstrated augmented endothelial function, with LnRHI as a predictor of 24 hr SBP, DBP, and SCP showing positive coefficients (R^2 0.12 - 0.21, all $P<0.05$, Table 3.5), in opposition to the hypothesis that endothelial dysfunction is associated with hypertension. This is explored in discussion (Section 3.4.2).

Measures of arterial stiffness (RHI-derived AI@75, PWV, and AIx from PWA) all demonstrated high R^2 values when considering the other stiffness variables as the response, also explaining a high proportion of the variation in estimates of central pressures and 24hr BP response. Of the three stiffness indices, AI@75 had the highest R^2 values across each of these responses, see Table 3.5. Conversely, SCP and DCP contributed a high proportion of the variance of measures of arterial stiffness (AI@75 43%, PWV 26%, PWA AIx 40%, all $p<0.001$), and of 24hr BP (SBP 53% and DBP 55%, $p<0.0001$).

With BP variables as predictors and cardiovascular measures as responses; 24hr SBP accounted for a low proportion of the variance in LnRHI (5%); moderate R^2 for AI@75, PWV and PWA AIx (35%, 22%, 27% respectively), and a high proportion in the variance of SCP and DCP (58% and 56% respectively); all P<0.005. Day rather than night-time mean BP, and diastolic rather than systolic BP, demonstrated greater R^2 values with regard to all arterial stiffness measures (R^2 values between 21% and 35%), central and 24 hour BP parameters (R^2 between 41% and 58%). SBP meanwhile demonstrated higher R^2 values for SD, % dip, and HR range variables. The systolic / diastolic difference is illustrated by Figure 3.10.

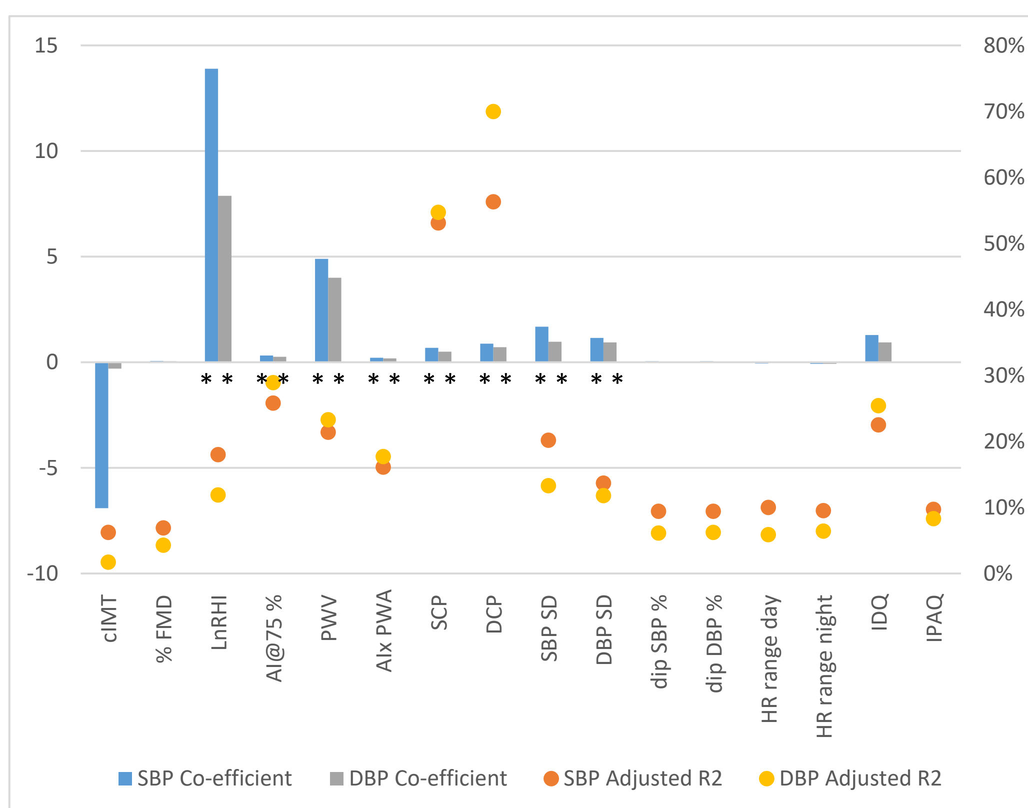


Figure 3.10. Beta coefficients (bars, left axis) for cardiovascular predictors and 24 hour systolic (SBP) and diastolic (DBP) responses, with R2 adjusted values (scatter plots, right axis). Both derived from multivariable regression adjusted for BMI (body mass index), age, sex, and physical activity). P<0.05 indicated by *; % FMD, percent flow mediated dilatation; LnRHI, logarithm of the reactive hyperaemia index; AI@75%, augmentation index adjusted to heart rate of 75 bpm; PWV, pulse wave velocity; PWA AIx, pulse wave analysis derived augmentation index; SCP, systolic central pressure; DCP, diastolic central pressure; HR, heart rate; cIMT, carotid intima-media thickness.

PREDICTORS	% FMD	R^2 adj	LnRHI	R^2 adj	AI@75 %	R^2 adj	PWV	R^2 adj	AIx PWA	R^2 adj
cIMT	-0.04 (3.57)	NS	-0.40 (0.32)	NS	14.3 (19.9)	NS	-1.38 (1.2)	NS	27.7 (20.9)	NS
% FMD	n/a	n/a	0.005 (0.007)	NS	0.33 (0.44)	NS	0.02 (0.28)	NS	0.054 (0.37)	NS
LnRHI	0.86 (1.1)	NS	n/a	n/a	n/a	n/a	-0.27 (0.37)	NS	-6.97 (5.0)	NS
AI@75 %	0.023 (0.02)	NS	n/a	n/a	n/a	n/a	0.019 (0.006)	0.24#	0.34 (0.07)	0.31*
PWV	0.24 (0.27)	NS	-0.016 (0.02)	NS	4.11 (1.3)	0.24#	n/a	n/a	2.88 (1.19)	0.23#
AIx PWA	0.003 (0.02)	NS	-0.0022 (0.0016)	NS	0.39 (0.10)	0.30*	0.014 (0.006)	0.21#	n/a	n/a
SCP	0.0018 (0.023)	NS	0.0036 (0.002)	0.02#	0.65 (0.09)	0.43%*	0.034 (0.007)	0.26*	0.54 (0.08)	0.40*
DCP	0.016 (0.028)	NS	0.0029 (0.002)	NS	0.83 (0.11)	0.45*	0.045 (0.009)	0.29*	0.64 (0.1)	0.39*
SBP24	0.0058 (0.02)	NS	0.005 (0.002)	0.05#	0.50 (0.10)	0.35*	0.027 (0.007)	0.22*	0.27 (0.09)	0.27#
DBP24	0.0092 (0.031)	NS	0.006 (0.002)	0.03#	0.82 (0.14)	0.39*	0.05 (0.010)	0.29*	0.55 (0.13)	0.32*
SBP SD	-0.15 (0.11)	NS	0.0095 (0.0089)	NS	1.54 (0.54)	0.22#	0.064 (0.04)	NS	1.07 (0.48)	0.24#
DBP SD	-0.028 (0.13)	NS	-0.003 (0.01)	NS	1.19 (0.60)	0.18#	0.088 (0.04)	0.17#	1.27 (0.53)	0.25#
Daytime SBP	0.0056 (0.02)	NS	0.0054 (0.0017)	0.06#	0.50 (0.10)	0.31*	0.025 (0.007)	0.22*	0.26 (0.09)	0.28#
Daytime DBP	0.0067 (0.03)	NS	0.0070 (0.0024)	0.05#	0.80 (0.15)	0.34*	0.047 (0.01)	0.27*	0.51 (0.13)	0.31*
Nighttime SBP	0.002 (0.023)	NS	0.0047 (0.0018)	0.04#	0.37 (0.11)	0.23#	0.025 (0.007)	0.21#	0.23 (0.10)	0.24#
Nighttime DBP	0.006 (0.03)	NS	0.0038 (0.0026)	NS	0.70 (0.15)	0.31*	0.043 (0.008)	0.35*	0.46 (0.13)	0.28*
dip SBP %	0.0035 (0.058)	NS	0.00027 (0.0047)	NS	0.37 (0.29)	NS	-0.001 (0.02)	NS	0.005 (0.25)	NS
dip DBP %	0.017 (0.05)	NS	0.0023 (0.0036)	NS	0.063 (0.23)	NS	-0.011 (0.01)	NS	-0.072 (0.20)	NS
HR range day	0.02 (0.029)	NS	0.00113 (0.0015)	NS	-0.007 (0.09)	NS	-0.005 (0.005)	NS	0.92 (0.31)	NS
HR range night	0.013 (0.03)	NS	-0.0011 (0.0026)	NS	-0.28 (0.16)	NS	0.0035 (0.010)	NS	-0.014 (0.15)	NS
IDQ	-0.017 (0.07)	NS	-0.0048 (0.0055)	NS	1.3 (0.32)	0.30*	0.041 (0.02)	NS	1.01 (0.28)	0.30*

Table 3.5 Multivariable regression analyses of vascular and blood pressure related parameters. Regression variables includes confounders (see Table 3.1). Reported as co-efficient (standard error), and R^2 (adjusted) value, * indicates P<0.0001, # indicates P<0.05. SBP24, 24 hour average systolic BP; DBP24, 24 hour average diastolic BP; MAP, mean arterial pressure; SBP SD, standard deviation i.e. variability of systolic BP; *continued next page*.

PREDICTORS	SCP	R^2 adj	DCP	R^2 adj	SBP24	R^2 adj	DBP24	R^2 adj	dip SBP %	R^2 adj	dip DBP %	R^2 adj
cIMT	8.3 (13.9)	NS	1.5 (11.4)	NS	-6.9 (15.4)	NS	-0.3 (10.7)	NS	-1.4 (6.4)	NS	-0.17 (7.6)	NS
% FMD	0.020 (0.32)	NS	0.14 (0.26)	NS	0.07 (0.32)	NS	0.05 (0.22)	NS	-0.032 (0.13)	NS	0.06 (0.2)	NS
LnRHI	9.45 (4.5)	0.21#	5.22 (3.6)	NS	13.9 (4.1)	0.18*	7.88 (2.95)	0.12#	-0.40 (1.7)	NS	0.77 (2.2)	NS
AI@75 %	0.46 (0.06)	0.43*	0.37 (0.05)	0.37*	0.33 (0.06)	0.26#	0.26 (0.04)	0.29*	0.042 (0.03)	NS	0.0072 (0.04)	NS
PWV	5.36 (0.93)	0.33*	4.59 (0.78)	0.26*	4.9 (0.98)	0.21#	4.0 (0.70)	0.23*	-0.03 (0.42)	NS	-0.2 (0.5)	NS
AIx PWA	0.39 (0.068)	0.35*	0.30 (0.06)	0.25*	0.22 (0.07)	0.16*	0.19 (0.05)	0.18*	0.004 (0.03)	NS	-0.015 (0.04)	NS
SCP	n/a	n/a	0.679 (0.037)	0.73*	0.69 (0.06)	0.53#	0.50 (0.04)	0.55*	0.05 (0.03)	NS	0.036 (0.04)	NS
DCP	1.04 (0.06)	0.76*	n/a	n/a	0.89 (0.07)	0.56#	0.71 (0.04)	0.70*	0.016 (0.04)	NS	-0.020 (0.05)	NS
SBP24	0.70 (0.06)	0.58*	0.585 (0.047)	0.56*	n/a	n/a	0.62 (0.03)	0.80*	0.009 (0.03)	NS	0.023 (0.04)	NS
DBP24	1.03 (0.08)	0.60*	0.95 (0.05)	0.71*	1.26 (0.05)	0.81#	n/a	n/a	0.005 (0.05)	NS	-0.016 (0.06)	NS
SBP SD	1.59 (0.41)	0.26*	1.10 (0.33)	0.16#	1.68 (0.39)	0.20#	0.97 (0.28)	0.13#	0.4 (0.16)	0.07#	0.37 (0.2)	NS
DBP SD	1.62 (0.46)	0.25#	1.40 (0.37)	0.18*	1.15 (0.45)	0.14*	0.95 (0.32)	0.12#	0.12 (0.18)	NS	0.14 (0.2)	NS
Daytime mean SBP	0.68 (0.06)	0.58*	0.55 (0.04)	0.53*	n/a	n/a	0.59 (0.03)	0.75*	0.056 (0.03)	NS	0.07 (0.04)	NS
Daytime mean DBP	1.02 (0.08)	0.60*	0.92 (0.05)	0.68*	1.22 (0.06)	0.77#	n/a	n/a	0.07 (0.05)	NS	-1.9 (0.04)	0.16*
Nighttime mean SBP	0.53 (0.07)	0.41*	0.49 (0.05)	0.43*	n/a	n/a	0.53 (0.04)	0.63*	-0.18 0.03)	0.25*	-0.18 (0.04)	0.15*
Nighttime mean DBP	0.72 (0.10)	0.41*	0.75 (0.07)	0.51*	0.95 (0.08)	0.54#	n/a	n/a	-0.23 (0.04)	0.21*	-0.38 (0.047)	0.33*
dip SBP %	0.35 (0.23)	NS	0.06 (0.19)	NS	0.047 (0.22)	NS	-0.005 (0.15)	NS	n/a	n/a	1.0 (0.06)	0.64*
dip DBP %	0.18 (0.18)	NS	-0.051 (0.14)	NS	0.056 (0.18)	NS	-0.03 (0.12)	NS	0.63 (0.04)	0.65*	n/a	n/a
HR range day	0.05 (0.07)	NS	0.003 (0.05)	NS	-0.059 (0.07)	NS	0.003 (0.05)	NS	0.029 (0.03)	NS	0.03 (0.03)	NS
HR range night	-0.088 (0.118)	NS	-0.05 (0.09)	NS	-0.069 (0.11)	NS	-0.065 (0.08)	NS	-0.11 (0.04)	0.06#	-0.11 (0.06)	NS
IDQ	1.2 (0.24)	0.31*	1.19 (0.18)	0.31*	1.29 (0.23)	0.23*	0.94 (0.16)	0.25*	-0.018 (0.1)	NS	0.004 (0.1)	NS

Table 3.5 continued: DBP SD, standard deviation i.e. variability of diastolic BP; SD, standard deviation; day, daytime average BP; noct, nocturnal average BP; dip, percentage reduction from day to night BP; RHI, reactive hyperaemia index; AIx, EndoPAT-2000-derived augmentation index; AI@75%, AIx adjusted for heart rate; HR, heart rate; PWV, pulse wave velocity; PWA, pules wave analysis; SCP systolic central pressure; DCP, diastolic central pressure.

Figure 3.11 demonstrates 24 hour SBP regression coefficients, adjusted R^2 value for the model, and statistical significance for normotensive and hypertensive groups analysed separately. Each model was adjusted for any significant confounding factors (BMI, age, physical activity and/or participant sex). Vascular parameters maintaining statistical significance with 24 hour SBP included LnRHI (P= 0.039) and AI@75 (P=0.002) in hypertension; PWV, SBP SD and DBP SD in normotension (P=0.021, 0.037 and 0.010 respectively); and SCP and DCP were significant for 24 hour SBP in both hypertensive and normotensive groups (all P<0.0001).

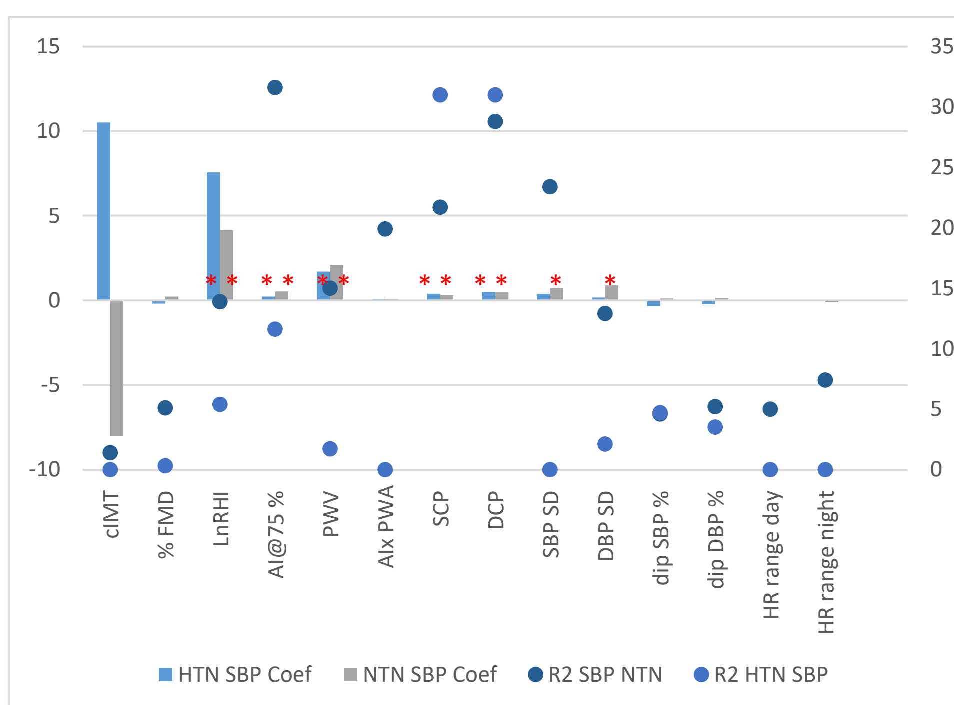


Figure 3.11. Beta coefficients (bars, left axis) for cardiovascular parameters by normotensive and hypertensive groups, with R^2 adjusted values (scatter plot, right axis). Both derived from multivariable regression models including BMI (body mass index), age, sex, and physical activity; P<0.05 indicated by *. % FMD, percent flow mediated dilatation; LnRHI, logarithm of the reactive hyperaemia index; AI@75%, augmentation index adjusted to heart rate of 75 bpm; PWV, pulse wave velocity; PWA AIx, pulse wave analysis derived augmentation index; SCP, systolic central pressure; DCP, diastolic central pressure; HR, heart rate; cIMT, carotid intima-media thickness.

Considering the second part of research question three; "Which cardiovascular parameters can discriminate these subgroups?" Figure 3.11 also demonstrates that some measures of arterial function have consistent R^2 values for both normotensive and hypertensive groups (CIMT, DCP, % dip DBP and SBP). Whereas

other parameters show highly divergent R^2 results; higher R^2 in normotension demonstrated by PWV (0.15 vs 0.02%), AI@75 (0.32 vs 0.12), AIx by PWA (0.20 vs 0.0), SBP SD (0.23 vs 0.02) and DBP SD (0.13 vs 0.02). SCP in contrast demonstrated higher R^2 adjusted in hypertension (0.31 vs 0.22).

3.3.4 Vascular phenotypes

To approach the third research question; "Are phenotypic subgroups apparent in hypertension? Which cardiovascular parameters can discriminate these subgroups?" I firstly consider widely accepted and well-defined subgroups with loss of nocturnal BP dip, white-coat hypertension, and masked hypertension, before reporting exploratory machine learning analyses combining a broader number of cardiovascular variables.

3.3.4.1 Nocturnal dipping

The proportion of the group displaying a nocturnal dip did not differ between normotensive and hypertensive groups (43/79 [59%] vs 44/73 [60%], see Table 3.1). Nor did mean values for percentage dip (% dip SBP 11.7 (±5.2) vs 12.0 (±6.3) mmHg; % dip DBP 15.5 (±6.2) vs 16.1 (±8.3) mmHg). Among the hypertensive subgroup, dippers comprised 34% (n=25/73), non-dippers 58% (n=42) and 8% had missing data (n=6). Nocturnal BP predictably differed between dippers (mean 109/64 ±14/9 mmHg) and non-dippers (120/73 ± 17/12 mmHg), $P<0.0001$. Between group testing (dipper vs non-dipper) demonstrated differences only for SBP SD (a measure of BP variability), dippers 10.9 ± 3.3 mmHg and non-dippers 9.7 ± 2.9 mmHg ($P=0.02$); and night-time maximum HR 77.6 ± 13.1 vs 84.5 ± 22.7 bpm (beats per minute), $P=0.007$.

3.3.4.2 White-coat and masked hypertension

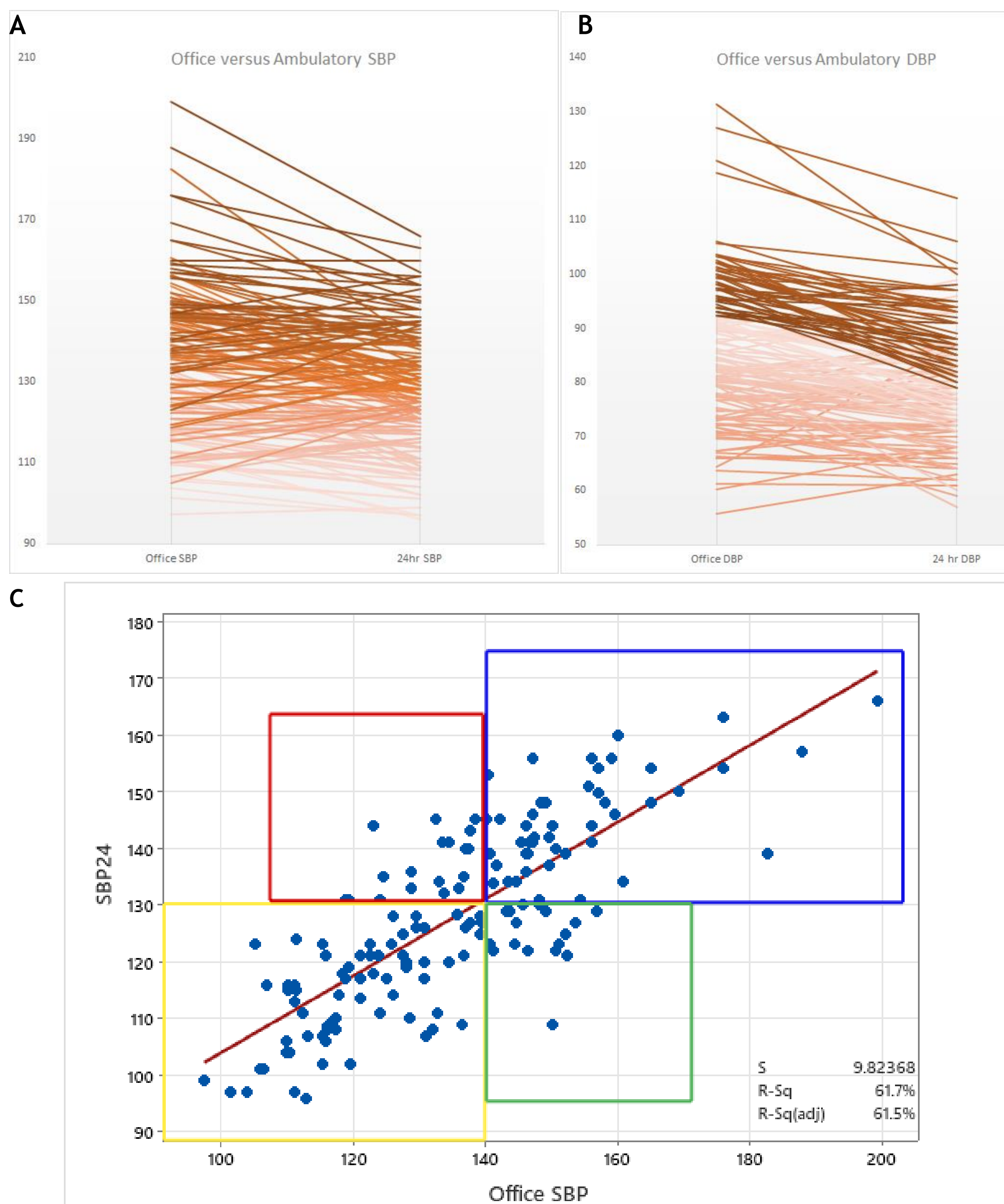


Figure 3.12. Participants' office blood pressure and 24hr SBP (ambulatory 24 hour average systolic) on the X axis; blood pressure in mmHg on the Y axis. Panel A, systolic; panel B, diastolic. Panel C: Regression analysis of Office SBP (mean of 3 readings) on the Y-axis, and 24-hour ambulatory SBP (SBP24) on the X axis, both mmHg. Green box indicates normotension, yellow encompasses white coat hypertension, orange is masked hypertension, and red is hypertension, based on office SBP threshold of 140 mmHg and 24-hour SBP of 130 mmHg.

Figure 3.12 demonstrates that for the majority of participants (116/152, 76%), mean values for office SBP were higher than average systolic ABPM results, as expected from large population studies.[226] The mean difference between office

and 24 hour ambulatory SBP was 7.9 ±11.1 mmHg in the normotensive group and 6.6 ±11.9 mmHg in the hypertensive group (P=0.52), DBP 8.1 ±12.6 vs 5.6 ±8.2 mmHg respectively (P=0.15).

Where this difference between office and ambulatory BP crosses the threshold for a diagnosis of hypertension i.e. where office untreated BP is elevated, but ABPM or home BP readings are normal, such individuals are labelled as 'white coat' hypertension (WCH).[217] Based on mean office SBP and 24 hour average SBP values, 13/152 participants (9%) had WCH. However, Figure 3.12 and Table 3.6 also illustrate that 34/152 (22%) of the group studied had 24 hour average SBP greater than (fasted) mean office SBP, so called 'masked hypertension' in cases where the 24 hour SBP result exceeds the hypertensive threshold of 130 mmHg (n=18/152, 12%). The same pattern in DBP (24 hour ABPM average greater than mean office value) occurred in 16% (24/152). These rates are similar to those reported by others.[227]

Table 3.6 demonstrates the demographic and cardiovascular features of masked and white-coat hypertensive subgroups. Age was well matched across NTN, HTN, WCH, and MHN subgroups; sex was distributed evenly other than a male dominance in MHN (15 of 18, 83%, P=0.04), and BMI was higher in the sustained HTN group at 29.1 kg/m^2, P=0.004. Measures of both arterial stiffness (PWV, PWA-AIx, EndoPAT-2000-derived AI@75) and central pressures demonstrated between group differences, all P≤0.001). Greatest arterial stiffening was apparent in the WCH subgroup, concordant across the different techniques e.g. WCH PWA-AIx 18.8 ±11.6, HTN 15.3 ±15.6, NTN 1.0 ±19.7, P<0.001, see Table 3.6. In contrast, MHN arterial stiffness values were in the same range as the NTN group (e.g. PWA-AIx 2.1% ±19.6). SCP and DCP for both WCH and MHN (123/79 and 136/83 mmHg respectively), demonstrated values intermediate to NTN and sustained HTN groups (114/71 and 144/90 mmHg respectively), P<0.001. Measures of endothelial function and cIMT did not differ between groups. Mean heart rate and BP variability as estimated by BP standard deviation demonstrated between group differences. Percent nocturnal BP dip did not differ between groups validating that WCH and MHN are not simply subgroups determined by nocturnal dipping status.

	NTN N=66 (43%)	HTN n=55 (36%)	WCH N=13 (9%)	MHN N=18 (12%)	P value	Rsq adj
Male sex (%)	35 (53)	25 (45)	7 (54)	15 (83)	0.04	N/A
BMI, kg/m2, mean (SD)	26.1 (4.7)	29.1 (4.2)	26.9 (4.7)	27.3 (3.1)	0.004	0.07
Age, years, median (IQR)	39 (8)	40 (9)	40 (8)	37 (8)	0.6	0.0
Office SBP, mmHg, Mean (SD)	120 (10)	153 (13)	148 (4)	129 (6)	<0.001	0.67
Office DBP, mmHg, mean (SD)	77 (9)	97 (11)	93 (7)	83 (5)	<0.001	0.48
Ever smoked N (%)	25 (38)	18 (33)	4 (31)	7 (39)	0.9	N/A
SBP24, mmHg, mean (SD)	114 (9)	144 (9)	123 (5)	136 (6)	<0.001	0.72
DBP24, mmHg, mean (SD)	71 (7)	90 (8)	79 (6)	83 (7.0)	<0.001	0.60
SBP SD, mmHg, median (IQR)	9.4 (3.4)	117 (3.7)	10.4 (2.8)	10.5 (3.9)	<0.001	0.10
DBP SD, mmHg, median (IQR)	8.2 (3.4)	9.5 (3.2)	9.9 (2.3)	8.6 (3.3)	0.04	0.04
% dip SBP mean (SD)	11.3 (5.9)	12.2 (7.7)	12.1 (6.8)	10.5 (5.2)	0.2	0.0
% dip DBP mean (SD	15.1 (7.3)	16.5 (9.7)	16.4 (7.6)	14.6 (7.8)	0.7	0.0
% FMD	6.0 (4.0)	6.7 (4.3)	7.0 (4.1)	5.9 (2.6)	0.7	0.0
LnRHI	0.7 (0.3)	0.8 (0.4)	0.7 (0.3)	0.8 (0.4)	0.2	0.01
Mean HR	62 (19)	68 (24)	68 (21)	64 (26)	0.02	0.05
AI@75 %	-2.9 (15.4)	13.1 (18.5)	22.3 (30.5)	-3.9 (11.9)	<0.001	0.18
cIMT	0.55 (0.28)	0.54 (0.28)	0.56 (0.30)	0.54 (0.27)	0.9	0.0
PWV	6.4 (2.0)	7.4 (2.6)	7.7 (3.2)	7.2 (0.7)	<0.001	0.12
AI % by PWA	**1.0 (19.7)**	**15.3 (15.6)**	**18.8 (11.6)**	**2.1 (19.6)**	<0.001	0.13
SCP	111 (25)	137 (23)	130 (36)	122 (7.2)	<0.001	0.46
DCP	76 (18)	95 (17)	88 (26)	86 (5.2)	<0.001	0.46
IDQ adjusted for HTN	6.7 (4.5)	8.5 (4.6)	9.1 (4.9)	7.6 (5.3)	0.1	0.02
IPAQ	3437 (2397)	2244 (2001)	2858 (1958)	4764 (4935)	0.005	0.07

Table 3.6. Comparison of Normotension (NTN), hypertension (HTN), white-coat hypertension (WCH), and masked hypertension (MH). NTN, 24 hour SBP (systolic blood pressure) <130 and office SBP <140 mmHg; HTN, 24 hour SBP >130 and office SBP >140 mmHg; WCH 24hr SBP <130 and office SBP >140 mmHg; MH 24hr SBP >130 and office SBP <140 mmHg. DBP, diastolic blood pressure; cIMT, carotid IMT; Rhi, reactive hyperaemia index; Aix, augmentation index; AI@75%, Aix adjusted for heart rate; SD, standard deviation; HR, heart rate; PWV, pulse wave velocity; PWA, pules wave analysis; SCP systolic central pressure; DCP, diastolic central pressure; IDQ, interheart diet score; IPAQ, International Physical Activity Questionnaire. ANOVA or Chi^2 testing.

Having identified parameters with between-group differences, analysis of those demonstrating the greatest discriminatory value was undertaken with a classification and regression tree (CART, Figure 3.13). CART illustrates that even without 24 hour APBM, the phenotypic subgroups of white coat and masked hypertension may be identifiable if brachial artery-derived estimates of central pressure are available. In individuals with office SBP ≥140 mmHg, the threshold best discriminating WCH from sustained hypertension was an office BP below 154 mmHg. Similarly, MHN was optimally identified from among normotensive participants with office BP readings ≤140 mmHg by a DCP above 78 mmHg, see Figure 3.13. This model categorised 100% of MHN and WCH participants correctly on training, and 94% and 92% respectively on testing. Area under the ROC curve was 0.90 for MHN and 0.89 for WCH.

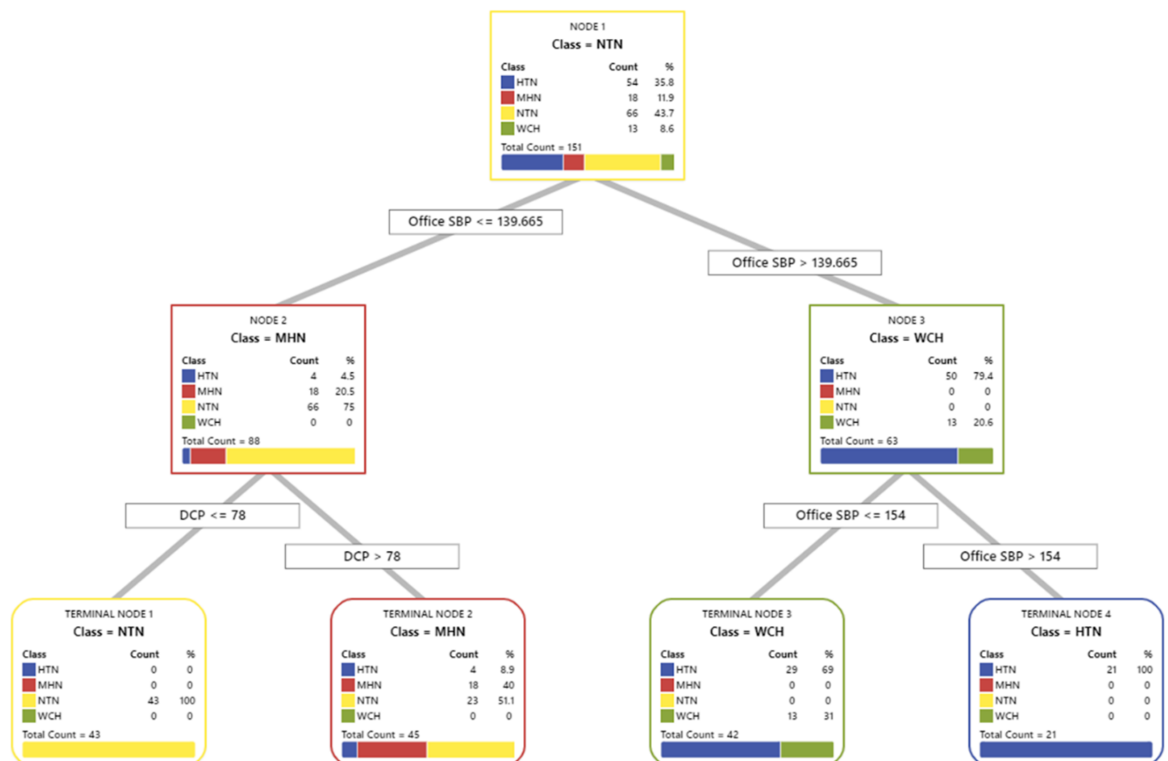


Figure 3.13. Classification and regression tree (CART) to classify normotension (Node 1), masked hypertension (2), and white-coat hypertension (3), and sustained hypertension (4), based on office SBP (systolic blood pressure) and systolic central pressure (SCP) and diastolic central pressure (DCP).

3.3.4.3 Bicluster and UMAP analysis

The goal of spectral biclustering was to use local similarities between demographic and cardiovascular variables to identify distinct phenotypic groups. Given the demonstrable differences in associations of vascular and BP parameters by hypertensive state (Figures 3.8 and 3.11), this was undertaken in the hypertensive group only.

Spectral biclustering identified three distinct groups of patients (rows), characterised by eight groups of features (columns) (Figures 3.13 and 3.14). Considering the vascular features, central BP columns associated with AIx measures by both techniques, but PWV as an alternative arterial stiffness measure affiliated more with measures of cardiovascular variability and age. These findings were consistent with Figure 3.8 correlation matrix. Proximity of BMI to IDQ was not surprising as IDQ score incorporates hip/waist ratio.

Comparing the three bicluster patient subgroups, Table 3.7 reports no between-group differences for FMD, LnRHI, or CIMT, consistent with aforementioned analyses. Among the parameters displaying between-group differences, nocturnal and central BP were the statistically strongest parameters (all P<0.0001, Table 3.7, figure 3.14); these and others follow.

Group 0 subjects (n=23) could be considered as ‘arterially stiffened’ hypertension. These patients trended toward being older (NS), 57% male, with a lower mean physical activity score (data and ANOVA analyses are reported fully in Table 3.7). Average BP values were highest for this group, seen across 24hr BP (149 ±9 / 95 ±8 mmHg, ANOVA P<0.0001), day and night mean values, office BP, and central BP (143 ±15 systolic, 101 ±11 diastolic, mmHg, both P<0.0001). BP variation (SD) was lowest, % nocturnal dip was reduced (systolic dip 9.3 ±4%), and a lower proportion of masked hypertension (MHN) patients were present (3 of 23, 13.0%). Arterial stiffening was apparent, with highest PWV (8.5 ±2.1 m/s, P=0.003) and AI@75 (17.4±17, P=0.039 (Table 3.7). FMD values were lowest (5.06 ±3.15%), but did not reach statistical significance (P=0.059).

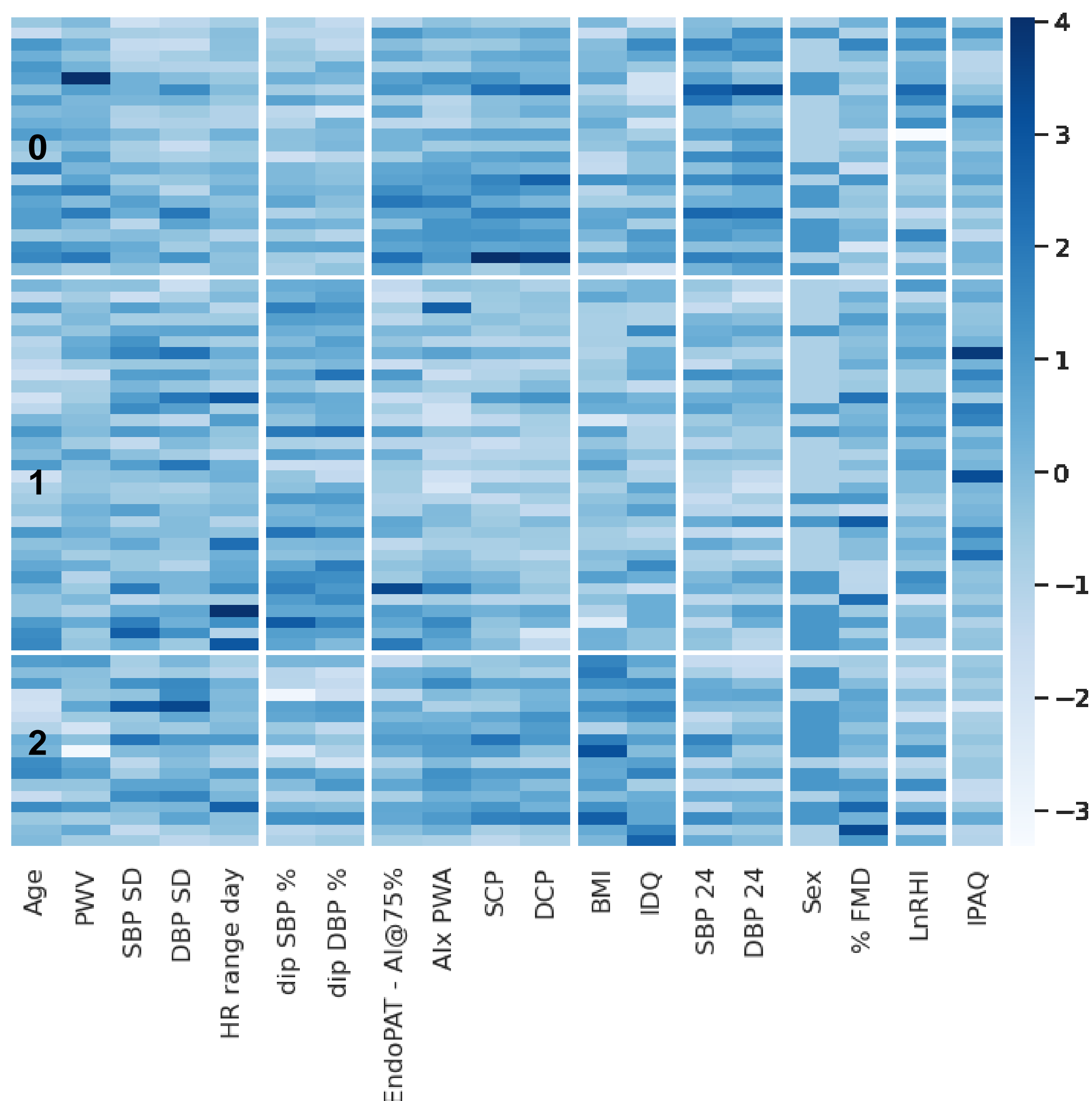


Figure 3.13. Biclustering of hypertensive group with division to 3 subgroups of patients and 8 groups of features. SBP24, 24 hour average systolic BP; DBP24, 24 hour average diastolic BP; SBP SD, standard deviation i.e. variability of systolic BP; DBP SD, standard deviation i.e. variability of diastolic BP; FMD, flow mediated dilatation; BMI, body-mass index; dip, percentage reduction from day to night BP; LnRHI, logarithmic reactive hyperaemia index; AIx, EndoPAT-2000-derived augmentation index; AI@75%, AIx adjusted for heart rate; HR range day, daytime heart rate range; PWV, pulse wave velocity; PWA, pulse wave analysis; SCP systolic central pressure; DCP, diastolic central pressure.

Group 1 included 33 subjects with pronounced nocturnal dipping, so far 'vaso-protected' from major vascular impairment. These predominantly male patients (67%) were on average fitter, with highest mean physical activity index, low resting HR, wider HR range, and SBP SD. Mean BP values (reported in Table 3.7) were lowest, including 24-hour BP 137 ±9 / 85 ±7 mmHg (both P<0.0001), day/night averages, office BP, and central BP; greatest nocturnal dip was also seen (systolic 15.4% ±4.2, diastolic 20.7% ±5.8, both P≤0.0001). MHN patients

were over represented (13 of 33, 39.3%, NS). This group showed lowest measures of arterial stiffness by PWV or AIx (Table 3.7).

Group 2 included 17 patients characterised as 'non-dippers', Table 3.7 demonstrating lowest average physical activity, female predominance, and more diverse racial background. They demonstrated intermediate BP measures across parameters of 24hr BP (140±9 / 86±6 mmHg, both P<0.0001), daytime BP, night-time BP, office BP, and central BP and had a low proportion of masked hypertension (MHN) patients (2 of 17, 11.7%, NS). Mean nocturnal dip values were low (9.0 ±7.1% systolic, 12.6 ±8.6% diastolic, both P≤0.0001). Regarding arterial stiffness, AIx derived from PWA was elevated, but resting HR was also higher and the HR-adjusted AI@75 as well as PWV were intermediate. Group 2 FMD values were highest but did not reach statistical significance. DBP SD was highest and SBP SD intermediate, despite reduced nocturnal dipping.

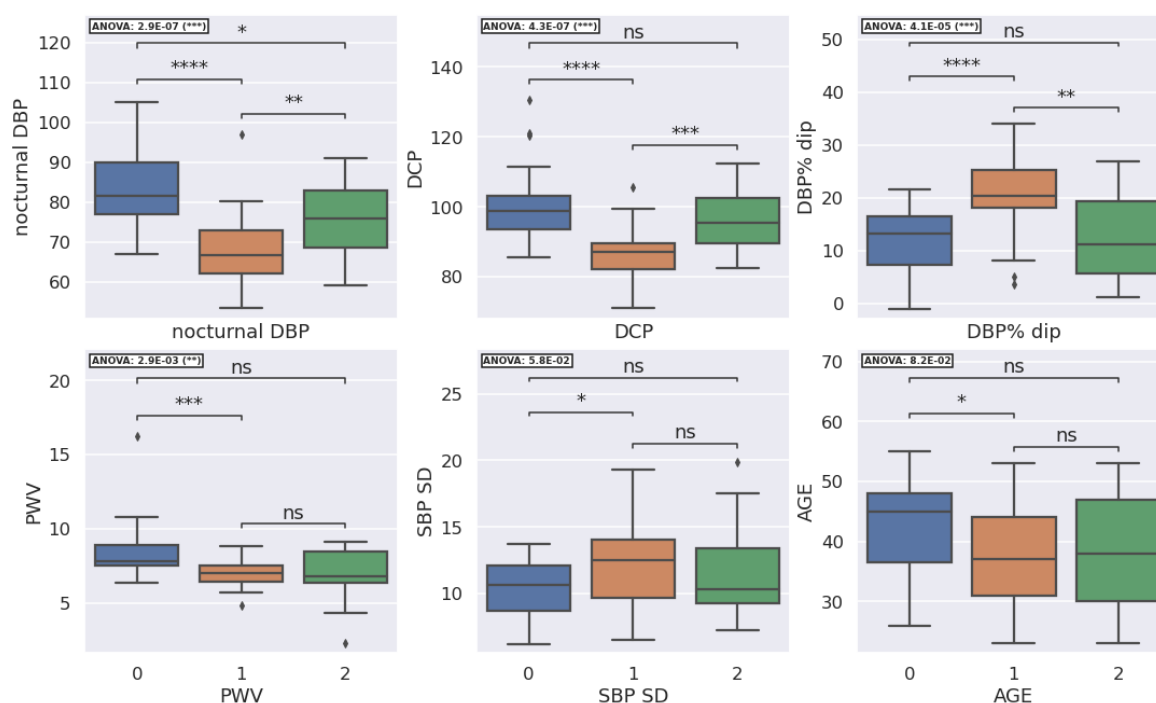
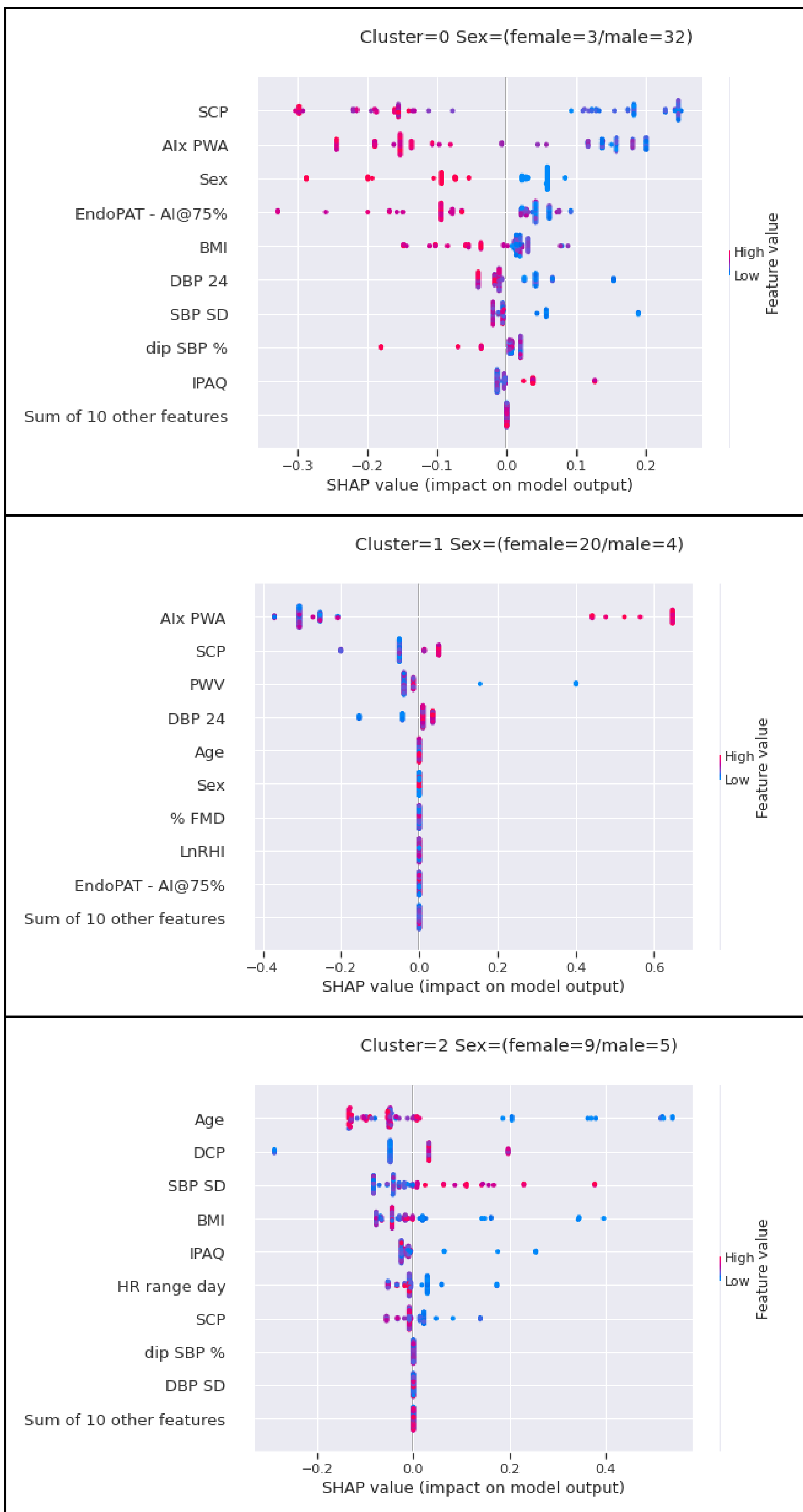


Figure 3.14. Boxplots of key features discriminating bicluster groups. DBP, diastolic blood pressure; SBP SD, standard deviation i.e. variability of systolic BP; % dip, percentage reduction from day to night BP; PWV, pulse wave velocity; DCP, diastolic central pressure.

Cluster group	FMD	LnRHI	CIMT	PWV	AI@75-EndoPAT	PWA-AIx	SCP	DCP	BMI	IPAQ	AGE	Female Sex	White Ethnicity
0 N=23	5.06 (3.15)	0.83 (0.37)	0.56 (0.13)	8.50 (2.13)	17.38 (16.95)	17.41 (14.91)	142.59 (15.19)	100.68 (11.28)	27.75 (3.22)	2336.39 (1727.67)	42.61 (7.76)	10 (43%)	18 (78%)
1 N=33	6.56 (3.88)	0.81 (0.24)	0.53 (0.11)	7.04 (0.86)	3.48 (22.03)	4.73 (18.93)	125.05 (8.00)	86.56 (6.59)	27.32 (3.01)	4150.06 (4087.56)	37.39 (8.71)	11 (33%)	32 (97%)
2 N=17	8.03 (4.38)	0.67 (0.35)	0.51 (0.08)	7.01 (1.88)	6.32 (13.83)	19.59 (12.00)	136.06 (13.35)	95.56 (8.62)	33.06 (4.21)	1077.81 (819.15)	38.12 (9.96)	11 (65%)	12 (71%)
ANOVA	0.0591	0.2892	0.6646	0.0029	0.0391	0.0035	<0.0001	<0.0001	<0.0001	0.003	0.0823	0.1061	0.1159

Cluster group	SBP24	DBP24	SBP day	DBP day	SBP noct	DBP noct	SBP % dip	DBP % dip	SBP SD	DBP SD	Mean HR	HR range day	HR range night
0	147.67 (8.71)	94.39 (7.45)	150.65 (9.06)	96.57 (7.61)	134.49 (10.30)	83.20 (9.58)	9.29 (4.17)	11.90 (5.77)	10.24 (2.05)	8.32 (2.48)	68.10 (9.22)	32.35 (6.66)	21.57 (10.29)
1	137.25 (6.52)	84.67 (6.56)	142.90 (6.76)	89.22 (6.99)	117.15 (10.86)	67.69 (8.78)	15.45 (5.73)	20.75 (7.35)	12.19 (3.08)	9.34 (2.50)	62.48 (9.52)	41.56 (19.87)	23.00 (9.33)
2	139.65 (8.99)	86.24 (5.49)	143.18 (9.07)	89.12 (6.05)	127.44 (13.73)	75.41 (8.70)	8.95 (7.06)	12.66 (8.55)	11.53 (3.53)	10.51 (2.98)	74.73 (10.09)	36.88 (14.31)	23.27 (11.74)
ANOVA	<0.0001	<0.0001	0.0016	0.0004	<0.0001	<0.0001	0.0001	<0.0001	0.0585	0.038	0.0007	0.0987	0.8503

Table 3.7. Comparison of hypertensive cluster groups. Mean value and standard deviation of cardiovascular parameters by cluster groups, with ANOVA assessment of between group difference and R^2 adjusted values indicating strength of the model. SBP24, 24 hour average systolic BP; DBP24, 24 hour average diastolic BP; SBP SD, standard deviation i.e. variability of systolic BP; DBP SD, standard deviation i.e. variability of diastolic BP; BMI, body-mass index; dip, percentage reduction from day to night BP; LnRHI, logarithmic reactive hyperaemia index; FMD, flow-mediated dilatation; AIx, EndoPAT-2000-derived augmentation index; AI@75%, AIx adjusted for heart rate; HR range day, daytime heart rate range; PWV, pulse wave velocity; PWA, pulse wave analysis; SCP systolic central pressure; DCP, diastolic central pressure.



Cluster=0 Sex=(female=3/male=32)
SCP
AIx PWA
Sex
EndoPAT - AI@75%
BMI
DBP 24
SBP SD
dip SBP %
IPAQ
Sum of 10 other features
−0.3
−0.2
−0.1
0.0
0.1
0.2
SHAP value (impact on model output)
High
Low
Feature value
Cluster=1 Sex=(female=20/male=4)
AIx PWA
SCP
PWV
DBP 24
Age
Sex
% FMD
LnRHI
EndoPAT - AI@75%
Sum of 10 other features
−0.4
−0.2
0.0
0.2
0.4
0.6
SHAP value (impact on model output)
High
Low
Feature value
Cluster=2 Sex=(female=9/male=5)
Age
DCP
SBP SD
BMI
IPAQ
HR range day
SCP
dip SBP %
DBP SD
Sum of 10 other features
−0.2
0.0
0.2
0.4
SHAP value (impact on model output)
High
Low
Feature value

Figure 3.15. Analysis of each cluster group by dimensional reduction using UMAP, with inclusion of SHAP values. SCP, systolic central pressure; DCP, diastolic central pressure; AI@75, augmentation index adjusted to heart rate 75 derived from reactive hyperaemia index (RHI); PWV, pulse wave velocity; AIx PWA, augmentation index derived from pulse wave analysis; dip DBP %, percentage nocturnal dip in diastolic blood pressure; BMI, body mass index; HR range day, range of daytime recorded heart rates.

For further validation of the machine learning model, SHAP values were computed from the dataset with UMAP dimensionality reduction techniques. This clustering pipeline generated three clusters based on global similarities (Figure 3.15). These were driven mostly by variables concordant with those identified in bicluster analysis. Central BP was a key driver across the three clusters, augmentation index in clusters 0 and 1, 24hr DBP in clusters 0 and 1; SBP % dip and cardiovascular variation (SD, heart rate range) in clusters 0 and 2; PWV in cluster 1, and demographic features in cluster 2.

3.4 Discussion

Bringing together data from the individual analyses, considering strengths and limitations, as well as validation or repudiation from scientific theory and published research, the research questions are hereby discussed in sequence.

3.4.1 Does cardiovascular function differ between incident hypertension versus healthy controls?

Participant demographics and blood pressure parameters: The inherent challenge of hypertension research is the dual systolic and diastolic components and continuous nature of BP, on which we base a threshold diagnosis. Thus, in order to compare hypertensive and normotensive individuals, I had to first assess whether recruitment had attained the specified groups. Separation of groups on BP variables was achieved (Table 3.1), though 21% demonstrated 'borderline' 24 hour average SBP of 125 to 135 mmHg, additional analyses of which supported validity of the between group comparisons. Another consideration in defining the hypertensive group was the differing strength of associations for systolic and diastolic BP. Specifically; measures of arterial stiffness were more closely linked to diastolic BP, and cardiovascular variability with systolic BP. The differences in R^2 values was not of great magnitude, but has been supported by other

published datasets[40,228] and highlighted that future research should either stratify data or employ mean arterial pressure.

Arterial stiffness: The data demonstrated that even in untreated, incident hypertension, in young individuals free from co-morbidities and HMOD, the process of arterial stiffening can be demonstrated through non-invasive surrogate markers. The validity of this statement is supported by the statistical strength of between group differences, biological plausibility, the 'dose-response', and consistency of findings across different techniques. The latter point being confirmed on correlation and regression analyses reported in Section 3.3.2. Published longitudinal studies also evidence arterial stiffening in newly diagnosed hypertensive populations with and without co-morbidities. Diverse measurement techniques studied include carotid-femoral and brachial-ankle PWV, augmentation index, and carotid elasticity, as determinants of longitudinal BP increase and as independent predictors of incident hypertension [229–232]. The relationship strengthens in established or uncontrolled hypertension, where 90% display elevated PWV [233]. Risk of cardiovascular events also rises with increasing arterial stiffness; [233,234] meta-analysis of 17 longitudinal studies suggests that this risk is amplified by other evidence of hypertension-mediated organ damage (HMOD) [235]. However, the sequential nature of arterial stiffening and hypertension remains contentious, [236,237] possibly reflecting data sampling periods if the interplay between them evolves over time. For example, UK BioBank data suggests midlife DBP as the strongest predictor of arterial stiffness progression, transitioning to increased stiffness and a falling DBP [228]. Capturing an incident hypertensive population such as described here is therefore critical when exploring early interactions of BP and vascular function.

Endothelial function: In contrast, whole cohort measures of endothelial function (FMD and LnRHI) are interesting in the absence of association with each other, with hypertension, and with arterial stiffness. Certain caveats apply; firstly, that percent FMD values were at the lower end of reported reference ranges, they were however similar to other studies performed in our centre [238] and heterogeneity across sites and populations is a known limitation of FMD.[239] Secondly, it is accepted that FMD and EndoPAT™-2000®

are not inter-changeable techniques [240], reflecting macro- and micro-vascular function respectively. Had only one measure demonstrated association, it may have been considered to be of superior sensitivity in an incident population. The concordance of the two techniques supports that no excess of endothelial dysfunction was presented among these otherwise healthy hypertensive individuals. Indeed, the direction of effect was the inverse of the hypothesis, with higher LnRHI values in the hypertensive group, and positive correlation and regression coefficients, possibly suggesting that the endothelium may demonstrate early compensation in hypertension.

Published longitudinal data does demonstrate increased prevalence of hypertension and elevated PWV in impaired FMD [241,242], but contrasting evidence from both healthy and untreated hypertensive individuals shows no association with incident hypertension [242–246], consistent with Inflammatension data. Possible explanations for the discordant findings across the body of published evidence include poor sensitivity of techniques early in hypertension, with most of the research validating these equipment in older participants or more long-standing hypertension.[247] Alternatively, environmental factors may have influenced data, as EndoPAT-2000 preceded FMD in the study protocol; EndoPAT-2000 cuff occlusion may have induced systemic nitric oxide release, potentially ‘priming’ the artery, resulting in higher baseline and rest readings and proportionally less dilatation. However, a minimal interval of 15 mins between cuff occlusions was always observed.

Multivariable analysis supports that LnRHI i.e. microvascular function, influences SCP and 24 hour BP variables; hence, that endothelial dysfunction may be a risk factor for hypertension, but does not appear to contribute significantly to BP determination in healthy individuals with incident,primary hypertension and may even show compensatory changes early on.

CIMT: No between group difference, correlation, or regression analysis supported association of CIMT with BP. This is despite well-established evidence supporting the relationship [248,249], if not necessarily the benefit of CIMT in cardiovascular risk prediction[217,250]. CIMT was not available for the whole group due to COVID-19 restrictions and this further limited sample size to detect change. More likely, the lack of association between CIMT and

hypertension is due to this healthy, incident population not yet developing clinically detectable atherosclerosis. This reconciles with the theory that in the natural history of HMOD, the relationship between arterial stiffening and atherosclerosis is complex (and reviewed in detail elsewhere) [251], but that the former can precede and contribute to the latter.[251,252]

Cardiovascular variability: HR range as one measure of cardiovascular variability showed no between-group differences. BP variability as measured by standard deviation (SD) was higher in hypertensive individuals and has been associated with increased cardiovascular risk. [40] Those with nocturnal dipping and with masked hypertension (MHN) i.e. subgroups defined by high BP variability, did predictably demonstrate increased BP SD, and differences were also seen between hypertensive phenotypes in cluster analysis, discussed in Section 3.4.3. Other studies have expanded theories on the mechanism of association; a 10-year follow up study suggested that BP variability may reduce nitric oxide availability, increase collagen to elastin ratio, and promote vascular smooth muscle proliferation, reducing compliance.[41] The broad range of determinants of BP variability, and heterogeneity across individuals makes delineating such pathophysiology very challenging.

Conclusion: Results advocate that hypertension research should employ both systolic and diastolic BP as continuous parameters and avoid reductionist approaches where possible. Additionally, hypertensive disease progression involves early arterial stiffness, already apparent in this newly diagnosed, young, primary hypertensive group. Atherosclerosis and impairment in microvascular function meanwhile are not detectable.

3.4.2 Do measures of cardiovascular function demonstrate clinically applicable associations?

Arterial Stiffness: The co-linearity of AI@75, PWV and AIx by PWA demonstrated in Sections 3.3.3 and 3.3.4, suggests that in studies of healthy versus hypertensive participants (under 50 years), one measure would suffice for estimation of arterial stiffness, with strong correlation if not interchangeability evidenced here and in the literature.[243,253] Considering if any one out-performed others in answering the research question, PWV

demonstrated the greatest between-group difference and is a more direct measure of arterial stiffness. However in multivariable regression (Table 3.5) AI@75 (EndoPAT-2000) accounted for the greatest proportion of variance of central and 24 hour BP. This arose as the strength of the regression model for measures of AIx (by either technique) increased with BP as a continuous rather than categorical variable, and due to the addition of confounding factors to the model. Published data supports AIx superiority in the young, but PWV over the age of 50.[254] If only one were to be employed, EndoPAT-2000-derived AI@75 demonstrated marginally higher R^2 values. SphygmoCor however is quicker, without requirement for single-use finger probes, is less uncomfortable for participants due to shorter cuff occlusion time, and provides centralised pressure data in addition to AIx. This equipment also provides the option to perform PWV (validated by invasive studies)[208], and central pressure estimates, which strongly associated with 24 hour BP (Table 3.4) and better estimates the pressure that organs are exposed to than brachial BP.[255]

Endothelial function: Section 3.4.1 discusses that only those with abnormal range LnRHI demonstrated a pattern of association with FMD, and neither variable was associated with arterial stiffness or cardiovascular variability. SCP and 24 hour BP demonstrated unexpected positive correlation with LnRHI, see earlier discussion (Section 3.4.1).

CIMT: No associations with other measures of cardiovascular function were demonstrated in correlation or regression, as discussed in Section 3.4.1.

Cardiovascular variability: both correlation and multiple regression demonstrated that BP variability (SD) more closely associated to arterial stiffness, central and 24 hour BP than HR variability (as estimated by HR range), and is supported by published data.[244,256,257] The potential mechanism of association was discussed in Section 3.4.1.

Conclusion: measures of arterial stiffness strongly correlate with each other. They are also associated with estimates of central BP and cardiovascular variability.

3.4.3 Are phenotypic subgroups apparent in hypertension? Which cardiovascular parameters can discriminate these subgroups?

Nocturnal dipping: despite Inflammatension demonstrating no correlation with cardiovascular measures beyond those directly linked (BP SD, maximum heart rate at night, and nocturnal BP); dipping status has been linked to risk of cardiovascular disease and arterial stiffness by other groups.[87,258] Consistent with this, dipping was pronounced in the 'vasao-protected' bicluster analysis group. Discordance night reflect the multiplicity of genetic and environmental determinants of nocturnal dip, resulting in being underpowered to detect correlation. Larger populations with older participants have demonstrated increased arterial stiffness in non-dippers, [87,259] either relating to larger sample size or more established vascular remodelling. The alternative theory would be confounding, as sleep disturbance due to ABPM is common, and the magnitude of effect on BP variable between participants. The Inflammatension study did not include any validated assessment of sleep quality during the ABPM assessment, so was unable to ascertain if this was a significant confounder. Maximum heart rate, which was demonstrated to be elevated in non-dippers, may be indicative of wakeful periods, or may support autonomic or endocrine dysfunction as the mechanism for loss of dip.

White-coat hypertension: Section 3.3.8 associated white-coat hypertension (WCH) and sustained hypertension with elevated values for measures of arterial stiffness and central pressure, but masked hypertension (MHN) values closer in range to normotension, see Table 3.6. These data might suggest that the arterial 'phenotype' of WCH resembles sustained hypertension and MHN vascular characteristics are akin to normotension. Alternatively, the pattern of office rather than ambulatory BP better associating with measures of arterial stiffness and central pressures may reflect that they are also office-based measures. Hence, this may be a limitation of the study protocol, and of office or research lab settings in general, with some patients displaying discordant arterial stiffness and central pressure values between clinical and 'ambulatory' settings, similarly to how their office BP fails to reflect their ambulatory BP. Other authors conducting office and ambulatory measures have concluded precisely this.[260]

Masked hypertension has been hypothesised to relate to environmental factors which are controlled for in the office and fasted setting, particularly salt, caffeine, exercise-avoidance, and sympathetic ‘stress’ response.[227] One outstanding question regards the validity of ‘loss of nocturnal dip’ or ‘masked hypertension’ diagnoses when considering the potential confounding effect of ABPM via associated sleep disturbance. No relationship was however demonstrated between nocturnal percent dip and masked hypertension status to support this explanation.

Our finding that office and central BP can be used to discriminate which ‘normotensive’ patients may have MHN, and which are likely to have WCH rather than sustained HTN offers potential clinical utility when ABPM availability is limited. Similar categorisation models have been attempted with PWV but with lower accuracy [261]. Given the ease and rapidity of central BP measures and the greater patient acceptance and concordance compared to ABPM, use in the office setting could provide supportive evidence of normotension or sustained hypertension, potentially without need for ABPM, in over 40% of patients. The model requires external validation, but is supported by proximity of 24-hour and central BP variables in correlation and cluster analysis, and by data published from other groups, including evidence of increased cardiovascular risk with elevated central BP, irrespective of brachial BP.[262–264]

Discriminatory capacity: Figure 3.11 suggests that the discriminatory value of measures of cardiovascular function depends on hypertension status, with a stronger association of arterial stiffness measures and BP in normotension than in hypertension. This might suggest that the vascular techniques presented are influenced by, or do not adequately adjust for, blood pressure. Or, that genetic, physiological, and environmental differences lead to hypertension in some individuals; thereafter, the degree to which arterial remodelling and loss of elastin occurs will further depend on genetic and environmental factors; causing the coefficient of association between BP and arterial stiffness to diverge. This process results in the pathophysiologic ‘phenotypes’ developing, as discussed below. The clinical implication of these parameters with divergent R^2 results is that the strength of association

(or potentially their value in predicting ambulatory SBP) appears to be dependent on hypertension status.

Machine learning techniques: Statistically significant between-group differences were present for almost all demographic and cardiovascular variables, confirming that biclustering was not determined by a single dominant factor. Furthermore, column variables grouped in a pattern consistent with preceding basic statistical analysis. The main discrepancies were firstly, that dipping status and nocturnal BP were key drivers of clustering, despite dipping status failing to demonstrate any significant associations in simple between group comparisons. Secondly, that PWV and AIx had consistently demonstrated a strong association, but between the biclusters they diverged. Despite their association, determinants of PWV and AIx do differ,[253,265] AIx being more susceptible to influence from heart rate, height, and female sex. Whole group analysis may have lacked the granularity of individual data presented in biclustering to detect these BP dip and AIx subgroups, or to show interactions e.g. the influence of age not being linear, but more pronounced in AIx under 50 years, and PWV over 50 years.[253]

The UMAP and bicluster machine-learning methodologies demonstrated results that were concordant concerning the key features of central and nocturnal BP values, nocturnal dipping pattern, and measures of arterial stiffness. The detected biclusters were also broadly consistent with results observed in basic statistical analysis. Such concordance supports the conclusions drawn from the data, and carries clinical implications as follows. Group 0 ‘arterially stiffened’ hypertension were sedentary, with greatest elevation in BP parameters, and both reduced nocturnal dip and BP variability. Sedentary lifestyle has been linked to increased arterial stiffness in other studies.[266] A longer duration of undiagnosed hypertension may also underlie this group’s characteristics. The clinical implication is that a lower threshold for commencing primary prevention (lifestyle and pharmacological) may benefit this group.

Group 1 could be surmised as ‘vaso-protected’ hypertension. They were fitter, with lowest BP measures, preserved nocturnal dip, least evidence of arterial stiffness. The group could represent hypertension earlier in its’

natural course, or the modifying and protective effects of physical activity and intact nocturnal dip.[266,267] ABPM was key for diagnosing hypertension as 39% had MHN i.e. office BP <140/90mmHg. Higher mean SBP variability and daytime HR range may reflect more physical activity in this group or alternatively a strong sympathetic drive, be it social stresses, or stimulants such as caffeine, which may be targeted as part of management. Group 2, 'non-dippers', also demonstrated elevated BP variability despite loss of nocturnal dip, but MHN was rare. Genotypic differences may be present across the groups, as group 2 were female-dominated in comparison to the other groups, and ethnically more diverse. Given that non-dipping status has been linked to both risk of cardiovascular disease and arterial stiffness in studies of older participants [87,258], lifestyle interventions and bedtime dosing of anti-hypertensives may reduce future cardiovascular risk.

Limitations: The restricted sample size limits robustness of the data, as does the higher BMI in the hypertensive group, which is known to influence cardiovascular function, hence inclusion of BMI in adjusted analyses. Furthermore, it is challenging to ascertain if associations between arterial indices are true relationships, or rather demonstrate co-dependency on BP. External validation would partially counter these limitations. Conversely, the strength of Inflammatension lies in the avoidance of pharmaceutical and co-morbidity confounders, the comprehensive vascular phenotyping, and in the application of unstructured advanced machine learning techniques – in comparison to other studies reporting hypertensive phenotypes, limited by their determination of defining characteristics *a priori* [268].

3.4.4 Conclusion

Hypertensive disease progression involves early arterial stiffness, already detectable in this newly diagnosed, young, primary hypertensive group in comparison to normotensive controls. Carotid atherosclerosis and impairment in endothelial function were not detected. WCH patients demonstrated arterial stiffening in excess of sustained hypertension; MHN vascular characteristics were akin to normotension; office and central BP values alone may be sufficient to identify these subgroups, though this requires external validation. In contrast, dipping status is a complex trait not well characterised by reductionist approaches of between group comparisons, but

a key parameter when assessing the phenotype of hypertensive patients. I further conclude that unsupervised machine learning is a valuable analysis tool, offering deeper clinical insights into nuances between hypertensive phenotypes, here driven by nocturnal and central BP, percent dipping, and arterial stiffness. Given the prognostic value of these parameters, such phenotypes may have important clinical implications for disease progression and individualised care.

Chapter 4 Flow Cytometry studies

4.1 Background

Section 1.3 surmises the existing evidence from animal models, human trials, and genetic studies of the link between the immune system and BP regulation, hypertension disease state, and HMOD. Evidence suggests that hypertensive stimuli, such as Ang II, promote a pro-inflammatory, polarised immune state, particularly in T lymphocyte and monocyte/DC subtypes, with immune cell infiltration of vasculature and organs. This inflammatory milieu exacerbates the hypertensive response, and contributes to endothelial dysfunction and organ damage. Leukocyte expression of BP mediator receptors, and non-pharmacological evidence linking hypertension and the immune system have provided clinical context and justification for the study design. From the evidence discussed arose the hypothesis that changes in circulating PBMCs and their expression markers would be detectable in the hypertensive group.

4.2 Chapter-specific methods

4.2.1 Blood sample preparation

To perform flow cytometry analysis, the whole blood sample was collected in tubes containing EDTA as an anti-coagulant. PBMCs were isolated by density gradient centrifugation using pre-filled 50ml LeucoSep™ tubes (Greiner) . Blood was transferred into the LeucoSep tube and centrifuged at 800g for 15 minutes, no brake, at room temperature. Thereafter, the layers from top to bottom were: i) plasma, ii) enriched cell fraction of PBMCs, iii) separation medium with porous barrier, iv) red cell and granulocyte pellet. PBMCs were collected by pipetting the PBMC interface into a 50ml centrifuge tube, and washing with room temperature dPBS. Cell number was counted and recorded using a hemacytometer before centrifugation at 300g for 10 minutes, room temperature; supernatant was discarded. PBMCs then underwent cell surface antibody staining for flow cytometry (Section 4.2).

For further lysis of erythrocytes and platelet wash, 10X Lysis Buffer (BD Bioscience, BD Pharm lyse) was diluted to 1X with distilled water and added to the PBMCs according to the manufacturer's instructions. Following 5 minutes incubation at room temperature, the tube was centrifuged at 200g for 7 minutes, the supernatant discarded, and cells washed by re-suspending in PBS and centrifuging at 400g for 5 minutes. Re-suspending in the correct volume obtained 1 x 10^7 cells/ml.

4.2.2 Cell surface antibody staining for flow cytometry

Pelleted PBMC were re-suspended in dPBS to give a final concentration of $1x10^7$ cells per ml. Flow cytometry tubes were then prepared for each flow cytometry panel (T cell, Chemokine, Monocyte, and B cell): i) Unstained, ii) fluorescence minus one (FMO, discussed below), iii) Mix antibodies for each flow cytometry panel.

To exclude dead cells, eFluor 506 Fixable Viability dye (Thermo Fisher Scientific) 1ul was added to 'FMO' and 'Mix' tubes of the T cell and Chemokine panel. 100ul of PBMC suspension was pipetted into all of the tubes ($1x10^6$ cells PBMCs total) before 30 minutes incubationat 4°C. Cells were washed by adding 2mls of FACS buffer (dPBS, 0.5% FBS, 2mM EDTA, 0.01%NaN3) to each tube and centrifuging at 600g for 6 min at 4°C, discarding supernatant and re-suspending cells.

Before staining with antibodies, Fc block (1ul, Miltenyi Biotec) was added to the Monocyte 'FMO' and 'Mix' tubes to block non-specific Fc receptor binding. The cell marker staining cocktails are included in Section 4.1.6. Staining cocktail was added to the relevant 'FMO' and 'Mix' tubes, then all tubes incubated for 20 minutes at 4°C. Cells were washed with 2mls FACS buffer centrifuged (600g for 6 min at 4°C), supernatant discarded and cells re-suspended in 350µl FACS buffer. FACS tubes were then wrapped in foil and remained refrigerated until cells were acquired with flow cytometry machine. All samples were run at 100µl/min with 300µl volume acquired, data collected and recorded.

4.2.3 Intracellular phosphorylated signalling proteins flow cytometry panels (Phospho-flow)

Whole blood samples collected in Sodium citrate vacutainer directly stimulated and stained for surface antibodies for 15 minutes in a 37°C water bath. 4 tubes were prepared The tubes were as follows: i. unstimulated surface only (isotype), ii. unstimulated panel, iii. stimulated surface only (isotype), iv. stimulated panel. Stimulation cocktail comprised IFN-alpha 40,000 units, IL-6 100ng, IL2 200ng, Cell stimulation cocktail (Thermo Fisher) 80nM, T cell activation beads (Miltenyi Biotech 5µl), IFN-gamma 25ng, IL-4 20ng, IL-23 20ng. Enough stimulation cocktail was prepared for all tubes required, and the surface staining cocktail was prepared (see Section 4.1.6). eFluor 506 Fix Viability dye (Thermo Fisher) 1ul was added into TH1 and TH17 tubes; 100ul whole blood and antibody cocktail for surface markers was added to all tubes. For each panel, stimulation cocktail was added to tubes marked i. ‘stimulated surface only (isotype)’ and ii. ‘stimulated panel’. All tubes were incubated at 37°C for 15 minutes. Two ml BD Phosflow™ Lyse/Fix Buffer (1X) was added, this had been warmed to 37°C and made fresh for each experiment by diluting 1 part Lyse/Fix with 4 parts deionized or distilled water. Tubes were then mixed by vigorously agitating for 30 seconds, and incubated in a 37°C water bath for 10 min.

The following steps were carried out on ice and centrifuged at 4°C; cells were centrifuged at 500x g for 8 min, the supernatant aspirated leaving 50 µL residual volume; 2ml of FACs washer buffer added to each tube and centrifuged at 600g for 6 min. Cells were permeabilized by adding 500 uL of chilled Perm Buffer II and vortexed to mix, then incubated 30 minutes on ice. Cells were washed twice by each time adding 4ml of FACS buffer to each tube and centrifuging at 600g for 6 minutes with supernatant removed leaving 50 µL of residual volume each time. Cells were then resuspended and for each panel the intracellular markers added to tubes marked ii. ‘unstimulated panel’ and iv. ‘stimulated panel’, these were mixed and incubated on ice for 30 minutes, protected from light. Cells were washed by adding 2mls of FACs buffer to each tube using a Pasteur pastette, these were centrifuged at 600g for 6 min at 4°C, the supernatant discarded and cells gently resuspended in 350µl FACS buffer; the rack containing the FACS tubes was wrapped in foil

and refrigerated until cells are put through the Attune NxT flow cytometer (Thermo Fisher), data collected and recorded.

4.2.4 Flow cytometry detail

The flow cytometer acts as a light detection device; a high dynamic detection range is achieved through careful design of optics, signal detection, and processing units. [269] Detailed technical detail of flow cytometers is not reproduced here, but in summary, forward scattered light (FSC) is proportional to cell surface size, whilst side-scattered light (SSC) reflects cell granularity, and fluorescent cell markers were added as described above. These three factors permit characterisation and quantification of separate immune cell sub-types, with a geometric mean fluorescence intensity (MFI) and histogram plot of fluorescence intensity and cell percentage being the output.

Fluorescence spillover refers to background signal, i.e. signal picked up by a detector specific for a different fluorochrome, and was compensated for through subtraction in order to accurately identify cell populations. In addition, titration was important when setting up flow cytometry panels to minimise non-specific staining, maximise sensitivity and specificity, and maintain a linear relationship between expression and staining intensity. Serial dilutions determined our optimal concentration of all antibodies, undertaken for each panel, as per flow cytometry guidelines. [269]

Non-specific binding of Fc receptors or unintended antigen binding is another source of potential error. This was controlled for by determining spread of fluorescence through the Fluorescence Minus One (FMO) approach, i.e. all antibodies were run *except* the control one to determine the threshold for positive staining. In this study, as we had a very large number of antibodies to analyse, instead of using a single FMO control for each antibody, we grouped two or three antibodies together based on our gating strategies. This approach proved to be more practical in determining the appropriate gating parameters for the antibodies we were interested in. These tubes were called FM control tubes. FM controls were employed for every sample and every panel to permit accurate gating and comparison across participants through.

A negative unstained and/or isotype stained control (cells that do not express the marker) was further employed to ensure specificity of immunofluorescent staining, or where a negative control was unavailable blocking or isotype controls were used. The samples were acquired by Attune NxT Acoustic Focusing Cytometery (Thermo Fisher).

4.2.5 Cell markers and gating strategies

Taking one panel as an example, the chemokine receptor panel included CD3+ T cells, CD4+ Th cells, CD8+ Tc cells, and TCR γ–δ receptor cells, with markers and FMO control approach illustrated in Table 4.0 and the immunological significance of markers outlined in Table 4.1. Other panel cell markers are described in Table 4.2.

Cell marker	Fluorescent label	Concentration (µl/sample) Mix Tube	Concentration (µl/sample) FM control 1 Tube	Concentration (µl/sample) FM control 2 Tube	Supplier	Cat. number
CD3	Alexa Fluor 700	2	2	2	Thermo Fisher	56-0038-42
CD8a	APC-eFluor 780	1	1	1	Thermo Fisher	47-0088-42
CD4	eFluor 450	1	1	1	Thermo Fisher	48-0049-42
CCR6	Alexa Fluor 488	5	X	5	Bio-legend	353414
CD161	PE-eFluor 610	1	1	1	Thermo Fisher	61-1619-42
TCR delta/ gamma	Brilliant Violet 605	1	X	1	BD	740415
CXCR3	PE-Cy7	2	2	2	Thermo Fisher	25-1839-42
CCR4	PerCP-Cy5.5	5	5	X	Bio-legend	359406
CCR10	PE	2	2	X	BD	563656
Fixable viability dye	eFluor 506	1ul added in separately before antibody mix	1ul added in separately before antibody mix	1ul added separately before antibody mix	Thermo Fisher	65-0866-18

Table 4.0. Chemokine panel cell markers, colour, and FM (fluorescence minus one) controls.

Cell subset	Surface markers	Function	Secrete
CD4+ Th1 CD8+ Tc1	CXCR3+CCR6- CCR4-	Pro-inflammatory against intracellular pathogens Tc1 express perforin and granzyme. Implicated in autoimmunity. Promotes opsonisation with B cells	IFN-γ, TNF-α
CD4+ Th2 CD8+ Tc2	CCR4+CXCR3- CCR6-	Immune response to extracellular pathogens Tc2 express granzyme Facilitates B cell isotype switch Induces eosinophils	IL-4, IL-5, IL-13

CD4+ Th9 CD8+ Tc9	CCR6+CCR4- CXCR3-	Anti-helminth and -malignant cell immunity Limited granzyme production / cytotoxicity Involved in atopy and autoimmunity	IL-9, IL-10
CD4+ Th17 CD8+ Tc17	CCR6+CCR4+ CXCR3-	Immune response against extracellular bacteria and fungi. Regulatory role	IL-17A, IL-22
CD4+ Th17.1 CD8+ Tc17.1	CCR6+CXCR3+ CCR4-	'Non-classical Th1' cells produce IL-17 and IFN Linked to autoimmune diseases	IFN- γ
Th17 precursor Tc17 precursor	CD161+CCR6+	As per Th17 cells	IL-17
CD4+ Th22 CD8+ Tc22	CCR10+CCR4+ CCR6+CXCR3-	Defensive role against skin / mucosal infections, extracellular pathogens, and malignant cells. Tc22 produce granzyme.	IL-22, IL-2, TNF-α

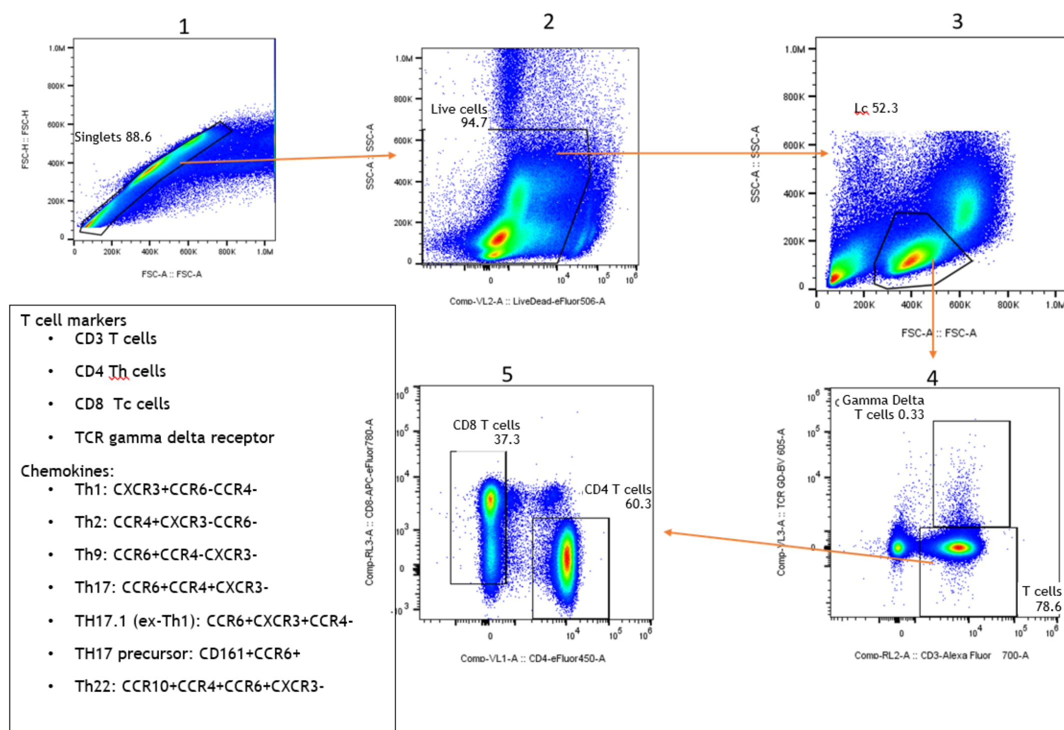
Table 4.1. Immunological significance of chemokine panel cells. [270]

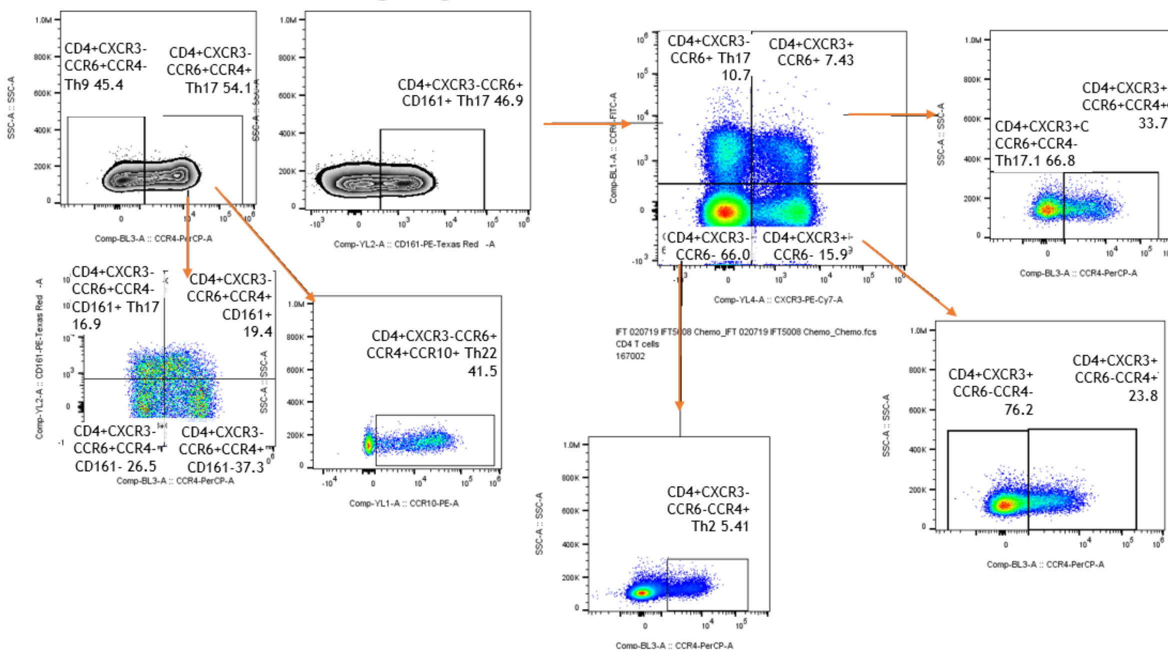
T cell Marker	Fluorescent label	**Concentration** (μl/sample) **Mix Tube**	**Concentration** (μl/sample) **FM control 1 Tube**	Supplier	Cat. number
CD3	Alexa Fluor 700	2	2	Thermo Fisher	56-0038-42
CD8a	APC-eFluor 780	1	1	Thermo Fisher	47-0088-42
CD4	eFluor 450	1	1	Thermo Fisher	48-0049-42
CD45RA	BV605	1	1	Thermo Fisher	MHCD5601
CD45RO	PE-Cy7	1	1	Thermo Fisher	61-0289-42
CD62L	PE	3	3	Bio-legend	304134
CD56	FITC	5	5	Thermo Fisher	17-1979-42
CCR7	APC	5	X	Thermo Fisher	46-1228-42
CD28	PE-eFluor 610	2	x	Thermo Fisher	25-0457-42
CD122	Per-CP eFluor 710	2	x	Thermo Fisher	12-0629-42
Fix viability dye	eFluor 506	1ul added in separately before surface antibody mix	1ul	Thermo Fisher	65-0866-18
B cell Marker	Fluorescent label	**Concentration** (μl/sample) **Mix Tube**	**Concentration** (μl/sample) **FM control 1 Tube**	Supplier	Cat. number
CD38 V450	V450	2	2	BD	646851
CD27 V500	V500	2	2	BD	561222
CD43 PE	PE	2.5	2.5	BD	560199
CD24 FITC	FITC	5	5	BD	555427
IgM PerCpCy5.5	PerCpCy5.5	5	5	BD	561285
CD19 APC	APC	5	5	Biolegend	302212
IgD APC-H7	APC-H7	5	5	BD	561305
CD10 PE-Cy7	PE-Cy7	5	x	BD	341112
CD5	AF700	5	x	Biolegend	300632
Monocyte Marker	Fluorescent label	**Concentration** (μl/sample) **Mix Tube**	**Concentration FM control 1 Tube**	Supplier	Cat. number

CD3	PE	1	1	Thermo Fisher	12-0038-42
CD56	PE	1	1	Thermo Fisher	12-0567-42
CD15	PE	1	1	Biolegend	63-0149-42
CD19	PE	1	1	Thermo Fisher	12-0199-42
HLA DR	PE-Cy7	1	1	Thermo Fisher	25-9952-42
CD141	APC	1	1	Thermo Fisher	17-1419-42
CD14	APC-eFluor 780	1	1	Thermo Fisher	47-0149-42
CD16	eFluor 450	1	1	Thermo Fisher	48-0168-42
Mannose Receptor	PE-CF594	2	x	BD	564063
CD303a	PerCP-eFluor 710	10	x	Thermo Fisher	46-9818-42
CD1c	FITC	3	3	Thermo Fisher	11-0015-42
CD192 (CCR2)	Brilliant Violet 510	3	x	Biolegend	357218
CD181 (CXCR1)	Brilliant Violet 605	3	x	BD	743421
CCR5	Alexa Fluor 700	3	x	Biolegend	359116
Lineage Marker	Fluorescent label	**Concentration** (µl/sample) **Mix Tube**	**Concentration FM control 1 Tube**	Supplier	Cat. number
CD3	Alexa Fluor 700	2	2	Thermo Fisher	56-0038-42
CD8a	APC-eFluor 780	2	2	Thermo Fisher	47-0088-42
CD4	eFluor 506	1	1	Thermo Fisher	69-0049-42
CD14	SuperBright 600	5	5	Thermo Fisher	63-0149-42
CD19	PE-Cy7	5	5	Thermo Fisher	25-0199-42
STAT1 (pY701)	Alexa Fluor 488	10	x	BD	612596
STAT3 (pY705)	Brilliant Violet 421	3	x	Bio-legend	651010
STAT6 (pY641)	PerCP-Cy5.5	5	x	Bio-legend	686010
Syk	PE	5	x	Thermo Fisher	12-6696-42
c-Cbl (pY774)	Alexa Fluor 647	10	x	BD	558103
CD3	Alexa Fluor 700	2	2	Thermo Fisher	56-0038-42
CD8a	APC-eFluor 780	2	2	Thermo Fisher	47-0088-42
CD4	eFluor 506	1	1	Thermo Fisher	69-0049-42
CD14	SuperBright 600	5	5	Thermo Fisher	63-0149-42
CD19	PE-Cy7	5	5	Thermo Fisher	25-0199-42

Table 4.2. T cell, B cell, monocyte/DC, and stimulation study panel cell markers, colour, and FM (fluorescence minus one) controls.

Flow cytometry data was analysed using FlowJo™ v10.8 (BD Life Sciences) version 10.8, confirmed by highly experienced researcher. Gating strategy for surface chemokine receptors of T cells is provided here as an example (Figure 4.2), for other flow cytometry panel gating strategies please see Appendix 5.





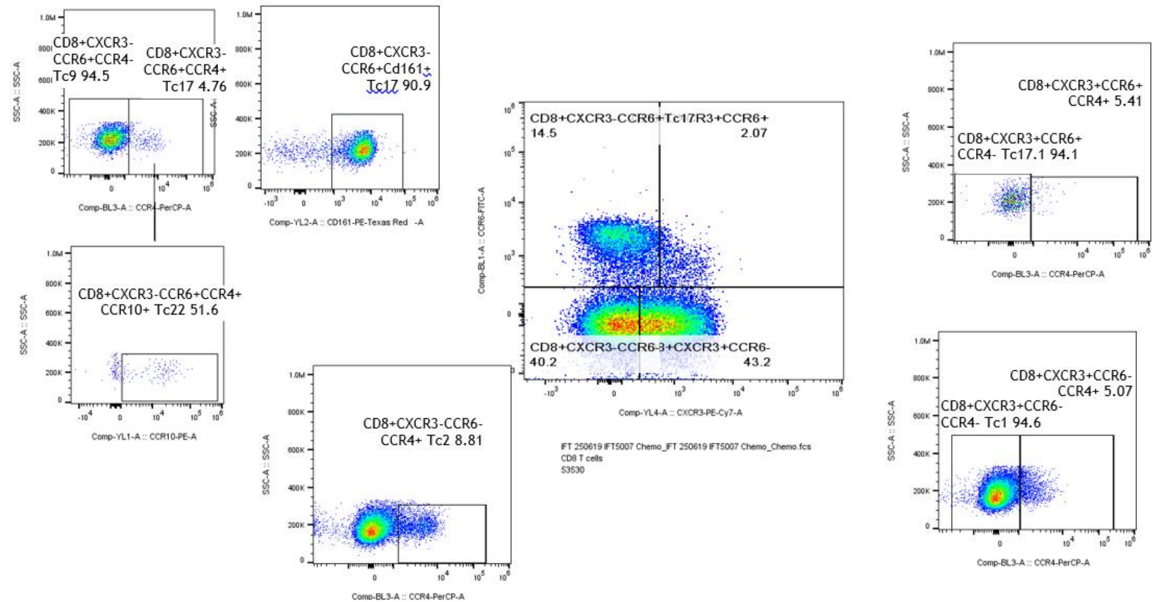


Figure 4.0. Gating strategy for the Chemokine cell panel. Th, T helper cell; Tc, cytotoxic T cell; SSC, side scatter.

4.2.6 Analysis and statistics

Of 154 recruited participants, 143 had sufficient flow cytometry data, the other 11 included two participant exclusions from final data set as were discussed in Chapter 3; three additional participant exclusions made on the basis of high BMI, known to affect circulating immune cells; six were not processed due to flow cytometer breakdown. For some panels the number of participants' data was lower as a consequence of lab restrictions following the COVID-19 pandemic (stimulation studies panel being additionally time-consuming), and optimisation issues early in the study (particularly monocyte/DC panel), see Table 4.3. One participant was removed specifically from the Monocyte analyses as a significant outlier (more than 3 standard deviations from the mean).

The threshold for determining normotensive (NTN) and hypertensive (HTN) groups was based on an ABPM 24hr mean value of 130 and/or 80 mmHg. Confounding by the high prevalence of 'borderline' 24hr BP results was considered, hence a tertile analysis of participants (normotensive, borderline and hypertensive) was undertaken to assess for this possibility; these BP thresholds were set at lower thresholds, based on evidence of immune system alterations at the upper end of the normotensive range.[271] Hence groups were set at NTN <121/78 mmHg, HTN >134/82 mmHg, with

'borderline group' in between, providing the following final numbers by flow cytometry panel:

Panel	**NTN : HTN** 24hr BP 130/80 mmHg threshold	**Tertiles 24hr BP^**	**Nocturnal Dipper : non-dipper** 10% of daytime average BP threshold
T cell N= 143	76 : 67	50 : 44 : 49	84 : 52
B cell N = 143	75 : 68	50 : 44 : 49	82 : 51
Chemokine N = 99	52 : 47	41 : 15 : 43	56 : 34
Phospho-flow N=120	66 : 54	42 : 40 : 38	68 : 42
Mono DC N=108	58 : 50	45: 17 : 46	59 : 49

Table 4.3. Sample numbers by panel and participant features. ^Tertile thresholds NTN <121/78, borderline 121/78 to 134/83, HTN >134/82 mmHg; WCH elevated clinical setting BP but 24hr average <130/80mmHg; MHN normal office BP but 24hr average >130/80mmHg; sustained HTN office and 24hr average BP both >130/80mmHg.

	Mean Age	Mean BMI	Mean Physical activity score
HTN male	37.3	28.2	50.2
HTN female	41.4	28.9	40.8
NTN male	39.8	26.7	55.9
NTN female	38.8	25.8	53.9
ANOVA p-value	0.29	0.026	0.015

Table 4.4. Assessment for confounding demographic variables.

Inflammatension statistical analysis was reported in Chapter 2.0 Methods, including analysis using Minitab (Version 19) with significance set at p<0.05, Bonferroni thresholds for statistical significance in the setting of multiple comparisons are reported where appropriate; data considerations specific to the flow cytometry data follow. Mean and standard deviation (SD) of mean fluorescence intensity (MFI) and percentage sub-sets are reported. Considering potential confounders, Table 4.4 demonstrates that age did not differ between hypertension and normotension groups, including when stratified by sex. BMI and physical activity levels did differ. Hence, adjusted models are also reported below, with 24hr SBP, age, BMI, physical activity score and the parameter of interest, to ascertain if t-test findings remained valid once potential confounders were adjusted for.

Stimulation studies are reported as 'U' (unstimulated levels), 'S' (levels after being stimulated with the stimulation cocktail), and 'S-U' (stimulated minus unstimulated). The latter ensures normalisation of cells with low level phosphorylation prior to stimulation, e.g. spontaneous phosphorylation, or that due to the cell processing techniques such as centrifugation. This enables reporting of only the phosphorylation occurring due to controlled stimulation. Parameters in which more than 25% failed to stimulate are not reported, as a quality control measure due to the possibility of a technical cause.

4.3 Results

4.3.1 Normotension versus hypertension

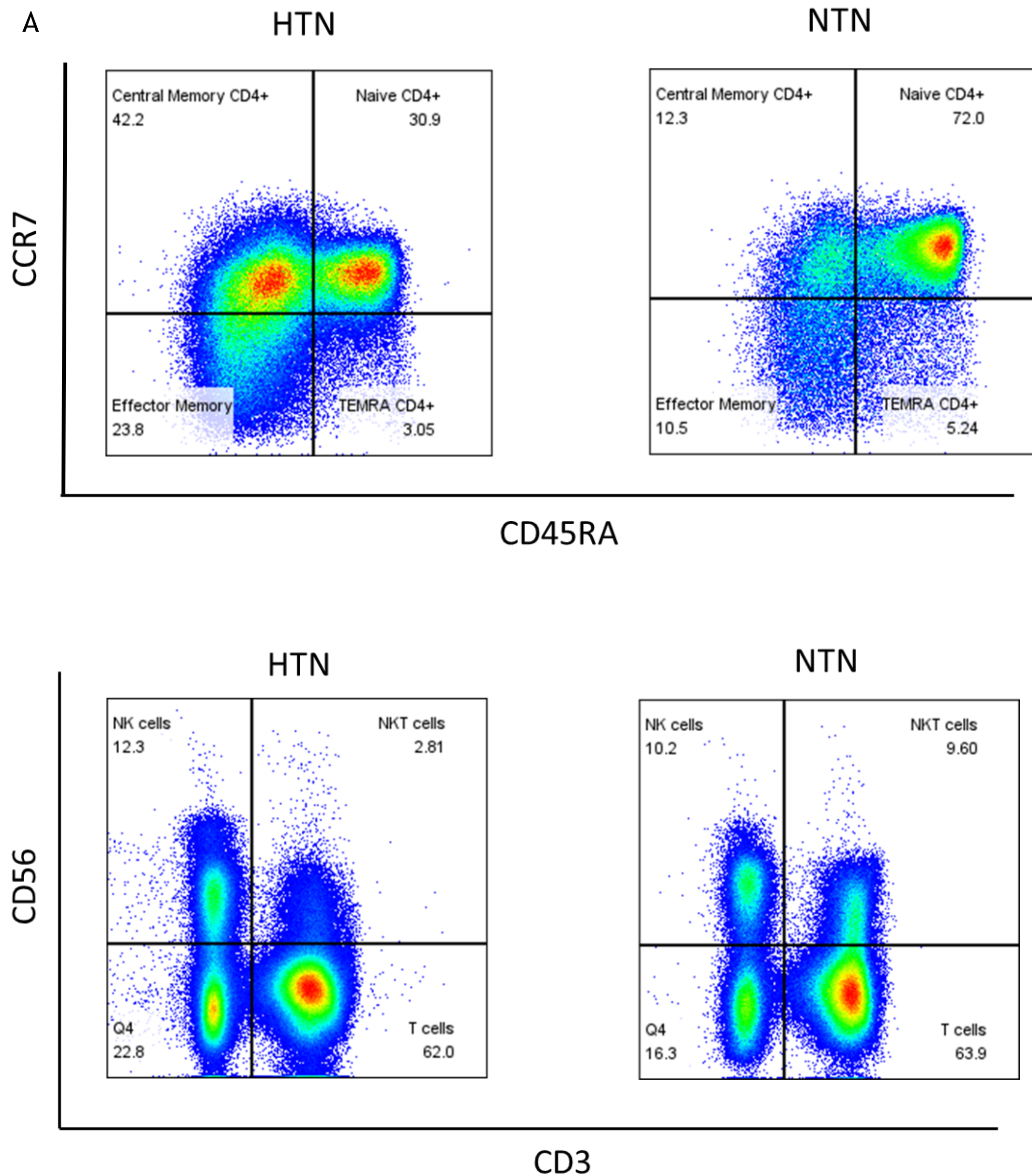
4.3.1.1 T cells and NK cells

Percentages of lymphocytes and monocytes were similar between NTN and HTN. Table 4.5 reports T cell subsets by normotensive and hypertensive groups, between group differences illustrated in Figure 4.1. CD4+ naïve T cells (CD45RA+CCR7+) were lower in HTN, but only the CD4+CD45RA+CCR7+ CD45RO+CD62l+ subset attained statistical significance (p= 0.039). Correspondingly, proportionally higher numbers of CD4+ Central Memory (TCM, CD45RA-CCR7+) cells were demonstrated in the HTN group, p=0.002. Regarding terminally differentiated T cell subsets, CD8+ TEMRA (CD45RA+ CCR7-) were fewer, but only CD8+CD45RA+CCR7-CD45RO-CD62l+ subgroup significantly so (p=0.036). Both NKT cells and CD122+NKT Lymphocytes were lower in the hypertensive group (p=0.026 and p=0.046 respectively), as were CD122+ T lymphocytes (p=0.019). The adjusted model column in Table 4.5 demonstrates that accounting for potential confounding factors expands the number of T cell subsets with statistically significant association with SBP to include NK cell subsets and both CD4+CD28+ and CD8+CD28+ T cells (less frequent in HTN group), among others (Table 4.5). However, none of the T cell panel remained significant following the multiple comparison adjustment of the threshold p-value to <0.0013.

T cell subset by Flow Cytometry cell markers, percent	NTN N=76	SD	HTN N=67	SD	T-test p value	Adjusted model P-value
Lymphocytes	77.6	12.0	78.3	8.9	0.650	0.623
CD122+ Lymphocytes	11.7	7.2	11.5	6.1	0.813	0.474
CD122+ NK cells	79.3	15.8	82.9	11.1	0.116	0.141
CD122+ NK cells (Lymphocytes)	9.3	5.5	9.7	5.5	0.605	0.068
CD122+ NKT cells	1.1	1.0	0.8	0.8	0.062	0.158
CD122+NKT cells (Lymphocytes)	0.13	0.0	0.08	0.1	0.046*	0.016*
CD122+ T cells	9.7	12.6	6.7	6.3	0.073	0.236
CD122+ T cells (Lymphocytes)	1.3	3.4	0.6	0.4	0.116	0.019*
NK cells	12.0	6.3	13.2	7.2	0.311	0.006*
Bright NK cells	7.6	6.3	6.1	5.3	0.110	0.005*
Dim NK cells	92.3	6.4	94.0	5.2	0.097	0.019*
NKT cells	3.6	3.5	2.5	2.4	0.026*	0.015*
Non-T Non-NK	14.3	5.0	16.0	5.6	0.051	0.012*
T cells	70.1	9.5	68.3	10.4	0.288	0.242
CD4+ Th	63.8	9.9	64.6	8.3	0.614	0.691
CD4+ Central Memory	23.1	7.2	27.7	10.0	0.002*	0.034*
CD4+CD45RO+CD62l- Central memory	8.3	3.4	9.1	4.0	0.231	0.686
CD4+CD45RO+CD62l+ Central memory	90.7	3.5	90.4	4.1	0.574	0.812
CD4+ Effector memory	16.8	8.4	17.3	6.8	0.685	0.251
CD4+CD45RA-CCR7-CD45RO+CD62l+	68.1	7.4	66.0	10.3	0.225	0.101
CD4+CD28+CD45RA-CCR7-CD45RO+CD62l+	96.9	5.4	95.6	7.9	0.166	0.100
CD4+CD28-CD45RA-CCR7-CD45RO+CD62l+	3.0	5.4	4.3	7.9	0.260	0.169
CD4+CD45RA-CCR7-CD45RO+CD62l- TEM	31.4	7.5	33.7	10.3	0.263	0.035*
CD4+CD28+ Effector memory	95.1	9.8	92.0	13.5	0.129	0.034*
CD4+CD28- Effector memory	4.9	9.8	8.0	13.4	0.124	0.095
CD4+ Naïve	57.1	12.9	52.4	13.9	0.124	0.321
CD4+CD45RA+CCR7+CD45RO+CD62l+	1.3	1.1	1.2	1.0	0.039*	0.708
CD4+ CD45RO-CD62l+Naive	98.3	1.2	98.2	2.3	0.512	0.115
CD4+ CD28+ Naïve	98.7	6.3	99.5	1.7	0.701	0.457
CD4+ TEMRA	3.1	2.9	2.7	2.2	0.321	0.883
CD4+CD28+CD45RA+CCR7-	83.0	17.7	82.0	18.0	0.325	0.611
CD4+CD45RA+CCR7-CD45RO+CD62l+	47.4	14.6	47.7	11.7	0.729	0.967
CD4+CD45RA+CCR7-CD45RO-CD62l+	39.3	16.0	37.4	13.5	0.862	0.372
CD4+CD45RA+CCR7-CD45RO+CD62l-	8.4	6.9	10.2	7.8	0.439	0.932
CD4+CD45RA+CCR7-CD45RO-CD62l-	5.0	5.1	4.7	3.5	0.142	0.883
CD4+CD28- TEMRA	16.9	17.7	18.0	18.0	0.692	0.696
CD8+ Tc	28.9	8.4	28.9	6.7	0.733	0.634
CD8+ Central Memory	6.7	3.7	7.8	4.8	0.974	0.352
CD8+ Effector Memory	34.2	13.9	36.3	14.7	0.156	0.069
CD8+CD45RA-CCR7-CD45RO+CD62l+	47.9	10.3	45.1	13.2	0.381	0.267
CD8+CD28+CD45RA-CCR7-CD45RO+CD62l+	70.8	15.3	72.7	13.5	0.167	0.870
CD8+CD28-CD45RA-CCR7-CD45RO+CD62l+	29.1	15.3	27.2	13.5	0.437	0.066
CD8+CD45RA-CCR7-CD45RO+CD62l- TEM	48.9	10.5	52.2	13.3	0.435	0.011*
CD8+CD28+ Effector memory	68.0	19.7	62.0	21.0	0.105	0.011*
CD8+CD28- Effector memory	32.0	19.7	37.9	21.0	0.082	0.582
CD8+ Naïve	44.8	17.5	43.6	19.7	0.082	0.176
CD8+CD45RO-CD62l+Naive	98.3	1.4	98.4	1.3	0.699	0.100
CD8+CD45RA+CCR7+CD45RO+CD62l+	1.5	1.4	1.3	1.2	0.651	0.199
CD8+ TEMRA	14.2	9.7	12.3	8.8	0.525	0.753
CD8+CD28- TEMRA	76.9	14.7	75.0	17.8	0.217	0.750

CD8+CD28+CD45RA+CCR7-	23.1	14.7	25.0	17.8	0.482	0.008*
CD8+CD45RA+CCR7-CD45RO+CD62l+	10.4	9.7	7.6	5.9	0.481	0.590
CD8+CD45RA+CCR7-CD45RO-CD62l+	52.2	14.5	50.2	16.1	0.036*	0.510
CD8+CD45RA+CCR7-CD45RO+CD62l-	4.3	4.4	4.7	4.8	0.436	0.033*
CD8+CD45RA+CCR7-CD45RO-CD62l-	33.1	15.4	37.6	15.6	0.662	0.052
DN T cells	6.3	3.2	5.7	3.1	0.086	0.075
DP T cells	0.9	1.1	0.8	0.5	0.265	0.217

Table 4.5. Flow cytometry T cell panel results by BP group. NTN, normotension; HTN, hypertension; SD, standard deviation; *p<0.05. Adjusted model accounts for potential confounding factors (age, BMI, physical activity) with partial correlation analysis of SBP and immune marker.



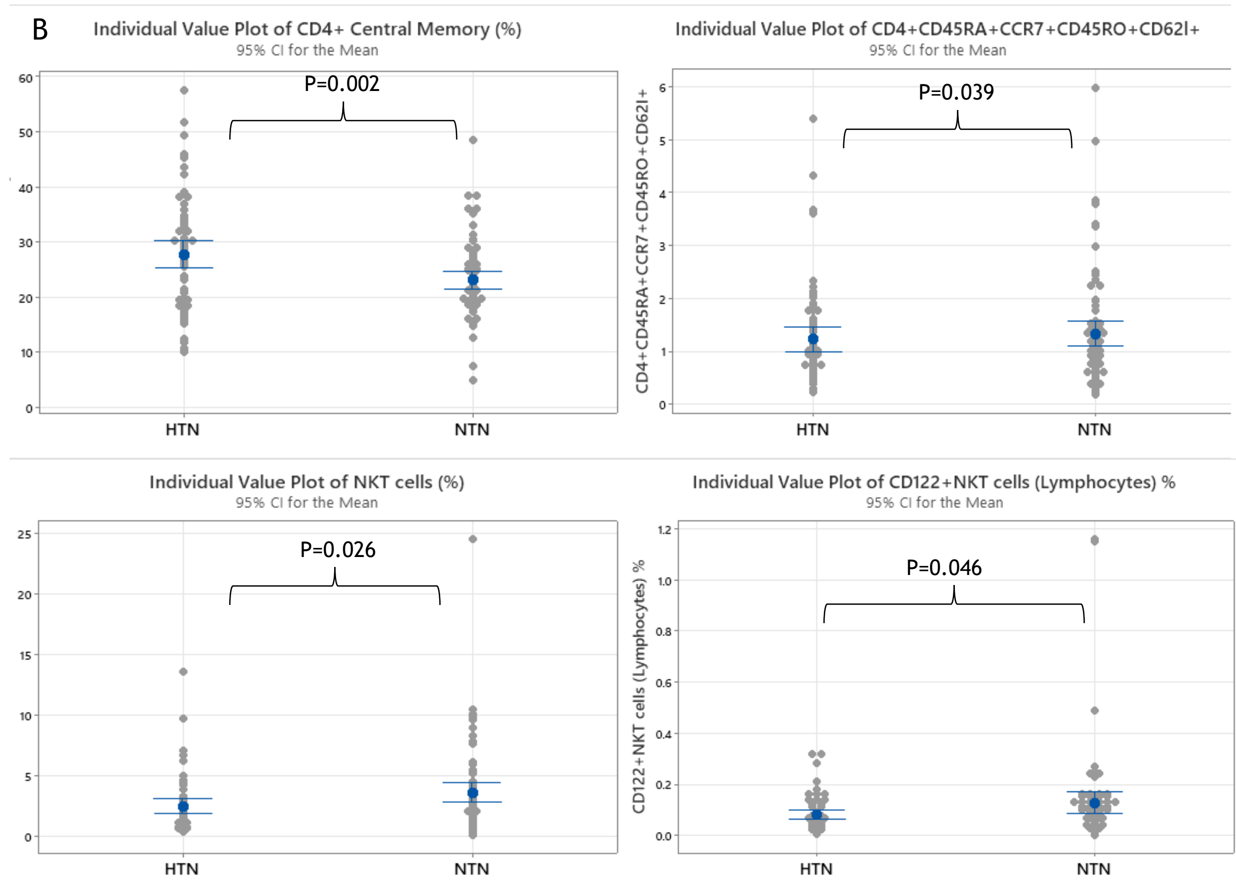


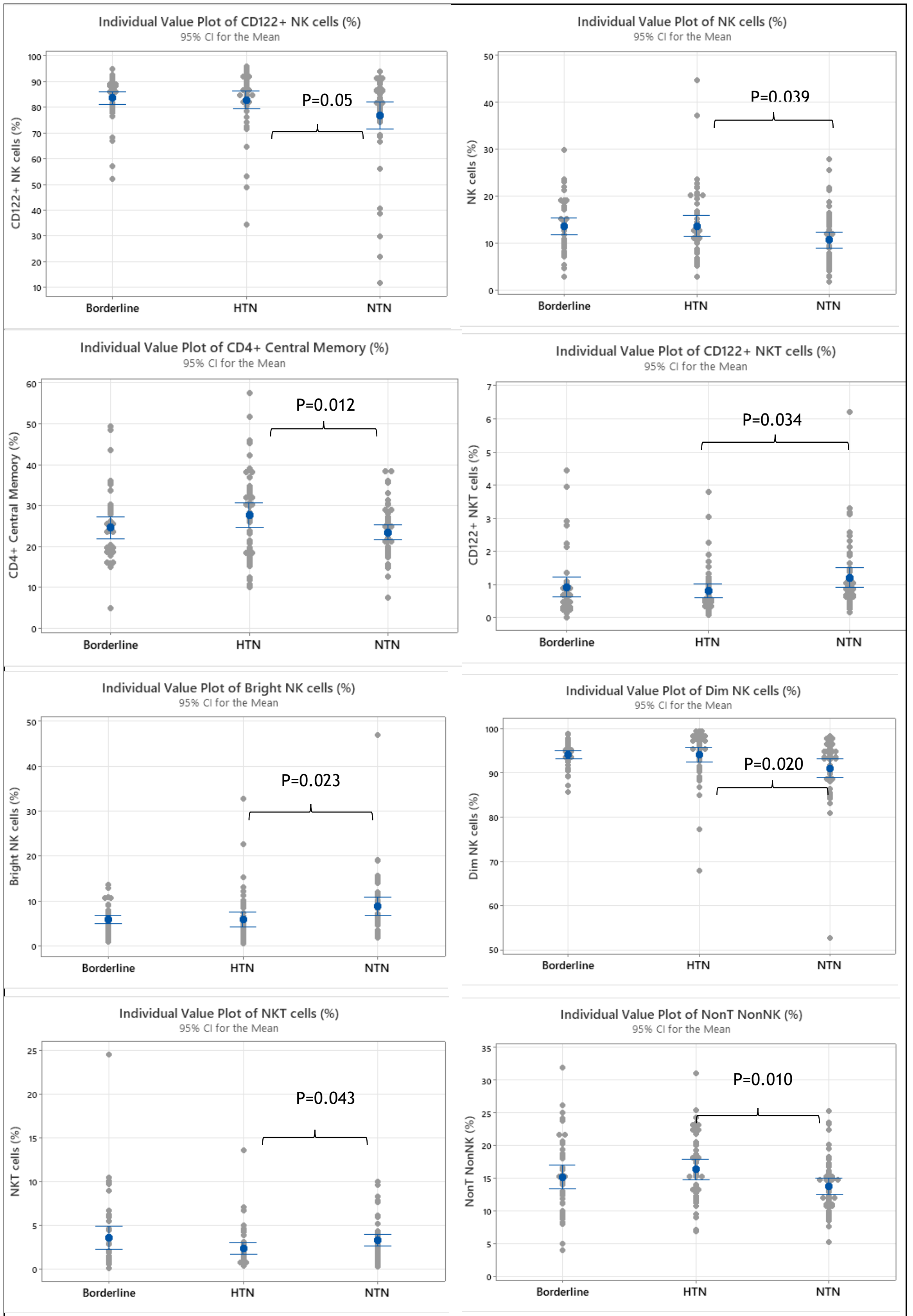
Figure 4.1. Flow cytometry T cell panel: A) representative images of T cell subset proportions; B) between blood pressure group individual value plots; mean and interval bar (standard deviation) depicted. NTN, normotension; HTN, hypertension.

Table 4.6 and Figure 4.2 present the tertile analysis of participants (normotensive, borderline and hypertensive). Additional cell subsets demonstrating between-group differences once the borderline participants were removed included a smaller circulating T cell compartment in the HTN group, though CD4+ and CD8+ did not differ. CD4+ TCM were again increased in HTN as illustrated in Figure 4.2, though no difference was apparent in CD4+ T_{EM} nor CD8+ T_{CM}; the ratio of CD8+CD28+ to CD8+CD28- effector memory T cells was however also lower in HTN. Circulating NK cells were higher in HTN, particularly cytotoxic $CD56+^{Dim}$ NK cells, concordant with the dichotomised BP group analysis.

T cell subset by Flow Cytometry cell markers, percent	NTN (SD) N=50	Borderline (SD) N=44	HTN (SD) N= 49	T-test P-value
CD122+ Lymphocytes	11.3 7.7	12.0 5.6	11.7 6.5	0.771
CD122+ NK cells	76.8 18.1	83.6 8.6	82.9 11.9	0.052
CD122+ NK cells (Lymphocytes)	8.4 5.3	10.1 5.2	9.9 6.0	0.189
CD122+ NKT cells	1.2 1.1	0.9 1.0	0.8 0.7	0.034*

CD122+ NKT cells (Lymphocytes)	0.1	0.2	0.1	0.1	0.1	0.1	0.063
CD122+ T cells	11.5	14.8	5.9	4.3	7.3	7.1	0.075
CD122+ T cells (Lymphocytes)	1.5	4.2	0.7	0.5	0.7	0.4	0.151
NK cells	10.6	6.0	13.5	5.7	13.6	7.9	0.039*
Bright NK cells	8.9	7.2	5.8	3.0	5.8	5.8	0.023*
Dim NK cells	91.1	7.3	94.2	3.0	94.2	5.8	0.020*
NKT cells	3.3	2.2	3.6	4.4	2.4	2.3	0.043*
NonT NonNK	13.7	4.4	15.2	6.0	16.3	5.4	0.010*
T cells	72.3	8.0	67.7	10.3	67.7	10.9	0.019*
CD4+ Th	62.8	9.9	65.0	9.7	64.8	7.9	0.274
CD4+ Central Memory	23.4	6.6	24.6	8.8	27.7	10.5	0.017*
CD4+CD45RO+CD62l- Central memory	8.3	3.2	8.6	4.1	9.1	3.9	0.256
CD4+CD45RO+CD62l+ Central memory	90.6	3.3	90.8	4.1	90.3	4.0	0.621
CD4+ Effector memory	16.0	6.5	18.4	9.9	16.8	6.3	0.522
CD4+CD45RA-CCR7-CD45RO+CD62l+	68.3	6.8	67.0	9.5	66.0	10.2	0.192
CD4+CD28+CD45RA-CCR7-CD45RO+CD62l+	97.4	4.5	95.1	7.1	96.3	8.0	0.442
CD4+CD28-CD45RA-CCR7-CD45RO+CD62l+	2.6	4.5	4.8	7.1	3.6	8.0	0.441
CD4+CD45RA-CCR7-CD45RO+CD62l- TEM	31.1	7.0	32.7	9.5	33.6	10.2	0.153
CD4+CD28+ Effector memory	95.7	8.4	92.2	12.7	92.8	13.6	0.205
CD4+CD28- Effector memory	4.2	8.4	7.7	12.7	7.2	13.6	0.204
CD4+ Naive	57.6	10.8	54.0	15.9	53.0	13.5	0.063
CD4+CD45RA+CCR7+CD45RO+CD62l+	1.4	1.2	1.3	0.9	1.1	0.9	0.216
CD4+ CD45RO-CD62l+Naive	98.3	1.3	98.0	2.7	98.6	1.0	0.260
CD4+ CD28+ Naive	98.3	7.7	99.6	1.0	99.4	2.0	0.341
CD4+ TEMRA	3.0	3.2	3.1	2.1	2.6	2.3	0.387
CD4+CD28+CD45RA+CCR7-	82.1	18.4	81.5	17.3	83.9	17.8	0.617
CD4+CD45RA+CCR7-CD45RO+CD62l+	47.5	14.4	48.2	14.4	47.0	11.3	0.833
CD4+CD45RA+CCR7-CD45RO-CD62l+	38.8	16.1	37.4	15.1	39.0	13.4	0.939
CD4+CD45RA+CCR7-CD45RO+CD62l-	8.9	7.8	9.0	6.6	9.8	7.7	0.578
CD4+CD45RA+CCR7-CD45RO-CD62l-	4.8	5.2	5.4	4.8	4.3	3.0	0.532
CD4+CD28- TEMRA	17.9	18.4	18.5	17.3	16.0	17.8	0.616
CD8+ Tc	29.5	8.3	28.2	8.0	29.0	6.7	0.756
CD8+ Central Memory	6.6	3.7	7.1	4.5	8.0	4.4	0.104
CD8+ Effector Memory	32.4	11.9	38.4	16.3	35.3	14.3	0.268
CD8+CD45RA-CCR7-CD45RO+CD62l+	49.8	10.0	44.2	11.6	45.4	13.1	0.060
CD8+CD28+CD45RA-CCR7-CD45RO+CD62l+	71.7	15.1	70.3	16.4	73.0	11.8	0.641
CD8+CD28-CD45RA-CCR7-CD45RO+CD62l+	28.2	15.1	29.7	16.4	26.9	11.8	0.642
CD8+CD45RA-CCR7-CD45RO+CD62l-	47.3	10.8	52.5	10.7	51.9	13.7	0.065
CD8+CD28+ Effector memory	70.1	18.6	63.5	21.9	61.7	20.2	0.035*
CD8+CD28- Effector memory	29.9	18.6	36.5	21.9	38.2	20.2	0.035*
CD8+ Naive	46.3	17.0	41.0	19.1	45.2	19.5	0.759
CD8+CD45RO-CD62l+Naive	98.2	1.3	98.1	1.6	98.6	1.1	0.179
CD8+CD45RA+CCR7+CD45RO+CD62l+	1.5	1.3	1.5	1.5	1.1	1.0	0.129
CD8+ TEMRA	14.8	10.5	13.5	9.2	11.6	7.9	0.094
CD8+CD28- TEMRA	75.7	14.6	78.7	16.1	73.9	17.6	0.568
CD8+CD28+CD45RA+CCR7-	24.3	14.6	21.3	16.1	26.1	17.6	0.566
CD8+CD45RA+CCR7-CD45RO+CD62l+	10.7	9.0	9.5	9.8	7.0	5.1	0.013*
CD8+CD45RA+CCR7-CD45RO-CD62l+	53.1	14.3	49.1	14.6	51.4	16.8	0.595
CD8+CD45RA+CCR7-CD45RO+CD62l-	4.4	4.3	4.7	4.8	4.4	4.7	0.952
CD8+CD45RA+CCR7-CD45RO-CD62l-	31.8	15.5	36.7	14.4	37.3	16.3	0.091
DN T cells	6.7	3.4	5.9	3.4	5.5	2.7	0.051

Table 4.6. Flow cytometry T cell panel results by BP tertile. NTN, normotension <121/78 mmHg, borderline 121/78 to 134/83 mmHg, HTN, hypertension >134/82 mmHg; SD, standard deviation; *p<0.05.


Individual Value Plot of CD122+ NK cells (%)
95% CI for the Mean
CD122+ NK cells (%)
P=0.05
Borderline
HTN
NTN
Individual Value Plot of NK cells (%)
95% CI for the Mean
NK cells (%)
P=0.039
Individual Value Plot of CD4+ Central Memory (%)
95% CI for the Mean
CD4+ Central Memory (%)
P=0.012
Individual Value Plot of CD122+ NKT cells (%)
95% CI for the Mean
CD122+ NKT cells (%)
P=0.034
Individual Value Plot of Bright NK cells (%)
95% CI for the Mean
Bright NK cells (%)
P=0.023
Individual Value Plot of Dim NK cells (%)
95% CI for the Mean
Dim NK cells (%)
P=0.020
Individual Value Plot of NKT cells (%)
95% CI for the Mean
NKT cells (%)
P=0.043
Individual Value Plot of NonT NonNK (%)
95% CI for the Mean
NonT NonNK (%)
P=0.010

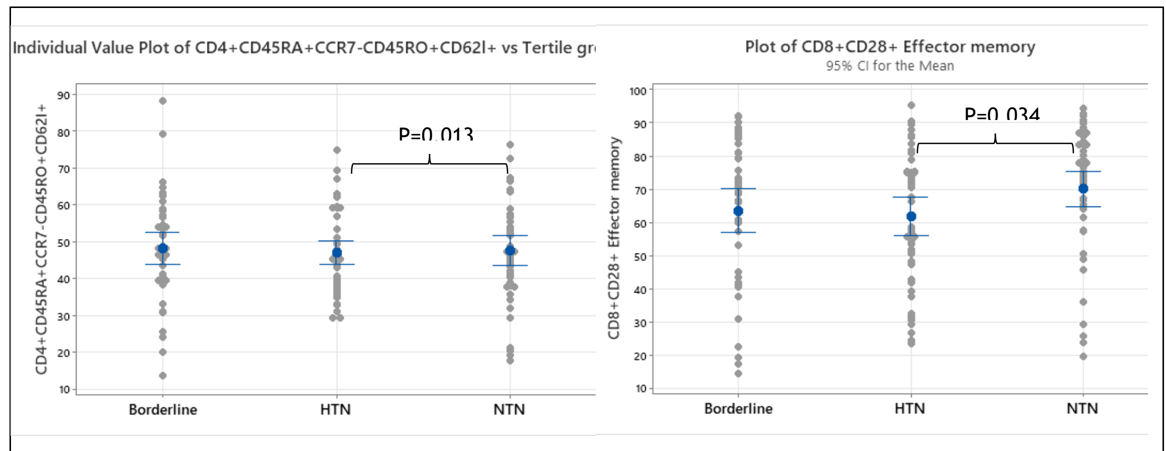


Figure 4.2. Flow cytometry T cell panel illustrative results by blood pressure tertile. NTN, normotension <121/78 mmHg, borderline 121/78 to 134/83 mmHg, HTN, hypertension >134/82 mmHg. Mean and interval bar (standard deviation) depicted.

4.3.1.2 Chemokines receptor T cell subsets

CD8+CXCR3+CCR6+ (Tc17.1), CD4+CXCR3+CCR6+ (Th17.1), and CD4+CXCR3+CCR6- (Th1) all demonstrated between group differences, with higher numbers of circulating cells expressing these markers in hypertension. CD4+CXCR3-CCR6- (Th2) conversely were lower in the hypertensive group, as Table 4.7 and Figure 4.3 illustrate. Tertile division of BP groups corroborated significance in these subsets with no additional differences detected (not shown), nor did SBP in a model adjusted for age, BMI and physical activity unmask additional associations.

Chemokine Receptor T cell subsets by Flow Cytometry , percent	NTN N=52	(SD)	HTN N=47	(SD)	T-test	Adjusted model
Gamma Delta T cells	0.75	0.70	0.62	0.49	0.323	0.077
CD4+CD161+CCR6+ Th17 precursor	8.99	3.65	10.70	5.49	0.068	0.199
CD4+CXCR3+CCR6+ Th17.1	7.47	4.24	10.05	5.78	0.011*	0.075
CD4+CXCR3+CCR6+CCR4+	33.56	9.50	34.54	11.94	0.640	0.788
CD4+CXCR3+CCR6+CCR4-	66.48	9.51	65.45	11.89	0.626	0.781
CD4+CXCR3+CCR6- Th1	10.93	4.94	13.10	5.01	0.027*	0.025
CD4+CXCR3+CCR6-CCR4+	26.68	6.41	25.97	6.74	0.587	0.353
CD4+CXCR3+CCR6-CCR4-	73.29	6.42	73.96	6.74	0.612	0.372
CD4+CXCR3-CCR6+ Th17	11.79	3.61	13.40	5.21	0.066	0.282
CD4+CXCR3-CCR6+CCR4+ Th17	60.50	8.32	61.67	8.94	0.484	0.851
CD4+CXCR3-CCR6+CCR4+CCR10+ Th22	41.83	8.92	40.50	10.13	0.438	0.353
CD4+CXCR3-CCR6+CCR4- Th9	39.06	8.34	37.88	8.90	0.481	0.827
CD4+CXCR3-CCR6+CD161+ Th17	44.90	9.33	44.30	10.94	0.725	0.941
CD4+CXCR3-CCR6+CCR4+CD161+	20.52	5.21	20.40	6.57	0.859	0.818
CD4+CXCR3-CCR6+CCR4+CD161-	43.03	7.81	44.52	8.93	0.341	0.778
CD4+CXCR3-CCR6+CCR4-CD161+	16.16	5.69	14.57	7.03	0.185	0.392
CD4+CXCR3-CCR6+CCR4-CD161-	20.31	5.18	20.50	6.97	0.810	0.477
CD4+CXCR3-CCR6- Th2	69.80	9.97	63.44	11.46	0.003*	0.021*
CD4+CXCR3-CCR6-CCR4+	10.91	5.50	11.88	5.45	0.283	0.992
CD8+CD161+CCR6+	12.95	8.66	11.10	7.48	0.229	0.116
CD8+CXCR3+CCR6+ Tc17.1	1.98	1.22	2.88	1.96	0.009*	0.203
CD8+CXCR3+CCR6+CCR4+	11.16	6.13	9.85	4.27	0.214	0.349
CD8+CXCR3+CCR6+CCR4-	88.84	6.13	90.14	4.26	0.219	0.361
CD8+CXCR3+CCR6- Tc1	42.67	17.09	47.48	17.52	0.129	0.231
CD8+CXCR3+CCR6-CCR4+	5.71	3.54	6.05	3.77	0.631	0.694
CD8+CXCR3+CCR6-CCR4- Tc1	94.27	3.53	93.88	3.77	0.585	0.657
CD8+CXCR3-CCR6+ Tc17	13.43	8.79	11.66	7.65	0.261	0.139
CD8+CXCR3-CCR6+CCR4+ Tc17	6.79	8.22	9.89	10.55	0.113	0.163
CD8+CXCR3-CCR6+CCR4+CCR10+ Tc22	45.72	16.94	47.36	19.23	0.509	0.381
CD8+CXCR3-CCR6+CCR4- Tc9	93.09	8.30	90.03	10.57	0.118	0.168
CD8+CXCR3-CCR6+CD161+ Tc17	90.94	8.95	86.94	12.49	0.062	0.050
CD8+CXCR3-CCR6- Tc2	41.92	18.88	37.98	16.54	0.228	0.543
CD8+CXCR3-CCR6-CCR4+ Tc2	5.87	4.54	7.29	6.83	0.204	0.578

Table 4.7. Chemokine panel T cell subsets by Flow cytometry, by normotension (NTN) and hypertension (HTN). SD, standard deviation. Adjusted model accounts for potential confounding factors (age, BMI, physical activity) with partial correlation analysis of SBP and immune marker. Multiple comparison adjustment to p-value threshold of 0.0016.

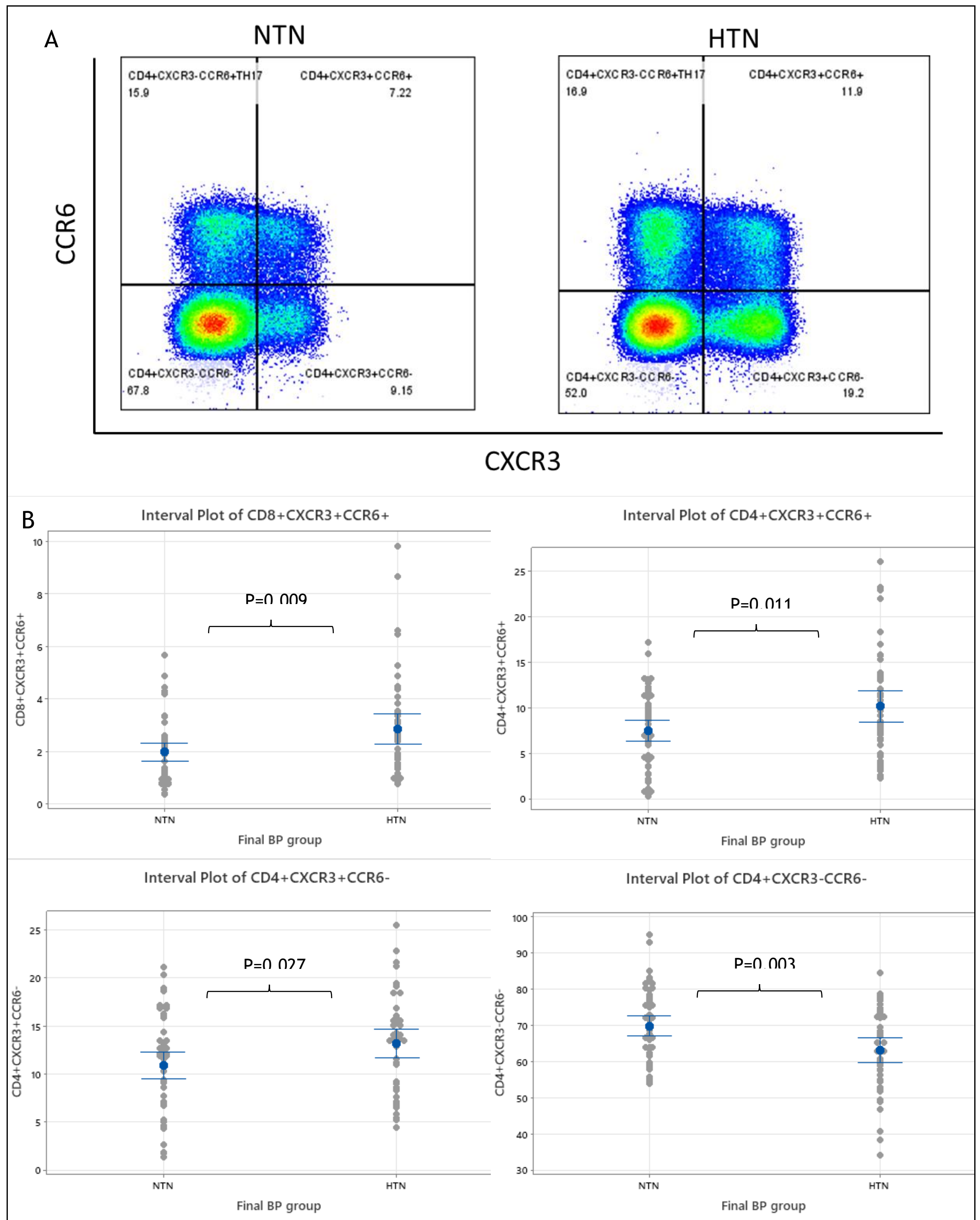


Figure 4.3. Flow cytometry Chemokine receptor panel: A) representative images of cell subset proportions; B) between blood pressure group individual value plots illustrating statistically notable results by blood pressure group. NTN, normotension; HTN, hypertension; mean and interval bar (standard deviation) depicted.

4.3.1.3 B cells

Although the total number of B cells in the hypertensive group was higher (Table 4.8), no differences were detected between normotensive and hypertensive patients across any of the B cell subsets. Nor were any

differences detected with the borderline group removed in tertile analysis, or in models adjusted for BMI, age and physical activity score (Table 4.8).

B cell subset by Flow Cytometry cell markers, percent	**NTN** N=76	SD	**HTN** N=67	(SD)	**T-test** p value	**Adjusted model P-value**
Lymphocytes	53.84	14.37	55.40	14.38	0.518	0.561
B cells	9.06	3.83	10.40	4.02	0.043*	0.086
CD5+B cells	0.67	0.35	0.76	0.50	0.216	0.197
Non-Transitional B cells	96.88	1.60	96.81	1.62	0.804	0.814
IgM memory	12.23	6.82	11.99	8.37	0.848	0.815
IgM memory B1 or activated	4.91	2.55	4.88	2.69	0.955	0.580
IgM memory plasma cell & blast	2.54	2.38	2.71	3.16	0.713	0.367
Memory IgM memory	92.58	3.88	92.44	4.69	0.854	0.378
Non-IgM memory	87.92	6.75	88.13	8.27	0.869	0.813
Non-IgM memory/CD27-IgD-	9.51	3.64	9.15	3.23	0.540	0.949
CD27-IgD- B1 or activated	7.42	4.28	6.72	4.50	0.344	0.206
CD27-IgD- memory	90.13	5.26	90.83	6.16	0.465	0.151
CD27-IgD- plasma cell & blast	2.47	1.77	2.46	2.81	0.971	0.232
Non-IgM memory/Naive	73.44	11.43	75.18	10.35	0.341	0.849
Naive activated	1.06	0.80	0.92	0.78	0.308	0.230
Naive naive	98.93	0.81	99.06	0.78	0.313	0.227
Non-IgM memory/Switched memory	12.93	7.77	11.61	6.71	0.276	0.948
Switched memory B1 or activated	8.17	3.51	8.08	4.29	0.884	0.396
Switched memory memory	90.03	4.59	90.35	5.15	0.692	0.443
Switched memory plasma cell	1.77	2.52	1.55	1.77	0.546	0.832
Un-switched memory	4.12	1.80	4.06	2.02	0.850	0.467
Unswitched memory B1/activated	6.19	2.77	6.58	3.34	0.451	0.740
Unswitched memory memory	92.75	3.41	92.50	3.86	0.684	0.390
Unswitched memory plasma cell	1.12	1.08	0.99	0.86	0.411	0.050
Transitional B cells	3.07	1.57	3.14	1.60	0.815	0.802
CD24+ Transitional	95.82	5.68	94.85	14.80	0.614	0.596

Table 4.8. B cell subset by Flow Cytometry cell markers. Adjusted model accounts for potential confounding factors (age, BMI, physical activity) with partial correlation analysis of SBP and immune marker. Multiple comparison adjustment to p-value threshold of 0.0019.

4.3.1.4 Monocytes and Dendritic cells

Table 4.9 demonstrates that no differences were detected in any DC subgroup between NTN and HTN patients. Neither did classical and non-classical monocytes differ between groups. Total intermediate monocyte (CD14++CD16+) compartment was also similar, though fewer intermediate monocytes expressed CCR2 in the HTN group, with both CCR2+CCR5- and CD97+CCR2+ populations smaller; correspondingly, there were more CCR5 and

CCR2 double positive and double negative cells in the HTN group, and more CD97+ intermediate monocytes were CCR2-. Table 4.9 and Figure 4.4 demonstrate that SBP was also associated with these same immune markers in an adjusted model, but not when applying a statistical significance level accounting for multiple comparisons (p<0.00049). Analysing the data in tertiles to separate out patients with borderline 24 hour average BP, the same markers remained statistically significant as demonstrating between group differences, and in addition non-classical monocyte CD14+CD16++ subset reached statistical significance (NTN 7.2 (3.1), borderline 10.8 (5.6), HTN 8.8 (4.6), p=0.010). See Figure 4.4.

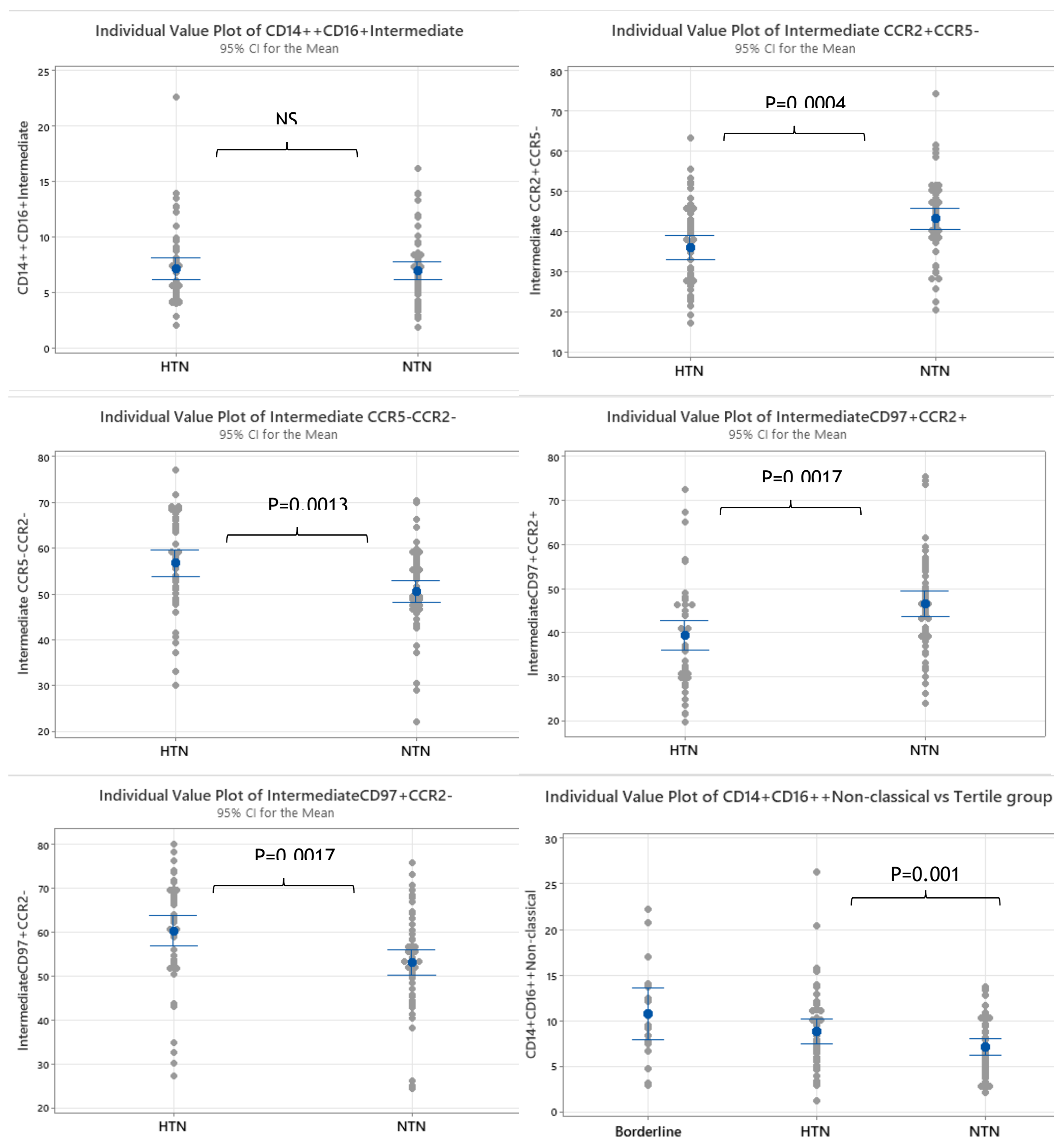


Figure 4.4. Flow cytometry Monocyte and DC panel illustrative results by blood pressure group. NTN, normotension; HTN, hypertension; mean and interval bar (standard deviation) depicted.

Monocyte/DC subsets by Flow Cytometry cell markers, %	NTN (SD) N=58		HTN (SD) N=50		T-test P-value	Adjusted model P-value
DCs	8.55	4.11	7.75	2.91	0.245	0.893
CD141+mDCs	2.56	1.32	2.63	1.01	0.759	0.110
CD1c-CD303a-	41.49	14.42	39.99	13.01	0.573	0.558
CD1c-CD303a- CCR2+CCR5-	25.57	12.25	26.79	10.32	0.576	0.552
CD1c-CD303a- CCR5+CCR2-	7.06	12.76	4.23	4.22	0.116	0.010*
CD1c-CD303a- CCR5+CCR2+	12.69	6.07	12.88	6.70	0.880	0.300
CD1c-CD303a- CCR5-CCR2-	54.68	14.08	56.11	10.41	0.548	0.434
CD1c-CD303a- CD97+	37.41	15.01	37.40	13.13	0.994	0.423
CD1c-CD303a- CD97-	62.54	14.93	62.53	13.07	0.996	0.651
CD1c-CD303a- CXCR1+	8.38	5.46	8.38	5.17	0.994	0.240
CD1c-CD303a- MR+	1.21	0.92	1.14	0.76	0.641	0.322
CD1c-CD303a- MR+ CXCR1+	0.20	0.21	0.21	0.20	0.936	0.346
CD1c-CD303a- MR- CXCR1-	90.20	5.58	90.28	5.34	0.941	0.129
mDCs	31.51	9.47	33.14	9.52	0.374	0.857
mDCs CCR2+CCR5-	84.82	13.22	84.65	13.64	0.945	0.863
mDCs CCR5+CCR2-	0.23	0.64	0.14	0.26	0.325	0.612
mDCs CCR5+CCR2+	12.51	12.27	12.98	13.03	0.849	0.255
mDCs CCR5-CCR2-	2.43	3.04	2.24	2.03	0.686	0.126
mDCs CD97+	96.48	4.46	97.03	2.90	0.442	0.127
mDCs CD97-	3.54	4.45	3.00	2.94	0.452	0.404
mDCs CXCR1+	6.49	6.19	6.35	5.94	0.904	0.058
mDCs MR+	11.40	6.80	12.26	7.89	0.546	0.401
mDCs MR+ CXCR1+	1.66	1.59	1.94	2.03	0.437	0.305
mDCs MR- CXCR1-	80.45	9.62	79.46	10.61	0.615	0.487
pDCs	25.72	10.46	25.54	10.66	0.928	0.429
pDCs CCR2+CCR5-	95.29	4.68	96.50	2.94	0.108	0.338
pDCs CCR5+CCR2-	0.12	0.35	0.09	0.12	0.476	0.846
pDCs CCR5+CCR2+	3.38	4.24	2.32	2.60	0.117	0.108
pDCs CCR5-CCR2-	1.21	1.25	1.10	1.20	0.636	0.752
pDCs CD97+	89.90	10.00	89.27	7.97	0.717	0.239
pDCs CD97-	10.43	10.26	11.18	8.27	0.675	0.254

Monocyte/DC subsets by Flow Cytometry cell markers, %	NTN (SD) N=58		HTN (SD) N=50		T-test P-value	Adjusted model P-value
pDCs CXCR1+	4.32	3.19	4.75	5.06	0.608	0.761
pDCs Mannose Receptor+	0.66	0.88	0.86	1.53	0.417	0.215
pDCs Mannose Receptor+ CXCR1+	0.11	0.16	0.10	0.19	0.755	0.435
pDCs Mannose Receptor- CXCR1-	94.91	3.20	94.29	5.30	0.475	0.444
Monocytes	91.43	4.11	92.22	2.92	0.246	0.269
CD14++CD16+Intermediate	6.94	3.03	7.19	3.57	0.700	0.648
Intermediate CCR2+CCR5-	43.20	9.86	35.44	9.79	0.000*	0.006*
Intermediate CCR5+CCR2-	1.51	1.42	1.88	1.59	0.206	0.032*
Intermediate CCR5+CCR2+	4.75	4.27	5.45	5.36	0.456	0.048*
Intermediate CCR5-CCR2-	50.54	8.98	57.22	9.95	0.000*	0.053
Intermediate CD97+	99.67	0.24	99.71	0.24	0.446	0.372
Intermediate CD97-	0.32	0.24	0.29	0.25	0.588	0.458
Intermediate CXCR1+	6.39	6.04	5.14	3.43	0.182	0.458
Intermediate MR+	2.35	1.54	3.00	2.53	0.120	0.399
Intermediate MR+CXCR1+	0.54	0.51	0.60	0.58	0.547	0.728
Intermediate MR-CXCR1-	90.72	6.61	91.26	5.14	0.637	0.813
CD14++CD16-Classical	84.32	5.69	82.98	7.50	0.306	0.154
Classical CCR2+CCR5-	96.93	1.36	96.87	1.56	0.851	0.687
Classical CCR5+CCR2-	0.16	0.16	0.13	0.09	0.131	0.205
Classical CCR5+CCR2+	1.15	0.81	1.08	0.97	0.696	0.963
Classical CCR5-CCR2-	1.76	1.07	1.92	1.28	0.510	0.597
pDCs CXCR1+	4.32	3.19	4.75	5.06	0.608	0.761
Classical CD97+	99.52	0.29	99.60	0.22	0.106	0.019*
Classical CD97-	0.48	0.30	0.41	0.22	0.170	0.035
Classical CXCR1+	9.21	6.83	9.06	5.49	0.899	0.360
Classical Mannose Receptor+	1.05	1.05	1.24	1.63	0.477	0.959
Classical Mannose Receptor+CXCR1+	0.16	0.15	0.18	0.16	0.409	0.777
Classical Mannose Receptor-CXCR1-	89.58	7.14	89.52	5.48	0.960	0.377

Cell markers	NTN (SD) N=58	HTN (SD) N=50	T-test P-value	Adjusted model P-value
CD14+CD16++Non-classical	7.88 3.92	9.07 4.79	0.163	0.041*
Non-Classical CCR2+CCR5-	2.46 2.17	3.16 3.32	0.202	0.746
Non-Classical CCR5+CCR2-	1.16 1.04	1.21 0.95	0.788	0.855
Non-Classical CCR5+CCR2+	0.14 0.18	0.12 0.14	0.397	0.244
Non-Classical CCR5-CCR2-	96.24 2.59	95.51 3.50	0.225	0.863
Non-Classical CD97+	97.17 2.73	96.97 3.09	0.716	0.910
Non-Classical CD97-	2.84 2.74	3.03 3.09	0.733	0.924
Non-Classical CXCR1+	3.91 3.35	3.57 2.54	0.550	0.302
Non-Classical MR+	1.73 0.77	2.05 1.16	0.103	0.096
Non-Classical MR+CXCR1+	0.27 0.28	0.33 0.39	0.358	0.468
Non-Classical MR-CXCR1-	94.09 3.63	94.04 3.02	0.945	0.964
CD97+CCR2+CD1c-CD303a-DC	29.04 13.98	28.63 10.43	0.862	0.403
CD97+CCR2- CD1c-CD303a-DC	17.33 8.91	18.22 7.80	0.578	0.914
CD97-CCR2+CD1c-CD303a-DC	10.03 4.40	10.96 5.12	0.316	0.931
CD97-CCR2-CD1c-CD303a-DC	43.59 14.69	42.18 9.90	0.556	0.341
CD97+CCR2+DC	53.44 19.26	54.13 14.74	0.834	0.691
CD97+CCR2- DC	6.79 4.69	6.81 3.34	0.979	0.419
CD97-CCR2+DC	19.37 14.34	21.54 11.65	0.387	0.441
CD97-CCR2-DC	20.41 12.65	17.52 8.10	0.155	0.169
CD97+CCR2+mDC	94.32 4.60	94.78 2.73	0.518	0.289
CD97+CCR2- mDC	1.15 0.97	1.52 1.23	0.092	0.090
CD97-CCR2+mDC	2.39 2.07	2.27 1.99	0.750	0.240
CD97-CCR2-mDC	2.15 3.10	1.43 1.58	0.127	0.221

Cell markers	NTN (SD) N=58	HTN (SD) N=50	T-test P-value	Adjusted model P-value
CD97+CCR2+pDC	60.82 21.12	56.49 18.54	0.259	0.467
CD97+CCR2- pDC	0.34 0.44	0.44 0.41	0.231	0.285
CD97-CCR2+pDC	38.44 21.31	42.78 18.71	0.263	0.447
CD97-CCR2-pDC	0.39 0.87	0.30 0.27	0.422	0.929
Intermediate CD97+CCR2+	46.51 10.89	38.78 11.63	0.001*	0.019*
Intermediate CD97+CCR2-	53.14 10.87	60.92 11.57	0.001*	0.018*
Intermediate CD97-CCR2+	0.11 0.11	0.08 0.08	0.051	0.566
Intermediate CD97-CCR2-	0.23 0.20	0.21 0.20	0.575	0.318
Classical CD97+CCR2+	99.38 0.32	99.46 0.28	0.155	0.173
Classical CD97+CCR2-	0.30 0.27	0.30 0.23	0.997	0.926
Classical CD97-CCR2+	0.19 0.12	0.16 0.11	0.226	0.252
Classical CD97-CCR2-	0.14 0.17	0.09 0.08	0.048*	0.029*
Non-Classical CD97+CCR2+	0.77 0.80	0.92 1.12	0.413	0.693
Non-Classical CD97+CCR2-	96.19 2.98	96.36 2.94	0.766	0.766
Non-Classical CD97-CCR2+	0.07 0.09	0.04 0.05	0.095	0.069
Non-Classical CD97-CCR2-	2.97 2.91	2.67 2.60	0.576	0.714
CD97+CCR2+Mono	87.39 4.76	85.56 6.66	0.110	0.054
CD97+CCR2- Mono	11.85 4.68	13.80 6.47	0.079	0.038*
CD97-CCR2+Mono	0.25 0.16	0.20 0.12	0.107	0.090
CD97-CCR2-Mono	0.52 0.39	0.43 0.38	0.196	0.421

Table 4.9. Flow cytometry Monocyte cell panel results by blood pressure group. NTN, normotension; HTN, hypertension; SD, standard deviation; DC, dendritic cell; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; MR, mannose Receptor; *$p<0.05$. Adjusted model accounts for potential confounding factors (age, BMI, physical activity) with partial correlation analysis of SBP and immune marker. Multiple comparison adjustment to p-value threshold of 0.00049.

4.3.1.5 Stimulation studies

The following parameters failed quality control checks, due to 25% or more participants having missing data, T cell Syk, CD8+ c-CBL, and CD8+ Syk failed on both unstimulated and stimulated analyses; CD4+Syk, DN Syk, and B cells c-CBL passed this quality control check unstimulated but failed stimulated. Values for these are not therefore reported.

Unstimulated cells did not differ between NTN and HTN groups. Table 4.10 reports that stimulation resulted in differences in only two cell subsets: the HTN group demonstrating lower B cell pSTAT1 levels (stimulated and S-U), and lower MO pSTAT1 stimulated (but not S-U). Once incorporated into adjusted models with demographic factors (Table 4.10), pSTAT1 levels lost statistical significance, but DN c-CBL acquired association with SBP, with a greater reduction in MFI values post-stimulation in hypertension. When the more stringent Bonferroni-corrected statistical threshold was used, none attained significance.

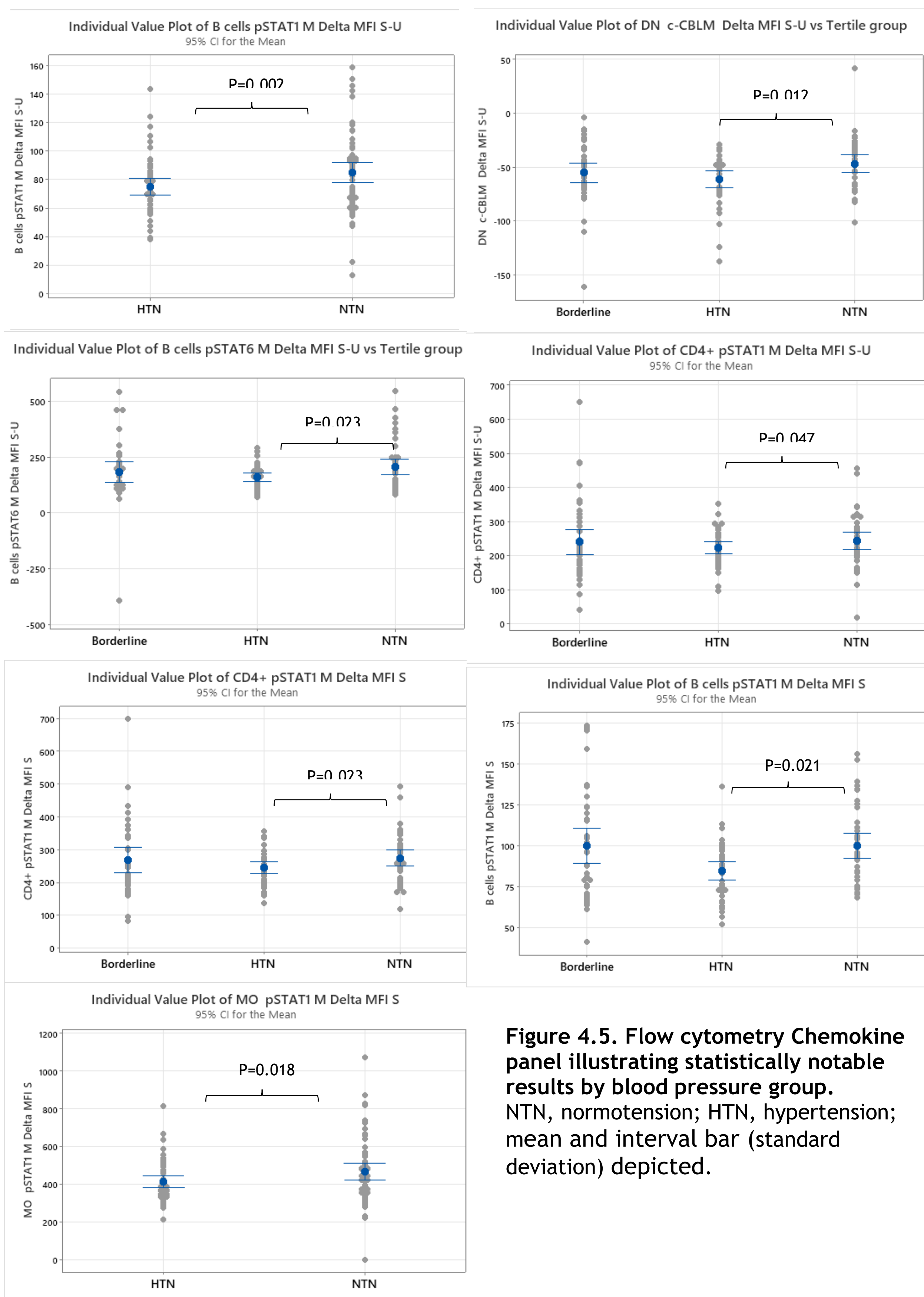


Figure 4.5. Flow cytometry Chemokine panel illustrating statistically notable results by blood pressure group. NTN, normotension; HTN, hypertension; mean and interval bar (standard deviation) depicted.

Stimulation studies	NTN (SD) N=66		HTN (SD) N=54		T-test P-value	Adjusted model P-value
B cells pSTAT1 M Delta MFI S-U	85.1	28.8	74.9	21.2	0.039*	0.510
B cells pSTAT1 M Delta MFI US	18.7	11.4	16.3	6.3	0.254	
B cells pSTAT1 M Delta MFI S	101.3	29.8	87.5	21.2	0.018*	
B cells pSTAT6 M Delta MFI S-U	196.3	133.9	168.9	69.0	0.603	0.101
B cells pSTAT6 M Delta MFI US	87.8	94.5	67.6	22.0	0.214	
B cells pSTAT6 M Delta MFI S	284.0	149.8	235.8	72.9	0.109	
B cells pSTAT3 M Delta MFI S-U	229.5	267.1	197.1	294.4	0.190	0.164
B cells pSTAT3 M Delta MFI US	779.7	661.7	906.3	537.2	0.251	
B cells pSTAT3 M Delta MFI S	1019.8	696.9	1099.7	595.3	0.853	
B cells Syk M Delta MFI S-U	2905.8	1021.0	2844.3	786.1	0.322	0.668
B cells Syk M Delta MFI US	6263.5	2125.7	6163.4	1664.7	0.232	
B cells Syk M Delta MFI S	9232.2	2704.2	9001.5	2200.9	0.214	
T cells pSTAT1 M Delta MFI S-U	187.5	91.0	183.6	63.3	0.721	0.453
T cells pSTAT1 M Delta MFI US	27.6	16.0	26.8	11.8	0.621	
T cells pSTAT1 M Delta MFI S	215.1	94.9	204.9	64.7	0.575	
T cells pSTAT6 M Delta MFI S-U	134.3	100.7	120.7	59.5	0.697	0.243
T cells pSTAT6 M Delta MFI US	25.9	47.0	18.8	10.2	0.194	
T cells pSTAT6 M Delta MFI S	167.8	94.2	144.3	60.9	0.182	
T cells c-CBL M Delta MFI S-U	-55.6	73.7	-54.9	30.5	0.350	0.441
T cells c-CBL M Delta MFI US	85.2	118.5	68.2	31.1	0.292	
T cells c-CBL M Delta MFI S	31.6	56.9	19.3	10.6	0.182	
T cells pSTAT3 M Delta MFI S-U	259.5	284.0	244.7	267.9	0.404	0.448
T cells pSTAT3 M Delta MFI US	877.0	745.4	1020.9	566.9	0.187	
T cells pSTAT3 M Delta MFI S	1159.2	722.7	1264.4	578.8	0.552	
CD4+ pSTAT1 M Delta MFI S-U	238.7	98.0	231.5	70.5	0.767	0.412
CD4+ pSTAT1 M Delta MFI US	29.5	17.4	29.3	13.3	0.772	
CD4+ pSTAT1 M Delta MFI S	269.8	101.2	254.2	71.6	0.600	
CD4+ pSTAT6 M Delta MFI S-U	150.7	109.0	135.5	64.5	0.718	0.267
CD4+ pSTAT6 M Delta MFI US	26.6	50.4	19.3	10.1	0.204	
CD4+ pSTAT6 M Delta MFI S	186.5	101.3	159.6	66.7	0.186	
CD4+ c-CBL M Delta MFI S-U	-64.4	38.8	-66.6	30.9	0.948	0.631
CD4+ c-CBL M Delta MFI US	78.8	67.4	73.8	37.3	0.498	
CD4+ c-CBL M Delta MFI S	25.6	44.2	19.3	11.5	0.314	
CD4+ pSTAT3 M Delta MFI S-U	369.0	323.7	354.5	286.9	0.389	0.500
CD4+ pSTAT3 M Delta MFI US	927.0	782.4	1076.7	616.2	0.221	
CD4+ pSTAT3 M Delta MFI S	1326.2	816.8	1429.6	648.6	0.642	
CD8+ pSTAT1 M Delta MFI S-U	112.8	50.8	108.6	43.5	0.880	0.441
CD8+ pSTAT1 M Delta MFI US	23.6	14.8	22.7	10.3	0.512	
CD8+ pSTAT1 M Delta MFI S	135.8	52.9	129.0	44.8	0.733	
CD8+ pSTAT6 M Delta MFI S-U	108.7	84.1	95.9	50.7	0.778	0.184
CD8+ pSTAT6 M Delta MFI US	25.9	43.1	19.5	10.7	0.215	
CD8+ pSTAT6 M Delta MFI S	138.0	77.4	118.0	50.3	0.217	

CD8+ pSTAT3 M Delta MFI S-U	88.0	228.6	54.1	235.0	0.214	0.366
CD8+ pSTAT3 M Delta MFI US	817.7	710.7	926.5	497.4	0.161	
CD8+ pSTAT3 M Delta MFI S	917.4	623.0	980.2	513.8	0.438	
DN pSTAT1 M Delta MFI S-U	83.4	41.2	79.9	35.0	0.392	0.690
DN pSTAT1 M Delta MFI US	20.8	12.7	18.8	9.1	0.319	
DN pSTAT1 M Delta MFI S	102.5	44.3	96.9	34.3	0.295	
DN pSTAT6 M Delta MFI S-U	78.8	74.2	69.6	41.6	0.842	0.208
DN pSTAT6 M Delta MFI US	25.2	43.6	17.5	8.9	0.159	
DN pSTAT6 M Delta MFI S	116.3	67.2	99.6	38.8	0.181	
DN c-CBL M Delta MFI S-U	-49.5	27.9	-60.0	24.5	0.105	0.033*
DN c-CBL M Delta MFI US	49.2	49.4	52.1	23.6	0.886	
DN c-CBL M Delta MFI S	17.1	47.5	9.8	7.0	0.292	
DN pSTAT3 M Delta MFI S-U	3.5	214.5	-32.8	202.3	0.206	0.479
DN pSTAT3 M Delta MFI US	688.2	531.3	802.3	457.3	0.261	
DN pSTAT3 M Delta MFI S	678.9	419.8	768.4	395.9	0.551	
MO pSTAT1 M Delta MFI S-U	271.3	127.6	234.7	92.7	0.156	0.556
MO pSTAT1 M Delta MFI US	214.4	108.6	196.9	72.4	0.174	
MO pSTAT1 M Delta MFI S	473.4	174.6	413.4	110.4	0.049*	
MO pSTAT6 M Delta MFI S-U	350.1	240.4	295.6	131.2	0.514	0.073
MO pSTAT6 M Delta MFI US	143.5	131.9	116.5	38.1	0.140	
MO pSTAT6 M Delta MFI S	483.5	310.0	392.1	139.0	0.191	
MO c-CBL M Delta MFI S-U	30.8	108.4	31.5	154.1	0.852	0.791
MO c-CBL M Delta MFI US	127.0	97.8	139.2	74.5	0.913	
MO c-CBL M Delta MFI S	63.8	115.8	54.0	26.3	0.434	
MO pSTAT3 M Delta MFI S-U	305.0	227.8	242.2	195.5	0.164	0.876
MO pSTAT3 M Delta MFI US	1055.4	679.2	1174.9	545.5	0.502	
MO pSTAT3 M Delta MFI S	1354.8	707.9	1398.5	576.6	0.965	
MO Syk M Delta MFI S-U	5913.7	2780.1	6020.4	2196.8	0.821	0.688
MO Syk M Delta MFI US	14676.4	4695.9	13970.0	4618.7	0.115	
MO Syk M Delta MFI S	20252.0	5239.1	19745.0	5843.8	0.220	

Table 4.10 Flow cytometry Phospho-flow cell panel results by blood pressure group. NTN, normotension; HTN, hypertension; SD, standard deviation; S-U, stimulated minus unstimulated; S, stimulated; U, unstimulated; STAT, signal transducer and activators of transcription; MFI, mean fluorescence intensity; DN, double negative i.e. CD4-CD8-; CBL, Casitas B Phospho-flow lymphoma; * indicates p<0.05. Adjusted model accounts for potential confounding factors (age, BMI, physical activity) with partial correlation analysis of SBP and immune marker. Multiple comparison adjustment to p-value threshold of 0.0025.

4.3.2 Nocturnal dipping status demonstrates leucocyte subset associations.

T cells: N=84 dippers, 52 non-dippers; between group differences were limited to CD4+ central memory compartment (CD4+CD45RO+CD62l-), 9.4 non-dipper (SD

4.3) versus 8.0 (3.3) dipper, p=0.04 ANOVA. CD4+ Effector memory compartment were correspondingly lower percentage, 15.3 (SD 6.0) vs 18.2 (8.7), p=0.02; thus the ratio of central to effector memory T cells was 1.88 (SD 0.77) in non-dippers vs 1.55 (0.78) in dippers, p=0.02. CD4+ TEMRA were proportionally reduced in non-dippers, 2.17 (SD 1.16) versus 3.35 (2.63), p=0.0005, see Figure 4.6; finally non-dippers showed higher numbers of CD8+CD45RA+CCR7+CD45RO+CD62l+ cells, 1.8 (SD 1.6) versus 1.2 (1.6), p=0.02. Of these results, only CD4+ TEMRA remained significant to a p-value threshold of p<0.0013 to account for multiple comparisons.

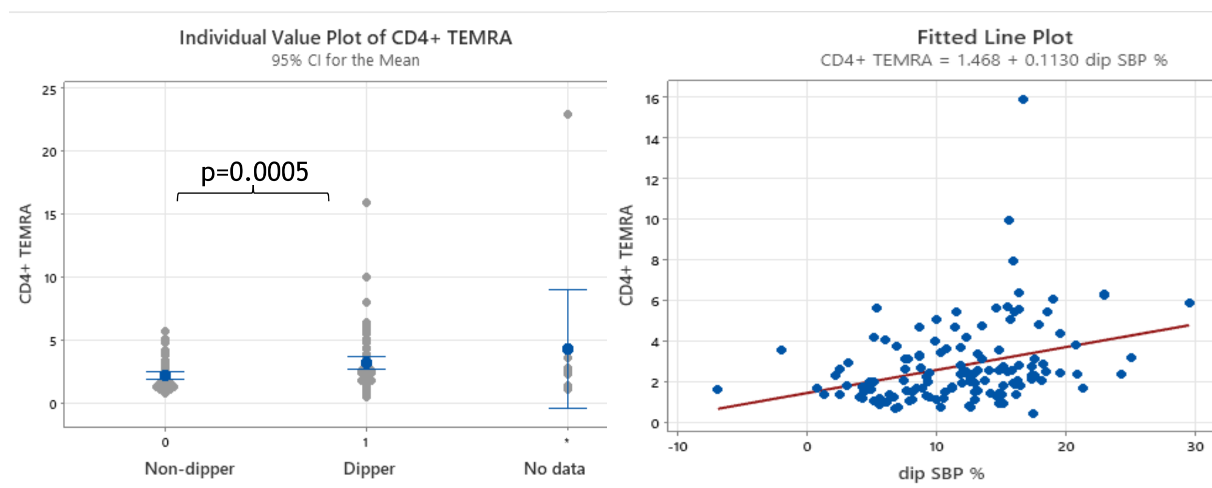


Figure 4.6. Individual value plot of CD4+ TEMRA cells by dipping status. Mean and interval bar depicted, along with regression analysis. TEMRA, terminally differentiated effector memory cells re-expressing CD45RA; dip SBP %, percent nocturnal dip in systolic blood pressure.

Chemokine receptor panel: 56 dippers and 34 non-dippers had chemokine receptor analysis. Of these, the following were significant at p<0.05 in univariate analysis; Th17 subgroups CD4+CXCR3-CCR6+CD161+ and CD4+CXCR3-CCR6+CCR4+CD161+ were higher in non-dippers (Figure 4.7), conversely CD4+CXCR3-CCR6+CCR4+CD161- were lower in the non-dipper group. Th2 cells expressing CXCR3-CCR6-CCR4+ were lower in non-dippers, as were Tc2 cells expressing CXCR3-CCR6- and CXCR3-CCR6-CCR4+. Tc1 expressing CXCR3+CCR6-, CXCR3+CCR6-CCR4-, and CD8+CXCR3+CCR6-CCR4+ were higher in non-dippers. Figure 4.7 illustrates these differences. None met the multiple comparison adjustment of p-value threshold of 0.0016.

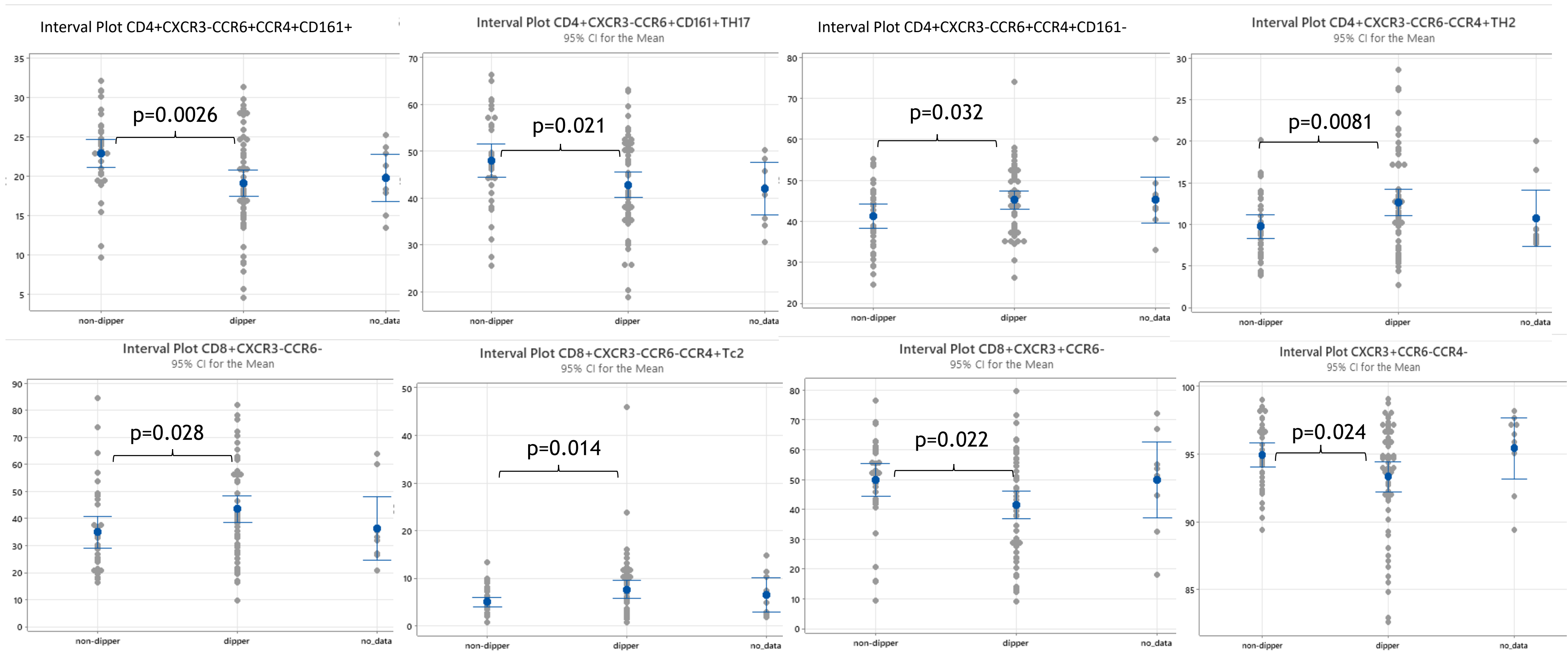


Figure 4.7. Individual value plot of chemokine receptor-related subsets with difference by dipping status. Mean and interval bar depicted.

B cells: data were available for 82 dippers and 51 non-dippers. The proportion between naïve activated (CD19+CD10-IgD+CD27-CD43+) and naïve non-activated (CD19+CD10-IgD+CD27-CD43-) differed (Figure 4.8); non-dippers demonstrating higher percent naïve activated, though SD was large (1.19 [SD 0.85] vs 0.866 [SD 0.74], p=0.020). Percentage CD27-IgD- memory (in proportion to CD27-IgD- activated) also reached unadjusted statistical significance, memory cells demonstrating lower levels in non-dippers (89.34 [SD 5.90] vs 91.34 [SD 5.20] respectively, P=0.036). No other differences were found, and none of these surpassed the lower multiple comparison p-value threshold of 0.0019.

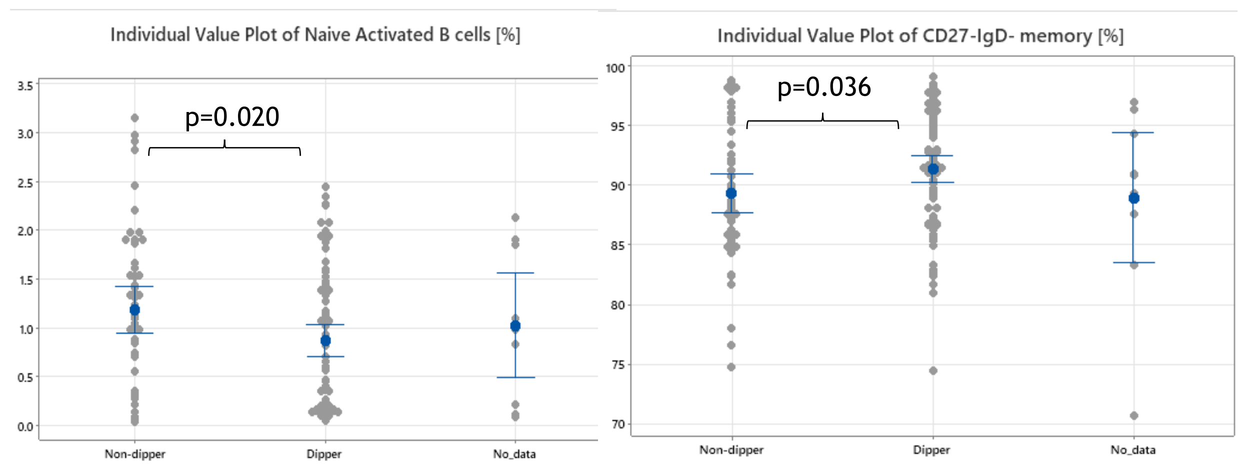


Figure 4.8. Individual value plot of B cell subsets with difference by dipping status. Mean and interval bar depicted.

Monocytes: N=59 dippers, 49 non-dippers, demonstrating between group differences in Mannose Receptor (MR)+ mDCs, with non-dipper 10.9 [SD 5.8] vs dipper 13.6 [SD 9.7], p=0.03; CD1c-CD303a- MR+ non-dippers 0.94 [SD 0.68] vs dippers 1.37 [SD 0.96] p=0.014. Plasmacytoid (p)DCs also differed regarding MR expression, with non-dipper MR+ pDCs 0.46 [SD 0.64] vs dipper 0.97 [SD 1.5], p=0.027; pDC MR+ CXCR1+ also a smaller compartment at 0.05 [SD 0.08] vs 0.15 [SD 0.22], p=0.002. Non-dipper pDC % CD97 expression was 86.3 [SD 11.7] vs dippers 91.3 [SD 6.6], p=0.018; % CD97- pDCs conversely 14.23 [SD 12.0] vs 9.02 [SD 6.9] respectively, p=0.018. Finally, the proportion between CCR2+CCR5- and CCR5+CCR2- classical monocyte subsets differed between by dipping status, though the magnitude of difference unlikely to be of any clinical or pathological significance, CCR2+CCR5- 97.2% [SD 1.3] non-dipper vs 96.7% [SD 1.6] dipper,

p=0.042 and CCR5+CCR2- 0.11% [SD 0.1] vs 0.17% [SD 0.2], p=0.023; Figure 4.9. Multiple comparison adjustment to p-value threshold of 0.00049 was not met for any comparison.

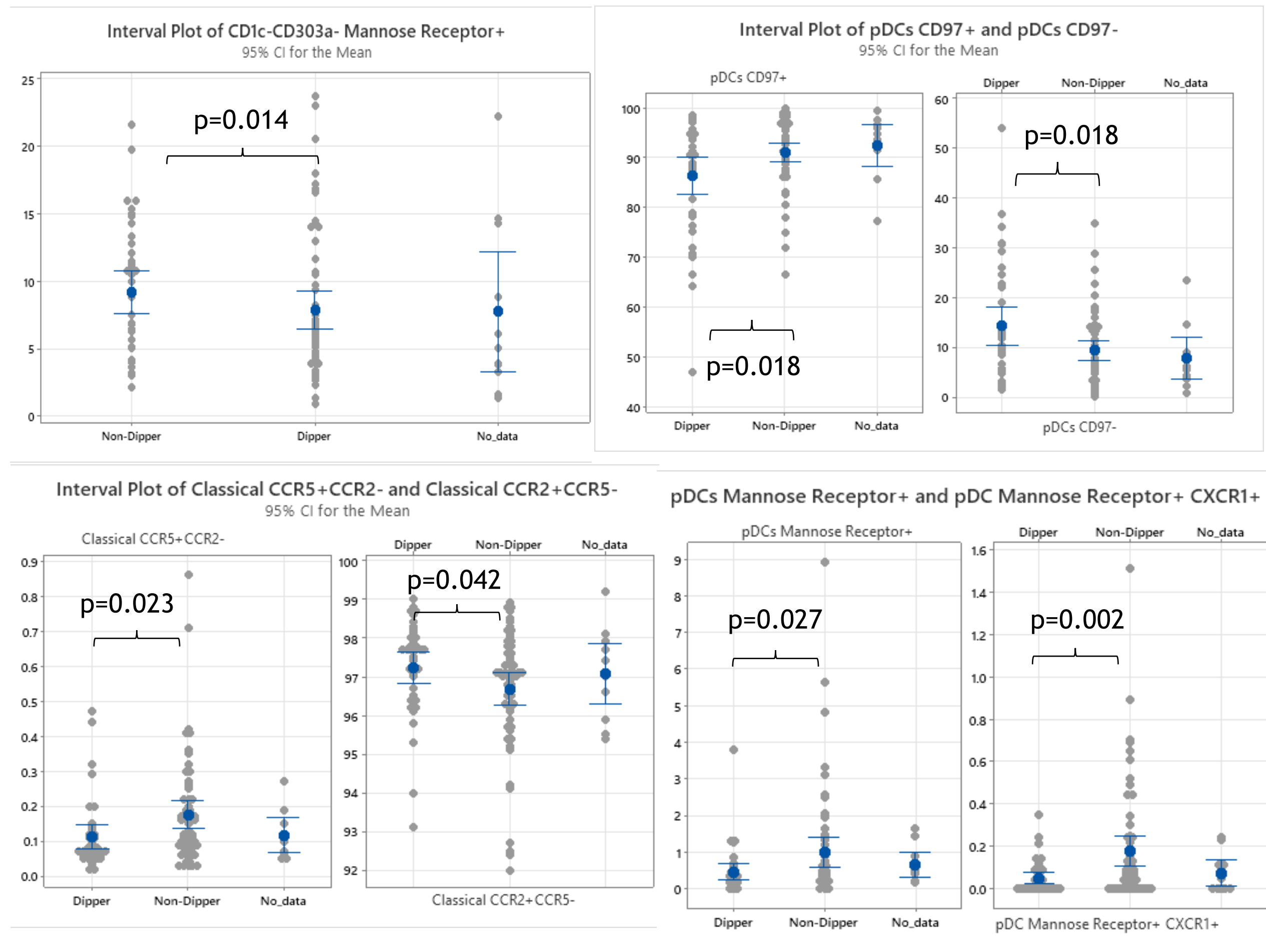


Figure 4.9. Individual value plot of Monocyte subsets with difference by dipping status, with mean and interval bar depicted.

Phospho-flow: N=68 dippers, 42 non-dippers; only between group differences on flow cytometry were CD4+ Syk US (dipper MFI 40.9 (SD 32.5), non-dipper 25.8 (SD 22.6), p=0.012) and DN Syk US (MFI 37.4 [SD 30.0] vs 25.3 [SD 20.2], p=0.027), Figure 4.10. Neither stimulated results are reported due to a high proportion of samples failing to stimulate. CD4+ pSTAT1 demonstrated differences following stimulation (MFI 247 [SD 83] vs 288 [SD 98], p=0.030). None surpassed the multiple comparison adjustment to p-value threshold of 0.0025.

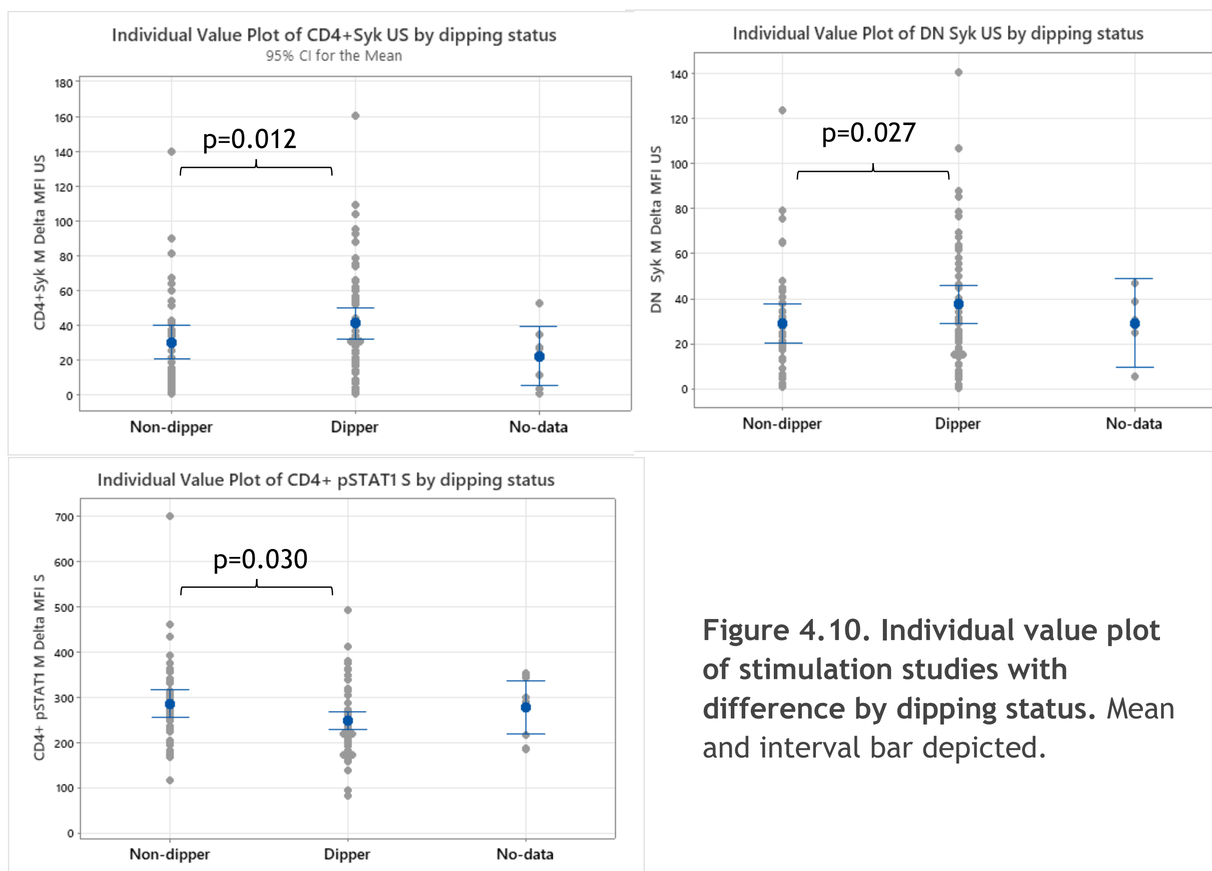


Figure 4.10. Individual value plot of stimulation studies with difference by dipping status. Mean and interval bar depicted.

4.4 Discussion

Inflammatension is a comprehensive flow cytometry study, with risk of confounding factors limited as far as possible through recruitment of otherwise healthy, untreated, incident-diagnosis patients, and environmental and demographic factors controlled for. The results demonstrate differently composed circulating innate and adaptive immune compartments between untreated, healthy hypertensive individuals and normotensive controls. This immune 'signature' of hypertension included CD4+ TCM over-representation; altered pattern of senescence and terminally differentiated cells; and effector T cell polarisation towards Th1/Tc1 and Th17.1/Tc17.1. Detectable changes in naïve T cells and B cells were restricted to the CD4+CD45RA+CCR7+CD45RO+ CD62l+ subset, though B cells had altered intracellular signalling following stimulation. Altered expression of NK and NKT cell markers were also demonstrated. The innate immune 'signature' was dominated by Mannose receptor downregulation on DCs, altered chemokine receptor expression on intermediate monocytes, and alterations in STAT1 and STAT6 phosphorylation cascades. PBMCs also differed by nocturnal dip, with some overlap to

hypertension but other unique patterns of immune cell marker expression. Consideration of these findings in the wider context of existing evidence is discussed below.

4.4.1 T cells

No difference in total lymphocyte percentages were observed, and although the circulating T cell compartment was proportionally smaller in the HTN tertile, CD4+ to CD8+ ratio were similar across groups, contrary to other groups observing increased CD4+/CD8+ ratio in the peripheral blood of patients with hypertension.[272] A limitation of this study was our inability to specifically quantify T regulatory cells; especially as other published work in hypertension has reported a protective role.[273,274]

In NK cell development, acquisition of CD122 indicates lineage commitment, it is also involved in IL-2 and IL-15 binding. [275] NKT lymphocyte subset express cell markers associated with NK cells (e.g. CD161), and in contrast to NK cells, also possess a T cell receptor (CD3). CD122+NKT Lymphocytes were lower in the hypertensive group (p=0.046), as were CD122+ T lymphocytes. Other evidence supports a regulatory central memory phenotype of $CD8^{+}CD122^{+}$ T cells, suppressing both allo- and auto-immune responses.[276] Proportionally lower numbers of these subsets thus supports the hypothesis of impaired regulation of immune responses and a pro-inflammatory state.

T cells are initially present in circulation as naïve lymphocytes; CD4+ naïve T cells were lower in the Inflammatension HTN group, as has been reported by others [277] and even predicting incident hypertension in women.[278] However, the only subset attaining statistical significance (but not confirmed in the adjusted model) was the small CD4+CD45RA+CCR7+CD45RO+CD62l+ compartment, featuring a combination of markers representing transition from naïve to memory state. CD62L (L-selectin) and CCR7 (chemokine receptor 7) are both lymphoid homing molecules also expressed by TCM, see Figure 4.11. Increased circulating memory T cells have been proposed as evidence of activation in hypertension. [279] As outlined in Chapter 1.3, memory T cells can be divided into TCM and TEM subsets with distinct functions and markers.[68] A larger CD4+ TCM compartment was demonstrated in our HTN group, and in non-dippers the TCM

CD4+CD45RO+CD62l- subset was similarly expanded, with non-dipper CD4+ TEM cells correspondingly lower i.e. non-dippers demonstrated a higher ratio of TCM to TEM. Elevated CD4+ TCM have also been reported in a small mass cytometry study of hypertension (CD4+CD45+CD45RO+CCR7+CD27+-)[277] and a study comparing circulating cells in human pre-eclampsia to healthy pregnant controls.[280] However, pre-eclampsia is distinct from HTN, and the association is not without equipoise as a further pre-eclampsia study demonstrated no difference in CD4+ TCM and reduced proportions of CD4+CD45RO+CCR7+CD69 activated TCM cells. [281] As TCM cells are uncommitted regarding effector cytokine production, their differentiation is influenced by the cytokine environment.[282]

The TEM compartment demonstrated significant results, but heterogeneous by marker expression, as Figure 4.11 illustrates. Other groups have also reported mixed findings, Gackowska *et al*. and Itani *et al*. [283] reporting an increase in circulating CD8+ TEM cells and Alexander *et al*. a decrease. [273] However, as is often the issue in comparing flow cytometry data, the groups differ on population and subgroup markers, the Gackowska reporting CD8+CD28+ and CD8+CD45RA+CCR7+ TEM cells in hypertensive adolescents with LVH, Itani CD8+CD45RO+ in hypertensive adults, and Alexander defining CD8+ TEM cells as CD45+CD3+CD8+CD45RO+; furthermore, within this data subclusters had varied associations with hypertension. [273] TEM cells have further been demonstrated as accumulating in the bone marrow and kidney in animal model hypertension.[284]

As reported by others (though with differences in employed markers)[277], CD8+ TEMRA were lower in the HTN group, but only the CD8+CD62l+ TEMRA subgroups attained statistical significance (Figure 4.11). The analysis by nocturnal dipping status further identified a smaller CD4+ TEMRA compartment in non-dippers. Clinical significance is suggested by the phenotype of TEMRA cells (terminally differentiated TEM cells re-expressing CD45RA); these demonstrate reduced TCR clonal diversity and TCR-dependent activation, but augmented sensitivity to innate inflammatory signals and aberrant cytokine production.[285] Increased circulating CD8+ TEMRA (and loss of CD28 from lymphocytes in general) reflects immune system aging. Interestingly, other subsets differed in hypertension based on CD28+ expression (Figure 4.11), a co-stimulatory molecule ubiquitous to lymphocytes in early life, with initially reversible, and then terminal, down-

regulation in aging and in T cell senescence. [286,287] The co-stimulation role of CD28 is critical to certain models of hypertension [288], and increased number of CD8+CD28- cells have been demonstrated in 19 treatment-naïve and newly diagnosed hypertensive patients, whilst other T cell subsets did not differ.[88] Inflammatension assessed CD28 expression separately in all subgroups (naïve, TEMRA, TCM etc.), rather than total CD8+CD28-; hence while the data are concordant, they are not directly comparable. The same group assess functionality and report that these immunosenecent cells remain pro-inflammatory and cytotoxic.[88] Evidence from other studies in hypertension is lacking.

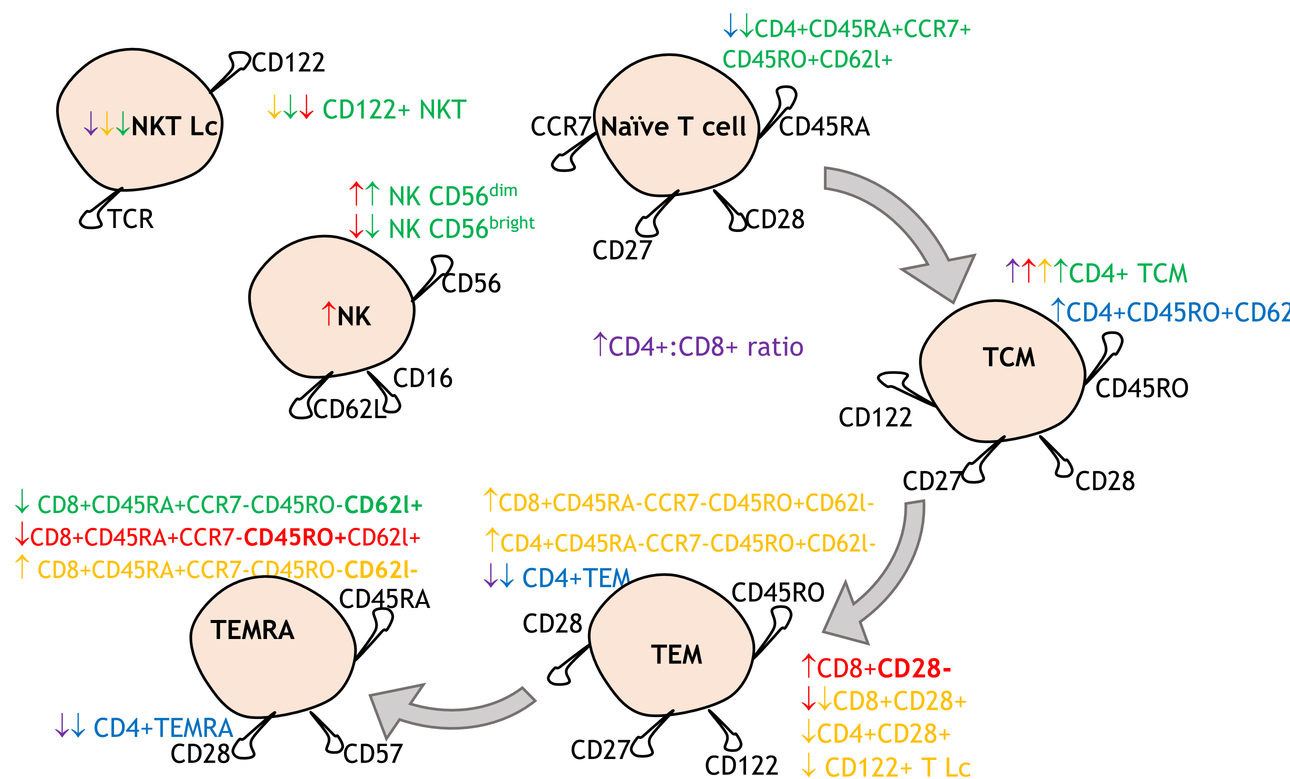


Figure 4.11. T and NK cell markers, differences demonstrated in hypertension, and functional phenotype. Green, HTN association in unadjusted between group comparison; Orange, HTN in adjusted analysis; Red, HTN in tertile analysis; Blue, non-dipper association, Purple, other research findings. NKT, natural killer T cell; TCM, T central memory; TEM, T effector memory; TEMRA, terminally differentiated effector memory cells re-expressing CD45RA;

Regarding NK cells, our findings support the association of CD56+^{Dim} NK cells with hypertension as has been reported in human hypertension [289] and in animal model studies, for example NK cell depletion demonstrating protection from Ang-II induced vascular dysfunction. [62,63] CD56+^{Dim} NK cells have a cytotoxic phenotype, with perforin and granzyme expression to induce cell lysis. They also

demonstrate early IFN-γ production, promoting rapid and comprehensive NK cell activity during the early phase of innate responses. [289] Correspondingly, the decreased content of inhibitory/regulatory CD56+^{Bright} NK cells in hypertension suggests an imbalanced, pro-inflammatory milieu in hypertension.

A human hypertension study supports the NKT data presented, demonstrating smaller NKT cell population in comparison with normotensive participants. [290,291] The protective role and potential clinical utility of this is alluded to by animal models in which NKT subsets abrogate Ang II-induced IL-6 and IL-17 production, hypertension, and vascular remodelling. [291,292]

4.4.2 Functional T cell subsets based on chemokine receptor expression

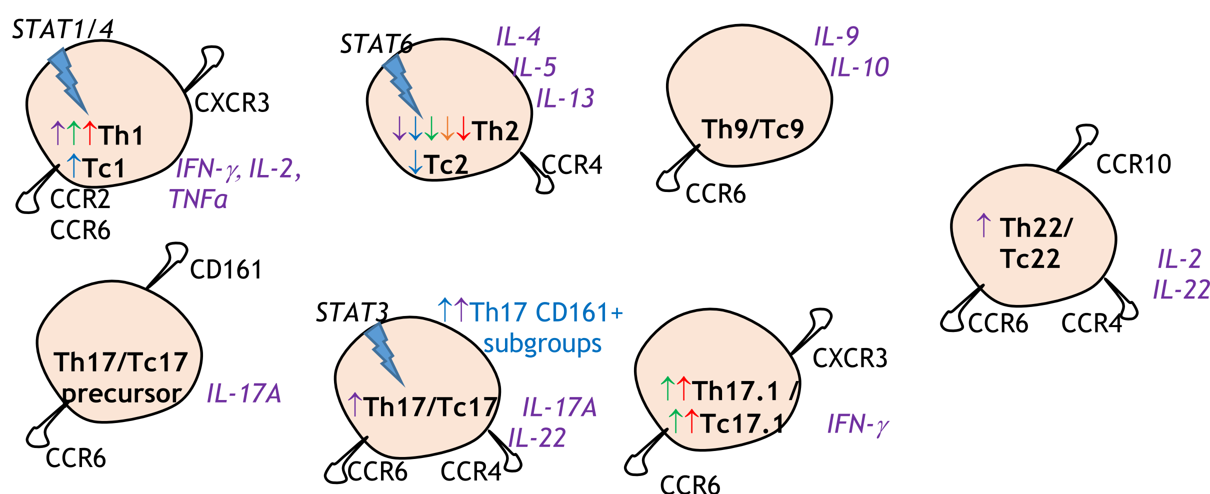


Figure 4.12 Chemokine panel cell markers and functional lymphocyte subsets. Th, T helper cell; Tc, cytotoxic T cell; IFN-¤, interferon gamma; IL, interleukin; CCR, C-C chemokine receptor; CXCR, CXC chemokine receptor. Black italic indicates intracellular signalling pathway involved; purple italic represents cytokines produced. Green arrow, HTN association in unadjusted between group comparison; Orange arrow, HTN in adjusted analysis; Red arrow, HTN in tertile analysis; Blue arrow, non-dipper association, Purple arrow, other research evidence.

Recruitment of T cells is dependent on their expression of chemokine receptors, CCR4, CCR6, and CXCR3 considered as critical chemokine receptors. Furthermore, differential expression of CXCR3 and CCR6 determines T cell phenotypes, as outlined above in Table 4.1 and Figure 4.12. Aberrant polarization of T helper (Th) cells has been reported in the context of vascular diseases, though this may vary by disease activity.[293,294]

Th1 (CD4+CXCR3+CCR6-) lymphocytes were found to circulate at higher levels in the Inflammatension hypertensive group. Furthermore, the Tc1 CD8+CXCR3+CCR6− compartment was expanded in non-dippers relative to those with preserved nocturnal dip. Th1/Tc1 cells display a pro-inflammatory function primarily targeting intracellular pathogens and characterised by cytolytic activity and IFN-γ and TNF-α production. Existing literature suggests that Th1 lymphocytes and levels of Th1-associated cytokines IFN-γ-inducible protein and TNF-α are elevated in hypertension, non-dipper phenotype, and in atherosclerosis [176,294,295], discussed further in Chapter 5. Also elevated in the hypertension group was the CXCR3+CCR6+ subset, again both CD4+ and CD8+ cells, though with no differential expression on CCR4 by BP group. These Th17.1 / Tc17.1 cells (also known as 'non-classical' Th17/Tc17 cells) have been demonstrated to inhibit infiltration of CD8+ T cells in the setting of malignancy [296]; they have not been robustly studied in hypertension, but have been linked to multiple sclerosis and a role in chronic infections. [297,298]

CD4+CXCR3−CCR6− Th2 cells are a subset directed predominantly at extracellular pathogens but also inducing isotype switch and eosinophil responses. This subset were reduced in our hypertensive patients and in non-dipper phenotype, again consistent with the findings of other research groups.[295,299] Similarly, Tc2 (CD8+CXCR3−CCR6−) circulated at lower levels in non-dippers. Finally, Th17/Tc17 cells (CXCR3−CCR6+) were higher in the hypertensive group, as demonstrated by others[295,300], but did not reach statistical significance, though CD161+ Th17 subgroups were elevated in non-dippers, a subgroup in which limited flow cytometry data is available in the existing literature. Data from hypertensive rats demonstrate infiltration into kidneys and aorta of CD161+ immune cells, with IL-17 secretion mediating hypertension[301], though another study in rats conversely found that IL17 infusion attenuated angiotensin-induced hypertension.[302] Elevated IL-17 has also been reported in uncontrolled human hypertension, and increased Th17 cells in both resistant hypertension and in hypertension-mediated organ damage [108,303,304], perhaps explaining why the otherwise healthy participants in Inflammatension did not achieve statistical significance with regards Th17 cells. Taken together, data suggest effector T cell polarisation differences exist in hypertension and in loss of nocturnal dip, towards a Th1/Tc1 and Th17.1/Tc17.1 milieu.

4.4.3 B cells

Some research groups have suggested an obligatory role for B cells in murine models of hypertension, Ang II infusion increasing CD86 expression, plasma cell numbers, and IgG levels, B-cell activating factor deficient mice lacking mature B cells or B cell depleting pharmaceuticals being protective.[305] Supporting this, mice with reduced numbers of B220+ B cells also demonstrated lower BP and greater resistance to DOCA-salt induced hypertension.[306] Antibody data have also been used as evidence of the role of B cells in hypertension, beyond the scope of this work and reviewed in detail elsewhere.[307] However, contradictory findings suggesting B cells and immunoglobulins are insufficient alone in leading to hypertension are also reported from murine models. Neither transfer of IgG from hypertensive animals, nor models unable to class switch to IgG production, influenced BP or organ damage. [308]

Our data demonstrated higher percentage of circulating B cells in the hypertensive group, consistent with the limited available human studies of B cells, Kresovich *et al*. for example suggesting B cell association with both baseline BP and risk of incident hypertension,[278] however statistical significance was lost in the adjusted model of Inflammatension data. Similarly, no robust associations with dipping status were demonstrated, and no other published data could be found to support or contest this.

4.4.4 Monocyte and DC subsets

DCs have been linked to the development of HTN [78] and endothelial damage through their role as antigen presenting cells, ability to produce ROS and cytokines, and to polarize T cells; however, no differences were detected in any DC subgroup between NTN and HTN patients. This contrasts data from small studies including mass cytometry of five hypertensive patients, and from hypertensive adolescent subjects demonstrating higher circulating pro-inflammatory myeloid (m)DC numbers [277,309] and lower frequency of plasmacytoid (p)DCs.[309] Other conditions involving vascular dysfunction and inflammation also arrive at heterogeneous conclusions; circulating mDCs were lower whilst pDCs remained unchanged in pulmonary HTN, [310] whilst SLE and systemic sclerosis demonstrate fewer peripheral pDCs, possibly due to migration

to inflamed tissue. The Inflammatension subgroup with loss of nocturnal dip did however demonstrate a smaller circulating immune compartment of both mannose receptor+ (MR+) mDCs and pDCs (Figure 4.13). Cell-bound MR (CD206) recognises terminal residues on micro-organism surfaces and has a role in endocytosis and antigen processing. Expression on immature DCs reflects activation status, regulated by cytokines and these microbial terminal residues. MR expression on APCs drives activated T cells towards a tolerogenic phenotype [311], and in gene and protein expression analysis of atherosclerosis, M2 macrophage markers including MR appear protective.[312] Thus, reduced MR expression suggests an association between loss of nocturnal dip and DC's activating T cells to pro-inflammatory rather than tolerogenic state, though directional effect cannot be inferred.

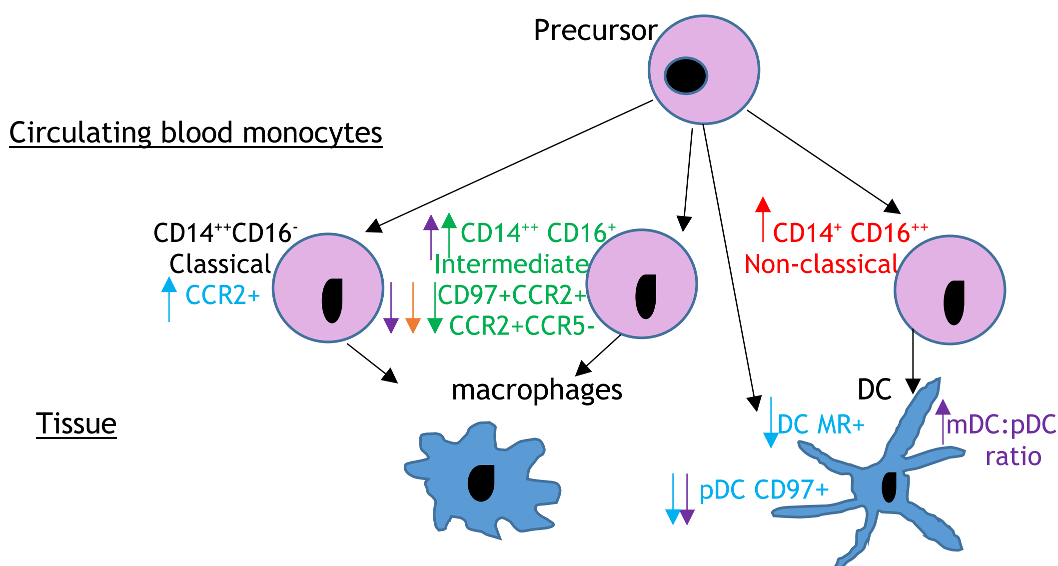


Figure 4.13 Monocyte and DC panel cell markers and functional subsets. Green arrow, HTN association in unadjusted between group comparison; Orange arrow, HTN in adjusted analysis; Red arrow, HTN in tertile analysis; Blue arrow, non-dipper association, Purple arrow, other research evidence.

Lower numbers of pDCs expressing CD97 were also associated with loss of nocturnal dip. CD97 has a role in cell adhesion, migration, and cell connection regulation, including stabilising the immunological synapse between T lymphocytes and DCs [313]; meta-analysis of gene expression signatures has implicated the CD97 gene in SBP, DBP, and hypertension [102], the role in BP regulation has not otherwise been studied, though our data would suggest that both mannose receptor and CD97 expression on DCs may be components of an immune 'signature' of hypertension (Figure 4.13).

Considering the implications of higher numbers of circulating intermediate monocytes (CD14++CD16+) in the HTN group, these cells usually comprise around 5% of monocytes, with features of both classical and non-classical monocytes including production of both anti- and pro-inflammatory cytokines (e.g. IL-10 and TNF-a). They are highly phagocytic and can produce high levels of reactive oxygen species (ROS). Inflammatension HTN group also found a lower proportion of this expanded intermediate monocyte subset expressed CCR2+CCR5- and CD97+CCR2+. CCR2 is the chemokine receptor for monocyte chemoattractant protein-1 (MCP-1), whilst CCR5 (CD195) is also involved in the recruitment and effector functions of immature DCs as well as TEM cells, and macrophages. Both were mentioned in Chapter 1 regarding evidence arising from animal models of hypertension.[79-81] In humans, CCR2 subsets have also been linked to hypertension and HMOD, elevated CCR2 expression on circulating monocytes subsequently reduced following Ang II receptor blocker therapy [314,315]; and to differential trafficking into atherosclerotic plaques, with statins demonstrating reductions in CCR2 expression. [316,317,318] A CCR5 polymorphism has been linked to establishment of BP levels in genetic study,[319] whilst findings in hypertension appear to vary with animal model or may be organ specific [79,320], and consistent with this, a dual CCR2/CCR5 inhibitor currently in undergoing clinical trials appears to show anti-inflammatory and anti-fibrotic effects, but no evidence in nocturnal BP control being identified.[321]

Although surface markers for intermediate monocytes lack specificity and historical variability in classification makes study comparisons challenging, our findings are congruent with others. [314,322] For example, a percentage increase in circulating intermediate monocytes being reported in association with hypertension, day 1 post following myocardial infarction, and prospectively been used to predict cardiovascular events.[277,323,324] Methodological differences also make direct data comparison challenging however, including treated HTN, and/or not reporting CCR5, CCR2 (MCP-1), and CD97 markers. Finally, Non-dippers also demonstrated classical monocyte subsets favouring CCR2 expression, those with preserved nocturnal dip expressing higher rates of CCR5. However, the effect size was small and statistical significance was negated by adjustment for BMI, age, and physical activity. Taken together, evidence suggests that intermediate monocytes are a component of the immune

phenotype of hypertension and the chemokine receptor expression may differ; but invites further analysis to explore heterogeneity in study methodology and results.

4.4.5 Stimulation studies

Phosphorylation / stimulation flow cytometry studies of immune cells is novel in the field of hypertension, permitting quantification of cell signalling. The main limitation of this phospho-flow panel however was that Syk signalling molecules did not consistently demonstrate phosphorylation, with T cell CD8+ and CD4+ subsets failing to activate Syk in sufficient participants; similarly CBL had high failure rates in B cells and CD8+ T cells. This may be because of spontaneous activation or priming *in vivo* in the hypertensive group, the subsequent response to laboratory stimulation thus reduced. Support for this hypothesis would be the earlier demonstration of altered T cell subsets, pro-inflammatory T cell polarisation, and as demonstrated by other groups in states of inflammation.[325] Alternatively, the findings may suggest that the stimulation cocktail was not optimised for Syk or CBL.

Unstimulated cells did not differ between NTN and HTN groups. Change in pSTAT1 (Signal transducer and activator of transcription 1) expression level following stimulation was smaller in the HTN group in both B cells (despite B cell panel demonstrating little association with hypertension) and in monocytes. STAT1 is the final protein of the intracellular phosphorylation cascade that transfers cell surface signals such as IFN-γ to the nucleus, acting as a transcription factor to regulate gene expression, including expression of pro-inflammatory and pro-fibrotic factors such as CCR2 [326], interleukin (IL)-1 and transforming growth factor (TGF)-ß. Linking stimulation and monocyte panels through pSTAT1, higher CCR2 expression was seen in classical monocytes of non-dippers. Early B lymphocyte development and antigen presentation are also processes in which STAT1 participates.[327] The STAT1 pathway has been linked to periodontitis [328], to pulmonary hypertension [329], and genetic variants of STAT1 associated with hypertension.[330] More specifically, work of Loperena et al. paradoxically associates hypertension with elevated levels of pSTAT1 and pSTAT3 phosphorylation on intracellular staining of intermediate monocytes

when co-cultured with endothelial cells under 10% stretch conditions.[322] Comparisons are limited however, as intracellular staining measures data distinct from stimulation flow cytometry studies; furthermore, Inflammatension reported pSTAT1 change in expression in monocytes as a whole, without intermediate monocyte subset data. It does invite further study to clarify the nature of these monocyte and pSTAT1 differences. No published B cell pSTAT1 data in hypertension was identified.

Tertile analysis additionally identified pSTAT6, though S-U MFI was lower in B cells from the HTN group, and higher in HTN group monocytes. STAT6-mediated signalling pathway results in transcriptional changes relating to immune cell proliferation and regulation, evidenced in B cells, Th2 cells, and macrophage M2 subtype activation. [331–333] In B cells, STAT6 is involved in IL-4 signalling, promotes maturation, differentiation, and class switching as evidenced by CD20 expression; deficiency impairs function and leads to morphological changes.[332] The significance of the findings are unclear, as STAT6 signalling may also mediated by AT1 receptor binding, and has further been linked to cardiac fibrosis,[334] and to vascular remodelling in hypertension via effects on macrophage MMP9 and MMP13 production. [335]

4.4.6 Non-dipper phenotype

Differences between those with preserved and reduced nocturnal dip have been discussed panel by panel; whilst the circulating PBMC signature of non-dipping BP has limited existing evidence, it is accepted that the immune system has a circadian rhythm and that sleep quality and duration can affect the immune response. [336] This has been demonstrated down to the level of distinct subset profiles, with naïve, TCM, and TEM cells showing a nocturnal peak, but terminally differentiated effector memory cells demonstrating a flattened or inverted pattern.[337] This relates (at least in part), to a varied responsiveness of subsets to cortisol levels.[337] Consistent with this, the collective immune differences identified in Inflammatension, fewer circulating CD4+ TEM and TEMRA were found in non-dipper participant group compared to BP dippers, whilst the chemokine receptor panel demonstrated expanded compartments of Tc1, Tc17.1 and Th17.1, with concomitantly fewer Th2 and Tc2 cells. Mannose receptor downregulation on DCs and a smaller compartment of pDCs expressing

CD97 were also associated with loss of nocturnal BP dip; classical monocytes meanwhile differed in chemokine receptor expression (non-dippers associating with CCR2, dippers favouring CCR5). The evidence that the circulating immune compartment does differ by nocturnal dip, leads to greater challenges when considering an immune signature reflecting dichotomised BP into normotensive and hypertensive.

4.4.7 Limitations

However, a limitation of the flow cytometry data remains the potential for confounding, particularly relating to the unforeseen COVID-19 pandemic. This is explored in Chapter 6, as it is relevant to the vascular, biomarker, and flow cytometry data. A further general limitation to this work relates to statistical significance in the context of multiple comparisons. Bonferroni thresholds are reported throughout; all results and conclusions must be interpreted remaining cognizant of this, and all require external validation.

Immunologically, defining circulating cell subsets remains a standard, but suboptimal, method of assessing the immune system for a number of reasons. Firstly, the circulating compartment is not synonymous with the cell numbers and activation state in other tissues, such as the vascular wall or the kidneys; secondly, flow cytometry will always be constrained by the number of markers and flourochromes, though the capacity of the machine was optimized, some important subsets are still missing, such as T-regulatory cells; thirdly, characterized phosphor-flows in the stimulation studies facilitate comparisons, but may not reflect a wider continuum of functional cell capacities. [338]

4.5 Conclusions

The data is exploratory, but does support differences in the composition of the circulating innate and adaptive immune compartment between untreated, healthy hypertensive individuals and normotensive controls. The immune ‘signature’ of hypertension appears dominated by T lymphocytes, particularly CD4+ TCM over representation, expanded TEM CD45RA-CCR7-CD62l-, and altered pattern of senescence and terminally differentiated cells. Furthermore, data suggest effector T cell polarisation in hypertension and in loss of nocturnal dip,

towards a Th1/Tc1 and Th17.1/Tc17.1 pro-inflammatory milieu, usually characterised by IFN-γ and TNF-α. There was no clear evidence of detectable changes in naïve T cell or B cell subsets, though the latter had altered characteristics of intracellular signalling following stimulation. NK cell $CD56^{+Dim}$ expression and fewer CD122-expressing NKT further suggests an imbalanced, pro-inflammatory milieu in hypertension. The innate immune 'signature' is characterised by intermediate monocytes with a differing pattern of CCR2 and CCR5 chemokine receptor expression, and alterations in STAT1 and STAT6 phosphorylation cascades in response to stimulation. Mannose receptor downregulation on DCs also characterised hypertension. Finally, the circulating immune compartment differs by nocturnal dip, with some overlap to hypertension but other unique patterns of immune cell marker expression.

Chapter 5 Circulating immune biomarkers and arterial hypertension

5.1 Background and aims

As outlined in Chapter 1.4, biomarkers are characteristics of biological or pathogenic processes that can be accurately measured and objectively evaluated, and relate to a clinical phenotype.[146,147] Hence, circulating protein biomarkers relating to pathogenesis may be of value in identifying hypertension, in assessing risk of progression, or of HMOD, as Figure 5.0 demonstrates; they therefore have potential to reduce morbidity and mortality. Many biomarkers have been proposed in hypertension, though few are used in routine clinical practice.[148,339] In particular, biomarkers relating to early hypertension and arterial dysfunction (as the Inflammatension participants are) may be most informative when considering the pathophysiology of hypertension, and potentially offer greatest utility if interventions could prevent progression.[339]

Hence, this chapter focuses on the circulating immune biomarkers relating to BP, hypertension, and arterial function; including cytokines, interleukins, soluble cell-adhesion molecules, leukocyte subgroups, and components of the clotting cascade. The research questions were as follows:

1) Do circulating immune biomarkers differ between incident patients with hypertension but no overt HMOD, versus healthy controls? If so, which are most discriminatory?

2) Which immune biomarkers demonstrate clinically relevant associations with BP, hypertension, and arterial function?

3) Relating to the immune milieu, are phenotypic subgroups apparent in hypertension?

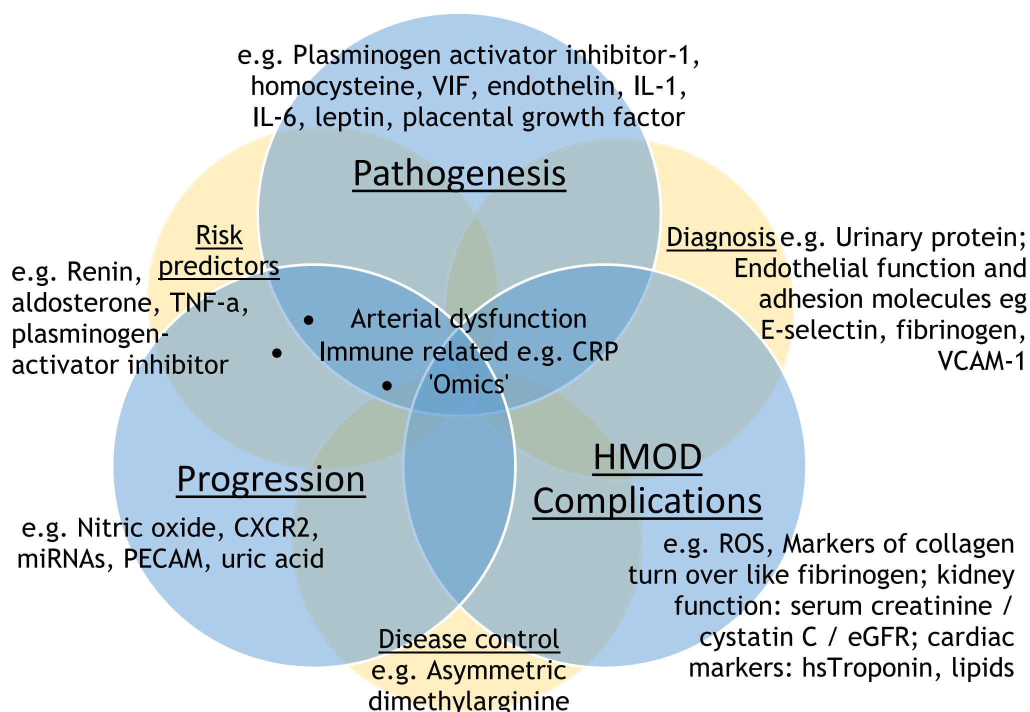


Figure 5.0. Schematic representation of proposed circulating biomarkers in primary hypertension, and aspects of hypertension relate to. HMOD, hypertension-mediated organ damage; ROS, Reactive oxygen species; IL, interleukin; VIF, vasoconstriction inhibiting factor; VCAM, vascular cell adhesion molecule; eGFR, estimated glomerular filtration rate; PECAM, platelet and endothelial cell adhesion molecule 1 (CD31); CXCR2, C-X-C motif chemokine receptor 2.

5.2 Study specific methods

5.2.1 Sample preparation

Fasting blood samples were obtained on the morning of the study visit and transported to the laboratory for same day processing and freezing, see Chapter 2.4. Plasma (EDTA) was centrifuged (1200g at room temperature for 10 minutes) before pipetting plasma into a plastic tube, leaving 0.5 ml of plasma above buffy layer. Five 0.5 ml aliquots were frozen at -80ºC. Subsequently, samples were thawed on ice, and transferred to 96 well PCR plates with samples randomly assigned to wells to remove positional associations. PCR plates were sent to Olink® Clinical Biomarkers Facility (Science for Life Laboratory, Uppsala, Sweden) on dry ice.

5.2.2 Olink®

Plasma samples of 61 normotensive controls, and 61 hypertensive participants were analysed for 384 protein biomarkers in whom plasma samples were available at the time point of sending, prior to recruiting the final participants.

Protein biomarkers were analysed with Olink® Explore Inflammation panel, a high-throughput multiplex proximity extension assay (PEA) technology.[340] PEA uses two highly specific oligonucleotide labelled antibodies per protein; when both antibodies bind to the surface of the target protein this permits formation of a PCR reporter sequence. Real-time PCR then quantifies the sequence. The value on the x-axis value where the fluorescent signal curve crosses the threshold is the Cycle threshold (Ct); indicating the number of cycles required to intersect the threshold line. Data derived from the Ct values are expressed as normalised protein expression (NPX) arbitrary units i.e. a relative signal strength in Log^2 scale. Normalisation is the application of an adjustment factor for each protein assay, applied to all measurements on one plate; this ensures data is comparable across plates, but as relative expression would not be comparable with studies ran separately.[341]

A feature of affinity based assays such as Olink® data, is the sigmoid-curve relationship with the true protein concentration. Below a certain threshold a sample risks being in the non-linear segment of the sigmoid curve, hence a 1x NPX difference may not equate to a 2x protein concentration at this level. A limit of detection (LOD) was therefore calculated for each Olink® assay and sample plate. Negative controls included on every plate allowed estimation of the background signal and setting of the LOD at three assay-specific standard deviations. Assays with >25% of samples below the LOD were excluded from analyses, as per Olink® recommendations. The full list of proteins included in the Olink® Inflammation panel are available in the literature.[341] C-reactive protein was unfortunately not analysed.

5.2.3 Statistical considerations

Data were analysed in Microsoft Excel (2013), RStudio, and Minitab (Version 19). Between-group comparisons employed Kruskal-Wallis test for non-parametric data distribution. Continuous variables were analysed with Pearson correlation. Statistical significance was assumed for $P<0.05$. Whilst each biomarker was tested independently, the number of biomarkers being assessed increased the risk of a type one error (false positive result), Bonferroni corrections are therefore also reported.

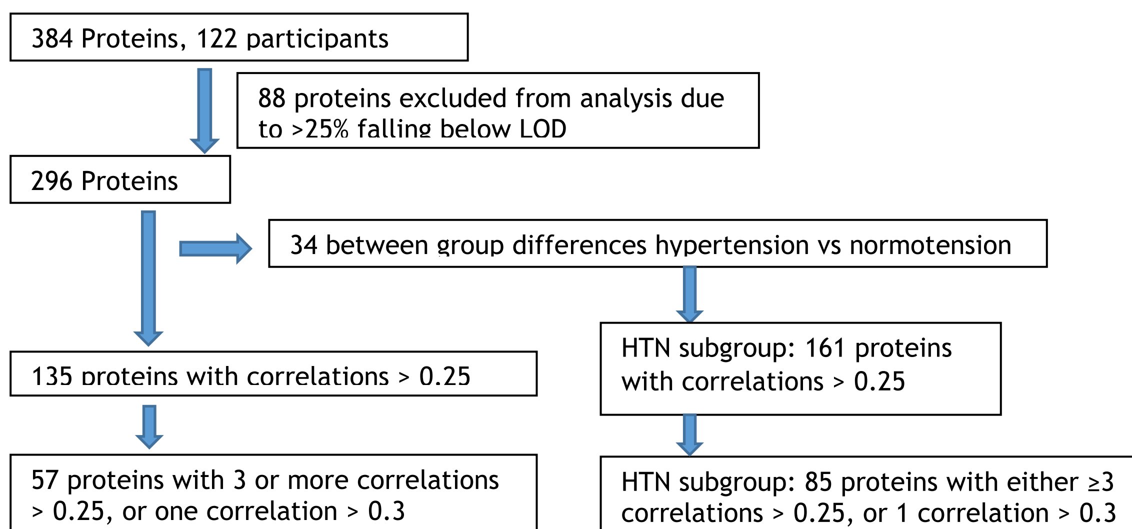


Figure 5.1. Flowchart detailing the participant and biomarker numbers at different stages of data checks and analysis. LOD, limit of detection; HTN, hypertension.

BP thresholds for hypertension were concordant with Chapter 3. Within the hypertensive group, distinct phenotypes were identified using Spectral Biclustering to identify homogenous patterns appearing in the data. [342] This technique assumes the data has a checkerboard structure, with the number of partitions in both dimensions as input. Each row is thus assigned to the same number of biclusters as the number of column partitions, and vice-versa. While classical clustering focuses on detecting ‘global’ similarities based on all features, biclustering reveals patterns containing ‘local’ subsets of features and subgroups of patients. Differences between groups identified in biclustering analysis were assessed with box-plots and ANOVA.

5.3 Results

5.3.1 Do circulating immune biomarkers differ between young, incident patients with hypertension but no overt cardiovascular disease, versus healthy controls?

Table 5.0 demonstrates that 34 of the 296 biomarkers suitable for assessment had between group difference that reached statistical significance of p<0.05 on unadjusted comparisons. However, all biomarkers lost statistical significance when the threshold p value of <0.00017 was applied based on the Bonferroni method to account for multiple comparisons. Of these 34 biomarkers in Table 5.0, 15 represent classical 'immune' biomarkers, dominated by cytokines and chemokines; six have an established role in cardiovascular disease or BP regulation, three have evidenced links to both, ten have broader roles in cell maintenance and cell cycle.[343]

Biomarker protein (assay name) *key association*	Normo-tension N=63	Hyper-tension N=59	Kruskal-Wallis p value
Hepatocyte growth factor (HGF) *cardiovasc*	-0.42	0.21	<0.001
Angiopoeitin 1 (ANGPT1) *cardiovasc & maintenance*	-0.29	0.37	0.010
C-C motif chemokine 4 (CCL4) *immune*	-0.30	0.01	0.036
Cellular communication network 2 (CCN2) *maintenance.*	-0.30	0.06	0.025
Cluster of differentiation 70 (CD70) *immune*	0.28	-0.22	0.041
Hydroxysteroid 11-beta dehydrogenase 1 (HSD11B1) *immune & maintenance*	0.27	-0.30	0.013
Cysteine rich with EGF-like domains 2 (CRELD2) *maintenance*	-0.36	0.09	0.022
C-X-C motif chemokine 14 (CXCL14) *immune*	0.11	-0.44	0.006
Interleukin 6 family receptor (LIFR) *immune & maintenance*	0.32	-0.30	0.009
Matrix metallopeptidase 1 (MMP1) *maintenance*	-0.15	0.21	0.012
WFIKKN2 *maintenance*	0.22	-0.33	0.037
Tripeptidyl-peptidase 1 (TPP1) *immune & maintenance*	-0.25	-0.01	0.004
Advanced glycosylation end product receptor (AGER) *cardiovasc*	0.33	-0.31	0.012
Corticotropin-releasing factor-binding protein (CRHBP) *cardiovasc & immune*	-0.11	0.35	0.005
C-C motif chemokine 7 (CCL7) *immune*	-0.27	0.11	0.004
Contactin-associated protein-like 2 (CNTNAP2) *cardiovasc*	0.21	-0.23	0.002
Serum paraoxonase/lactonase 3 (PON3) *cardiovasc*	0.28	0.01	0.017
Phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP) *maintenance*	-0.24	0.17	0.022
Interleukin-8 (CXCL8) *immune*	-0.27	0.17	0.003
C-C motif chemokine 3 (CCL3) *immune*	-0.41	0.19	0.008
Secretoglobin family 3A member 2 (SCGB3A2) *immune*	0.32	-0.19	0.004

C-C motif chemokine 13 (CCL13) *immune*	-0.18	0.12	0.008
Ectonucleotide pyrophosphatase/ phosphodiesterase family member 7 (ENPP7) *maintenance*	-0.16	0.20	0.028
Interleukin-24 (IL24) *immune*	0.05	-0.25	0.016
MHS class I polypeptide-related sequence (MICB_MICA) *immune*	0.16	0.40	0.041
Pappalysin (PAPPA) *immune & maintenance*	0.26	-0.20	0.042
Serpin B8 (SERPINB8) *maintenance*	-0.32	0.11	0.006
Endothelial cell-specific molecule 1 (ESM1) *immune*	0.00	-0.25	0.050
Serine Protease 8 (PRSS8) *cardiovasc*	-0.27	0.10	0.025
Erythropoietin (EPO) *cardiovasc & immune*	-0.36	-0.02	0.042
Secretogranin III (SCG3) *maintenance*	0.23	-0.34	0.011
Kynureninase (KYNU) *cardiovasc*	-0.32	0.13	0.014
2'-Deoxynucleoside 5'-Phosphate N-Hydrolase 1(DNPH1) *maintenance*	-0.16	0.18	0.034
Interleukin 18 Receptor 1 (IL18R1) *immune & cardiovasc*	-0.24	0.39	0.029

Table 5.0 Biomarker comparison between normotensive and hypertensive groups. *Cardiovasc,* cardiovascular. Nominal p values are shown. Bonferroni correction for multiple comparisons sets a significance p value of 0.00017; none of the above reach this significance level.

5.3.2 Which immune system biomarkers are associated with BP, hypertension, and arterial function?

Correlation analyses were then undertaken on the basis that although the participants could be dichotomised into normotensive and hypertensive, BP is a continuous parameter. A correlation matrix was selected as hypertension diagnosis can involve consideration of multiple variables, including systolic, diastolic, day, and night-time values. Further, inflammatory protein biomarkers may relate to BP, may only be seen in the disease state of hypertension but not healthy controls, or may link to other aspects of vascular function as demonstrated in Table 5.1.

<u>Blood pressure</u>: Table 5.1 includes the 57 biomarkers with one BP (SBP24, DBP24, SCP, DCP) or vascular correlation >0.3, or three or more correlations of >0.25. LnRHI and %FMD columns are not included in Table 5.1, as neither demonstrated any correlation >0.25 with the 57 biomarkers included, this is explored in the discussion. Of the 57 biomarkers in Table 5.1, 20 were also represented in the 34 parameters with between Kruskal-Wallis group differences analysis in Table 5.0, supporting their potential biomarker role. Eleven of these 20 correlated >0.25 (positively or negatively) with one or more of SBP24, DBP24, SCP, and DCP, and seven with augmentation index or pulse wave velocity. Table 5.1 illustrates that correlations *not* demonstrating differences between normotension and hypertension were mostly for demographic features, followed by augmentation index as measured by either PWA or PAT, as outlined below.

Systolic and diastolic 24 hr BP had a high degree of concordance in biomarker associations, though only HGF correlated >0.35 in analysis with all participants, with the following correlating >0.25 for both systolic and diastolic 24 hr BP: TPP1, CCL7, CCL11, and CCL21 positively; IL18R1, and KYNU negatively. Table 5.1 demonstrates that many of the same biomarkers also demonstrated stronger correlation with central systolic and diastolic pressure (HGF, ENPP7, TPP1, CCL7, IL18R1), but also many more biomarkers correlated >0.25, particularly for DCP.

Hypertension: Protein biomarkers of hypertension may however only be apparent in the disease state and not in healthy controls, if they are involved in the pathogenesis or are a consequence of the disease. Therefore, correlation analyses were also performed in only the hypertensive patients. Table 5.2 illustrates that among the HTN subgroup, more numerous (85) and greater strength correlations emerged than had been apparent when normotensive participants were included. In particular, 24hr DBP correlated with CCL21 (r = 0.45), LGALS4 (0.40), and SELPLG (r = -0.36), all demonstrating slightly weaker correlations with SBP; whilst central SBP and DBP demonstrated TLR3, PON3, CCL21, and CSF3 all with r > 0.35. Percent nocturnal dip demonstrated negative correlation stronger than -0.35 with CTRC (protease with enhanced expression in basophils), EPHA1 (angiogenesis, cell proliferation and adhesion, enhanced expression in T cells), LGALS4 (lactose-binding protein with that may modulate cellular interactions), SIT1 (negatively regulates T cell and NK cell receptor signalling), and SMOC (enriched in plasmacytoid DCs); whilst 18 more (Table 5.2) arose between r values -0.25 and -0.35 notably, IL18 (epithelial cell repair and pro-inflammatory cytokine) and TNFSF11 (DC survival factor and augments T cell activation).

Of these 85 biomarkers demonstrating correlation though, fewer (16) were among those in Table 5.0 as also demonstrating with between group differences, whilst 41 overlapped with the 57 biomarkers demonstrating correlations as specified among the whole cohort. These included a number of chemokines and proteases. See Table 5.1. The biomarkers demonstrating correlations with demographic, vascular, or BP parameters in the HTN subgroup which had failed to demonstrate any correlation at whole cohort level were dominantly immune-

related. Specifically, a number of cluster of differentiation molecules mediating cellular interactions, activation, and differentiation (CD200R1, CD22, CD58, CD6, CD70), as well as cytokines and interleukins (IL-5, -11, -15, -18, and -32, IL-1RL2, IL-22RA1, and IL-5RA). See Table 5.2.

Demographic variables: Age in Table 5.1 (all participants) demonstrated strongest negative correlations ($r > -0.35$) with COL9A1, MEPE, and NPPC (along with r -0.25 to -0.35 for CDON, TNFAIP8, ROBO1); strongest positive correlations ($r > 0.35$) with IL17D, CXCL14, WNT9A, and CXCL17 (moreover, r 0.25 to 0.35 for CXCL10, TNFSF13, CXCL9, MLN, and FLT3LG). These findings were largely replicated in the hypertensive patient subgroup in Table 5.2 with IL15 ($r = 0.44$), and PGF ($r=0.39$) in addition.

BMI in all participant analysis demonstrated negative correlation greater than r -0.35 for SCGB3A2, HSD11B1, WFIKKN2; and -0.25 to -0.35: SCG3, OMD, GAL, PON3, AGER. A total of 36 circulating biomarkers demonstrated positive correlation, those with r over 0.35 being ERBB3, ANGPTL2, TNFRSF11A, CCL3, IL18R1, TREM2, HGF, LGALS9, IL1RN. The hypertensive participants additionally demonstrated the following with $r > 0.35$: CSF3, IL1RL2, OSM, and PLAUR, as reported in Table 5.2. IDQ demonstrated 24 correlations in Tables 5.1 and 5.2, but mostly weak (r 0.25 to 0.35), only CTSO and HGF greater than 0.35 in all participants and none in hypertensive subgroup.

Protein	Age	BMI	IDQ	AI@75%	PWA AIx	PWV	SBP SD	DBP SD	SBP 24	DBP 24	SCP	DCP	CIMT	Dip SBP%	Dip DBP%	IPAQ
COL9A1	-0.669*	-0.209	0.008	-0.283	-0.410*	-0.296	-0.073	0.101	-0.033	-0.084	-0.200	-0.135	0.318	-0.079	0.010	0.234
CNTNAP2	-0.055	-0.118	0.014	0.217	0.084	-0.144	-0.052	-0.010	-0.316*	-0.255#	-0.152	-0.163	0.174	0.133	0.058	-0.141
GAL	-0.061	-0.302*	-0.194	0.017	-0.090	-0.049	-0.083	-0.151	-0.097	-0.088	-0.080	-0.107	0.035	0.200	0.209	0.097
HSD11B1	0.073	-0.419*	-0.182	0.126	-0.135	-0.047	-0.134	-0.093	-0.162	-0.101	-0.091	-0.054	0.016	0.086	0.059	0.009
SCG3	-0.087	-0.347*	-0.103	0.087	-0.168	-0.192	-0.119	-0.052	-0.185	-0.138	-0.162	-0.145	0.103	0.071	0.012	-0.068
OMD	-0.178	-0.313*	-0.139	-0.075	-0.226^	-0.118	-0.012	0.026	-0.055	-0.068	-0.150	-0.129	0.301*	0.061	0.053	0.051
AGER	-0.128	-0.250#	-0.029	-0.054	-0.188	-0.006	-0.243	-0.104	-0.247#	-0.200^	-0.263#	-0.262#	-0.112	-0.032	-0.019	0.042
PON3	0.014	-0.274#	-0.169	-0.087	-0.209	-0.167	-0.155	-0.238	-0.293*	-0.304*	-0.280*	-0.360*	0.015	0.214	0.249	0.127
WFIKKN2	-0.104	-0.392*	-0.137	-0.108	-0.211	-0.122	-0.152	-0.048	-0.192	-0.179	-0.210	-0.214	0.048	0.132	0.148	0.010
SCGB3A2	0.100	-0.454*	-0.152	0.035	-0.106	-0.191	-0.245	-0.163	-0.225	-0.215	-0.238	-0.215	0.072	0.162	0.229	0.066
AMBN	-0.060	-0.172	0.006	0.051	-0.058	-0.099	-0.186	-0.112	-0.139	-0.094	-0.127	-0.120	0.341*	0.076	0.094	-0.026
MEGF10	-0.202	-0.080	0.039	-0.239#	-0.272#	-0.212	-0.104	-0.092	-0.036	-0.103	-0.115	-0.130	0.056	0.056	0.082	0.304*
MEPE	-0.383*	-0.067	0.045	-0.281#	-0.242#	-0.156	-0.155	-0.009	0.014	-0.036	-0.067	-0.109	0.130	-0.018	0.059	0.148
ITGB6	-0.071	-0.208	-0.165	-0.232#	-0.306*	-0.086	-0.193	-0.097	-0.101	-0.113	-0.186	-0.174	0.051	-0.126	-0.015	0.219
CD58	-0.096	-0.038	-0.026	-0.243#	-0.316*	-0.146	-0.198	-0.187	-0.128	-0.159	-0.196^	-0.271#	-0.075	0.006	0.073	0.131
NPPC	-0.350*	0.000	0.202	-0.255#	-0.193^	-0.166	-0.061	0.077	0.069	0.049	-0.065	0.016	0.118	-0.192	-0.077	0.142
CDON	-0.297*	0.136	0.291*	-0.193	-0.156	0.012	0.104	0.178	0.095	0.048	0.025	0.079	0.062	-0.162	-0.108	0.101
PSPN	-0.053	0.159	0.184	-0.293*	-0.127	0.017	-0.166	-0.228	0.095	0.039	-0.002	0.002	-0.059	-0.082	0.006	0.158
WNT9A	0.508*	-0.134	0.005	0.073	0.096	0.180	-0.090	-0.112	-0.056	-0.047	0.054	-0.048	-0.010	0.219	0.134	-0.084
IL17D	0.385*	-0.045	-0.124	0.143	0.059	0.013	-0.132	-0.187	-0.150	-0.167	0.029	-0.104	0.001	0.142	0.031	-0.063
MICB_MICA	0.021	0.073	-0.068	0.113	-0.038	0.089	0.014	0.049	0.140	0.097	0.141	0.130	-0.454*	0.083	0.070	-0.028
SIT1	-0.179	0.250	0.227	0.007	0.067	0.000	-0.053	0.016	0.169	0.147	0.078	0.180	0.008	-0.294#	-0.327*	-0.187
SMOC2	-0.036	0.258#	0.188	-0.311*	-0.026	0.059	-0.019	0.100	0.223	0.153	0.188	0.129	-0.098	-0.159	-0.057	-0.013
CRHBP	-0.050	0.308*	0.218	-0.155	0.033	0.126	0.022	-0.021	0.215	0.106	0.091	0.035	-0.236	-0.108	-0.132	0.000
CTSO	0.089	0.285*	0.350*	-0.057	0.133	0.140	0.069	0.107	0.175	0.111	0.215	0.176	-0.122	-0.139	-0.106	-0.044
ANGPTL2	0.099	0.366*	0.292#	-0.059	0.024	0.104	-0.001	-0.003	0.106	0.029	0.029	0.032	-0.056	-0.142	-0.115	-0.063
LAIR1	0.148	0.331*	0.177	0.074	0.088	0.094	-0.030	0.055	0.068	0.073	0.062	0.007	-0.353*	-0.050	-0.083	-0.151

	Age	BMI	IDQ	AI@75%	PWA AIx	PWV	SBP SD	DBP SD	SBP 24	DBP 24	SCP	DCP	CIMT	Dip SBP%	Dip DBP%	IPAQ
FSTL3	0.163	0.325*	0.147	0.053	0.156	0.204	0.055	0.167	0.069	0.061	0.137	0.029	-0.240	-0.070	-0.170	-0.234
MLN	0.334*	0.107	0.090	-0.010	0.098	0.013	-0.001	0.019	-0.049	-0.017	0.056	0.041	-0.123	-0.122	-0.256#	-0.028
CXCL10	0.252#	0.312*	0.127	0.043	0.244	0.138	0.161	0.056	0.121	0.070	0.142	0.110	-0.222	-0.057	-0.124	-0.021
PGF	0.239	0.231	0.044	-0.119	-0.022	0.300*	-0.163	-0.165	0.135	0.065	0.134	0.008	-0.151	0.091	0.013	-0.083
TNFRSF11B	0.234	0.017	0.181	0.216	0.155	0.211#	0.336*	0.279#	0.156	0.105	0.241^	0.183	-0.064	0.088	0.042	-0.005
CCL21	0.060	0.126	0.129	0.163	0.101	0.164	0.005	0.078	0.257#	0.288*	0.182^	0.265#	-0.025	0.029	-0.005	-0.031
TREM2	0.220	0.387*	0.305*	0.165	0.294*	0.178	0.345*	0.395*	0.081	0.073	0.210^	0.189^	-0.189	0.047	-0.074	-0.200
LGALS4	-0.059	0.048	0.147	0.095	0.114	0.097	0.183	0.215	0.208	0.259	0.143	0.265	0.035	-0.248	-0.291	-0.110
KYNU	-0.078	0.329*	0.334*	-0.081	0.071	0.122	0.198	0.218	0.256#	0.221^	0.212^	0.267#	0.082	-0.193	-0.178	-0.156
FABP1	-0.070	0.290*	0.330*	0.008	0.085	0.111	0.237#	0.269#	0.206^	0.228#	0.169^	0.267#	-0.119	-0.200	-0.251^	-0.182
LGALS9	0.203	0.460*	0.293#	0.030	0.285#	0.279#	0.083	0.146	0.167	0.155	0.179	0.172	-0.149	-0.105	-0.186	-0.240
IL1RN	0.113	0.486*	0.315*	0.023	0.280#	0.144	0.198	0.173	0.092	0.068	0.148	0.141	-0.117	-0.059	-0.097	-0.228
CCL3	0.052	0.381*	0.334*	0.084	0.143	0.113	0.202	0.173	0.187	0.152	0.145	0.192	-0.064	-0.087	-0.147	-0.120
ERBB3	0.007	0.362*	0.346*	-0.018	0.197	0.213	0.163	0.225	0.183	0.123	0.120	0.141	-0.230	-0.139	-0.121	-0.116
TNFRSF11A	0.039	0.371*	0.217	0.149	0.258#	0.205	0.033	0.100	0.145	0.178	0.215	0.177	-0.012	0.024	-0.082	-0.192
CCL4	0.077	0.264#	0.301*	0.220	0.276#	0.132	0.066	0.183	0.141	0.192	0.207^	0.274#	-0.039	-0.105	-0.185	-0.171
AGRN	0.076	0.199^	0.198^	-0.137	0.001	0.032	-0.257#	-0.153	-0.016	0.013	-0.101	-0.069	-0.192	-0.265#	-0.270#	-0.229#
EPHA1	0.061	0.213^	0.190	-0.142	-0.035	0.197^	-0.150	-0.038	0.108	0.121	0.069	0.080	-0.353*	-0.231#	-0.311*	-0.125
CCL23	0.219^	0.196	-0.133	-0.075	0.046	0.302*	-0.123	-0.074	-0.071	-0.079	0.010	-0.123	-0.086	0.031	-0.017	-0.002
HGF	0.059	0.444*	0.355*	0.330*	0.356*	0.326*	0.309*	0.222#	0.382*	0.324*	0.462*	0.400*	0.075	-0.106	-0.108	-0.185
FLT3LG	0.347*	0.072	-0.055	0.088	0.233	0.107	0.074	0.039	0.017	0.007	0.134	0.069	-0.119	-0.088	-0.202	-0.157
CXCL17	0.623*	0.027	0.183	0.254^	0.348*	0.245^	-0.120	-0.042	-0.056	0.006	0.111	0.115	-0.101	-0.028	-0.173	-0.083
CXCL14	0.417*	0.057	0.134	0.048	0.198^	0.011	-0.171	-0.121	-0.113	-0.141	-0.020	-0.095	-0.101	-0.015	-0.058	-0.019
PRSS8	0.053	0.291#	0.263#	0.132	0.165	0.352*	0.073	0.173	0.236*	0.209#	0.309*	0.247#	-0.224	-0.129	-0.143	0.002
CCL7	0.106	0.327*	0.156^	0.121^	0.286#	0.259*	0.184	0.112	0.300*	0.250#	0.238#	0.274#	-0.117	-0.057	-0.097	-0.198
CCL11	0.117	0.132	0.116	0.114	0.265#	0.219^	0.147	0.142	0.259#	0.168	0.262#	0.233	-0.059	-0.079	-0.142	-0.044

	Age	BMI	IDQ	AI@75%	PWA AIx	PWV	SBP SD	DBP SD	SBP 24	DBP 24	SCP	DCP	CIMT	Dip SBP%	Dip DBP%	IPAQ
IL18R1	0.154	0.383*	0.269#	0.127	0.251#	0.289*	0.327*	0.274#	0.286*	0.214#	0.356*	0.329*	-0.029	-0.115	-0.159	-0.122
ENPP7	0.059	0.205^	0.290*	0.121	0.117	0.165	0.360*	0.374*	0.343*	0.297*	0.233#	0.332*	-0.127	-0.051	-0.005	-0.038
CXCL8	0.000	0.225^	0.112	0.143^	0.308*	0.091	0.154	0.199	0.274#	0.280*	0.297#	0.336*	0.006	-0.202	-0.239	-0.154
TPP1	0.031	0.295*	0.346*	0.239^	0.264#	0.129	0.141	0.192	0.331*	0.315*	0.319*	0.382*	-0.027	-0.178	-0.204	-0.192
CCL20	-0.044	0.157	0.335#	0.208	0.210	0.066	0.288#	0.313*	0.170	0.189	0.201	0.246#	-0.017	-0.118	-0.227^	-0.103

Table 5.1 Correlation of 57 biomarkers with BP and/or vascular variables in 122 participants (normotension and hypertension). ^p<0.05, #p<0.01, *p<0.001, weak correlation (0.25 to 0.35) in green, moderate (0.35 to 0.4) orange, and strong (>0.4) in pink. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CIMT, carotid intima-media thickness; LnRHI, natural logarithmic scaled reactive hyperaemia index; AIx, augmentation index; AI@75%, AIx adjusted for heart rate; PWV, pulse wave velocity; PWA_AIx, pules wave analysis derived augmentation index; SCP systolic central pressure; DCP, diastolic central pressure; IDQ (INTERHEART diet score), cardiovascular risk score adjusted for hypertension; IPAQ, International Physical Activity Questionnaire; SBP24, 24 hour average SBP; DBP24, 24 hour average DBP; SBP SD, standard deviation of SBP readings i.e. BP variability; DBP SD, standard deviation of DBP readings. Only biomarkers with one correlation >0.3, or three or more >0.25 included. For full protein names see product literature.[341]

	BMI	Age	% FMD	PAT AI@75%	PWV	AIx PWA	SCP	DCP	IDQ Adjusted	SBP SD	DBP SD	dip SBP %	dip DBP %	HR range day	SBP 24	DBP 24
AGRN	0.145	0.085	0.085	-0.099	-0.074	-0.066	-0.079	0.024	0.156	-0.313	-0.180	-0.256	-0.287	-0.028	0.091	0.091
AMN	0.335	0.027	0.053	-0.234	-0.001	-0.150	-0.178	-0.106	0.061	0.141	0.315	-0.007	0.010	0.051	-0.256	-0.282
CCL20	0.161	-0.153	-0.010	0.230	0.012	0.305	0.153	0.315	0.308	0.278	0.399	-0.167	-0.232	0.194	0.109	0.136
CCL21	-0.010	0.081	0.112	0.343	0.141	0.124	0.351	0.433	0.074	0.078	0.284	0.041	-0.054	0.108	0.432	0.451
CCL3	0.300	0.006	-0.054	0.052	-0.030	-0.026	-0.163	-0.078	0.272	0.142	0.222	-0.043	-0.042	-0.033	-0.105	-0.133
CCL4	0.337	-0.061	0.058	0.042	-0.057	-0.010	-0.080	-0.064	0.159	-0.002	0.137	-0.151	-0.130	-0.198	-0.050	-0.073
CCL7	0.207	-0.037	-0.088	0.333	0.124	0.215	0.229	0.227	0.174	0.260	0.224	-0.181	-0.257	-0.028	0.172	0.061
CD200R1	0.083	-0.169	0.179	0.117	-0.006	-0.009	-0.037	0.003	0.158	0.230	0.313	0.002	-0.024	0.064	-0.042	-0.111
CD22	0.318	-0.269	0.035	0.065	-0.145	0.194	0.167	0.196	0.212	0.250	0.414	-0.140	-0.083	0.225	0.103	0.081
CD58	-0.040	-0.141	0.140	-0.324	-0.144	-0.373	-0.266	-0.290	-0.089	-0.185	-0.268	-0.148	-0.010	-0.106	-0.039	-0.150

	BMI	Age	% FMD	PAT AI@75%	PWV	AIx PWA	SCP	DCP	IDQ Adjusted	SBP SD	DBP SD	dip SBP %	dip DBP %	HR range day	SBP 24	DBP 24
CD6	0.071	-0.138	-0.010	0.261	0.120	0.010	0.083	0.169	-0.035	0.215	0.290	-0.143	-0.168	0.264	0.164	0.141
CD70	0.176	0.331	-0.185	0.203	0.131	0.352	0.224	0.123	-0.051	-0.006	0.221	0.093	-0.111	-0.036	-0.029	-0.014
CDON	-0.016	-0.273	0.241	-0.125	-0.031	-0.268	-0.016	0.099	0.322	0.090	0.188	-0.215	-0.128	0.222	0.031	0.003
CLEC4C	0.150	0.002	0.299	0.117	0.077	0.076	0.215	0.303	-0.006	-0.093	0.115	-0.114	-0.218	0.081	0.148	0.098
COL9A1	-0.204	-0.668	0.313	-0.324	-0.322	-0.448	-0.269	-0.076	0.031	0.023	0.148	-0.134	-0.018	0.082	0.040	0.034
CSF3	0.372	0.076	-0.153	0.334	0.126	0.245	0.373	0.254	-0.018	0.111	0.141	0.041	-0.015	0.003	0.249	0.206
CTRC	0.073	-0.164	0.048	-0.088	0.055	0.098	0.023	-0.014	-0.074	0.021	-0.049	-0.401	-0.254	-0.083	0.162	-0.021
CXCL1	0.100	-0.020	-0.090	-0.095	0.101	-0.085	-0.028	0.024	-0.055	-0.178	-0.033	-0.335	-0.231	-0.039	0.009	0.121
CXCL10	0.300	0.191	-0.034	0.124	0.126	0.193	0.099	0.064	0.029	0.148	0.206	-0.103	-0.162	-0.069	0.071	-0.041
CXCL14	0.263	0.375	-0.036	0.021	0.039	0.248	-0.084	-0.111	0.180	-0.121	-0.076	-0.033	-0.134	-0.163	-0.106	-0.199
CXCL17	0.180	0.654	-0.248	0.155	0.343	0.262	0.308	0.186	0.087	-0.090	0.018	-0.025	-0.185	0.125	0.239	0.112
ENPP7	0.161	0.034	-0.123	0.103	0.036	0.051	0.092	0.094	0.036	0.366	0.356	0.023	0.058	-0.032	0.244	0.077
EPHA1	0.062	0.021	0.024	-0.136	0.109	-0.050	0.053	0.218	0.043	-0.302	-0.058	-0.380	-0.393	-0.070	0.098	0.190
ERBB3	0.369	-0.123	0.098	0.037	0.060	0.153	0.129	0.164	0.317	0.356	0.395	-0.124	-0.092	0.172	0.182	0.029
ESM1	-0.286	0.276	0.063	-0.027	0.134	-0.010	-0.139	-0.203	-0.299	-0.246	-0.245	0.155	0.098	-0.030	-0.071	-0.088
GAL	-0.390	0.088	0.126	0.257	0.122	0.155	0.216	0.154	-0.077	-0.017	-0.192	0.184	0.108	0.351	0.107	0.167
GMPR	-0.100	-0.150	-0.108	-0.209	-0.116	-0.221	-0.191	-0.252	-0.264	-0.192	-0.253	-0.044	0.115	-0.105	-0.160	-0.189
HGF	0.423	-0.038	-0.014	0.409	0.204	0.289	0.343	0.164	0.161	0.252	0.141	-0.030	-0.053	-0.046	0.156	0.002
IFNγ	0.079	0.167	0.014	0.274	0.100	0.276	0.250	0.192	-0.170	0.197	0.332	0.183	0.113	0.084	0.174	0.083
IL11	0.232	0.330	0.020	-0.094	0.143	0.039	-0.017	0.027	0.076	-0.243	-0.125	-0.167	-0.157	-0.134	0.058	-0.010
IL15	0.082	0.442	-0.173	0.075	0.270	0.243	0.204	0.083	-0.312	-0.055	-0.025	0.154	0.054	-0.194	0.094	0.014
IL17D	0.039	0.429	-0.077	0.147	0.078	0.096	0.153	0.015	-0.083	-0.164	-0.145	0.108	-0.041	-0.061	-0.043	-0.131
IL17RB	-0.051	0.029	0.173	-0.260	0.077	-0.337	-0.278	-0.235	-0.043	-0.069	0.004	0.021	0.149	0.008	-0.134	-0.172
IL18	0.095	-0.033	-0.030	-0.062	0.069	0.028	0.072	0.133	-0.123	0.030	0.129	-0.237	-0.321	-0.037	0.054	0.004
IL18R1	0.384	0.120	-0.056	0.180	0.159	0.110	0.248	0.228	0.138	0.217	0.289	-0.139	-0.169	0.154	0.197	0.081
IL1RL2	0.451	0.116	-0.116	0.132	0.136	0.008	0.035	-0.090	0.159	-0.037	-0.099	0.021	0.000	-0.143	0.008	-0.191
IL1RN	0.579	-0.085	0.266	0.114	0.051	0.194	0.230	0.207	0.331	0.285	0.432	-0.117	-0.081	-0.042	0.088	0.001
IL22RA1	0.061	-0.092	-0.013	0.269	0.170	0.258	0.270	0.234	-0.050	0.305	0.337	0.044	-0.048	0.059	0.260	0.171

	BMI	Age	% FMD	PAT AI@75%	PWV	AIx PWA	SCP	DCP	IDQ Adjusted	SBP SD	DBP SD	dip SBP %	dip DBP %	HR range day	SBP 24	DBP 24
IL32	0.139	-0.011	0.007	0.178	0.159	0.072	0.152	0.230	0.158	0.331	0.424	-0.066	-0.039	0.119	0.197	0.183
IL5	0.054	-0.019	-0.005	-0.040	0.041	0.008	0.083	0.180	0.343	-0.079	0.009	-0.036	0.007	0.002	-0.082	-0.019
IL5RA	0.008	0.117	-0.071	0.314	0.161	0.349	0.242	0.145	-0.004	-0.016	0.029	0.145	-0.077	0.136	0.030	0.003
ITGA11	-0.314	-0.070	-0.025	-0.101	0.013	-0.262	-0.194	-0.244	-0.043	-0.054	-0.154	0.024	0.079	0.072	-0.085	-0.190
ITGB6	-0.192	-0.098	0.028	-0.323	0.012	-0.302	-0.195	-0.175	-0.177	-0.241	-0.090	-0.296	-0.097	-0.083	-0.074	-0.058
JCHAIN	0.096	-0.077	0.023	0.023	0.062	0.050	0.044	0.084	0.172	0.265	0.351	0.099	0.040	0.196	0.230	0.199
JUN	0.060	-0.132	0.068	-0.226	-0.106	-0.085	-0.085	-0.059	0.062	-0.335	-0.183	-0.098	-0.029	0.006	-0.170	-0.123
KLRD1	0.102	0.000	0.018	0.053	-0.042	0.074	-0.151	-0.197	-0.041	0.194	0.242	0.146	0.033	0.217	-0.234	-0.342
LAIR1	0.327	0.119	0.144	0.130	0.034	0.069	0.047	0.017	0.094	-0.112	0.091	-0.069	-0.054	0.012	-0.002	0.007
LAMP3	0.139	0.196	-0.016	-0.150	0.153	-0.109	-0.094	-0.138	-0.265	-0.240	-0.077	-0.270	-0.242	-0.281	0.067	-0.026
LGALS4	0.008	-0.103	0.017	0.077	0.189	0.102	0.170	0.339	0.067	0.078	0.219	-0.373	-0.421	-0.095	0.367	0.404
LGALS9	0.436	0.132	-0.173	0.129	0.147	0.173	0.138	0.112	0.244	0.159	0.316	-0.162	-0.220	0.024	0.059	0.023
LGMN	0.187	-0.178	-0.029	0.095	0.051	-0.039	0.076	0.144	0.287	-0.001	0.056	-0.326	-0.263	0.031	0.033	0.039
MEGF10	-0.146	-0.068	-0.089	-0.213	-0.221	-0.324	-0.157	-0.161	0.136	-0.168	-0.089	-0.061	-0.040	0.123	-0.128	-0.142
MLN	0.204	0.333	-0.044	-0.042	0.068	0.208	0.153	0.087	0.065	-0.057	0.005	-0.061	-0.174	-0.280	-0.014	-0.014
NFATC3	0.267	-0.001	0.134	0.018	0.208	0.049	0.142	0.121	0.192	0.290	0.375	-0.013	-0.042	0.205	-0.028	-0.105
NPPC	-0.090	-0.317	0.083	-0.314	-0.155	-0.236	-0.057	0.080	0.178	-0.068	0.083	-0.293	-0.140	0.184	0.049	0.135
OSCAR	0.227	0.101	0.123	0.281	0.019	0.340	0.235	0.177	0.054	0.005	0.178	-0.083	-0.266	-0.132	0.198	0.058
OSM	0.477	0.196	0.040	0.237	0.092	0.229	0.198	-0.002	-0.073	0.135	0.000	0.116	0.040	-0.228	0.039	-0.138
PADI2	0.186	-0.238	0.131	-0.328	-0.082	-0.112	-0.022	0.056	0.172	-0.118	0.030	-0.133	-0.061	0.154	-0.164	-0.165
PAPPA	-0.132	0.075	-0.361	-0.100	0.068	-0.158	-0.053	-0.082	0.037	-0.141	-0.142	-0.133	-0.188	-0.044	0.032	-0.013
PCDH1	0.086	0.099	0.051	-0.097	0.029	-0.166	-0.099	-0.156	-0.046	0.076	0.122	0.045	0.086	0.014	-0.151	-0.315
PGF	0.112	0.394	-0.011	-0.035	0.207	-0.071	0.072	0.007	-0.030	-0.189	-0.093	-0.018	-0.057	-0.011	0.112	0.005
PKLR	0.152	-0.135	0.100	0.071	-0.326	0.072	-0.007	-0.033	0.015	0.096	0.065	0.090	0.178	0.004	-0.128	-0.100
PLAUR	0.407	0.151	0.066	0.259	0.058	0.252	0.212	0.119	0.248	0.042	0.142	-0.050	-0.030	-0.139	0.156	0.058
PNPT1	0.222	0.015	-0.030	0.023	0.064	-0.075	0.187	0.113	-0.004	0.143	0.178	-0.011	0.003	-0.041	0.302	0.175
PON3	-0.119	0.045	0.003	-0.132	-0.068	-0.103	-0.298	-0.393	-0.036	-0.001	-0.220	0.139	0.214	-0.009	-0.171	-0.242
PREB	0.004	0.088	-0.273	-0.024	0.023	0.119	-0.010	-0.039	-0.054	-0.199	-0.252	-0.041	-0.109	-0.065	-0.265	-0.113

	BMI	Age	% FMD	PAT AI@75%	PWV	AIx PWA	SCP	DCP	IDQ Adjusted	SBP SD	DBP SD	dip SBP %	dip DBP %	HR range day	SBP 24	DBP 24
PROK1	-0.015	-0.272	0.028	-0.337	0.234	-0.204	-0.042	0.041	0.053	-0.131	-0.043	-0.312	-0.083	0.062	0.041	-0.011
PSPN	-0.106	-0.139	-0.097	-0.366	-0.083	-0.267	-0.259	-0.126	0.187	-0.210	-0.160	-0.129	-0.014	-0.089	-0.183	-0.182
ROBO1	0.277	-0.274	0.162	0.041	0.061	-0.044	0.008	0.032	0.197	0.182	0.256	-0.235	-0.170	0.104	0.134	-0.047
SCGB1A1	-0.153	0.012	-0.099	-0.081	-0.144	-0.134	-0.301	-0.216	-0.042	-0.159	-0.232	0.035	0.078	-0.130	0.005	0.012
SCGB3A2	-0.306	0.134	-0.001	-0.137	-0.067	-0.177	-0.254	-0.286	-0.175	-0.140	-0.241	0.107	0.147	-0.074	-0.066	-0.154
SELPLG	0.185	-0.110	0.038	-0.120	-0.106	-0.059	-0.333	-0.291	-0.046	0.253	0.269	0.178	0.071	0.090	-0.298	-0.358
SIT1	0.294	-0.221	0.053	0.055	-0.127	-0.044	0.073	0.142	0.132	-0.152	0.042	-0.402	-0.361	-0.149	0.036	0.072
SMOC2	0.150	0.066	-0.086	-0.144	-0.042	-0.010	0.176	0.139	0.145	-0.046	0.153	-0.357	-0.254	0.130	0.219	0.141
SULT2A1	0.254	-0.129	0.107	-0.074	0.020	-0.092	0.015	0.164	0.286	0.259	0.335	-0.155	-0.077	0.102	0.062	-0.019
TLR3	0.025	-0.090	0.203	0.274	-0.161	0.182	0.275	0.351	0.199	0.242	0.303	-0.081	-0.158	0.155	0.155	0.228
TNFAIP8	-0.100	-0.350	-0.021	-0.115	-0.147	-0.168	-0.073	0.027	-0.136	0.013	-0.032	-0.247	-0.190	-0.086	0.041	0.069
TNFRSF1 1B	0.185	0.223	-0.058	0.147	0.297	0.246	0.275	0.156	0.261	0.341	0.324	0.029	-0.003	0.225	0.212	0.032
TNFSF11	0.049	-0.224	0.038	0.075	-0.223	-0.097	-0.043	0.118	0.038	-0.090	0.102	-0.308	-0.266	0.058	0.094	0.185
TNFSF13	0.058	0.319	-0.290	0.085	0.151	0.120	0.217	0.108	-0.093	-0.136	-0.143	0.016	-0.029	0.019	0.055	-0.027
TPP1	0.317	-0.042	0.015	-0.001	0.053	0.096	0.026	0.096	0.208	-0.081	-0.052	-0.300	-0.289	-0.125	0.144	0.077
TPSAB1	-0.028	0.030	0.218	-0.155	-0.015	0.026	0.041	0.023	-0.037	-0.281	-0.063	-0.274	-0.349	0.011	0.031	0.107
TREM2	0.514	0.163	0.042	0.118	0.167	0.194	0.191	0.130	0.281	0.338	0.462	0.062	-0.041	0.081	0.042	-0.074
WFIKKN2	-0.399	-0.044	0.001	-0.061	0.031	-0.027	-0.032	0.009	-0.085	-0.114	-0.099	0.055	0.079	0.064	-0.049	0.096
WNT9A	-0.097	0.542	-0.190	-0.035	0.263	0.111	0.151	-0.019	-0.104	-0.199	-0.155	0.137	0.093	0.086	-0.034	-0.034

Table 5.2 Correlation of 85 biomarkers with BP and/or vascular variables in 59 hypertensive participants. Name in italic indicates biomarker meets correlation criteria within HTN group AND within whole cohort. Weak correlation (0.25 to 0.35) in green, moderate (0.35 to 0.4) orange, and strong (>0.4) in pink. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CIMT, carotid intima-media thickness; LnRHI, natural logarithmic scaled reactive hyperaemia index; AIx, augmentation index; AI@75%, AIx adjusted for heart rate; PWV, pulse wave velocity; PWA_AIx, pules wave analysis derived augmentation index; SCP systolic central pressure; DCP, diastolic central pressure; IDQ (INTERHEART diet score), cardiovascular risk score adjusted for hypertension; IPAQ, International Physical Activity Questionnaire; SBP24, 24 hour average SBP; DBP24, 24 hour average DBP; SBP SD, standard deviation of SBP readings i.e. BP variability; DBP SD, standard deviation of DBP readings. Only biomarkers with one correlation >0.3, or three or more >0.25 included. For full protein names see website.[341]

Arterial function: as reported in Table 5.1 (all participants) COL9A1 demonstrates a negative correlation with PAT-derived AI@75, PWA-AIx, and PWV, as well as a positive correlation with CIMT. HGF had a positive correlation of r >0.3 in AI@75, PWA-AIx, and PWV, and the following demonstrated correlation of r >0.25 across 2 of the 3 measures of vascular stiffness (in either direction): CXCL17, CKMT1A_CKMT1B, IL18R1, CCL7, LGALS9. Among the hypertensive subgroup, augmentation index (by PWA or PAT) was associated with CD58, CD70, and PSPN (surpassing correlation of 0.35), in addition to COL9A1 (r = -0.45) and HGF (r = 0.41) already mentioned. No biomarkers demonstrated correlation r >0.3 for % FMD or LnRHI in the analysis including all participants (Table 5.1), and only PAPPA and COL9A1 for %FMD among the hypertensive group (r = -0.36 and 0.31 respectively). CIMT meanwhile had a number of biomarkers with negative correlation >-0.35 in Table 5.1; EPHA1, LAIR1, MICB_MICA. Others with weaker correlations reported in Table 5.2.

BP standard deviation as a measure of variation correlated positively (>0.25) for both systolic and diastolic BP with ENPP7, CCL11, CCN2, SERPINB8, FABP1, LGALS4, FST, CRHBP, as reported in Table 5.1. Among hypertensive participants (Table 5.2), 11 biomarkers demonstrated correlation >0.35 with cardiovascular variability, with CD22, IL1RN, IL32, and TREM2 all r >0.4.

Key biomarkers: Hepatocyte growth factor (HGF), advanced glycosylation end products (AGE), and CCL21 demonstrated strongest statistical significance for between group differences and also correlated with multiple BP or vascular parameters. Their results are now explored in greater detail. HGF attained strongest statistical significance in Table 5.1, higher levels were demonstrated in the hypertensive group, but in Table 5.2 also correlated with arterial stiffness (AIx measured by PAT or PWA, and PWV), BMI and cardiovascular risk score, 24hr and central systolic and diastolic BP, and BP variation. SCP was notably a strong positive correlation of 0.46. Further exploration can be found in the discussion (Section 5.4).

AGE accumulate in vascular tissue through aging, accelerated by diabetes mellitus, and with AGE-receptor (AGER / RAGE) mediating vascular inflammation. Tables 5.0 and 5.1 illustrate lower circulating levels of AGER in

the hypertension group and negative correlation with central BP and BMI. Aforementioned CCL21 correlated positively with 24 hour BP and central BP parameters across all participants, with stronger associations in the hypertensive group, reaching 0.45 for DBP 24 (Tables 5.1 and 5.2) and additional association with AI@75 derived from PAT. Interpretation and implications are covered in Discussion (5.4.2).

5.3.3 Relating to the immune milieu, are phenotypic subgroups apparent in hypertension?

Chapter 3 demonstrated that heterogeneous phenotypic groups could be identified within hypertension, based on bicluster analysis of vascular function parameters and demographics. Two analyses were therefore performed; firstly to see if biomarkers were significantly different between vascular phenotypic groups identified in Chapter 3; secondly, to assess if biomarkers demonstrate their own clusters and what participant phenotype characterises them.

Ten of the 57 biomarkers demonstrating correlation with vascular parameters (and 16 of the 85 biomarkers in HTN) also differed between the vascular phenotypic groups identified in Chapter 3 (ANOVA, $P<0.05$). These are included in Table 5.3, many relating to immune cell migration and cellular interactions. Interpretation and potential significance of these biomarkers follows in the discussion (Chapter 5.4). Existing literature was also considered, and biomarkers with robust evidence of association with vascular function were also compared by vascular phenotypic group, to explore if associations within certain subgroups may be masked when considered across all participants.

Biomarker	Linked to	Group 0 ‘arterially stiffened’ Mean NPX (SD) N=16	Group 1 ‘vaso-protected’ Mean NPX (SD) N=29	Group 2 ‘non-dipper’ Mean NPX (SD) N=11	P value (ANOVA)
CCL20	BP SD, IDQ, PWA-AIx	-0.128 (0.57)	-0.616 (0.93)	0.653 (1.33)	0.001
CCL7	HTN, AI@75%	0.424 (0.45)	0.031 (0.43)	0.642 (0.68)	0.001
CXCL8	HTN, PWA-AIx, 24hr BP, SCP/DCP	0.953 (0.58)	0.521 (0.47)	1.115 (0.76)	0.006
EPHA1	CIMT, % dip, SPB SD	0.431 (0.70)	-0.291 (1.05)	0.204 (1.02)	0.033
ERBB3	BMI, IDQ, BP SD	-0.061 (0.70)	0.011 (0.91)	0.804 (1.49)	0.049
IL1RN	BMI, IDQ, PWA-AIx, BP SD	-0.082 (0.64)	-0.248 (0.72)	1.15 (1.25)	<0.0001*
LGALS4	24hr BP, % dip, DCP	0.424 (0.89)	-0.261 (0.86)	0.015 (1.00)	0.039
LGALS9	BMI, IDQ, PWA-AIx, PWV	0.124 (0.88)	-0.202 (0.69)	0.896 (1.50)	0.0076
PON3	HTN, BMI, 24hr BP, central BP	-0.608 (1.20)	0.121 (0.86)	-0.379 (0.95)	0.046
TREM2	BMI, IDQ, BP SD,	-0.172 (0.75)	-0.219 (0.69)	0.947 (1.84)	0.005
CD22	BMI, age, DBP SD	-0.197 (0.99)	-0.125 (0.75)	0.838 (1.12)	0.007
ESM1	HTN; BMI; age, IDQ	0.040 (0.87)	-0.052 (0.94)	-0.085 (0.85)	0.025
IL18	DBP %dip	0.215 (0.78)	-0.339 (0.98)	0.364 (1.05)	0.047
KLRD1	DBP24	-0.444 (1.07)	0.207 (0.89)	0.383 (0.75)	0.027
PLAUR	BMI, AI@75%, PWA-AIx	0.007 (0.79)	-0.341 (0.71)	0.542 (1.06)	0.012
SELPLG	24hr BP, central BP, BP SD	-0.57 (1.37)	0.239 (0.67)	0.618 (0.84)	0.003
Classical Biomarkers of cardiovascular inflammation based on existing evidence					
IL-6 [90,176,344]	Correlation demonstrated but failed LOD	0.799 (0.73)	0.587 (0.74)	1.263 (0.77)	0.043
CXCL1[345]	Only HTN group and only correlated with %dip	0.464 (1.08)	-0.115 (0.83)	0.646 (1.49)	0.068
IFN-γ* [114,346]	Only HTN group and only correlated with AIx	0.079 (0.92)	-0.111 (0.91)	0.113 (0.54)	0.662
IL-1β [176,177,339,347]	No associations within Inflammatension data.	0.296 (1.30)	-1.06 (0.94)	0.227 (0.70)	0.382
IL-2[348]	No associations within Inflammatension data.	0.167 (1.20)	-0.215 (0.72)	-0.265 (0.89)	0.311
IL-7	No associations within Inflammatension data.	0.282 (1.15)	0.021 (0.87)	0.269 (1.33)	0.653
IL-10 [349,350]	No associations within Inflammatension data.	-0.118 (0.71)	-0.151 (0.74)	0.573 (1.39)	0.063
TGF-β* [173,176,347]	No associations within Inflammatension data.	0.497 (1.10)	-0.10 (0.832)	-0.003 (1.47)	0.152

Table 5.3. Biomarkers correlating with vascular parameters and also demonstrating between group differences based on vascular phenotypic group; plus additional biomarkers based on existing research. HTN, normotensive vs hypertensive group difference; biomarker in italic, correlated in all participant and hypertensive subgroup analyses; * maintained statistical significance after Bonferroni correlation (P<0.00059)

Secondly, biclustering generated from the biomarker NPX levels of hypertensive participants (N=59) is shown in Figure 5.2. Cluster 1 visually demonstrated greatest differentiation between the participant groups (Figure 5.2). Some biomarkers clustered by functionality, for example biomarkers of the IL family

were predominantly in Cluster 0. Others such as C-C and C-X-C motif chemokines (CCL and CXC) were distributed across clusters 0, 2, 3, and 4, with a relative dearth of CCL and CXC chemokines and interleukins in Cluster 1. Instead, Cluster 1 had a dominance of cell maintenance biomarkers, particularly kinases, and immune biomarkers relating to activation and regulation of both the innate and adaptive immune systems. For example, CD40 and its' ligand CD40LG, which co-stimulate T cells to induce proliferation and cytokine production, including IL-4 and IL-10. Also IRAK-1 and IRAK-4, kinases involved in TLR signalling and innate immune cell activation. Eight biomarkers in Cluster 1 related to cell survival (CASP2, TRAF2, BCL2L11, BID, DFFA, MAP2K6, TBC1D5, NT5C3A).

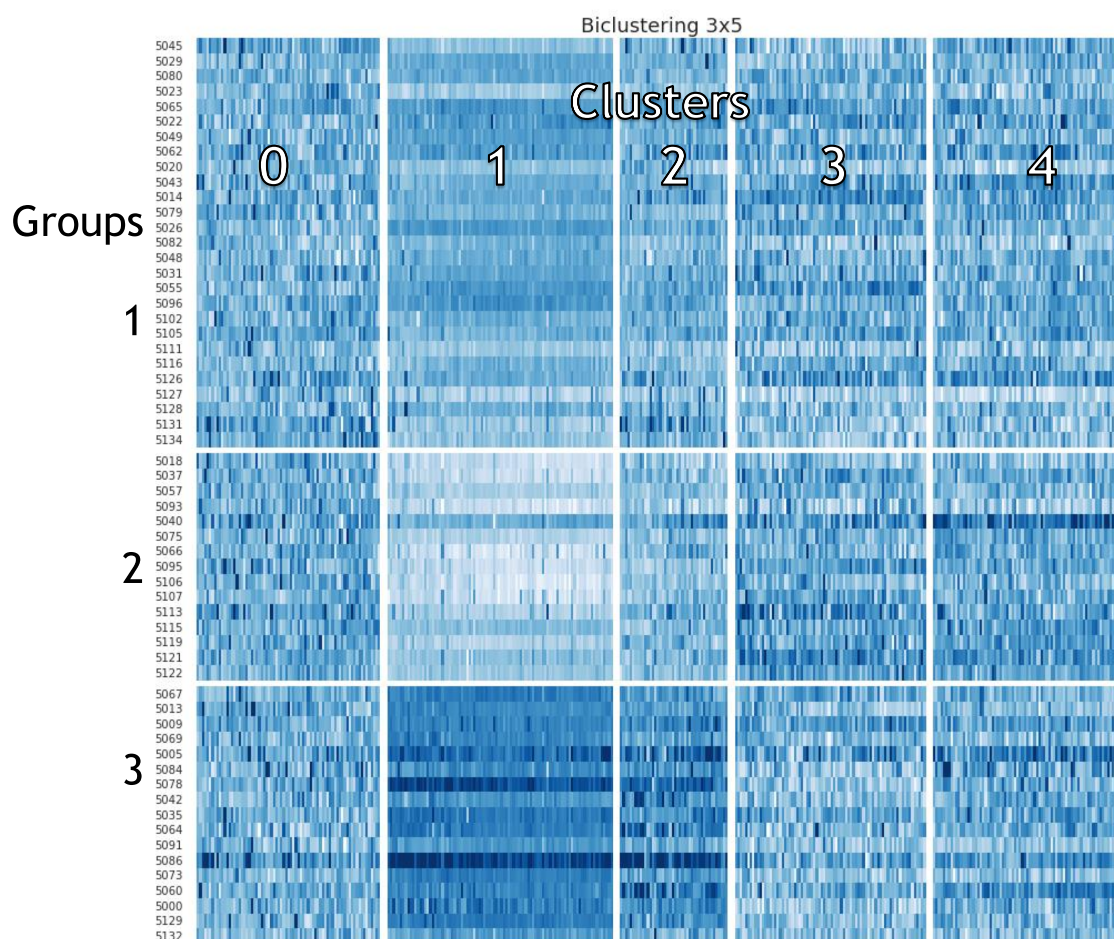


Figure 5.2. Raw data 3x5 biclustering of biomarkers (Y axis clusters) in incident hypertension, by participant ID number (X axis groups), n=59 participants. Full list of biomarkers in each cluster reported in Appendix 6.

These proteins driving biomarker cluster 1 were distinct from those significant for NTN vs HTN between-group differences and for correlations of vascular and biomarker parameters. Corroborating this, vascular and demographic features did not differ significantly between these three biomarker cluster groups (Figure 5.3). Hence, protein

biomarkers differentiating the immune phenotypes of incident hypertension (discussed below), appear to be distinct from those associated with vascular function.

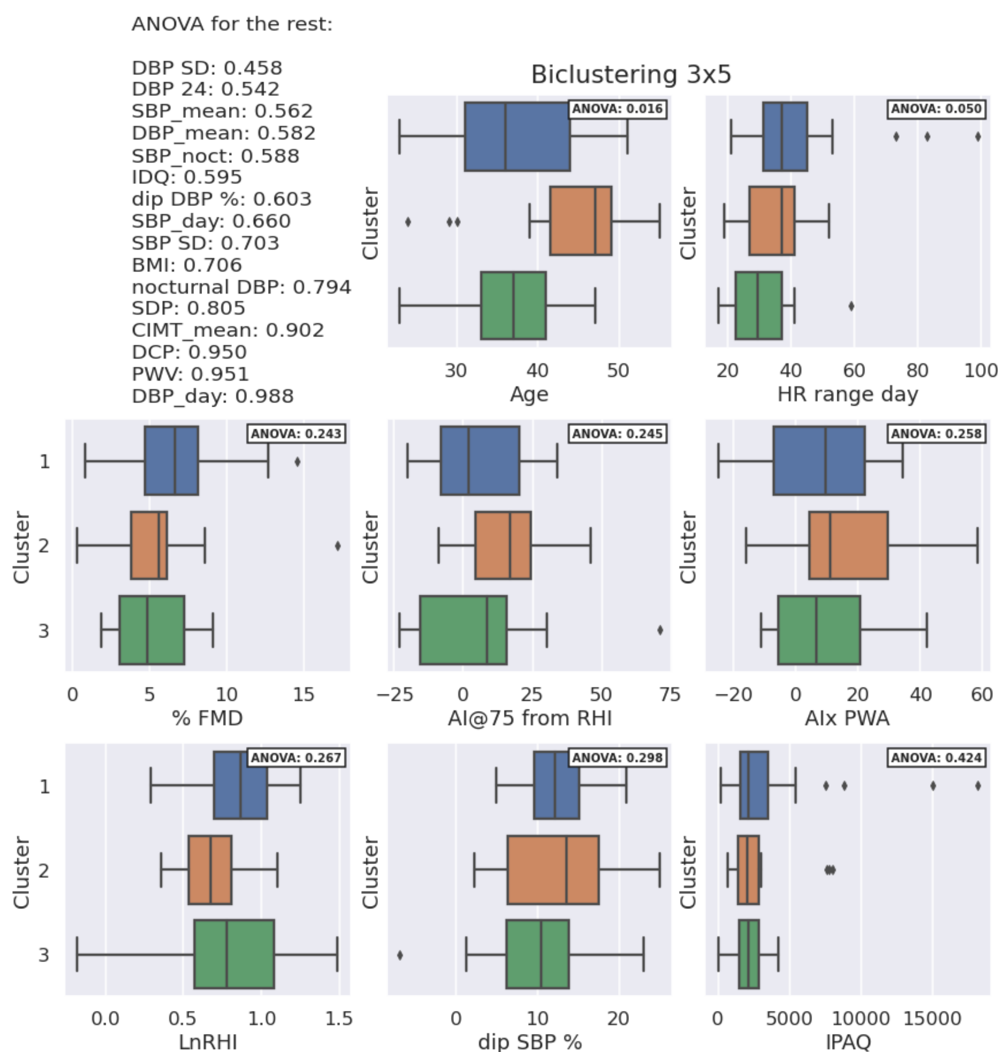


Figure 5.3. Boxplots and ANOVA reports of differences in arterial function and demographic factors between Groups 1-3 identified in Figure 5.2.

5.4 Discussion

The discussion follows the structure of the results section, drawing data from analyses together and interpreting results in the context of the existing literature to consider the implications of the data. Discussion integrating data from multiple chapters, such as biomarker levels compared across the arterial phenotypic groups, is reserved for Chapter 6.

5.4.1 Do circulating immune biomarkers differ between young, incident patients with hypertension but no overt cardiovascular disease, versus healthy controls?

Approximately 1 in 10 of the available biomarkers demonstrated difference between normotensive and hypertensive groups. Although applying a Bonferroni-adjusted significance value of $P<0.00017$ resulted in loss of significance, many of these biomarkers have an evidential basis supporting that the study may have been underpowered to overcome the demands of Bonferroni method. These biomarkers with established links to cardiovascular disease, and those demonstrating greatest between group differences that were also replicated in the correlation analyses, were dominated by immune mediators and regulators. These included HGF, Endothelial cell-specific molecule 1 (ESM1), cytokine and chemokine molecules. However, biomarkers relating to endothelial dysfunction, and to cell cycle / cell maintenance also demonstrated association, namely AGER and TPP1 respectively; hereby discussed in more detail.

HGF acts as an immunoregulatory cytokine favouring tissue repair and resolution of inflammation as figure 5.4 illustrates.[351] HGF is also a growth factor for a diverse range of cell lineages. HGF promotes cell survival, vasodilatation, and anti-fibrotic effects through inhibition of fibroblast to myofibroblast and endothelial-mesenchymal transition.[352] Higher levels in hypertension and strong correlations with various vascular function measures is consistent with the body of evidence, linking immunologically-active HFG not only with hypertension and vascular tone, but also arterial stiffness, arteriosclerosis, heart failure, insulin resistance, and pulmonary hypertension, and HGF has been explored as a therapeutic option for hypertension.[353-357]

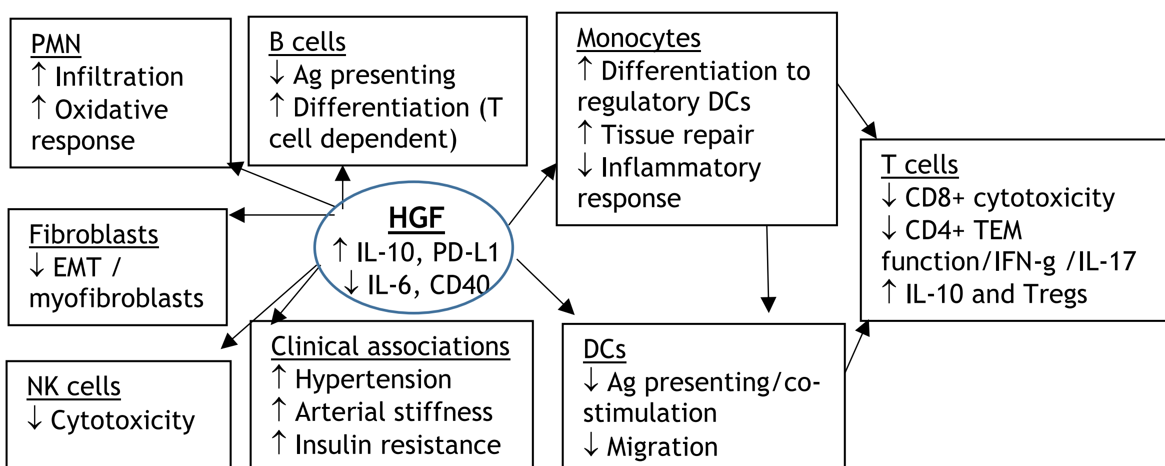


Figure 5.4. The functional roles of Hepatocyte growth factor (HGF). DC; dendritic cells, Tregs, T regulatory cells; PMN, polymorphonuclear leukocytes; NK, natural killer cells. Adapted from Molnarfi et al. 2015 and wider evidence.[351,353,357]

Interestingly, ESM1, also known as Endocan, is a positive regulator of HGF receptor signalling pathway, is involved in angiogenesis, and has a potential role in leucocyte interactions with endothelial cells. In this cohort, ESM1 demonstrated lower levels in hypertension, and correlated with age positively, BMI and IDQ (cardiovascular risk score) negatively, but not with vascular function measures. For reasons not established, this contrasts with other work demonstrating elevated levels in hypertension and lower levels following treatment.[358,359] TPP1 meanwhile has defined functions as a lysosomal protease with a ubiquitous role in telomere function. Tables 5.0 and 5.1 illustrated higher levels of TPP1 in hypertension, and correlation with multiple BP parameters, BMI, cardiovascular risk score, and PWA. It has not previously been linked to hypertension and vascular function in published literature and is thus a novel finding requiring validation, though was associated with sepsis severity and need for vasopressors in one study.[360]

Inflammatension demonstrates lower circulating levels of AGER in the hypertension group and negative correlation with central BP and BMI. AGER mediates vascular inflammation via signalling pathways that regulate inflammatory cytokines, oxidative stress, and endothelial dysfunction. Hence, AGER has a theoretical pathophysiological link to hypertension and vascular dysfunction, supported by genetic, animal, and biomarker studies.[361,362] Existing evidence is heterogeneous however, and interpretation is complicated by expression of AGER variants, two of which are secreted as soluble (s)- and endogenous secretory (es)-AGER that acts as a decoy receptor for AGE. Whilst McNulty *et al* describe higher levels of AGER in hypertension [362], sAGER conversely appears lower in hypertension, diabetes, and arterial stiffness [363,364] possibly reflecting shifts in cleavage e.g. by MMPs. Methodology may explain differences in findings, if studies are unable to distinguish different soluble isoforms.

Molecules stimulated by hypoxia featured in Table 5.2, such as ANGPT1 involved in angiogenesis and endothelial cell survival through reorganization of the actin cytoskeleton; and ESM1 discussed above, which stimulates angiogenesis in

addition to promoting the HGF receptor signalling pathway. Hypoxia drives hypertension through various systems including activation of RAAS, and drives a pro-inflammatory state, hence such biomarker associations have theoretic grounds.[365] C-C motif, C-X-C motif, and interleukins were also particularly well represented in Table 5.2, such as elevated CXCL8 (chemoattractant for neutrophils and T cells) and CCL7 (monocyte chemoattractant) in the hypertensive group. Cytokine and chemokine molecules also demonstrated some of the most robust correlations, CCL21 discussed in Section 5.3.2 associating with 24 hour and central BP. CCL21 has a dual role in inhibiting haematopoiesis and stimulating chemotaxis of thymocytes and activated T cells, but not B cells or granulocytes. This role may extend to T cell homing to secondary lymphoid organs, as CCL21 binds to lymphocyte-expressed CCR7, permitting migration into the lymph node. Existing evidence suggests that CCL21 binding CCR7 promotes atherogenesis and has been linked to risk of CAD and cardiovascular events in the context of myocardial infarction.[366,367] Controversy persists regarding CCL21/CCR7 however, as expression may be demonstrated as either increased or decreased in atherosclerotic plaques, and CCR7 deficiency has been shown both to retard, and conversely to promote, atherosclerosis in experimental models.[368] These discordant results may be due to the pleiotropic cytokine effects, multiple cellular sources, and diverse methodologies of research.

In summary, circulating levels of a number of immune biomarkers differ between healthy normotensive and incident hypertensive participants, though the study was underpowered to overcome the demands of multiple comparisons.

5.4.2 Which immune system biomarkers are associated with demographic features, BP, hypertension, and arterial function?

5.4.2.1 Observed correlations with demographic features:

Both age and BMI demonstrated a number of correlations with circulating immune biomarkers. This highlights the importance of controlling for both in immune-related research, or making statistical adjustments for differences. The pro-inflammatory contribution of excess adipose tissue has been widely studied; IL-18 and IL-1β for example have been linked [369], supporting the IL18R1, IL1RL2, and IL1RN findings above. Tables 5.2 and 5.3 demonstrate that these biomarkers

are distinct from those associating with 24hr SBP and DBP, despite the clear epidemiological associations of age and obesity with hypertension and cardiovascular disease.[35,369,370] How these demographic, immune, and BP interactions triangulate is an area for ongoing interest, particularly for promoting maintenance of a healthy weight.

5.4.2.2 Observed correlations with BP:

HGF, CCL21, and TPP1 have already been discussed in 5.4.1. Additional notable biomarker correlations include PON3 and CNTNAP2. PON3 negatively correlated with 24 hour and central BP. It acts as an antioxidant in circulation and can associate with HDL, inhibit oxidation of LDL, and hydrolyse lactones. The link to BP is a novel one, though low levels of PON3 in 'vaso-protected', and higher levels in 'arterially-stiffened' and 'non-dipper' hypertensive phenotypic subgroups align with existing data linking PON3 to peripheral artery disease and coronary artery disease.[371] CNTNAP2 also showed negative correlation with 24 hour BP, and has been linked to BP through GWAS and longitudinal genetic data models.[372,373]

Both CXCL8 and ENPP7 demonstrated positive correlation with 24 hour and central BP, as well as BP variability for ENPP7. CXCL8 is chemotactic for neutrophils but also T cells and granulocytes, and induces endothelial cell proliferation. Levels were higher in the hypertensive group. Within the biomarker biclustering CXCL8 located to Cluster 2 along with a number of other chemokines for different immune cells. Existing evidence links CXCL8 to animal models of hypertension, with expression induced by Ang II, supressed by losartan, and CXCL8 inhibitor lowering BP.[374] Human studies also link CXCL8 to hypertension, coronary artery disease, and cardiovascular events.[375] ENPP7 has a role in intestinal fatty acid absorption and lipid metabolism, but also inactivates platelet-activating factor. No significant evidence links ENPP7 to BP or arterial function, other than a genetic study associating it with BP response to hydrochlorothiazide.[376] Other than ENPP7, biomarkers correlating with BP variability were largely distinct from those correlating with central and 24hr BP measures, possibly suggesting a distinct interaction. Little data has been published looking at BP variability and biomarkers, though stress-related GDF-15 (growth/differentiation factor 15) has been identified by others.[377]

5.4.2.3 Observed correlations within the disease state of hypertension:

Some immune biomarkers correlated with BP, vascular, or demographic parameters in the HTN subgroup, having failed to demonstrate any correlation at whole cohort level (Table 5.2); in particular cytokines and interleukins (IL-5, -11, -15, -18, and -32, IL-1RL2, IL-22RA1, and IL-5RA). This potentially signifies an immune system association restricted to pathological-range BP, a possibility previously suggested by others.[76] Various studies evidence the association of the interleukins with hypertension and HMOD,[195,378] discussed in Section 5.4.2. Cluster of differentiation molecules were also heavily represented, mediating cellular interactions, activation, and differentiation, including CD58 (Leukocyte-function antigen 3) and SELPLG (selectin P ligand, CD162) discussed here in more detail. Co-stimulatory CD58 has an established role in adhesion and activation of T lymphocytes; soluble CD58 supresses T and NK cell-mediated immune responses by competing for the ligand CD2; hence the negative correlation with central BP and AIx may suggest a lack of co-stimulation regulation, though published evidence is lacking.

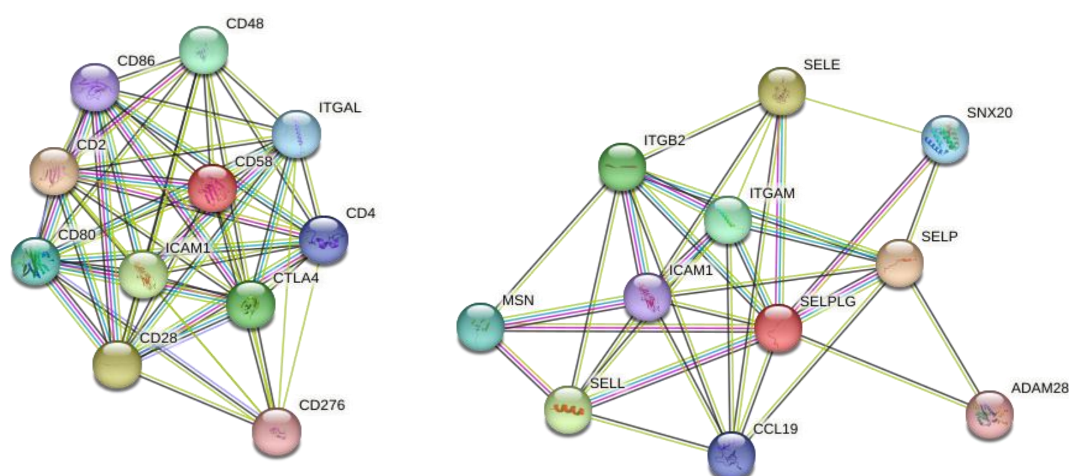


Figure 5.5 Protein networks of CD58 (left) and SELPLG (right). Cyan and pink lines indicate known interactions; blue, red, and green indicate predicted interactions. Generated from https://string-db.org/

SELPLG functions to permit leukocyte rolling / margination, has overlapping functional partners with CD58 in network analysis (Figure 5.5), and similarly demonstrated negative correlation with central BP and 24hr BP. This is consistent with other research groups reporting reduced levels in hypertension

[379], and polymorphisms have been linked to incident coronary artery disease and stroke.[380] Related to SELPLG, P-selectin expression has correspondingly been demonstrated as elevated in resistant hypertension.[190] Given that CD58 and SELPGL mediate immune cell interaction with the vascular endothelium, these associations with BP in hypertension support the role of the immune system in arterial function as a component of hypertensive disease.

5.4.2.4 Observed correlations with measures of arterial function:

LnRHI and %FMD did not show association with biomarkers other than PAPPA and COL9A1, and only within the hypertensive group. Other studies have linked impaired vasodilatation to elevated levels of selectins, TIMP-1, MCP1, and VEGF. [150,187] Though none have gone beyond association to demonstrate direction of causality. Given COL9A1 is an alpha chain of type IX collagen, negative correlation with age and arterial stiffness (PWA-AIx) makes theoretic sense, and other research links it to arterial vessel wall remodelling and dissection.[381,382]

HGF had a positive correlation of >0.3 in AI@75, PWA-AIx, and PWV, and its' functional role regulating tissue repair and existing evidence have already been discussed in this chapter. Among the hypertensive subgroup, augmentation index (by PWA or PAT) was also associated with CD58 (negative correlation) and CD70 (positive correlation), both with roles in T cell function, CD58 has already been discussed; CD70 cytokine binds to CD27 expressed on activated T cells, the co-stimulatory interaction being important for T cell proliferation of cytotoxic lymphocytes. Whilst the link to arterial function appears to be a novel finding; Markousis *et al* [383] demonstrate association of CD70 and T cell co-stimulation with heart failure, and Itani *et al* [284] demonstrate that hypertensive stimuli can increase APC expression of CD70, mice deficient in CD70 did not accumulate TEM cells, nor develop hypertension or renal damage. Inflammatension demonstrated positive correlation of CD70 with augmentation index, but lower circulating levels in hypertension. The underlying explanation for these differences is unclear.

5.4.2.5 Expected but not observed

IL-6 demonstrated positive correlations with measures of augmentation index and DCP, as well as differences across hypertensive bicluster groups. Evidence

from other published literature supports both BP and vascular function links.[89,173,174,176,344,384] IL-6 is not however included in the above results however as it demonstrated over 25% of samples below the LOD, increasing the risk of compromised reliability, though Inflammatension data were concordant with these other data. TGF-β binding to its' receptor regulates cell cycle and proliferation and through modifying gene expression can influence production of other growth factors and cytokines. TGF-β demonstrated no between group differences and no correlations with vascular parameters, despite association being supported by the work of others, hypothesised as being mediated via aldosterone.[173,176,347] One possibility is differential effects in early or incident versus well established hypertension, as has been demonstrated in cardiac remodelling.[385]

IL-10 is another immune regulatory cytokine with anti-inflammatory functions to limit excessive tissue disruption. When IL-10 binds to its receptor, this leads to JAK1 and STAT2-mediated phosphorylation of STAT3. STAT3 in turn translocates to the nucleus to promote expression of anti-inflammatory mediators. IL-10 has been associated with hypertension and arterial stiffness [386], though with varied conclusions, Gordon *et al* [171] demonstrating inverse association with risk of hypertension, Barbaro *et al* conversely identifying elevated levels in resistant hypertension.[349] Our data did not demonstrate difference in levels between normotensive and hypertensive groups, nor correlation with BP or vascular parameters, and was only significant for a correlation with heart rate. It may be that no differences in IL-10 were seen in hypertension as production is in part regulated by TGF-β (also no differences, as above).

IFN-γ is produced by T cells and NK cells, and via a JAK/STAT pathway induces transcription to promote activation of effector immune cells, and enhancement of antigen presentation. Ang II had been demonstrated to drive greater IFN-γ production with increasing lymphocyte age, promoting inflammation and fibrosis.[346] Inflammatension data demonstrated weak correlation with AI@75% by PAT, AIx by PWA, and SCP within the hypertensive patients, but no assiation when normotensive participants were included, nor any difference between phenotypic groups identified (Table 5.3). As with other biomarkers correlating with BP, but restricted to participants with hypertension, one explanation is that

altered levels only occurs in the disease state, as discussed above. Whilst one would hope that the between group comparison of normotension and hypertension would detect these, it is unlikely that the threshold for a 'pathological' state is biologically consistent and concordant with diagnostic categories across individuals.

CXCL1 (also known as fractalkine) acts as a chemokine for neutrophils. In inflammation it exerts its effects on endothelial cells in an autocrine fashion. Inflammatension data only demonstrated correlation with percent nocturnal dip, and only within the hypertensive group. Other research suggests that CXCL1 may mediate hypertensive organ damage to various tissues, with a role in cardiac monocyte infiltration and in hypertensive retinopathy, both animal studies with Ang-II induced hypertension. [345,387]

5.4.3 Relating to the immune milieu, are phenotypic subgroups apparent in hypertension?

The immune biomarkers have not been well studied in the available literature regarding nocturnal dipping as a phenotype of hypertension. This is despite known impacts of sleep deprivation and loss of circadian rhythm on the immune and cardiovascular systems.[267,388] Specifically, leukocyte trafficking has been demonstrated as regulated by circadian rhythm; increased circulating numbers but impaired functionality of NK cells occurs with prolonged wakefulness, with monocytes, NK and B cell numbers then decreasing following sleep; and pro-inflammatory cytokine secretion increases in sleep deprivation.[336,389] This may in part relate to cortisol diurnal variation.[390] Neutrophil-Lymphocyte ratio has been associated with percent nocturnal dip [87,391,392], and GWAS report an association with BCL11B gene and the protein it encodes. Table 5.2 demonstrates negative correlation between percent systolic nocturnal dipping with biomarkers relating to immune cells and cellular adhesion (CTRC, EPHA1, LGALS4, SIT1, SMOC, IL-18 and TNFSF11). Of these, IL-18 was among biomarkers identified as being associated with ABPM in Carranza-Leon's study utilising the Olink® inflammation panel; [90] whilst data is lacking for the others and these novel findings from exploratory data should inform validation studies. Further integration of data from Chapters 3 and 5 is covered in Chapter 6 Discussion.

The biclustering of circulating biomarkers themselves did not generate participant groups demonstrating phenotypic differences in arterial function or BP parameters. This may suggest that inter-individual differences are greater than group differences; or may reflect the use of all available biomarkers. Use of bicluster analysis in untreated and otherwise healthy individuals has not previously been attempted; it remains to be seen if larger population sizes, or restricted numbers of biomarkers will identify immunologically-based subgroups of hypertension.

5.4.4 Limitations and strengths

This chapter reports on circulating levels of biomarker proteins, but many factors are likely to influence levels. A study strength arises from the significant effort taken to keep confounding demographic and environmental factors to a minimum. However, BMI in particular demonstrated biomarker correlations and did differ between normotensive and hypertensive groups. The relationship of plasma to tissue expression of biomarkers is also not always linear, or even necessarily clearly elucidated, counter-regulation and redundancy of systems can also complicate interpretation, as can existence of isoforms. The findings must therefore be interpreted within the wider body of evidence, as I endeavoured to do. Panels or combined scores have been proposed as potential methods to overcome some of these limitations, hence the importance of techniques such as the biclustering to identify which biomarkers cluster together in untreated hypertension.[179]

Existing evidence also highlights biomarkers that were not included in the Olink® panel. As an example, MCP-1 (CCL2) acts as a chemokine for monocytes and basophils, but not neutrophils or eosinophils. It has been hypothesised as having a role in arterial wall infiltration in the process of atherosclerosis.[90,91,347,393,394] Unfortunately MCP-1 was not included in Inflammatension panel. Similarly, C-reactive protein, neutrophil to lymphocyte ratio, and urinary protein measures were lacking where other evidence supports association. [148]

Furthermore, quantifying circulating protein biomarkers is a pragmatic approach, but not one which mechanistically elucidates their role in

hypertension or arterial function. Circulating levels can demonstrate association but not causation; nor determine varying effects on local tissues, e.g. vascular endothelial versus renal tubular mechanisms. Again, this requires consideration of the data within the context of other human and animal studies. As with the other chapters, sample group sizes were small and standard deviations high, particularly once restricted to the hypertensive patients in which biomarkers were available. While interesting observations, no conclusions can be drawn without external validation.

5.5 Conclusions

With reference to the Chapter aims, a number of circulating immune biomarkers of both the innate and adaptive immune systems were differentially present between normotensive and hypertensive groups, but none robust enough to surpass correction for multiple comparisons. Correlation with arterial stiffness (but not endothelial function) and BP was apparent for many circulating biomarkers, including HGF, AGER, and CCL21. Additional ones, cluster of differentiation (CD) molecules and interleukins especially, appear to correlate with arterial function only in the disease state i.e. hypertension. Many of the same biomarkers differed across the phenotypic hypertension groups identified in Chapter 3. Bicluster analysis of biomarkers themselves failed to demonstrate clinically-relevant phenotypic groups. HGF in particular appears to be a robust biomarker for both BP and arterial function, internally validated across a number of analyses and external validation should be sought.

Chapter 6 Discussion and Conclusions

6.1 Summary

The objective of this thesis was a comprehensive analysis of incident patients with untreated hypertension, in comparison to a control group; and an exploration of immune and vascular characteristics of any identified phenotypic subgroups. The data comprised of vascular studies, circulating PBMC flow cytometry, and protein biomarkers. Each of these results chapters is structured by the pre-determined research questions, and Inflammatension data are reported and interpreted alongside published evidence. This ensures that the aims of the thesis have been met. This chapter therefore focusses on the interface between chapters, integrating the data on arterial function, circulating immune cells, and biomarkers to offer additional insights into how they may relate to each other, to BP regulation, and to primary hypertension, as it is these interactions that remain the elusive gaps in scientific understanding.

6.1.1 Arterial stiffness can be detected early in hypertension, with biomarker associations

Even in ‘healthy’ individuals with incident hypertension, increased arterial stiffness (but not endothelial dysfunction or increased CIMT) was demonstrated relative to normotensive controls. Chapter 3 discusses the published data that supports these Inflammatension findings, further substantiated by the co-linearity of data by different measurement techniques, and correlation with central systolic and diastolic BP. Measures of arterial stiffness additionally demonstrated circulating biomarker associations in Chapter 5, both negative (COL9A1, CD58) and positive (HGF, CD70). Mechanistically, COL9A1 and HGF act via arterial wall remodelling, and regulation of fibroblasts and tissue repair; CD28 and CD70 meanwhile suggest a lack of co-stimulation regulation as a component of arterial stiffening. Furthermore, within the hypertensive ‘arterially stiffened’ group, biomarker differences demonstrated were also consistent with the theme of immune cell interactions and adhesion, with lowest mean NPX levels of SELPLG (WBC rolling, discussed in Chapter 5), KLRD1 (MHC recognition), and PON3 (anti-oxidant), and highest mean levels of LGALS4, EPHA1 (T cell proliferation and adhesion), and ESM1, discussed in Chapter 5 as

having a role in angiogenesis, leucocyte – endothelial cell interactions, and HGF regulation (Table 5.3). Existing evidence regarding inflammatory markers linked to measures of arterial stiffness has already been discussed in Chapter 5.

6.1.2 Diastolic BP and estimates of central BP have clinical value.

Within the hypertensive patients, diastolic 24hr BP demonstrated stronger correlation in adjusted models than systolic 24hr BP with regards both measures of arterial stiffness and studied circulating biomarkers, and supported by BioBank data.[228] SBP 24 in comparison was more strongly linked with variability measures of BP and heart rate in Chapter 3, with BP variability also showing a distinct set of biomarkers in correlation analyses. Furthermore, central systolic and especially central diastolic BP demonstrated strong correlation with arterial stiffness and was a key driver of WCH/MHN phenotypes and dimensionality reduction techniques in Chapter 3, as well as surpassing SBP 24 and DBP24 in number of correlating biomarkers (Chapter 5). In clinical practice, diastolic BP is often considered ancillary to systolic, though BP profile is accepted to evolve as we age.[395] Inflammatension participants had a mean age of 39 years which may account for the findings, but nevertheless highlights the importance of DBP. Central BP is less commonly evaluated, but in assessing cardiovascular risk does appear to have value above brachial BP measurements, and potentially in identifying masked hypertension where ABPM is unavailable, as outlined in Chapter 3.[262–264,396]

6.1.3 The immune signature of hypertension is a pro-inflammatory one, dominated by T memory cells, T cell polarisation, and distinctive monocyte and DC surface marker expression.

Differences in both adaptive and innate immune systems were detected. The adaptive immune ‘signature’ of hypertension appears dominated by T lymphocytes, particularly CD4+ TCM and also both CD4+ and CD8+ CD45RA-CCR7-CD62l- TEM subsets circulating at higher frequencies, as has also been demonstrated in mouse models. [284] Changes in CD8+ memory T cells have also been found in human studies, though comparisons can be limited by divergences across subclusters, a decrease in CD45+CD3+CD8+CD45RO+ cells for example in

hypertension, whilst other subsets were not significantly different compared to normotension.[273]

Figure 6.0 summarises the altered pattern of senescence and terminally differentiated cells demonstrated in hypertension, consistent with other data,[88,285,286,288] and already discussed in Chapter 4. Although no significant difference was found in IFN-γ or IL-17 levels as demonstrated by others,[108,397] effector T cells polarised in the hypertensive group towards a Th1/Tc1 and Th17.1/Tc17.1 pro-inflammatory milieu, consistent with existing flow cytometry evidence from studies of hypertension,[295,303] and similar to other inflammatory states such as rheumatoid arthritis.[398] Whilst others have demonstrated proportionally lower naïve T cells, [277] Inflammatension did not demonstrate statistically significant changes in naïve T cell or B cell subsets (other than proportionally lower CD4+CD45RA+CCR7+CD45RO+CD62l+ naïve T cells). Increased NK cell CD56+Dim expression and fewer CD122-expressing NKT further suggests an imbalanced, pro-inflammatory milieu in hypertension, with CD56+Dim NK cells linked to pronounced IFN-γ production,[289] tying the NK cell results to the T cell polarisation discussed above.

The innate immune 'signature' was characterised by intermediate monocytes with a differing pattern of chemokine receptor expression, and alterations in pSTAT1 and pSTAT6 phosphorylation cascades in response to stimulation, as previously linked to hypertension as well as immune regulation, as discussed in Chapter 4. Mannose receptor downregulation on DCs also characterised loss of nocturnal dip, potentially favouring T cell activation as discussed in Chapter 4.3. Experiments by Kirabo *et al* and Barbaro *et al* [78,399] offer hypothetical links between some of these immune findings, as well as potential influence of dietary factors, and a possible inciting trigger. The group report that sodium enters DCs via an amiloride-sensitive channel, high sodium concentrations promoting DCs to form ROS and isolevuglandin-protein adducts.[399] A further publication by the same group demonstrate that in response to isoketal-modified proteins, DCs of angiotensin-II treated mice induced highly proliferative CD8+ memory T cells, producing IFN-γ and IL-17A. To a lesser extent, CD4+ memory T cells also proliferated, but not naïve T cells. Isoketal accumulation was also

associated IL-6, IL-1ß, and IL-23 production from DCs, and increased expression of costimulatory CD80 and CD86 molecules, and with hypertension.[78]

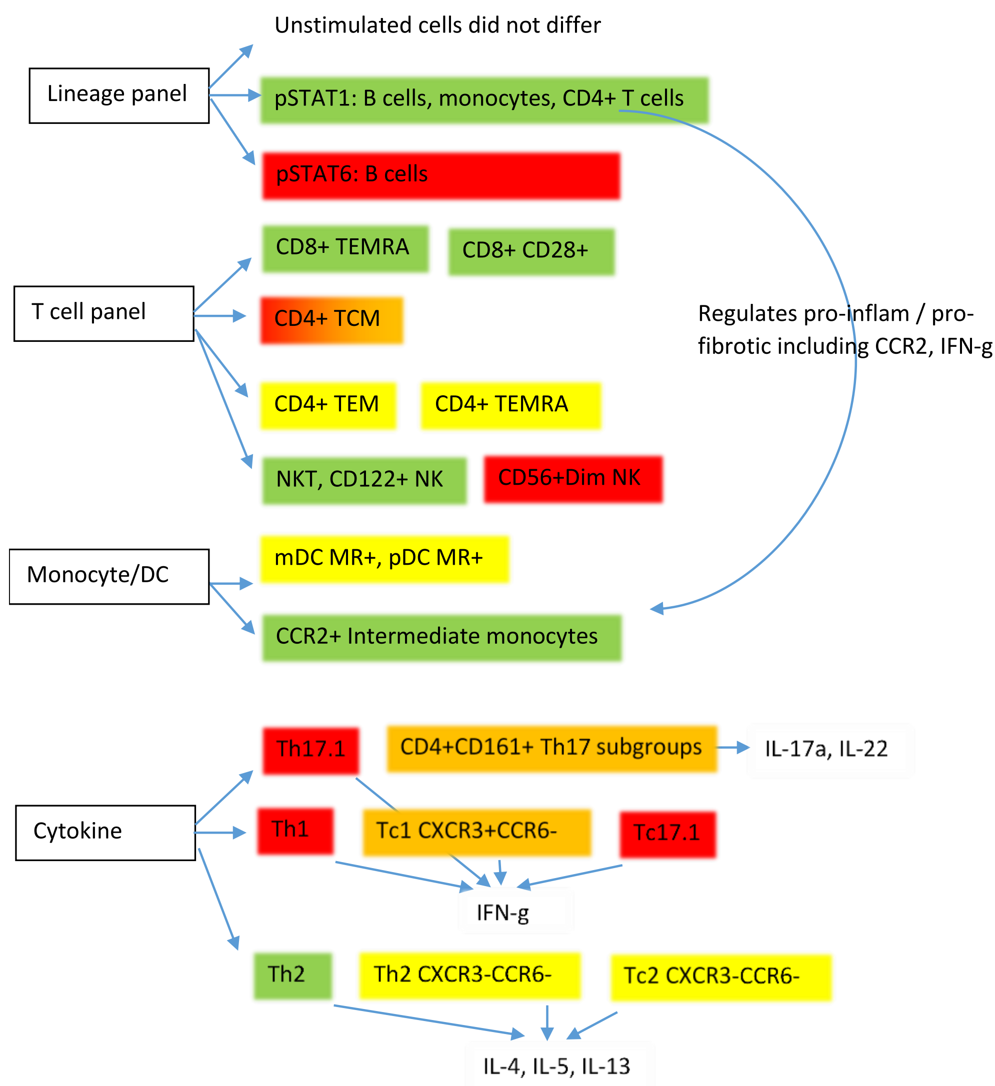


Figure 6.0. Summary of flow cytometry results reported in Chapter 4. Green = lower in hypertension; Red = elevated in hypertension; Yellow = lower in non-dipper; Orange = elevated in non-dipper.

6.1.4 Some measures of arterial function and circulating biomarkers correlate across the range of BP, others only demonstrate association in the disease state of hypertension.

Some arterial and BP parameters only demonstrated association in the hypertensive subgroup, but not in the whole population (Chapter 3). A similar pattern was observed in the analysis of circulating immune biomarkers (Chapter 5.4.2), particularly cluster of differentiation molecules mediating cellular

interactions, activation, and differentiation, as well as cytokines and interleukins. This may be a reflection of changes that only occur in the disease-state of hypertension, or may be a false positive arising from underpowered subgroups and multiple comparisons. The vascular phenotypic groups of only hypertensive participants identified in Chapter 3 also demonstrated differences in their circulating biomarkers, particularly related to immune cell migration and interaction (Table 5.3). This was not surprising, given that bicluster groups differed on many of the parameters also demonstrating biomarker association, such as BP, dipping status, and arterial stiffness, but the pattern of immune differences requires further exploration. Interestingly, HGF strongly associated with various BP and arterial measures, but did not differ across hypertensive bicluster subgroups, suggesting its' association relates to BP more than to a specific phenotype.

6.1.5 Different vascular phenotypes of hypertension also have immune biomarker variances.

Analysing the biomarkers of Chapter 5 by the vascular phenotypic groups in Chapter 3; eight biomarkers demonstrate a consistent pattern of lowest levels in the vaso-protected Group 1 and highest in the non-dipper Group 2, their functional roles supporting the validity of the findings as follows. Three are chemotactic; CCL20 binds CCR6 with a role in lymphocyte and DC chemotaxis and recruitment of Th17 and Treg cells; CCL7 is involved in chemotaxis of monocytes and eosinophils, induces gelatinase B, and is degraded by MMP2; CXCL8 didn't reach the same statistical significance, but is a third chemotactic factor, attracting T cells, basophils, and neutrophils, and has already been discussed in Chapter 5 regarding various BP associations. TREM2 may have a role in chronic inflammation through inducing inflammatory cytokine production; IL-18 is also a pro-inflammatory cytokine, polarising toward Th1 and NK cell responses. In contrast, IL-1RN inhibits IL-1α and IL-1β and modulates the acute phase response; whilst LGALS9 polarises toward a Th2 response [400], promotes T regulatory cells, and macrophage bactericidal activity.[400,401] Whether the group is 'vaso-protected' leading to less activation of the immune system, or 'immuno-protected' resulting in less arterial remodelling and dysfunction, or indeed if the

'non-dipper' inflammatory association is the dominant driver of phenotypic groups, cannot be ascertained from this data. One caveat is the potential confounding of BMI, which was also elevated in phenotypic Group 2. Further research and validation studies are required, but the findings are consistent across Tables 5.3 and 5.4 and do support the existence of hypertensive phenotypic subgroups with differing arterial characteristics, demographics, and inflammatory biomarkers, with non-dipper demonstrating elevated chemotactic, pro-inflammatory, and modulatory markers in comparison to a seemingly vaso-protected group.

6.1.6 Nocturnal BP non-dipping is associated with immunological changes.

Percent nocturnal dipping did not differ between hypertensive and normotensive groups, nor were vascular parameters different by dipping status, though others have demonstrated an association with arterial stiffness as discussed in Chapter 3.[87] In phenotypic subgroups of hypertension however, "non-dipper" status appeared to be a defining characteristic of Group 2 in bicluster analysis. Others have associated non-dipper status (or BP variability) with central BP and arterial stiffness.[87,256] Some differences in circulating PBMCs by nocturnal dipping status overlapped with hypertension versus normotension analysis and have already been discussed, such as polarisation towards Th1/Tc1 and Th17.1/Tc17.1, higher proportion of CD4+CD45RO+CD62l- TCM cells. Other findings were specific to dipping state, such as a lower proportion of MR+ mDCs, reduced CD4+TEMRA and increased CD4+CD161+ Th17 in non-dippers. The circulating T lymphocyte population is not well described on the basis of dipping status, and these findings are novel, though consistent with understanding of the circadian rhythm of the immune system.[336,337] Numerous immune biomarkers also demonstrated correlation with percent nocturnal dip in systolic and diastolic BP, discussed in Chapter 5. Furthermore, Table 5.3 reported biomarkers differing across the vascular phenotypic groups (including EPHA1, LGALS4, and IL-18), of which dipping status was a key discriminator. The strong association of circadian rhythm with immune function, but limited published flow cytometry and immune biomarker data in BP dipping was already discussed in Chapter 5.4.3. Interpretation of the data included consideration that the intrinsic flaw of percent nocturnal dip remains confounding by ABPM-disturbed sleep, though how

to objectively quantify and best adjust for sleep disturbance remains a challenge.

Genetic determinants have not been found to be a strong influence on nocturnal BP dip, and although lifestyle factors are, these were all aspects controlled for in Inflammatension data.[402] Taking vascular, biomarker, and cellular findings combination, one might hypothesise that loss of nocturnal dip is a reflection of impaired sleep quality and circadian rhythm disturbance, that mediates an increase in cardiovascular risk through inflammatory mechanisms, at least initially, as in this incident group of patients we did not detect evidence of endothelial dysfunction or arterial stiffening by nocturnal dipping status to account for the accepted increased risk of adverse outcomes.[27]

6.1.7 The importance of the obesity epidemic

Chapter 3 demonstrates BMI associations with arterial function and BP, and Chapter 5 discussed the strong correlations of various circulating biomarkers with BMI, distinct from BP associations. BMI was also included in the adjusted models analysing PBMC in hypertensive and normotensive groups (Chapter 4). The differences in intermediate and classical monocytes, Th2, and CD4+ TCM were unchanged by adjustment for demographic features; whilst Th1, Th17.1, and B cells lost statistical significance, and other subsets gained significance following adjustment for patient demographics. Similar to the biomarkers, this highlights that high BMI, assumed here to reflect increased adiposity, had a heterogeneous effect across leukocyte subsets. How these demographic, immune, and BP interactions triangulate is an area for ongoing interest, particularly for promoting maintenance of a healthy diet and weight.

6.1.8 The complexity of biological systems

The comprehensive nature of the data, and ability to link vascular, circulating immune compartment and protein biomarkers, overcomes the issue commonly encountered in research where one factor is linked to hypertension in isolation, despite the known complex interactions of these systems. In doing so, Inflammatension expands understanding of the immune system in early

hypertension and in clinical phenotypes such as dipping status, and offers potential exploratory insights into the pathophysiology perpetuating high blood pressure and the associated medical consequences.

Integrating the vascular, immune cell, and biomarker data, along with other published results, emphasises hypertension as a highly complex disease syndrome. Inter-individual variability in the relative involvement of lifestyle and demographic, arterial, and immune factors was generally greater than between-group differences, despite the considerable efforts to control for as many confounding factors as possible.

6.1.9 General Limitations

Potential limitations have been reported within the respective results chapters, but general issues affecting interpretation of Inflammatension to remain cognisant of are as follows. Firstly, whilst analysis of the data identify associations, the directionality of cause and effect is not assumed, nor can be inferred from the data generated. Furthermore, despite targeting an incident group of participants, Inflammatension does not answer the question of what the inciting events in the development of hypertension are, if indeed there are commonalities in ‘triggering’ events between individuals. If anything, the heterogeneity between participants and demonstrated complexities of regulatory systems suggests that it is likely to be small effect sizes of a multitude of factors that determines risk and progression of hypertension.

Furthermore, normotensive and hypertensive groups were defined by their 24 hour ABPM. Although this is considered the gold standard measurement technique, it remains susceptible to confounding relating to heterogeneity between individuals as to the degree of sleep disturbance and impact this has on nocturnal BP, as discussed previously.

A limitation to the reliability of all the data, but especially the flow cytometry results remains the potential for error to arise relating to COVID-19. The Inflammatension study was halted during the pandemic, and once re-initiated

COVID-19 disease or vaccination in the preceding three weeks was adopted as an additional exclusion to reduce the risk of immune system activation influencing the analyses. Despite this, a number of possible confounding factors remain. Firstly, that people may have had asymptomatic or undiagnosed COVID-19 (or undisclosed vaccination) prior to their study visit and blood sampling; secondly, the lab set up and sample processing was done by a new member of staff and on new batches of antibodies following the hiatus associated with the pandemic; thirdly, the three week embargo following COVID-19 disease or vaccination may have been insufficient and immune system changes may have persisted beyond this.[403]

Considerable efforts were made to control for other potential confounding factors, though a slight BMI difference between normotensive and hypertensive groups persisted. The potential implications of this have already been discussed in the respective chapters. Certain data were lacking from the Inflammatension study which may have assisted in interpreting and integrating data. For example, CRP, urine protein creatinine ratio (though protein on urinalysis was an exclusion factor, as it can indicate underlying kidney disease), T regulatory cell flow cytometry, MCP-1 (CCL2) circulating biomarker, and neutrophil to lymphocyte ratio. The Inflammatension data set is comprehensive, and boundary setting will always lead to some parameters not featuring, but in retrospect these gaps, especially T regulatory cells and CRP, leave some unanswered questions.

Lastly, the number of participants was appropriate for exploratory work, and but all results and conclusions are reported with the caveat that external validation on an independent population group is still required.

6.2 Future directions

External validation in a similar incident population is required. Endeavours were made to obtain a comparable data set from Italy to do just this, but this is yet to come to fruition. A broad range of interesting findings are identified which do merit further investigation. The following recommendations arise from the Inflammatension data, and from the process of undertaking recruiting participants, study visits, and analysis. They span simple technical

considerations easily adopted into hypertension research or clinical practice, though to more esoteric ones relating to how such research is conducted and the population context.

On one hand, BP is a continuous variable and many parameters discussed demonstrated correlation across the range of average BP; other associations (such as interleukins with arterial function) were only demonstrated in the hypertensive range i.e. associated with the disease state, and supporting the traditional cut off dichotomising patients into normotensive and hypertensive. It is likely that both scenarios do occur *in vivo*, hence the in similar research, both possibilities should be considered and findings reported accordingly to determine physiology from pathophysiology.

Similarly, systolic and diastolic brachial BP appear to relate to different aspects of arterial function and circulating biomarkers, suggesting differences in regulation or in effect. Central BP measures also appear to offer additional value to brachial measures. This thesis supports measuring and reporting both systolic and diastolic is emphasised in research studies, and not just reporting of mean arterial pressure, and that central BP measures should be made more readily available in clinical practice.

Inflammatension data supports the view that hypertension as a diagnostic entity doesn't fully describe the patient phenotype, consideration of which may have implications for risk of cardiovascular disease and for optimal management of the individual patient. It is therefore recommended that when caring for patients with hypertension, time might be invested in assessing their 'phenotype' (e.g. masked and white-coat hypertension, vaso-protected, arterially stiffened, non-dipper) in addition to traditional cardiovascular risk scores.

Considering future studies, a very interesting next step would be a follow up visit with the same parameters measured in the same patients, but in the context of controlled BP. Influence of different anti-hypertensives would need to be adjusted for, but such a study could assess if the arterial, immune signature, and circulating biomarkers altered toward a 'normotensive' pattern, or if persisting differences were apparent, and if this varied between individuals.

HGF appears to be a robust circulating biomarker for hypertension and arterial stiffness within Inflammatension, and from published literature also associates with various manifestations of HMOD and cardiovascular risk. The role of this molecule in the immune system is fairly well elucidated (Figure 5.4), consequently, the recommendation would be that future research assess it for clinical utility in identification, categorisation, or risk quantification regarding hypertension and HMOD.

Considering the diverse variances identified in the immune systems of those with hypertension, in nocturnal loss of BP dip, and across hypertensive phenotypes, as well as the inherent complexities of the immune system and its' in-built interactions and redundancies, pharmacological therapies targeting the immune system are unlikely to be a successful avenue for treatment of hypertension. Of greater therapeutic potential may be targeting of the hypothesised demographic and lifestyle factors contributing to the aberrant immune responses. This would be consistent with a public health or population level approach aimed at prevention. Weight management, diet, and exercise are already cornerstones of hypertension recommendations, and the phenotypic groups identified in chapter 3 supports the protective or beneficial effects of lower BMI and increased physical activity. Currently, little financial backing supports lifestyle interventions, relative to costs of pharmacological treatment options. It should be recommended that ongoing efforts at targeting the social determinants of health is key to prevention of hypertension and cardiovascular disease.

It is clear that hypertension research in which a single 'system' is studied in isolation can be informative, but greater understanding and clinical applicability arises when the interactions between different regulatory systems can also be analysed. Balanced against the significant challenge in recruiting study participants who met the restrictive criteria; it is recommended that clinical trials endeavour to collate data on diverse aspects of hypertension to provide as fully comprehensive data sets as possible, but also that this data is then available to other study groups to maximise utility of all generated data.

6.3 Conclusions

As exploratory work, the Inflammatension study and this thesis do not propose any definitive conclusions, only report the findings and hypothesise their significance based on interpretation of the known underlying science and evidence base. Despite these limitations, Inflammatension remains a unique and comprehensive study of hypertension in a young, incident population, without influence of co-morbidities or pharmaceuticals. In this incident population, measures of different aspects of arterial function demonstrated arterial stiffness (but not endothelial dysfunction or increased CIMT) in hypertensive patients. This was most pronounced in the WCH group, whilst MHN resembled normotension with regards to arterial function, though whether valid findings or an office phenomenon of "white-coat arterial stiffness" remains unclear. DBP was more closely associated with arterial stiffness, SBP with cardiovascular variability. Estimates of central BP demonstrated strong links with ABPM, vascular parameters, and immune biomarkers.

No single cell subtype or biomarker was an adequate 'label' for hypertension in incident patients. Differences were not seen across the whole immune system, but rather T cells dominated the adaptive immune 'signature' of hypertension, particularly subsets of TCM and TEM; with senescence markers and terminally differentiated cells also differing. The hypertensive group polarised towards a Th1/Tc1 and Th17.1/Tc17.1 pro-inflammatory milieu. NK cell marker expression also differed. The innate immune 'signature' was characterised by DCs and intermediate monocytes with altered chemokine receptor expression and phosphorylation cascades.

Consistent with the pro-inflammatory T cell and monocyte signature, cytokines and chemokines dominated the biomarkers differing between normotension and hypertension, and also correlated with arterial stiffness, though not surpassing Bonferroni-adjusted statistical thresholds. Associations were concordant across systolic and diastolic BP; TPP1, CCL7, CCL11, and CCL21 positively correlating; IL18R1, and KYNU negatively. These relationships were more pronounced in the hypertensive subgroup, especially CD molecules and cytokines and furthermore, biomarkers also differed between the 'arterially stiffened', 'vaso-protected', and 'non-dipper' phenotypic groups. This possibly implies that different aspects

of cardiovascular physiology and characteristic traits or phenotypes of hypertension have distinct associations with circulating immune biomarkers.

Across the whole study group, nocturnal dipping was not associated with other cardiovascular measures, though within hypertensive patients, non-dipping status was a key driver of both UMAP and bicluster machine learning techniques, and was one of the three phenotypic groups identified, along with arterial stiffened, and vaso-protected. Chapters 5 and 6 link the vascular and biomarker data and demonstrate broadly similar deviances in circulating PBMCs by nocturnal dipping status as in hypertension. Numerous immune biomarkers also demonstrated correlation with percent nocturnal dip. Taking all the results together, the role of the immune system may be more strongly associated with loss of nocturnal BP dip than with hypertension *per se*, and may be mediated by circadian regulation of the immune system and steroid hormone production.

The Inflammatension study was fraught with challenges to recruitment and restrictions arising from the COVID-19 pandemic, and the limitations have been emphasised throughout, but does corroborate much of the existing evidence in this field of study, and offers a number of novel findings worthy of further investigation.

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Appendix 1: Chapter 1. Patient Information Sheet

Participant Information Leaflet

The InflmmaTENSION Study

A study of the roles of the immune and inflammatory systems in hypertension.

Investigators: Professor Tomasz Guzik FRCP, FACP & Professor Christian Delles MD, FRCP

Your safety is our primary concern and we will comply with Government, Health Board and Health Research Authority Guidelines on clinical research visits during the COVID-19 pandemic.

1. **Title: A study of the roles of the immune and inflammatory systems in hypertension.**

2. **Why have I been invited to take part**

We would like to invite you to take part in this research study. You have been diagnosed with hypertension and our research aims to look at how your high blood pressure has affected your blood vessels, kidneys, brain, and immune system.

Before you decide to take part or not it is important for you to understand why this research is being done and what it will involve if you agree to take part. Please take time to read the following information carefully and don't hesitate to contact us if there is anything that is not clear or if you would like more information. Please discuss with family and friends if you would like.

3. **What is the Purpose of the Study?**

High blood pressure, known as hypertension, is a very common disease occurring in 30% of adults in the UK (with another 30% of people being at very high risk of the disease). If untreated it can lead to serious consequences such as heart attacks, strokes, heart failure, kidney failure and dementia. Since the 1980's no new medicines or tests have been successfully introduced to the routine treatment of hypertension.

A team of scientists working at the University of Glasgow have discovered a new important mechanism of hypertension related to the immune system not working properly. This system protects us from infections but when not working it may lead to disease. We propose to perform

this research study to understand changes in immune system in patients with high blood pressure and whether these changes are related to the changes in blood vessel properties and kidney function that are seen in people with hypertension.

4. **Do I have to take part?**

No, taking part is entirely voluntary. You are free to withdraw your participation and/or samples or data at any time and without giving a reason. Not taking part or withdrawing from the study will not affect your current or future care.

5. **What will happen to me if I take part?**

We will provide an in depth non-invasive investigation of your hypertension, assess you kidney function and look at how healthy your blood vessels and immune system are. Many of these investigations are part of tests you would normally get as a patient with hyertension while some are additional but we hope will provide further valuable information about your hypertension. The InflammaTENSION investigator will organise for you to attend:

A. **Baseline Visit at the Clinical Research Facility, Queen Elizabeth Hospital, Glasgow (expected duration 1 hour):**

In order to gain insight into your high blood pressure, this visit needs to take place before your new medication is started (if any is required), and therefore will be scheduled as soon as possible after you agree to take part in the study. This will not delay you starting on your new medicine as we will make sure the baseline visit is made between your routine hypertension clinic visit (needed before medication is started) and your next clinic visit.

- The InflammaTENSION investigator will explain the study to you and answer any of your questions relating to the study.
- If you are eligible and wish to take part in the study, you will be asked to sign a consent form.
- The following study procedures will be performed;

Height and Weight: Your height and weight will be measured and recorded.

- **Urine Sample**: You will be asked to provide a sample of your urine (approx. 10ml).
- **Blood Samples**: Blood, approximately 50 ml in total (this is approximately 8 teaspoons),.will be taken at each clinic visit
- **Heart Rate and Blood Pressure**: Your heart rate and blood pressure will be measured 3 times after a period of 5 minute rest through the inflation of a blood pressure cuff.
- If a **24-hour Ambulatory Blood Pressure Monitoring** (ABPM) has not been done as part of your routine clinic visits within the past 3 months, we will perform an ABPM measurement and you will be fitted with a monitor at this visit.
    - If you have never had a 24 hour long measurement of blood pressure, this is a routine measurement that allows your doctor to choose the most suitable medicine to treat your hypertension. A blood pressure cuff connected to a small portable device will be placed on your arm, which does not limit everyday activities. Measurements will be performed every 30 minutes during the day and every hour during the night. You will need to return the ABPM machine to the hospital after 24 hour measurements are finished.

B. **Visit 1 at the Clinical Research Facility at the Queen Elizabeth Hospital, Glasgow (expected duration 1.5-2 hours):**

During this visit you will return the ABPM monitor and the study procedures described below will be performed;

a. **Measurements of blood pressure and blood flow**: You will be asked to lie down for the test and roll up your sleeves. A blood pressure cuff will be wrapped around your arm. An ultrasound probe is used to locate and measure the artery in your arm. Later, the blood pressure cuff will inflate for 5 minutes. When the blood pressure cuff is inflated, blood flow to the hand and fingers will be temporarily stopped. After 5 minutes the blood pressure cuff will be released, the strong flow of blood will stimulate the arteries to widen and deliver more blood to the hand and fingers. Some participants can report temporary sensation of 'pins and needles' within their hands when the blood flow is returning to their hands and fingers, but this will resolve when the blood pressure cuff is released. We will then repeat the ultrasound probe measurements of the artery in your same arm. This test will take approximately 20-25 minutes.

Another similar test will also be done while you are lying down. Once again a blood pressure cuff will be wrapped around your arm, however this time fingertip sensors will be placed on your index fingers of both hands. The blood pressure cuff will inflate for 5 minutes. When the blood pressure cuff is inflated, blood flow to the hand and fingers will be temporarily stopped. After 5 minutes the blood pressure cuff will be released, the strong flow of blood will stimulate the arteries to dilate and deliver more blood to the hand and fingers. Blood flow will be measured by the finger sensor to assess how well the arteries dilate. Some participants can report temporary sensation of 'pins and needles' within their hands when the blood flow is returning to their hands and fingers, but this will resolve when the blood pressure cuff is released. This test will take approximately 15 minutes.

We will also use an ultrasound probe to take measurements of the blood flow and thickness of the artery in your neck. This test will take approximately 25 minutes.

b. **Vascular Stiffness**: Vascular stiffness will be studied using SphygmoCOR system which involves special probes being placed on the skin near your arteries which measure pulse wave (stiffness) in these vessels. This test will take approximately 15 minutes to perform.

c. **4 Questionnaires assessing your diet, physical and mental activity/ability** will be offered to you, which should not take longer than 30 minutes to complete.

You may wish to combine these visits into one 2.5-3h visit during which procedures described in above in sections (A) and (B) will be performed during one visit to the hospital although you will need to return ABPM monitor to the hospital nurse at convenient time within the next 48 hours.

C. Letter contact/Telephone contact.
3-4 months after your Baseline visit we will contact you in order to ask about changes to your health status regarding your hypertension and any problems with your heart or kidneys. We may either send a letter by post or email you and/or contact you by phone – whichever you prefer. This contact should not take more than 5 minutes.

6. **Is there anything I need to do before each study visit?**
We ask that 12 hours before each study visit you refrain from drinking alcohol or drinking caffeinated products and we ask that you refrain from smoking for 3 hours prior to study Visit 1.

7. **How long does each study visit take?**
Baseline visit will not take more than 1 hour of our time and Visit 1 – 1.5-2 hours. Both visits can be combined into 1 visit if you would find this easier for you. Visits will take place at the Clinical Research Facility, Queen Elizabeth University Hospital Glasgow.

8. **What are the possible disadvantages and risks of taking part?**
When blood samples are taken in some circumstances bruising may occur at the site of the needle, if this does occur the bruising usually subsides over 1-14 days. Some participants can feel faint or

dizzy while blood is taken, if this happens we will stop the procedure and lie you down on the examination couch and check your heart rate and blood pressure. We will then check that your symptoms have resolved before asking you to sit up. Some participants can report temporary sensation of 'pins and needles' within their hands when the blood flow is returning during the blood pressure and blood flow tests but this will resolve when the blood pressure cuff is released.

9. **What are the possible benefits of taking part?**

We will perform several vascular investigations, results of which may be useful for your doctor. We will perform in depth analysis of your blood vessel and kidneys and will analyse how your hypertension is affecting your physical and mental activity. If any of these studies were to identify any incidental findings we will notify your GP and hospital doctor by letter and/or refer you for specialist follow up as appropriate.

The information that is collected during the study will give us a better understanding of high blood pressure and the effects it has upon the heart, blood vessels and kidneys. This information will also help us develop larger scaled studies, which together will provide possible new methods of treatment of hypertension and prevention of its complications.

10. **Will my taking part in this study be kept confidential?**

All information which is collected about you during the course of the research will be kept strictly confidential. Your contact details will also be recorded on an NHS database, but this is secure and only accessible to the InflammaTENSION investigators. During the study we will access your medical records to verify what is already known about the causes and treatments of your hypertension. Medical records will also be reviewed by representatives of the sponsor of the study and NHS GGC where it relates to taking part in the study to ensure that study is carried out correctly. You will be identified by a study unique ID number, and any information about you will have your name and address removed so that you cannot be recognised from it. Please note that assurances on confidentiality will be strictly adhered to unless evidence of serious harm, or risk of serious harm, is uncovered. In such cases the University may be obliged to contact relevant statutory bodies/agencies.

NHS GGC will use your name, CHI number and contact details to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Individuals from NHS GGC and regulatory organisations may look at your medical and research records to check the accuracy of the research study. The only people in NHS GGC who will have access to information that identifies you will be people who need to contact you to or audit the data collection process. The people who analyse the information will not be able to identify you and will not be able to find out your name, CHI number or contact details.

NHS GGC will keep identifiable information about you from this study for a minimum of 10 years. This is in accordance with the General Data Protection Regulations 2018 (GDPR).

11. **What if there is a problem or I have a complaint?**

If you have any problems with the study in the first instance you should contact Professor Tom Guzik the InflammaTENSION chief investigator on 0141 330 7590 or email tomasz.guzik@ glasgow.ac.uk. If you would like to discuss the study with someone outwith the research team you can contact Dr Gemma Currie, Clinical Lecturer, on 0141 330 2627 or gemma.currie@glasgow.ac.uk.

This study is sponsored by NHS Greater Glasgow & Clyde. The sponsor has insurance cover for negligent harm. provided under the Clinical Negligence and Other Risks Indemnity Scheme (CNORIS). Non-negligent harm relating to the design of the study will be covered by the University of Glasgow.

If you believe that you have been harmed in any way by taking part in this study, you have the right to pursue a complaint and seek any resulting compensation through the NHS Greater Glasgow and Clyde who are acting as the research sponsor. Details about this are available from the research team. Also, as a patient of the NHS, you have the right to pursue a complaint through the usual NHS process. To do so, you can submit a written complaint to the Patient Liaison Manager, Complaints Office by telephoning 0141 201 4500 or email complaints@ggc.scot.nhs.uk. Note that

the NHS has no legal liability for non-negligent harm. However, if you are harmed and this is due to someone's negligence, you may have grounds for a legal action against NHS Greater Glasgow and Clyde but you may have to pay your legal costs.

12. **What will happen to samples that I give?**

You will donate blood and urine samples for research purposes. Some tests will be done on these straight away whilst others will be done at a later stage when we have collected more samples from other participants. We will measure molecules involved in the development of hypertension and inflammation. We will isolate DNA to assess genetic changes related to high blood pressure. At this stage all investigations will be anonymised. We will also store some samples long-term in secure University of Glasgow storage to perform additional tests if required. Further tests on stored samples will again require review and approval by the ethics committee. Samples will be destroyed after maximally 15 years after collection.

13. **What will happen to the results of the research study?**

The anonymised results for the InflammaTENSION study:

- Will contribute to the understanding the causes of high blood pressure and its effects upon the heart, blood vessels and kidneys.
- Will be used to develop more advanced studies.
- Will be published in a scientific report or publication
- May be used to develop healthcare policies relating to the use of electronic cigarettes.

14. **Who is organising and funding the research?**

The British Heart Foundation Centre of Research Excellence Award at the University of Glasgow is hosting the research which is funded by the European Research Council. The study is being Sponsored by NHS Greater Glasgow and Clyde.

15. **Who has reviewed the study?**

The West of Scotland Research Ethics Service REC, which has responsibility for scrutinising all proposals for medical research on humans, has examined the proposal and has raised no objections from the point of view of research ethics. It is a requirement that your records in this research, together with any relevant medical records, be made available for scrutiny by monitors from NHS Greater Glasgow and Clyde whose role is to check that research is properly conducted and the interests of those taking part are adequately protected.

16. **Contact for Further Information**

If you wish to take part in the InflammaTENSION study, please contact the research nurse team at the clinical research facility in the Queen Elizabeth University Hospital on 0141 232 7600. Alternatively by email or phone;

InflammaTENSION phone: 07825829526; InflammaTENSION email: cams-ins-inflammatension@glasgow.ac.uk
Professor Tom Guzik phone: 0141 330 7590 Email: tomasz.guzik@glasgow.ac.uk

Appendix 2 Study protocol and work book.

The *InflammaTENSION* Study
A study of the roles of the immune and inflammatory systems in hypertension.

1. Background

The overarching theme of the proposal Hypertension is a common disease impacting 1 billion people worldwide, which leads to catastrophic cardiovascular complications, including heart failure, dementia, myocardial infarction and stroke - all of which carry a severe socioeconomic burden. In spite of many years of research, the cause of primary hypertension remains unknown and this disease is uncontrolled in a large proportion of patients. By interrogating the key hypothesis that inflammatory dysregulation fundamentally controls development of hypertension and vascular remodelling, **InflammaTENSION** provides a new paradigm for the management of the disease, with the potential to lead to the identification of novel therapeutic targets to control blood pressure and limit target organ damage. InflammaTENSION will result in the discovery of novel biomarkers, which may identify patients who could benefit from such immune targeted therapies. Importantly, we already made the seminal observation that the immune system not only mediates target organ damage, but defines the roles of pro-inflammatory T cells, monocytes, as well as anti-inflammatory T regulatory cells in the disease process[1-4]. However, our current knowledge remains very fragmented and so far has not been applied to human pathology. InflammaTENSION will for the first time advance the knowledge procured in rodent models into human studies. By combining clinical translational and model mechanistic studies it will identify novel inflammatory factors that can control immune mechanisms of hypertension. In detail, over the course of the programme we will: (1) characterize the immunophenotypic signature of human hypertension; (2) define key concepts in cytokine biology of hypertension and (3) understand how chronic cytokines implicated in hypertension regulate the T cell dependent mechanisms of hypertension. InflammaTENSION will also go beyond current state-of-the-art diagnostic methods,with comprehensive combination of immunology and cardiovascular disease to create a new understanding of how the immune system may lead to human hypertension and vascular remodelling. Such a coordinated and integrative programme to better understand the role of dysregulation of the immune system in human hypertension will have major impact on the field, enabling translation of these exciting findings to clinical practice.

Unmet need to be investigated: There is an urgent need for a better understanding of the mechanisms of hypertension (HTN), as it remains a major cause of death and disability in Europe and worldwide and the prediction is that its prevalence will increase by 60% over the next 25 years. The disease affects 30% of adults, with an additional 30% considered at high risk of hypertension[1]. Its prevalence increases with age with 70% of adults older than 70 being afflicted with this disease. Thus within the ageing society, the socioeconomic consequences are particularly marked[5], with many elderly patients developing severe cardiovascular complications such as heart failure, stroke, myocardial infarction, vascular dementia and renal failure. Drug therapy for hypertension improved dramatically between 1975 and 1985, with the addition of angiotensin receptor antagonists, angiotensin converting enzyme (ACE) inhibitors and calcium channel blockers. Since the mid-1980s, however, no new classes of drugs have been successfully introduced to treat hypertension. This is unfortunate, because up to 40% continue to have elevated blood pressure despite the use of multiple antihypertensive agents. While partially related to poor treatment compliance, this highlights our insufficient understanding of the underlying mechanisms of this disease. While some cases of hypertension are due to single gene mutations[6], or underlying correctable causes such as renal artery stenosis, pheochromocytoma or adrenal adenoma[7], these are uncommon and the cause of the majority of cases of adult hypertension are unknown. In these cases, neurohumoral factors such as angiotensin II play key roles, as drugs interfering with this pathway are anti-hypertensive. Systemic vascular resistance is generally elevated in hypertension[8-10], and vasodilators lower blood pressure, which would suggest that hypertension, is a *vascular disease*. In contrast, the genetic disorders causing hypertension often affect sodium transport in the distal nephron[6]. Therefore we would like to assess genetic difference in cytokine and chemokines in the study cases and controls. Transplant of kidneys lacking the angiotensin II AT1a receptor into wild-type mice causes resistance to angiotensin II-induced hypertension[11] and diuretics are effective anti-hypertensive agents[12], which in turn suggests that *the kidney* is a major cause of high blood pressure. Finally, there is ample evidence that the *central nervous*

system, and in particular the circumventricular organs surrounding the third ventricle, play a critical role in hypertension[13]. These seemingly disparate roles of the vasculature, the kidney and the CNS make the aetiology of hypertension very difficult to comprehend. Importantly, we have made an observation that helps link the vasculature, the kidney and the CNS in the genesis of hypertension, as all appear to be related to a common inflammatory mechanism. These studies have initiated a new research area, which over the past few years has led to the better understanding of inflammation in hypertension.

While the evidence for the role of immune mechanisms in hypertension has been obtained primarily in rodent models, epidemiological and genetic evidence strongly supports this. Blood pressure increases with the quartile of C-reactive protein[14],[15] and cytokine levels such as TNF-α and IL-6 are consistently increased in hypertension[16-18], and may convey risk of developing the disease[16]. Hypertension is highly prevalent in immune mediated diseases such as rheumatoid arthritis or psoriasis[19], [20]. A recent small study has shown an increase of senescent CD8+T cells (CD28null) in peripheral blood and target organs in human hypertension[21]. The T cell modulating agent, mycophenolate mofetil, as well as anti-TNF-α treatments lower blood pressure not only in rodents[22],[23], but also in humans[24], suggesting that immune targeted interventions may pose a feasible future approach, if we are able to identify its mechanisms. Importantly, both GWAS studies[25] and gene expression signatures of hypertension[26] strongly point towards the role of the immune system and inflammation. SH2B3/LNK gene encoding T cell activation modulator is a top key driver of hypertension in recent systems biology analysis[25].

2. Rationale

Understanding of the inflammatory and immune nature of hypertension is currently based on studies in rodent models of hypertension, but is supported by human epidemiological and GWAS studies. It is now essential to identify key checkpoints and mechanisms of inflammatory mechanism(s) of human hypertension in comprehensive and sufficiently powered studies, which will then be able to guide subsequent in-depth hypothesis-driven mechanistic studies. This approach may provide the basis for future randomized clinical trials (RCTs).

By interrogating the key hypothesis that inflammatory dysregulation fundamentally controls development of hypertension and vascular remodelling, **InflammaTENSION** provides a new paradigm for the management of the disease, with the potential to lead to the identification of novel therapeutic targets to control blood pressure and limit target organ damage.

We hypothesize that primary hypertension is associated with distinct pattern of changes within immune/inflammatory systems consisting of cytokines/chemokines and individual immune cell populations.

The data generated from the InflammaTENSION study will be invaluable for identification of novel biomarkers and therapeutic targets in hypertension.

Biomarker analysis will be carried out using standard laboratory techniques for cytokines and chemokines as follows;

Luminex (Invitrogen Cytokine/Chemokine/Growth Factor 45-
Plex Human ProcartaPlex™ Panel 1
Target List: BDNF; Eotaxin/CCL11; EGF; FGF-2; GM-CSF; GRO alpha/CXCL1; HGF; NGF beta; LIF; IFN alpha; IFN gamma; IL-1 beta; IL-1 alpha; IL-1RA; IL-2; IL-4; IL-5; IL-6; IL-7; IL-8/CXCL8; IL-9; IL-10; IL-12 p70; IL-13; IL-15; IL-17A; IL-18; IL-21; IL-22; IL-23; IL-27; IL-31; IP-10/CXCL10; MCP-1/CCL2; MIP-1 alpha/CCL3; MIP-1 beta/CCL4; RANTES/CCL5; SDF-1 alpha/CXCL12; TNF alpha; TNF beta/LTA; PDGF-BB; PLGF; SCF; VEGF-A; VEGF-D

O-Link:Proseek® Multiplex Inflammation I96×96 is a high-throughput, multiplex immunoassay enabling analysis of 92 inflammation-related protein biomarkers across 96 samples simultaneously. This high level of multiplexing is a chieved without any compromise on data quality, thanks to our proprietary Proximity Extension Assay (PEA) technology

If the data generated from such a trial demonstrate that hypertension has a very distinct immune signature – it can be used to stratify patients for future therapies and may provide proof-of-concept for immune targeted therapies thus leading to the development of long term studies.

3. Prior Experience of Intervention in Cardiovascular Disease

In a small pilot study published in July 2016 we were able to identify that hypertension is associated with key changes of memory T cells and with changes of immune factors (IL-17, IFN-g)[27]. This justifies performing a comprehensive investigation to identify detailed immune signature of hypertension.

1.4 Study hypothesis

We hypothesize that primary hypertension is associated with distinct pattern of changes within immune/inflammatory systems consisting of cytokines/chemokines and individual immune cell populations and that this immune signature corresponds with vascular, renal or cognitive phenotypes of hypertension.

2. Study Objectives

The InflammaTENSION pilot study is an observational study aiming to define the cytokine and cellular immune signature of primary hypertension.

- **Primary Endpoint**

  Cytokine and cellular immune signature of primary hypertension

- **Secondary Endpoints**

1. Relationships and predictive value of the immune signature of hypertension and clinical phenotypes of hypertension
   - Predictive value of immune signature for blood pressure parameters measured by ambulatory blood pressure measurements (ABPM)
   - Predictive value of immune signature for endothelial function assessed by Endo-PAT2000 and flow mediated dilatation (FMD) both complementary non-invasive techniques.
   - Predictive value of immune signature for vascular stiffness and central pressure assessed by Sphygmocor
   - Predictive value of immune signature for renal function parameters
   - Predictive value of immune signature for cognitive function
2. To define genetic determinants of immune signature of hypertension which could be used for future mendelian randomization studies.

3. Study Design

The InflammaTENSION study will be performed according to the Research Governance Framework for Health and Community Care (Second edition, 2006).

3.1 Study Population

120 hypertensive subjects and 120 controls will be recruited into the trial (both male and female).

3.2 Inclusion criteria

- Age between 18-55 years
- Cases: Office blood pressure ≥140 and/or ≥90.

  Controls: Office blood pressure <140 and <90 and age, sex and BMI patching to cases

3.3 Exclusion criteria

(a) Age >55 years old;

(b) Secondary hypertension (including e.g. adrenal tumours, pheochromocytoma, renal artery stenosis; thyroid disease)

(c) Acute inflammatory disorders incl. flu, rhinitis, sinusitis etc. within 3 weeks; hospitalization with an inflammatory condition within the past 3 months; Life expectancy of < 3 years; History of alcohol/substance abuse

(d) Allergic disorders; chronic infections, COPD, tuberculosis; hepatitis B or C; pneumonitis, bronchiectasis; pericardial or pleural effusion, ascites; liver disease;

(e) Chronic inflammatory/autoimmune conditions such (e.g. SLE, rheumatoid arthritis, ulcerative colitis/Crohn's disease; non-basal cell malignancy or myelo- or lymphoproliferative disease within the past 5 years; known HIV+; Immunizations (3 months); pulmonary hypertension;

(f) Pregnancy, nursing;

(g) History of symptomatic coronary artery disease (events) or heart failure;

(h) BMI>35, diabetes/glucose intolerance (fasting glucose, HbA1; testing, glucose challenge where indicated);

(i) Known albuminuria/microalbuminuria; GFR<60mL/min/1.73m2.

(j) Any chronic concurrent treatment. Use of systemic or local steroids/immunosuppressive agents (within 6 months) of the inclusion; current (within past 3 months) use of anti-hypertensive medication;
(k) Major depressive illness or other psychiatric conditions.
(l) Participants who decline participation in the study or who are unable to provide informed consent

3.4 Identification of Participants and Consent

3.4.1. Participant Identification

Participants will be identified through:

- Potential participants will be identified by members of the direct clinical hypertension care team at *QEUH Glasgow Blood Pressure Clinic* (where PI – Prof Guzik and Prof Delles are part of the team), supported by the research nurse team
- Other secondary care routine out-patient appointments where potential participants and controls can be identified and invited to participate.
- Participants may also be identified though SHARE, primary and secondary care data bases and direct identification of potential participants through the SPCRN/GGC primary care team.
- Potential participants for the age/sex/BMI matched control group will be identified by a member of the direct clinical pre-OP orthopaedics care team (from among subjects with planned minor surgeries (e.g. arthroscopy), supported by the research nurse team
- Potential participants may also be identified through traditional and social media including newspaper advertisement, press release, posters in NHS and public sites as well as word of mouth and referral from colleagues.

Potential participants will be approached in one of two ways: 1) approach in person by the clinical care team at a routine outpatient appointment. Those who demonstrate interest will be given the patient information leaflet (PIL) and asked for verbal consent for their details to be passed to the research team. The PIL will contain a contact email address and telephone number to allow patients to “opt in” to the study or get in contact for further information. If there has been no contact from a patient 48 hours after being given the PIL, the patient will be telephoned by the clinical fellow or research nurse to ascertain interest in participation in the study; 2) letter drop: letters posted to the patient by the principal investigator with information about the study. The same contact information will be provided for patients contacted by letter drop to allow them to opt in to the study. These patients will also be contacted 5-7 days after posting the letter by the clinical fellow to assess interest in participating in the study.

Interested participants will be able to register their interest by contacting the InflammaTENSION investigators by:

- Email
- Telephone
- Posting the attached reply slip in the prepaid envelope

Both the poster and letters will contain contact registration details:

- Study specific email address:gg-uhb.inflammatension@nhs.net
- InflammaTENSION investigator study specific mobile number:
- InflammaTENSION investigator telephone number: 0141 330 7590

3.4.2. Participant Registration

The telephone registration process will include:

- Obtaining participants contact details: Name, Date of Birth, Address, Telephone and Email
- Explanation of the study
- Initial Eligibility Screen (with particular focus on verification of lack of known exclusion criteria)
    - Ineligible participants will be asked if they would like a referral to *Glasgow Blood Pressure Clinic, QEUH*.
- Eligible participants will be invited for a baseline visit, and an appointment date and time will be made, a participant information leaflet (PIL) will be sent via post or email; depending on the participants’ preference.

3.4.3. Consent at Baseline Visit

All participants will have to provide written consent to take part in the study, the consent taken will be inclusive for the InflammaTENSION study and must be obtained before any of the study procedures can commence. All participants will be provided with a signed copy of their consent form. .
The Chief Investigator (PI) will retain overall responsibility for the informed consent of participants and will ensure that any person delegated responsibility to participate in the informed consent process is duly authorized, trained and competent to participate according to the ethically approved protocol, principles of Good Clinical Practice (GCP) and Declaration of Helsinki.
Informed consent will be obtained prior to the participant undergoing procedures that are specifically for the purposes of the study and are out-with standard routine care.
The right of a participant to refuse participation without giving reasons will be respected.
Before consent is taken the InflammaTENSION investigator will:

- Ensure that the participant has read the PIL and has had ≥ 24 hours to reflect upon the information
- Explain the study to the participant
- Go through detailed inclusion and exclusion criteria for the study**
- Answer any of their questions relating to the study
- Explain that participation in the study is entirely voluntary and they do not have any obligation to take part in the study and they can leave the study at any point***

The process of consent will involve:

- a discussion between the potential participant and an individual knowledgeable about the research about the nature and objectives of the trial and possible risks associated with their participation
- the presentation of written material (e.g., information leaflet and consent document which must be approved by the REC and be in compliance with GCP, local regulatory requirements and legal requirements)
- the opportunity for potential participants to ask questions
- Assessment of capacity: for consent to be ethical and valid in law, participants must be capable of giving consent for themselves. A capable person will:
    - understand the purpose and nature of the research
    - understand what the research involves, its benefits (or lack of benefits), risks and burdens
    - understand the alternatives to taking part
    - be able to retain the information long enough to make an effective decision
    - be able to make a free choice
    - be capable of making this particular decision at the time it needs to be made (although their capacity may fluctuate, and they may be capable of making some decisions but not others depending on their complexity)
    - where participants are capable of consenting for themselves but are particularly susceptible to coercion, it is important to explain how their interests will be protected

A person is assumed to have the mental capacity to make a decision unless it is shown to be absent. Mental capacity is considered to be lacking if, in a specific circumstance, a person is unable to make a decision for him or herself because of impairment or a disturbance in the functioning of their mind or brain. In practice for participants with mental incapacity this means that they should not be included in clinical trials if the same results can be obtained using persons capable of giving consent and should only be included where there are grounds for expecting that their taking part will be of direct benefit to that participant, thereby outweighing the risks.

3.5 Withdrawal of Subjects

The participant will remain free to withdraw at any time from the trial without giving reasons and without prejudicing his/her further treatment and will be provided with a contact point where he/she may obtain further information about the study and where they can receive further needed care. Patient's withdrawn and needing clinical care will be referred for a visit to *Glasgow Blood Pressure Clinic at QEUH*. Referrals to *Glasgow Blood Pressure Clinic* will be a written letter and participants will be directed to NHSGGC Prof Guzik/Prof Delles led clinic.
Where a participant is required to re-consent or new information is required to be provided to a participant, the PI will ensure this is done in a timely manner.
The investigator can withdraw participants from the study in the event of inter-current illness or identification of a secondary cause for hypertension. Participants who withdraw from the study will be replaced with additional participants until 120 participants/arm have attended the final study visit.

***Verbal consent will be obtained from the participants prior to eligibility screening and/or referral to NHSGGC to QEUH General Medicine/Hypertension Clinic*

****Participants who are either ineligible or no longer wish to take part in the study will be asked if they would like a referral to QEUH General Medicine/Hypertension Clinic for further diagnosis and treatment, and they will be provided with the contact details for QEUH General Medicine/Hypertension Clinic. Referrals to QEUH Glasgow Hypertension Clinic will be a written letter and participants will be directed to NHSGGC Prof Guzik/Prof Delles led clinic.*

4. Study Schedule and Trial procedures

NB:

- ***Please refer to the InflammaTENSION Manual of Study Operations for an in-depth detail of the study procedures***
- ***Please refer to Table 1: Study Schedule***

1. **Recruitment**

Once identified and consented as described in section 3.4 the study visit activities can commence.

4.3 Study Visit Information

- **Location:** All study visits will take place at Glasgow Clinical Research Facility 5th Floor, Neurosciences Building Queen Elizabeth University Hospital Campus 1345 Govan Road Glasgow G51 4TF
- Number of Study Visits: All participants will attend a baseline study visit and visit 1 -either separately or combined depending on the participants wishes and availability of ABPM within past 3 months.
- An additional telephone contact and verification will be performed 3.5months +/- 2 weeks after final study visit to ensure secondary causes of hypertension would have not been identified in subsequent months.
    - In case of clinical need, and if subjects are agreeable, they will be referred to QEUH General Medicine/Hypertension Clinic, led by NHSGGC Prof Guzik/Prof Delles for further diagnosis and treatment..

4.4 Study Schedule and Trial Procedures

Table 1: Schedule of InflammaTENSION Study procedures

Study Procedure	Baseline Visit 0 at CRF	Visit 1 at CRF	Telephone visit/Letter Drop
Timeframe	V0	V0+(0-72h)	V1+12m±2w
Demographic History	X	-	x
Medical History	X	-	x
Height and Weight	X	-	x
Urine Sample	X	-	-
Heart Rate	X	-	-
Office Blood Pressure measurement – 3 times following 15 minutes of rest	X	-	-
Venous Blood Sample (40ml) for Blood Tests and Immune signature/Biomarker analysis	X	-	-
Venous Blood Sample (10ml) for storage for subsequent genetic analysis	X	-	-
Ambulatory Blood Pressure Monitor Installation if no ABPM performed within past 3 months	X	-	-
MCQ	-	X	-
MoCA	-	X	-
DSS	-	X	-
IDQ	-	X	-

IPAQ	-	X	-
ABPM monitor will be returned	-	X	-
Carotid Intima-Media Thickness	-	X	-
Flow Mediated Dilation of Brachial Artery	-	X	-
Endo-PAT2000	-	X	-
Sphygmocor study	-	X	-

Key	• Not performed	X Performed

h hours w – weeks m - months

General Considerations

In hypertensive subjects' study visit(s) will be arranged as soon as possible after initial diagnosis of hypertension and before initiation of the treatment for hypertension. The study visit will not delay initiation of therapy in any way and will be fitted in between the hypertension work-up and subsequent clinic visit. In subjects recruited from GHBPC (QEUH) this will be organized following initial diagnostic ABPM which is routinely performed prior to patient being seen by a consultant. In patients recruited from elsewhere – this will be arranged as soon as possible after identification and GP will be informed about ABPM which will be performed as part of the study by a letter from InflammaTENSION clinical team member.
Secondary hypertension will be excluded as per routine clinical practice. We also planned a letter drop questionnaire or telephone visit/data review 3-4 months after initial visit in order to verify secondary/primary status of hypertension.
While we have planned baseline visit and Visit 1 separately with ABPM being performed in between, subjects who do not require ABPM (it is available within past 3 months) we will offer an option to combine these visits in one day for the patients.

Baseline Visit (Visit 0)

Meeting: The InflammaTENSION investigators will meet the participants at reception of the CRF.
Estimated Duration of Baseline Visit: Approximately 0.5 - 1 hours
Recording of Study Information: The data from the study procedures will be recorded in the electronic case report form (e.g. CASTOR EDC).

Study Procedures Performed at the Baseline Visit will include:
(Please refer to the InflammaTENSION Study Manual of Procedures for more details relating to the study procedures)

- Demographic History
- Medical History
- Height and Weight
- Urine Sample
- Office Blood Pressure measurement – 3 times following 5 minutes of rest
- Venous Blood Sample (40ml) for Blood Tests and Immune signature/Biomarker analysis
- Venous Blood Sample (10ml) for storage for subsequent genetic analysis
- Ambulatory Blood Pressure Monitor Installation if no ABPM performed within past 3 months
- Subjects will be given an IDQ questionnaire (The Interheart Diet Questionnaire) to be returned during visit 1

All procedures will be performed in both Cases and Control groups including ABPM as it will ensure that hypertension is not missed by office blood pressure readings.

Anonymisation

- Patient data will be pseudo-anonymised with a study number given to the patient. This study number will be used for blood tubes. The study specific number will be used for all storage of electronic and paper data relating to the;
    - Vascular function determinations
    - Immune signature determinations (cytokine/chemokine and cellular)
    - Cognitive function questionnaires
    - Will be blinded towards hypertension status and group allocation.

- Only the Chief Investigator, and designated and trained research fellow, who is a member of the direct care team, will have access to the participants' personal data during the study. These procedures will comply with the 1998 Data Protection Act. Biological samples collected from participants as part of this study will be transported, stored, accessed and processed in accordance with national legislation relating to the use and storage of human tissue for research purposes and such activities will meet the requirements as set out in the 2004 Human Tissue Act and the 2006 Human Tissue (Scotland) Act.

Visit 1 (CRF)

Study Procedures performed at Visit 1 at CRF (either in combination with baseline or up to 72h after baseline Visit)

NB: There is no need for a study specific ABPM if a routine care ABPM has been performed within the past 3 months.
This visit will take approximately 2-3.5 the following study procedures will be performed at the final visit.

- Questionnaires
  - MCQ The Mild Cognitive Impairment Questionnaire
  - MoCA Montreal Cognitive Assessment
  - DSS Digital Symbol Substitution
  - IDQ The Interheart Diet Questionnaire
  - IPAQ The International Physical Activity Questionnaire
- ABPM monitor will be returned
- Carotid Intima-Media Thickness
- Flow Mediated Dilation of Brachial Artery
- Endo-PAT2000
- Sphygmocor study

Participants will be thanked for taking part in the InflammaTENSION study.

Letter drop / Telephone contact (3.5 months +/- 14days)

A follow - up letter will be sent by post or e-mail to all participants asking for filling in a questionnaire regarding the key changes in medical status since Visit 0. It will also announce possible telephone contact if no response is received to initial letter
The telephone registration process will include:

- Obtaining information on participant health status changes; changes in demographics; changes in medical history and in particular verification if any cause for secondary hypertension has been identified.

4.5 Study Outcome Measures

4.5.1 Primary Outcome Measure

Cytokine and cellular immune signature of primary hypertension.
Association between hypertensive status assessed by ABPM and immune signature analysed as a whole as well as individual pro- and anti- inflammatory cytokine levels and discreet immune cell populations.

4.5.2 Secondary Outcome Measure

- Association of selected cytokine/chemokine levels with vascular phenotypes
  - endothelial function assessed by Endo-PAT2000, a non-invasive technique.
  - Large vessel endothelial function assessed by brachial artery flow mediated dilatation, a non-invasive technique.
  - Changes in cardiovascular parameters through non-invasive hemodynamic measurements
- Association of selected cytokine/chemokine levels with renal phenotypes
  - GFR; renin levels; aldosterone levels
- Association of selected cytokine/.chemokine levels with cognitive dysfunction
  - Results of MCQ, MoCA, DDS
- Association of selected cytokine/.chemokine levels with genetic variation assessed by GWAS

6. Incidental findings

Any incidental findings observed during the research procedures will be acted upon: this means that the PI takes responsibility to notify the patients GP and refer on for specialist follow up as appropriate and with the patient or participants permission.
Patients who would benefit from hypertension/cardiovascular risk factor management will be referred to and provided with contact details for NHSGGC Prof Guzik/Prof Delles led clinic *at the QEUH General Medicine/Hypertension Clinic for further diagnosis and treatment.*

6. Statistics and Data Analysis Plan

6.1 Statistical Analysis Plan

The InflammaTENSION study will have a comprehensive Statistical Analysis Plan, which will govern all statistical aspects of the study, and will be authored by the Study Statistician (Dr John McLure). To gain additional insight, variable selection analysis will be used to identify the best predictors of hypertension and its vascular outcomes (endothelial function/vascular compliance). To do this we will draw the expertise of local bioinformaticians (Dr John McClure) and of Professor Andrew Yates, a theoretical immunologist at University of Glasgow with experience in the modeling of cytokine networks and in machine learning approaches to TCR repertoire analysis. We anticipate using exploratory unsupervised approaches such as Principle Component Analysis and clustering may be used on the cytokine profiles to identify signature patterns of cytokine expression that may allow a reduction of dimensionality. One can also associate each patient's cytokine profile with a score - either one dimensional measure of disease severity or perhaps multi-factorial measures, combining severity with demographic data, for example, and use supervised learning approaches such as support vector machines or neural networks to generate classifiers or predictors of outcomes.

6.2 Sample Size

The sample size will comprise of 120 participants per study arm (120 cases + 120 controls).
Based on preliminary data: at least 116 subjects will allow us to detect an IL-6 difference of 25% at a two-sided alpha of 0.1% and power of 90% (multiple markers were tested, giving similar or smaller group; TNF-α – 58 subjects). 87 subjects/group for a 25% difference in CD8+CD25+ T cell (α=0.1%, power - 90%). These analyses take into account multiple comparisons which are foreseen for immune signature determination.
We believe that these projected effect sizes would potentially be clinically meaningful and credible.

7. Study Closure or Definition of End of Trial

The study will end when:

- Final participants attend last study visit OR

i. The planned sample size has been achieved;
ii. There is insufficient funding to support further recruitment, and no reasonable prospect of additional support being obtained;
iii. Recruitment is so poor that completion of the trial cannot reasonably be anticipated.

8. Data Handling

8.1 Case Report Forms / Electronic Data Record

An electronic case report form (e-CRF) will be used to collect study data. The e-CRF will be developed by the study data centre at the CASTOR EDC (https://castoredc.com) and access to the e-CRF will be restricted, with only authorised site-specific personnel able to make entries or amendments to their Participants' data. It is the investigator's responsibility to ensure completion and to review and approve all data captured in the e-CRF.

All data handling procedures will be detailed in a Study Specific Data Management Plan. Data will be validated at the point of entry into the e-CRF and at regular intervals during the study. Data discrepancies will be flagged to the study site and any data changes will be recorded in order to maintain a complete audit trail (reason for change, date change made, who made change).

8.2 Record Retention

As discussed above in patient recruitment procedures, subjects will be initially identified by a member of the patient's existing clinical care team who has access to patient records in relation to clinical service. They will inform the patient about the possibility of participating in a study.
Patient data will be pseudo-anonymised with a study number given to the patient. This study number will be used for blood tubes. To enable evaluations and/or audits from regulatory authorities, the investigator agrees to keep records, including the identity of all participating subjects (sufficient

information to link records), all original signed informed consent forms, in accordance with ICH GCP, local regulations, or as specified in the Clinical Study Agreement, whichever is longer. Data will be retained at the Data Centre for a minimum of 10 years.
Only the Chief Investigator, who is a member of the direct care team, will have access to the participants' personal data during the study. These procedures will comply with the 1998 Data Protection Act. Biological samples collected from participants as part of this study will be transported, stored, accessed and processed in accordance with national legislation relating to the use and storage of human tissue for research purposes and such activities will meet the requirements as set out in the 2004 Human Tissue Act and the 2006 Human Tissue (Scotland) Act.
Personal data will be stored on NHS password protected computers, which are secured as per local NHS protocols. No personal patient details will be on the University computers.
Manual files with personal information will be kept in a secure location in the Clinical Research Facility, containing copies of the consent forms and a sample collection record.
Only members of the study team will have access to any of the data generated.

To enable evaluations and/or audits from regulatory authorities, the data will be retained at the Data Centre for 15 years. The data records will include including the identity of all participating subjects, (to ensure the subjects information can be linked to records), all original signed informed consent forms, source documents, and detailed records of treatment disposition in accordance with ICH GCP, local regulations.

9. Protocol Amendments

Any change in the study protocol will require an amendment. Any proposed protocol amendments will be initiated by the principle investigators and any required amendment forms will be submitted to the regulatory authority, ethics committee and sponsor. The principle investigators will liaise with study sponsor to determine whether an amendment is non-substantial or substantial. All amended versions of the protocol will be signed by the principle investigator and Sponsor representative. Before the amended protocol can be implemented favourable opinion/approval must be sought from the original reviewing Research Ethics Committee and Research and Development (R&D) office(s).

10. Ethical Considerations

10.1 Ethical conduct of the study

The study will be carried out in accordance with the World Medical Association Declaration of Helsinki (1964) and its revisions (Tokyo [1975], Venice [1983], Hong Kong [1989], South Africa [1996] and Edinburgh [2000]).
Favourable ethical opinion will be sought from an appropriate REC before Participants are entered into this clinical trial. Participants will only be allowed to enter the study once either they have provided written informed consent
The principle investigator will be responsible for updating the Ethics committee of any new information related to the study.

10.2 Informed consent

Written consent will be obtained from each trial participant; each participant will receive a signed copy of the consent form. Participants unable to provide written informed consent will be excluded from the study.
The Research Nurse or investigator will explain verbally and in writing the exact nature of the study in writing with the provision of Participant information sheet. This will include the known side-effects that may be experienced, and the risks of participating in this clinical trial. Trial participants will be informed that they are free to withdraw their consent from the study or study treatment at any time.

10.3. Assessment and management of risk

The small amounts of venous blood which will be obtained are safe and do not pose any additional risk. All procedures for venepuncture will be fulfilled to ensure safety of participants and the procedure will be carried out by trained medical practitioner, member of InflammaTENSION team.
The study is classified as Type A = No higher than the risk of standard medical care

10.4. Blinding

Patient data will be pseudo-anonymised with a study number given to the patient. This study number will be used for blood tubes. These numbers will be used for blinding. All investigators performing clinical and laboratory assessment will be blinded towards hypertension status and group allocation of individual subjects.

Only the Chief Investigator and research fellow trained who is a member of the direct care team will have access to the participants' personal data during the study. These procedures will comply with the 1998 Data Protection Act. Biological samples collected from participants as part of this study will be transported, stored, accessed and processed in accordance with national legislation relating to the use and storage of human tissue for research purposes and such activities will meet the requirements as set out in the 2004 Human Tissue Act and the 2006 Human Tissue (Scotland) Act.

11. Insurance and Indemnity

The InflammaTENSION study is sponsored by NHS GGC. The sponsor will be liable for negligent harm.. NHS indemnity is provided under the Clinical Negligence and Other Risks Indemnity Scheme (CNORIS). Non-negligent harm from the protocol, design of the study and which is therefore non-negligent, will be covered by the University of Glasgow.

The NHS has a duty of care to Participants treated, whether or not the Participant is taking part in a research study, and the NHS remains liable for clinical negligence and other negligent harm to Participants under its duty of care.

12. Funding

Funding for the InflammaTENSION study will be from the European Research Council and the BHF Centre of Excellence Award.
Grant Funder: European Research Council and British Heart Foundations
Grant award number: ERC Grant agreement in negotiations; BHF - RE/13/5/30177

13. Dissemination of Findings

The research findings will be disseminated to healthcare professionals and public health specialists through newsletters; to members of the public by newspapers and other media as well as by information sessions; and to the wider scientific community through peer reviewed publications and presentations. No identifiable participant information will be detailed within the research findings

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Appendix 3 IPAQ (International Physical Activity Questionnaire) short version and InterHeart Risk Score Questionnaire.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE
We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the last 7 days. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.
Think about all the vigorous activities that you did in the last 7 days. Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.
1. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, aerobics, or fast bicycling?
_____ days per week
No vigorous physical activities Skip to question 3
2. How much time did you usually spend doing vigorous physical activities on one of those days?
_____ hours per day
_____ minutes per day
Don’t know/Not sure
Think about all the moderate activities that you did in the last 7 days. Moderate activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.
3. During the last 7 days, on how many days did you do moderate physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.
_____ days per week
No moderate physical activities Skip to question 5
4. How much time did you usually spend doing moderate physical activities on one of those days?
_____ hours per day
_____ minutes per day
Don’t know/Not sure
Think about the time you spent walking in the last 7 days. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.
5. During the last 7 days, on how many days did you walk for at least 10 minutes at a time?

_____ days per week
No walking Skip to question 7
6. How much time did you usually spend walking on one of those days?
_____ hours per day
_____ minutes per day
Don't know/Not sure
The last question is about the time you spent sitting on weekdays during the last 7 days. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.
7. During the last 7 days, how much time did you spend sitting on a week day?
_____ hours per day
_____ minutes per day
Don't know/Not sure
This is the end of the questionnaire, thank you for participating.

Enter your birth date:

Year

Month

Day

Enter your gender:
- Male
- Female

MEDICAL HISTORY AND MEDICATIONS:
1. Past Medical History:
   a) Diabetes:
   - No
   - Yes

   b) High Blood Pressure:
   - No
   - Yes

2. Have either or both of your biological parents had a heart attack:
- No or unsure
- Yes

TOBACCO:
3. Which best describes your history of tobacco use:
- Never
- Former smoker (>12 months of not smoking)
- Current

 # cigs/day

4. Over the past 12 months what has been your typical exposure to other peoples smoke:
- Never
- Yes

hours per week

STRESS:
5. How often have you felt stress in the past year:
- Never Experienced Stress
- Several Periods of Stress
- Some Period of Stress
- Permanent Stress

6. During the past twelve months, was there ever a time when you felt sad, blue, or depressed for two weeks or more in a row:
- No
- Yes

PHYSICAL ACTIVITY:
7. How active are you during your leisure time:
- Mainly sedentary (e.g. sitting, reading, watching television)
- Mild exercise, minimal effort (eg. yoga, archery, sport fishing, easy walking)
- Moderate exercise (eg. walking, bicycle riding, or light gardening at least 4 hours per week)

Strenuous exercise (heart beats rapidly e.g. running/jogging, football, vigourous swimming)

DIET:

8. Do you eat salty food or snacks one or more times a day:

- No
- Yes

9. Do you eat deep fried foods or snacks or fast foods 3 or more times a week:

- No
- Yes

10. Do you eat fruit one or more times daily:

- No
- Yes

11. Do you eat vegetables one or more times daily:

- No
- Yes

12. Do you eat meat and/or poultry 2 or more times daily:

- No
- Yes

PHYSICAL MEASUREMENTS:

13. Waist Circumference Measurement (This measurement is taken at a specific spot along your side. Slide your thumb down your side until you find your hip bone. Place the measuring tape over that spot where your thumb found your bone and wrap the measuring tape around your middle. While making sure the measuring tape is level all around, please note your waist circumference in either centimetres or inches (even if this is not your usual waistline)):

Cm OR

inches

14. Hip Measurement (While standing in front of a mirror, look for the largest point of your buttocks and place the measuring tape at that position and wrap it around your middle. While making sure the measuring tape is level all around, please note your hip circumference in either centimetres or inches):

Cm OR

inches

Submit Reset

Appendix 4 Laboratory protocol.

INFLAMMATENSION Sample Handling And Processing Manual

Chief Investigator: Tomasz Guzik

IRAS project ID: 224036

REC Reference Number: 1 7/WS/0115

Sponsor: European Research Council

	Name	Signature	Date
Compiled by:	**Eleanor Murray/ Hannah Bialic**		**18/04/2019**
Approved by PI:	**Tom Guzik**		
CI acceptance:			
Review date:	**25th March 2020**		

1. Purpose

To describe the procedures involved in collection, transport and processing blood and urine samples for the *Inflammatension* study.

Blood and urine samples will be obtained from participants (subjects and controls) as per the Inflammatension Manual of Operations, and as approved by Ethics and R&D. These samples will be analysed by flow cytometry and frozen for later analysis with the purpose of immunophenotyping those with hypertension compared to those with normal blood pressure, ie the overall purpose of obtaining samples is to study mechanisms of hypertension.

2. **Contact Information**

	Name & contact information
Project Manager	Eleanor Murray xxxxxxxxxxx eleanor.murray@glasgow.ac.uk
Laboratory technician (e.g. sample receipt)	Ryszard Nosalski xxxxxxxxxx Ryszard.nosalski@glasgow.ac.uk Hannah Bailic 07496483664, 0141 330 8134 Hannah.Bailic@glasgow.ac.uk Aysin Tulunay Virlan 0141 330 8132 Aysin.TulunayVirlan@glasgow.ac.uk
Courier Details	Glasgow Taxis / Zedify couriers

3. Roles and responsibilities

The processing of clinical trial samples in accordance with this manual will be overseen by the above named individuals who assume responsibility for the conduct of this work at the trial site. The Principal Investigator will ensure that site personnel are appropriately trained and qualified to perform the roles and responsibilities assigned to them (see delegation log) and that the site has the necessary resources to process and store the samples.

4. Summary Sample Collection

Biological sample	**Purpose**	**Baseline**	**Label**
Plasma (EDTA)	Protein Arrays/ELISA	6ml	A_5001
Serum (SST)	Cytokines	5ml	B _5001
PBMC (EDTA)	FACS & *ex vivo* studies	20ml	C _5001
PBMC for phosphflow (Sodium citrate)	Phospho-FACS	4ml	D _5001
RNA (Tempus)	Transcriptional studies	3ml	E _5001
Total volume blood		**38 ml**	
Urine (universal container)	For proteomics/ metabolomics	5-10ml	F_5001

INFLAMMATENSION sample collection packs will contain all the relevant vacutainers for *university* samples, pre-printed barcode labels and sample transfer form.

Please note, vacutainers for routine blood tests (U&Es, CRP) are not included, so please remember to take a separate SST tube IF NOT ALREADY DONE, label as per TrakCare, send to the NHS laboratory, and record results in the eCRF.

For each sample processed, a Sample Transfer Form V1.1 should be completed (see appendix). This documents the type and number of samples, type of collection vials,

processing, and storage. Completed Sample Transfer Forms should be stored for monitoring purposes.

Sundry Items

Sample collection transfer form (supplied)
Sample labels (supplied)
Alcohol swab (not supplied)
Cotton swab (not supplied)
Sharps bin (supplied by CRF)
12 inch blood collection set eg. BD Vacutainer® Push Button (supplied)
Sealable sample bag for transfer(supplied)
Cool bag or cool pack for transfer of specified samples (from CRF)

5. Patient IDs, Time Point Identifiers and Sample Tube Labels

Each participant will be assigned a study ID recorded in the eCRF, sample collection record log and laboratory information management system (LIMS). All samples will be anonymised and only the Study ID used when labelling. Label will include information on:

- *Participant ID*
- *Study ID*
- *Time & Date collected*
- *Staff member collecting*

6. Sample Collection technique

1. Draw into the appropriate blood bottles in the following order:
   a. SST tubes (gently invert tube 5 times)
   b. EDTA tubes (gently invert tube 8 times)
   c. Sodium citrate (gently invert 4 times)
   d. Tempus RNA tube (shake vigorously for 10 seconds to ensure that the Applied Biosystems Stabilizing Reagent makes uniform contact with the sample).

NB. Prevention of Backflow with Tempus RNA: The Tempus RNA blood collection tube contains chemical additives. **It is important to avoid possible backflow from the tube, with the possibility of adverse subject reaction**:

1. When drawing blood from participant:
   a. Use a blood collection set such as the BD Vacutainer® Push Button Blood Collection Set or BD Vacutainer® Safety-Lok™ Blood Collection Set.
   b. Place limb in a downward position.
   c. Hold tube with stopper upper-most.
   d. Release tourniquet as soon as blood starts to flow into the tube.
   e. Make sure that tube additives do not touch the stopper or the end of the needle during venepuncture.

7. Sample transfer

All samples will be couriered**.** A date and time of transfer will be pre-specified and agreed between the named individual responsible for obtaining the sample, the taxi/courier, and the person delegated to receive the sample.

Study specific blood samples along with the sample transfer form will be sent to Hannah Bialic and Aysin Tulunay Virlan, Sir Graeme Davies Building, 126 University Place, BHF for aliquoting, freezing, and storage.

NOTE: please ship all blood at room temperature and ship **as soon as possible** to the GBRC labs to allow processing of the bloods. Urine and plasma sample ('B' 6ml EDTA) should be transported in a cool bag or on ice.

If any potential errors on samples or labelling are detected by staff receiving the sample, they will discuss same day with staff member who obtained the sample, and will record on Sample Transfer Form. Any errors or notes should be marked clearly on the Sample Transfer Form and signed and dated.
Receiving staff member will acknowledge receipt, by email to the staff member obtaining sample stating study ID and "receipt of sample" in the subject title.

8. Storage at Site

Samples will be processed same day for Flow cytometry analysis (see IFT Lab Manual V1.1 and Protocol preparation V1.4), or stored in -80 degrees Celcius initially, then moved into liquid nitrogen for long-term storage at the University of Glasgow in a secure location, identified only by the Participant ID/lab number.

9. Withdrawal of participant consent

In the case of a withdrawal of consent, all samples will be destroyed, though any data already generated will be kept (if the participant is in agreement). Samples will be destroyed and witnessed according to local policy.

10. Non compliances and potential serious breaches in GCP

Risk	Actions to reduce risk of occurrence	Action taken if occurs
Loss of sample in transit	1. Single approved courier to be used (if cannot be taken by staff identified on delegation log) 2. Use of sample transfer form with sample details and destination 3. Requirement for staff obtaining sample to inform lab staff that sample will be arriving 4. Use of electronic calendar to forewarn lab staff of visit	1. Lab staff communicate with individual obtaining/sending sample to highlight if the sample does not arrive. 2. Courier to be contacted to identify if transported and to where. 3. All reasonable attempts at retrieval made 4. Highlighted to CI 5. Clear documentation of events and analysis to prevent recurrence
Data loss	1. Data to be stored on secure cloud storage, meeting University of Glasgow/ NHS standards 2. Paper workbooks (where used), to be transcribed to cloud storage at first opportunity (within max 72 hrs), then stored securely	1. Identify events resulting in data loss and what data involved 2. Highlight to CI 3. All reasonable attempts made to retrieve data. 4. Clear documentation of events and analysis to prevent recurrence

Data breach	1. Data to be stored on secure cloud storage, meeting University of Glasgow and NHS standards 2. Paper workbooks (where used), to remain in the secure Clinical research facility 3. All data to be anonymised – only identified by participant ID 4. No data to be stored on unencrypted USB or other storage device	1. Identify events resulting in data breach and what data involved 2. Highlight to CI 3. Clear documentation of events and analysis to prevent recurrence

11. Protocol Amendments

Any protocol amendments made in subsequent versions will be documented in this section inclusive of how the amendments will be distributed and implemented.

12. Sample processing - conducted by the laboratory staff at the GBRC.

A) 5ml SST vacutainer for SERUM (x1)

Local CRF:

1. Note collection time of the 6ml EDTA tube in the vascular study workbook & eCRF

GBRC:

2. Note arrival time of samples in the sample collection record log
3. The vacutainer should be allowed to stand for at least 30 minutes from collection before being centrifuged.
4. Centrifuge SST vacutainer at 1200g at room temperature for 10 minutes
5. Aliquot serum into 5 x 500µl aliquots in pre-labelled FluidX tubes (maximum capacity of 750ul).
6. Seal each FluidX with an **ORANGE** lid and scan into the LIMS system
7. Place FluidX tubes into 96 well box, then into -80°C freezer.

B) 6ml EDTA vacutainer for plasma (x1)

Local CRF:

1. Note collection time of the 6ml EDTA tube in the vascular study workbook & eCRF

GBRC:

2. Note arrival time of samples in the sample collection record log
3. Centrifuge blood sample at 1200g at room temperature for 10 minutes
4. Pipette plasma into plastic tube, leaving 500µl of plasma above buffy layer (take care not to disturb it).
5. Aliquot plasma from plastic tube into 5 x 500µl aliquots in pre-labelled FluidX tubes (maximum capacity of 750ul).
6. Seal each FluidX tube with a **BLUE** lid and scan into the LIMS system
7. Place FluidX tubes into 96 well box, then into -80°C freezer.

C) 10ml EDTA vacutainers for FACs and Ex Vivo studies (x2)

Local CRF:

1. Note collection time of each 10ml EDTA tube in sample collection record log & eCRF.

GBRC:

2. Note arrival time of samples in the sample collection record log

3. Reagents for FACs and Ex Vivo storage

- Pre-filled 50ml Leucosep tubes (Greiner, Cat. No: 227288)
- Dulbeccos's PBS (dPBS, Ca++ and Mg++ free).
- FACS Buffer: DPBS + 0.5% BSA + 1mM EDTA + 0.01% NaN3
    - ♣ 50ml DPBS (Ca2+/Mg2+ free)
    - ♣ 0.25g BSA
    - ♣ 0.1 ml endotoxin free 0.5M EDTA
    - ♣ 50μl 10% NaN3

    Store at 4°C for 2 months.
- eFluor 506 Fix Viability (Thermo Fisher, Cat. No.: 65-0866-18)
- Fc Block (Miltenyi Biotec, Cat. No.: 130—59-901)
- Bambanker (Alpha Labs, Cat. No.: 320-14681)
- All antibodies are outlined in tables 2-5 below, including manufacturer and Cat. No.

4. Preparation of peripheral blood mononuclear (PBMC) cells from whole blood.

- Bring 1 leucosep tube and dPBS up to room temperature.
- Pulse leucosep tube in centrifuge to move all lymphoprep below the frit.
- Label the leucosep tube IFT, Date, Patient ID.
- Record the volume of blood in each tube in the Lab Record Log.
- Transfer the blood into the leucosep tube.
- Centrifuge the leucosep tube at 800g for 15 minutes, no brake, at room temperature using a swinging bucket rotor.
- Following centrifugation the sequence of layers from top to bottom is:
    - i. plasma
    - ii. enriched cell fraction of PBMCs (PBMC interphase)
    - iii. separation medium with porous barrier
    - iv. red cell and granulocyte pellet (below the barrier)

From each leucosep tube:

- Collect the PBMC interphase by removing and discarding the plasma up to 1cm before the PBMC interphase, then pipette the remained plasma and PBMC interphase into a new 50ml collection tube.
- Top up the volume of the 50ml tube with sterile, room temperature dPBS.
- Count and record total cell number (See Section 8: Cell Counting).record the cell count in the Lab Record Log.

- Centrifuge tube at 400g for 10 minutes at room temperature, with full brake applied.
- Discard the supernatants.

Experimental protocols using PBMCs:
-cell surface antibody staining for FACs (see section 5)
-cell processing for future ex vivo or FACS studies (see section 6)

5. Preparation of PBMCs for FACS staining

- Resuspend the pelleted PBMC in dPBS to give a final concentration of $1x10^6$ cells per 100ul
- Remove 2ml of the cell suspension and place in a separate 15ml falcon tube.
    - For the remaining cell please process according to section 6.
- Dilute the Lysis Buffer (BD Bioscience, BD Pharm lyse, lysing buffer cat no. 55899) 1:10 (add 1ml buffer to 9ml water)
- Add 10ml of the diluted lysis buffer onto the 2ml volume of cells in the 15ml falcon tube.
- Incubate for 5 minutes at room temperature.
- Centrifuge at 200g for 7 minutes
- Discard the supernatant and wash the cells by re-suspending the cells in 5ml PBS.
- Centrifuge at 400g for 5 minutes.
- Re-suspend in the correct volume to obtain 1×10^6 cells/100 ul

- Prepare FACs tubes for each FACs panel (T cell, Chemokine, Monocyte and B cell):
    i. Unstained
    ii. FMO (2 FMO tubes for chemokine panel)
    iii. Mix

- Add 1ul of eFluor 506 Fix Viability dye to ‘FMO’ and ‘Mix’ tubes of the T cell and Chemokine panel
- Add 100ul of PBMC suspension into all of the tubes ($1x10^6$ cells PBMCs total)
- Vortex to mix
- Incubate tubes in fridge for 30 minutes
- Wash the cells by adding 2mls of FACs buffer to each tube
- Centrifuge at 400g for 5 min at 4°C, then discard supernatant and gently resuspend cells.
- Add 1ul Fc block into the Monocyte ‘FMO’ and ‘Mix’ tubes.
- Prepare staining cocktail (Mix and FMO) for each panel: chemokine (Table 2); T cell (Table 3); Monocyte (Table 4); and B cell (Table 5).

Table 1: Chemokine Panel

Marker	Color	Panel volume for 1 test (µl)	FMO 1	FMO 2	Cat No	Manufacturer
CD3	Alexa Fluor 700	2	2	2	56-0038-42	Thermo Fisher
CD8a	APC-eFluor 780	1	1	1	47-0088-42	Thermo Fisher
CD4	eFluor 450	1	1	1	48-0049-42	Thermo Fisher
CCR6	Alexa Fluor 488	5	x	5	353414	Biolegend
CD161	PE-eFluor 610	1	1	1	61-1619-42	Thermo Fisher
TCR delta/gamma	Brilliant Violet 605	1	x	1	740415	BD
CXCR3	PE-Cy7	2	2	2	25-1839-42	Thermo Fisher
CCR4	PerCP-Cy5.5	5	5	x	359406	Biolegend
CCR10	PE	2	2	x	563656	BD
eFluor 506 Fix Viability	eFluor 506 Fix Viability	1ul added in separately before antibody mix	1ul added in separately before antibody mix	1ul added in separately before antibody mix	65-0866-18	Thermo Fisher
	Total volume	20	14	13		

Table 2: T cell Panel

Marker	Color	Panel volume for 1 test (µl)	FMO	Cat No	Manufacturer
CD3	Alexa Fluor 700	2	2	56-0038-42	Thermo Fisher
CD8a	APC-eFluor 780	1	1	47-0088-42	Thermo Fisher
CD4	eFluor 450	1	1	48-0049-42	Thermo Fisher
CD56	FITC	5	5	MHCD5601	Thermofisher
CD28	PE-eFluor 610	2	x	61-0289-42	Thermo Fisher
CD45RA	PerCP-Cy5.5	1	1	304134	Biolegend
CCR7	APC	5	x	17-1979-42	Thermo Fisher
CD122	Per-CP eFlour 710	2	x	46-1228-42	Thermo Fisher
CD45RO	PE-Cy7	1	1	25-0457-42	Thermo Fisher
CD62L	PE	3	3	12-0629-42	Thermo Fisher
eFluor 506 Fix Viability	eFluor 506 Fix Viability	1ul added in separately before antibody mix	1ul added in separately before antibody mix	65-0866-18	Thermo Fisher
	Total volume	23	14		

Table 3: Monocyte/DC Panel

Marker	Color	Panel volume for 1 test (µl)	FMO	Cat No	Manufacturer
CD3	PE	1	1	12-0038-42	Thermo Fisher
CD56	PE	1	1	12-0567-42	Thermo Fisher
CD15	PE	1	1		Biolegend
CD19	PE	1	1	12-0199-42	Thermo Fisher
HLA DR	PE-Cy7	1	1	25-9952-42	Thermo Fisher
CD141	APC	1	1	17-1419-42	Thermo Fisher
CD14	APC-eFluor 780	1	1	47-0149-42	Thermo Fisher
CD16	eFluor 450	1	1	48-0168-42	Thermo Fisher
Mannose Receptor	PE-CF594	2	x	564063	BD
CD303a	PerCP-eFluor 710	10	x	46-9818-42	Thermo Fisher
CD1c	FITC	3	3	11-0015-42	Thermo Fisher
CD192 (CCR2)	Brilliant Violet 510	3	x	357218	Biolegend
CD181 (CXCR1)	Brilliant Violet 605	3	x	743421	BD
CCR5	Alexa Fluor 700	3	x	359116	Biolegend
	Total volume	32	11		

Table 4: B cell panel

Marker	Color	Panel volume for 1 test (µl)	FMO	Cat No	Manufacturer
CD38 V450	V450	2	2	646851	BD
CD27 V500	V500	2	2	561222	BD
CD43 PE	PE	2.5	2.5	560199	BD
CD24 FITC	FITC	5	5	555427	BD
IgM PerCpCy5.5	PerCpCy5.5	5	5	561285	BD
CD19 APC	APC	5	5	302212	Biolegend
IgD APC-H7	APC-H7	5	5	561305	BD
CD10 PE-Cy7	PE-Cy7	5	x	341112	BD
CD5	AF700	5	x	300632	Biolegend
	Total volume	36.5	26.5		

- Add the staining cocktail to the relevant 'FMO" and 'Mix' tubes.
- Incubate all tubes for 20 minutes in the fridge
- Wash the cells by adding 2mls of FACs buffer to each tube using a Pasteur pipette
- Centrifuge at 400g for 5 min at 4°C, then discard supernatant and gently resuspend cells in 350µl FACs buffer
- Wrap rack containing FACs tubes in foil and place in fridge until cells are put through FACs machine (all samples run at 100µl/min with 300µl being acquired), data collected and recorded.
- Data will be transferred onto the Inflammatension hard drive and uploaded onto Castor EDC
    - Data will be named as follows: Date_patientID_ panel
    - Example: 170922_501 _chemokine

6. Preparation of PBMCs for future FACS or ex vivo studies

- Top up the remaining cells to 50ml in dPBS.
- Centrifuge the tube at 200g for 10 minutes at room temperature to remove any contaminating platelets.
- Remove and discard the supernatant and resuspend the cells in 10ml dPBS.
- Centrifuge the cells at 600g for 10minutes at room temperature.
- Remove the supernatant completely and discard.
- Resuspend cell pellet gently in 1 ml of sterile Bambanker solution (Alpha Labs, cat no. 302-14681) and mix with care. Do not vortex.
- Aliquot the cell suspension into one FluidX vials (orange top, maximum capacity of 2ml).
- Seal each FluidX tube and scan into LIMS system.
- Freeze overnight at -80°C
- The next day transfer the FluidX tube into the liquid nitrogen storage box and mark position in LIMS.

D) Sodium citrate 2ml vacutainer (x2)

Local CRF:

1. Note collection time of tubes in sample collection record log & eCRF

GBRC:

2. Note arrival time of samples in the sample collection record log

3. Reagents for phosphoFACs
    - BD Phosflow lyse/fix buffer (5X concentrate, BD, Cat. No: 558049)
    - Dulbeccos's PBS (dPBS, Ca++ and Mg++ free).
    - FACS Buffer: DPBS + 0.5% BSA + 1mM EDTA + 0.01% NaN3
        - ♣ 50ml DPBS (Ca2+/Mg2+ free)
        - ♣ 0.25g BSA
        - ♣ 0.1 ml endotoxin free 0.5M EDTA
        - ♣ 50µl 10% NaN3

        Store at 4°C for 2 months.

- Perm Buffer II (BD, Cat. No: 558052): Ensure that Perm Buffer II is chilled to between -20°C and 4°C.
- Cell Stimulation Cocktail (500X) (eBioscience, Cat. No: 00-4970-03)
- eFluor 506 Fix Viability (Thermo Fisher, Cat. No.: 65-0866-18)
- T Cell Activation/Expansion Kit (Miltenyi, Cat no. 130-091-441, once prepared, store at 2–8 °C for up to 4 months).
- All cytokines are outlined in tables 5 (including manufacturer, Cat. No.)
- All antibodies are outlined in tables 6 (including manufacturer, Cat. No.)

4. PhosphoFACs-preparation of Intracellular FACs panels

- Prepare 35mL lyse/fix buffer per patient (28mL dH20 + 7mL 5X lyse/fix) and put in 37°C water bath
- Transfer 8mL Perm Buffer II (per patient) in a tube and put it on ice, protected from light
- Prepare 4 tubes
    - i. Unstimulated surface only (isotype)
    - ii. Unstimulated panel
    - iii. Stimulated surface only (isotype)
    - iv. Stimulated panel

- Prepare enough stimulation cocktail for all tubes required (see table 6)
- Prepare surface staining cocktail (see table 7)
- Add 100ul whole blood to each tube
- Add antibody cocktail for surface markers to all tubes
- For each panel: add stimulation cocktail to tubes marked i. '**stimulated surface only (isotype)' and ii. 'stimulated panel'.**
- Incubate all tubes at 37°C for 15 minutes
- Add 2ml of BD Phosflow™ Lyse/Fix Buffer (1X) which has been pre warmed to 37°C and has been prepared freshly for each experiment by diluting 1 part Lyse/Fix with 4 parts deionozied or distilled water. (For 4 tubes prepare: 2ml Lyse/Fix + 8ml dH20)
- Cap tubes and mix well by vigorously agitating the tubes for 30 seconds
- Incubate the tubes in a 37°C water bath for 10 min.

The following steps are carried out on ice and centrifuged at 4°C

- Centrifuge the cells at 500x g for 8 min at 4°C, aspirate the supernatant leaving no greater than 50 µL of residual volume.
- Add 2ml of FACs buffer to each tube and centrifuge at 600g for 6 min.
- Permeabilize the cells by adding 500 µL of chilled Perm Buffer II
- Vortex to mix and incubate the tubes for 30 minutes **on ice,** protect from light by covering with tinfoil
- Wash the cells by adding 4ml of FACs buffer to each tube and centrifuge at 600g for 6 minutes.
- Remove the supernatant, leaving no greater than 50 µL of residual volume.

- Wash the cells again by adding 4ml of FACs buffer to each tube and centrifuge at 600g for 6 minutes.
- Resuspend the cells and for each panel: add the intracellular markers (marked in **orange** and **bold** in table 10) to tubes marked ii. 'unstimulated panel' and iv. 'stimulated panel', mix and incubate on ice for 30 minutes, protected from light by covering with tinfoil.
- Wash the cells by adding 2mls of FACs buffer to each tube
- Centrifuge at 600g for 6 min at 4°C, then discard supernatant and gently resuspend cells in 350µl amount of FACs buffer
- Wrap rack containing the FACs tubes in foil and place in fridge until cells are put through FACs machine and data collected and recorded.
- Data will be transferred onto the inflammatension hard drive and uploaded onto Castor EDC
  - Data will be named as follows: Date_patientID_Panel
  - Example: 170922_5001 _Lineage/pSTAT

Table 10: Lineage/pSTAT

Marker	Color	Panel volume for 1 test (µl)	FMO	Cat No	Manufacturer
CD3	Alexa Fluor 700	2	2	56-0038-42	Thermo Fisher
CD8a	APC-eFluor 780	2	2	47-0088-42	Thermo Fisher
CD4	eFluor 506	1	1	69-0049-42	Thermo Fisher
CD14	SuperBright 600	5	5	63-0149-42	Thermo Fisher
CD19	PE-Cy7	5	5	25-0199-42	Thermo Fisher
STAT1 (pY701)	**Alexa Fluor 488**	**10**	**x**	**612596**	**BD**
STAT3 (pY705)	**Brilliant Violet 421**	**3**	**x**	**651010**	**Biolegend**
STAT6 (pY641)	**PerCP-Cy5.5**	**5**	**x**	**686010**	**Biolegend**
Syk	**PE**	**5**	**x**	**12-6696-42**	**Thermo Fisher**
c-Cbl (pY774)	**Alexa Fluor 647**	**10**	**x**	**558103**	**BD**
Total volume per test	**surface**	15			
	intra	33			

E) Tempus RNA tube

Local CRF:

1. Note collection time of tube in sample collection record log & eCRF

GBRC:

2. Note arrival time of sample in the sample collection record log
3. Tempus RNA vacutainers should be kept upright, at room temperature for precisely 3 hours after blood collection and then transferred to -80°C freezer.
4. Record any deviations from this time point in the Lab Record Log.
5. Place the Tempus RNA tube, upright in storage box, and then place into -80°C freezer.

F) Mid-Stream Urine Sample Collection.

1. Provide patient with a labelled white top urine collection pot.

2. Ask the patient to wash their hands before and after urine collection. Instruct how to collect the sample using the following steps:

   i. Remove the lid from the urine collection cup
   ii. Void a small amount of urine into toilet then stop midstream.
   iii. Continue to pass urine into the collection cup until the cup is at least half full.
   iv. Replace the lid of the urine collection cup and ensure it is screwed on tightly.
   v. Transfer urine from collection cup into yellow capped urine vaccutainer.

Local CRF:
1. Note collection time of urine sample in sample collection record log & eCRF

GBRC:
2. Note arrival time of samples in the sample collection record log.
3. Aliquot urine from 15ml tube into 3 x 1.5 ml aliquots in pre-labelled FluidX tubes (2 mL maximum capacity).
4. Seal each FluidX tube with a yellow lid and scan into the LIMS system
5. Place FluidX tubes into 42 well box, then into -80°C freezer.

8. Counting cells using haemocytometer

- Prepare the glass haemocytometer and coverslip by cleaning both with 70% alcohol. Moisten the coverslip with water and affix to the haemocytometer. The presence of Newton's refraction rings under the coverslip indicates proper adhesion
- Gently swirl the centrifuge tube containing your cells resuspended in 25ml of dPBS, to ensure that the cells are evenly distributed.
- Add 60µl of 0.4% trypan blue trypan blue to a well of a 96-well plate (round bottom)
- Add 20µl of cell suspension to the trypan blue in the 96-well plate and MIX THOROUGHLY
- Gently pipette 10µl of the cell/trypan solution into the chamber underneath the coverslip, allowing the cell suspension to be drawn out by capillary action
- Using a microscope, focus on the grid lines of the haemocytometer with a 10X objective

- Using a hand tally counter, count the live, unstained cells (live cells do not take up trypan blue) in one set of 16 squares (labelled A in figure 1). Move the haemocytometer to the next set of 16 squares (labelled B in figure 1), and then repeat for the other 2 remaining sets of 16 squares (C and D in figure 1)

- Take the average cell count from each set of 16 squares: (A+B+C+D)/4
- Multiply by 10,000 (10^4)
- Multiply by 4 to correct for the 1:4 dilution from the trypan blue addition
- The final value is the number of viable cells/ml in the original cell suspension
- Multiply this value by 25 to calculate the total number of cells in a 25ml cell suspension
- Record this in the IFT record sheet

INFLAMMATENSION

Sample Record Log

Site Name	Patient ID Number	Visit Number			
Glasgow CRF	Baseline	Visit 1	Follow up		

1. **Samples Collected: Insert either Yes/No/NA** (NA = Not Applicable at this visit)

Tube Type	Collected Yes/No/NA
SST 5ml (serum cytokines) X1 tubes 'A' Barcodes	
EDTA 6ml (plasma) x1 tube KEEP COOL 'B' Barcodes	
EDTA 10ml (FACS & ex vivo) X2 tubes 'C' Barcodes	
Sodium citrate 4ml (phospho FACS) tube 'D' Barcodes	
RNA Tempus (RNA) x1 tube 'E' Barcodes	
Urine – KEEP COOL x1 tube 'F' Barcodes	

Dispatch samples to lab immediately following collection	
Sample collection:	Date:
	Time:
Sent by: (print name)	

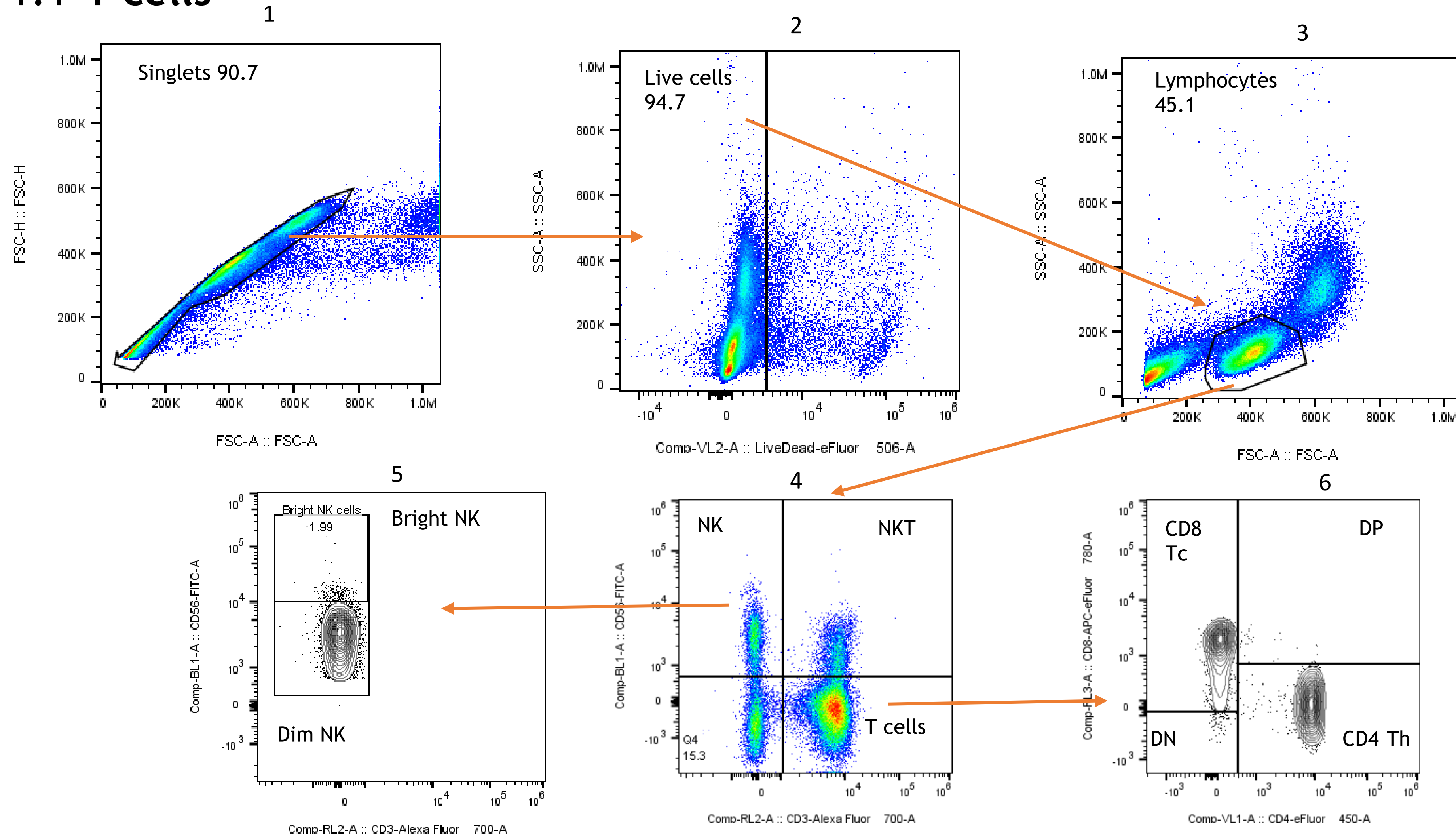
2. **Time samples received at central processing laboratory**

3. **Keep Tempus RNA sample at room temperature for 3 hours then place in -80 C**

Appendix 5: Chapter 4

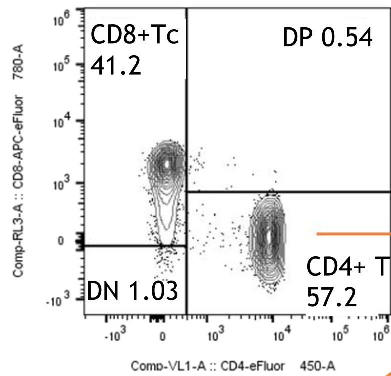
Flow cytometry cell markers and gating strategies

1.1 T cells

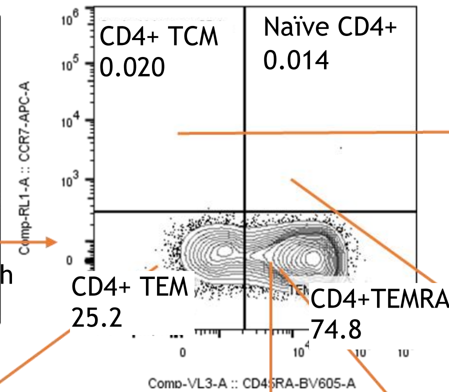


CD4+ Sub-populations

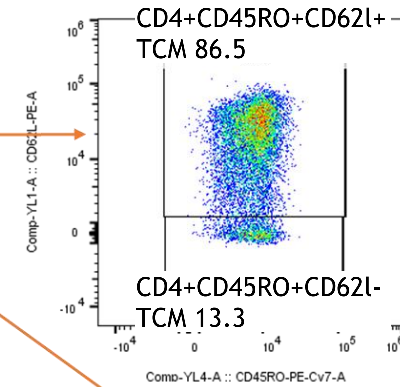
6) Under CD4+



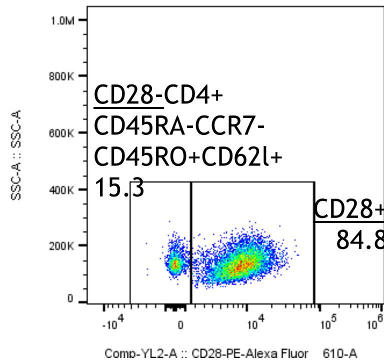
7) Use FMO tube first then copy the gate to the mix tube



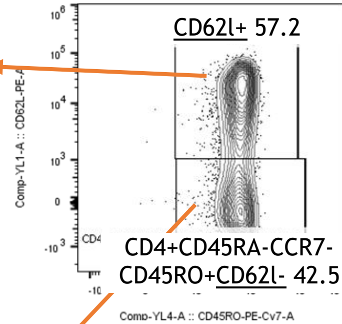
8) Under central memory



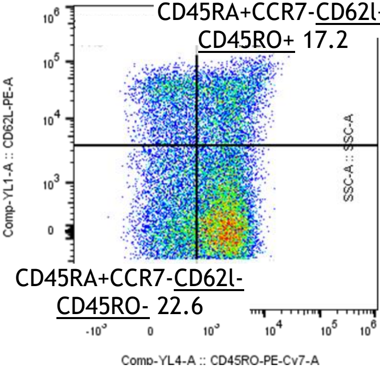
13) Effector memory



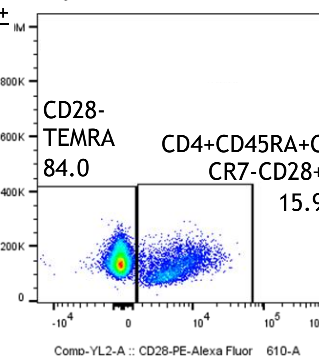
11) Under Effector memory



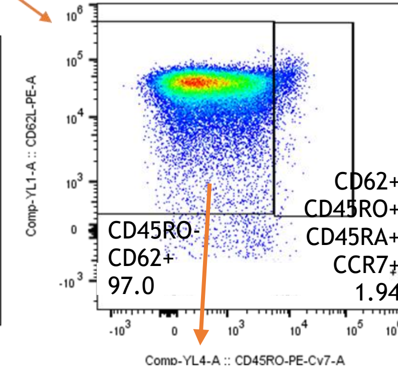
10a) Under TEMRA



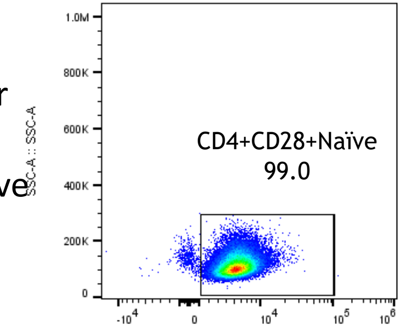
10b) Under TEMRA

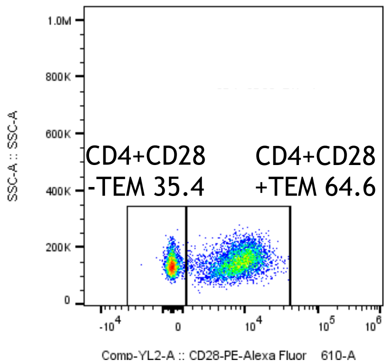


9) Under naive (CD4+)



12) Under CD45RO-CD62l+Naïve





CD8+ Sub-populations

14) Under CD8+ (step 6)

CD8+Tc 41.2

DP 0.54

DN 1.03

CD4+Th 57.2

15) FMO tube first then gating copied to the mix tube

CD8+TCM 8.33

CD8+Naive 34.3

Effector Memory CD8+ 45.2

TEMRA CD8+ 12.1

CD8+TEM 45.2

CD8+TEMRA 12.1

16) Under naive (CD8+)

CD45RO- CD62l+Naïve 89.9

CD45RO+ CD45RA+ CCR7+9.23

CD28-CD45RA- CCR7-CD45RO+ CD62+39.8

CD28+CD45RA- CCR7- CD45RO+

18) Under Effector memory

CD45RA-CCR7-CD45RO+CD62+ 41.3

CD45RA-CCR7-CD45RO+CD62- 58.4

CD8+CD28- TEM 55.2

CD8+CD28+ TEM 44.9

17a) Under TEMRA

CD45RA+CCR7- CD62l+CD45RO- 14.4

CD45RA+CCR7-CD62- CD45RO- 34.3

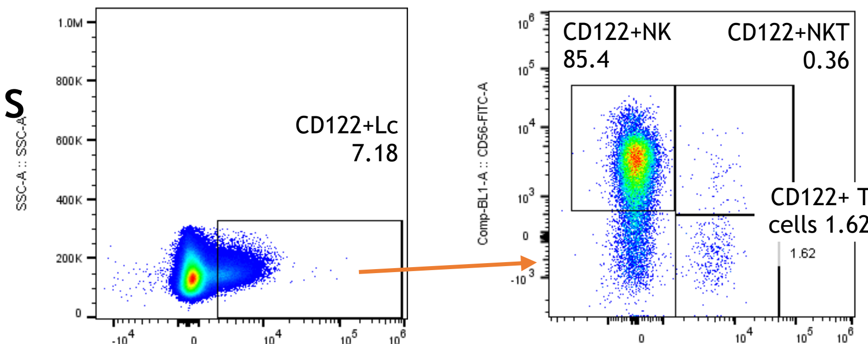
17b) Under TEMRA

CD8+CD28- TEMRA 86.6

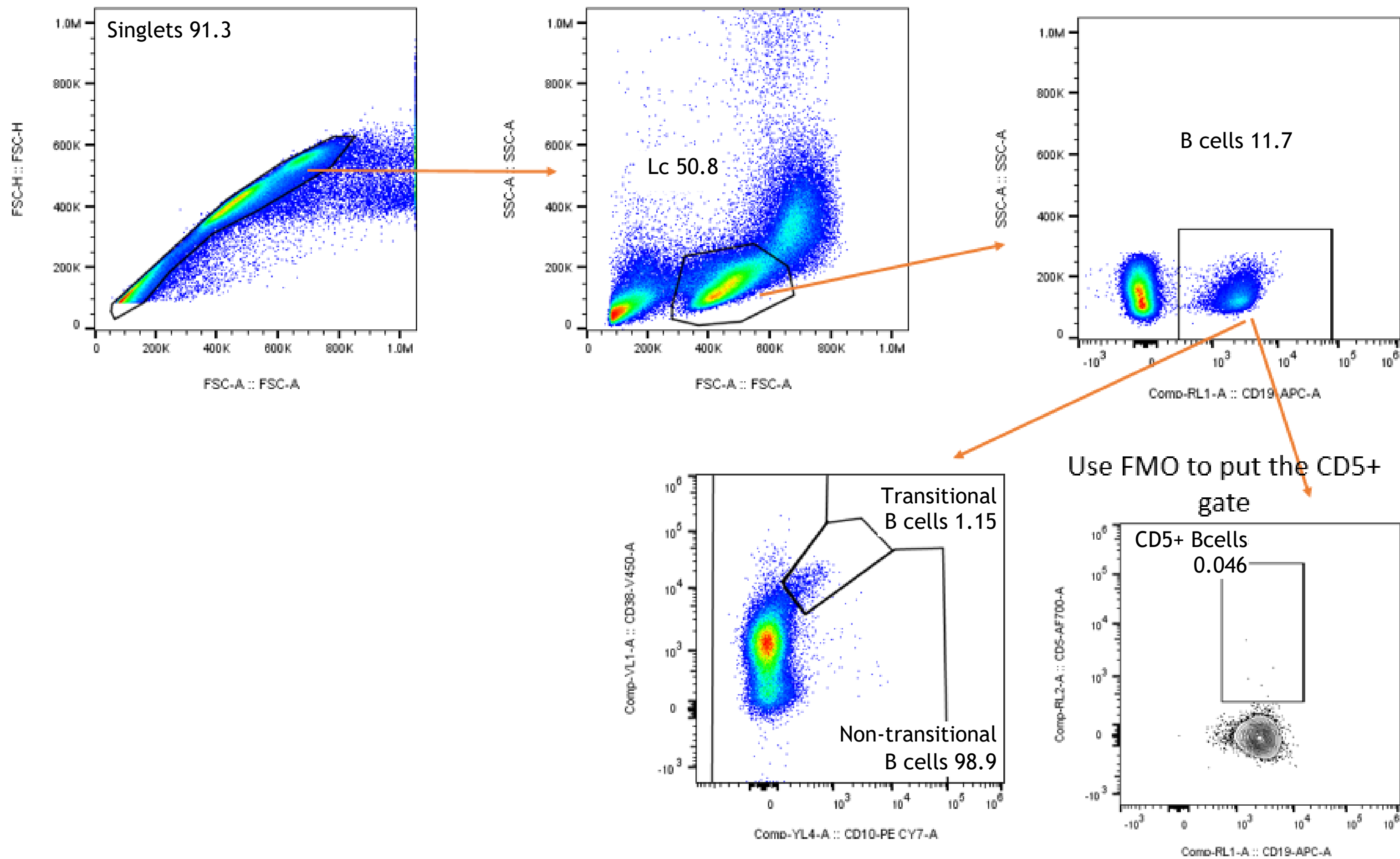
CD45RA+CCR7- CD28+ 13.5

CD122+ Sub-populations

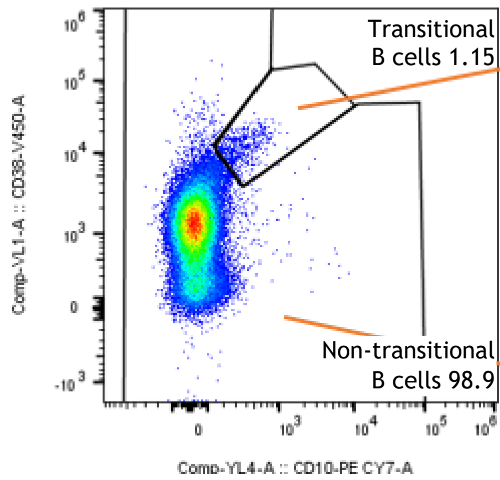
17) Under Lc step 3, use FMO to out the gate

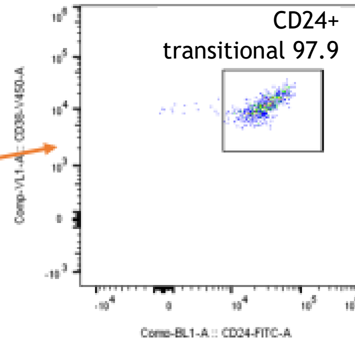


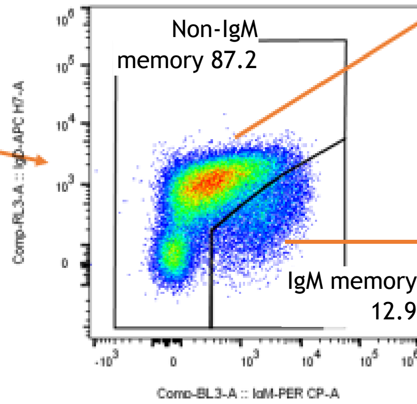
1.2 B cells



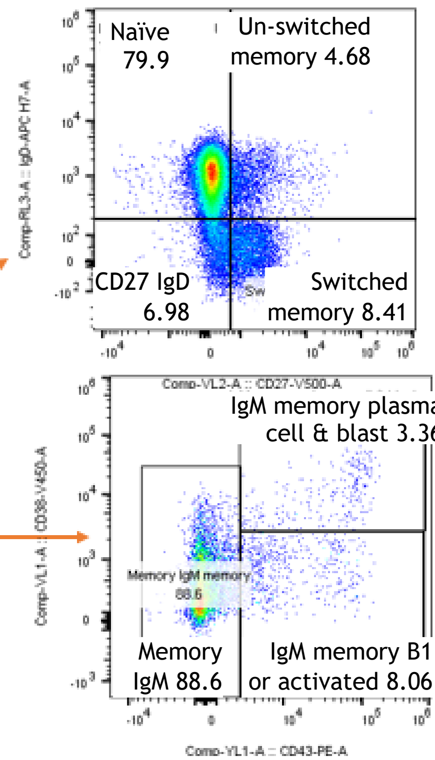
Transitional & Non-Transitional



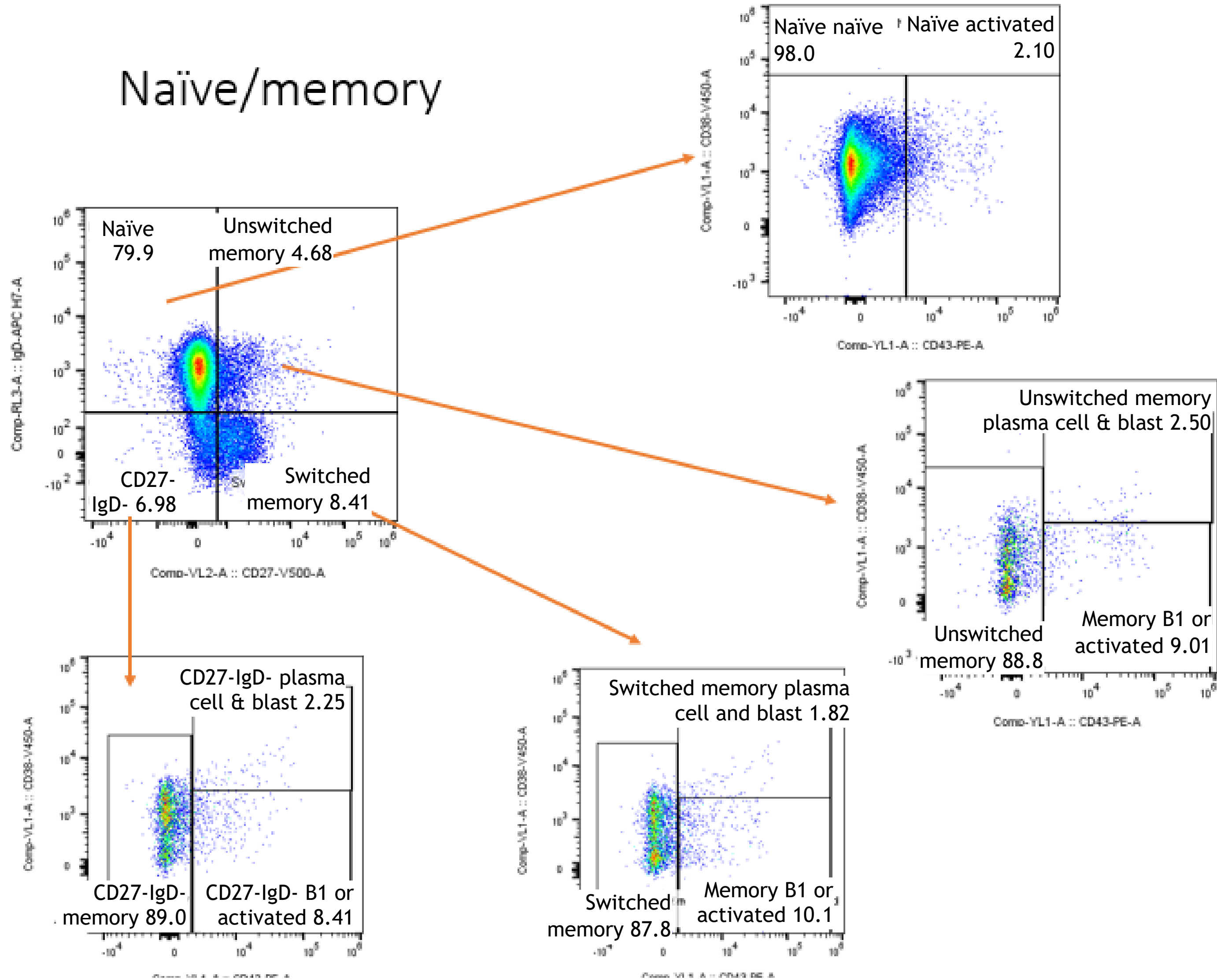




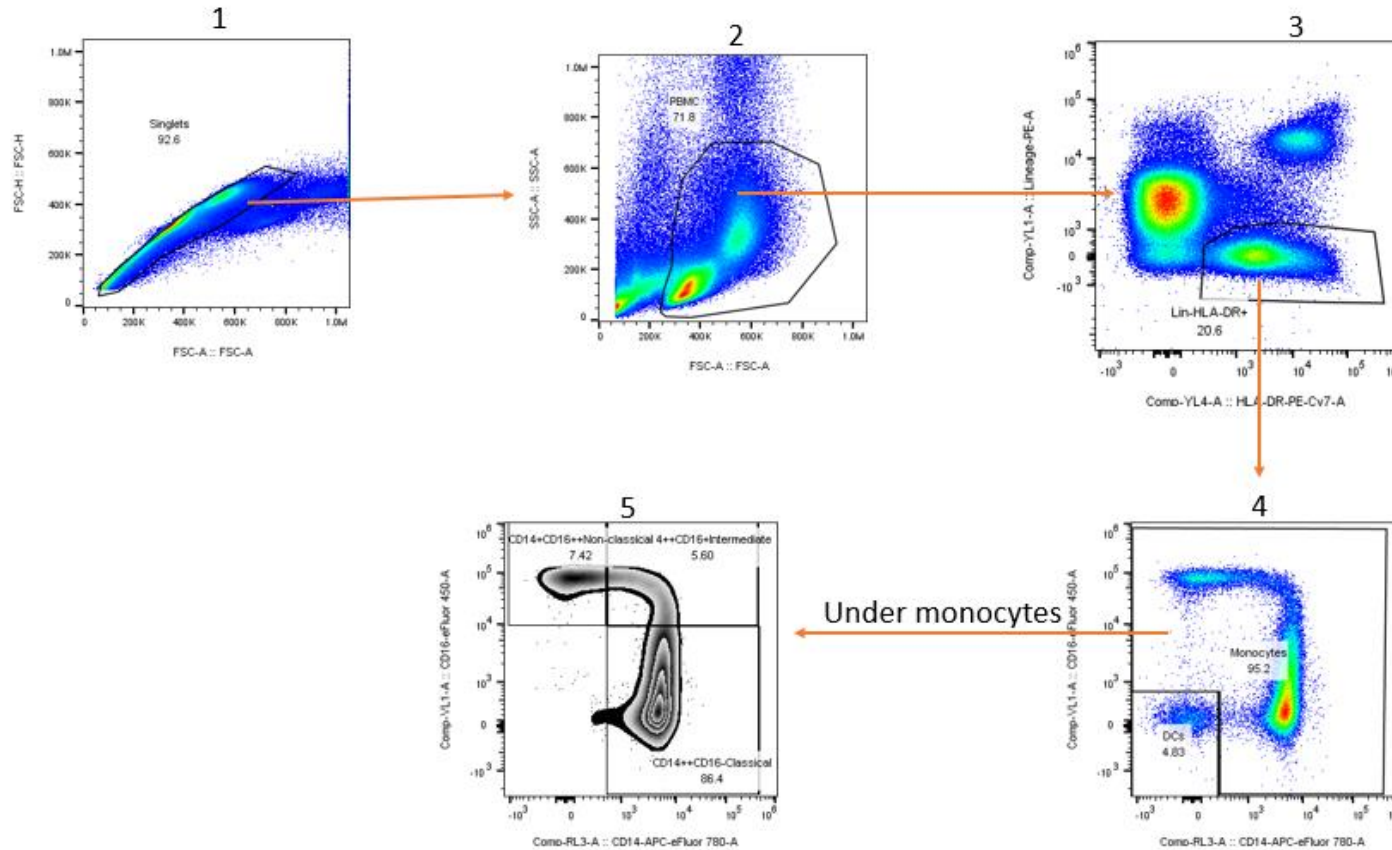
IgM memory



Naïve/memory



1.3 Monocytes

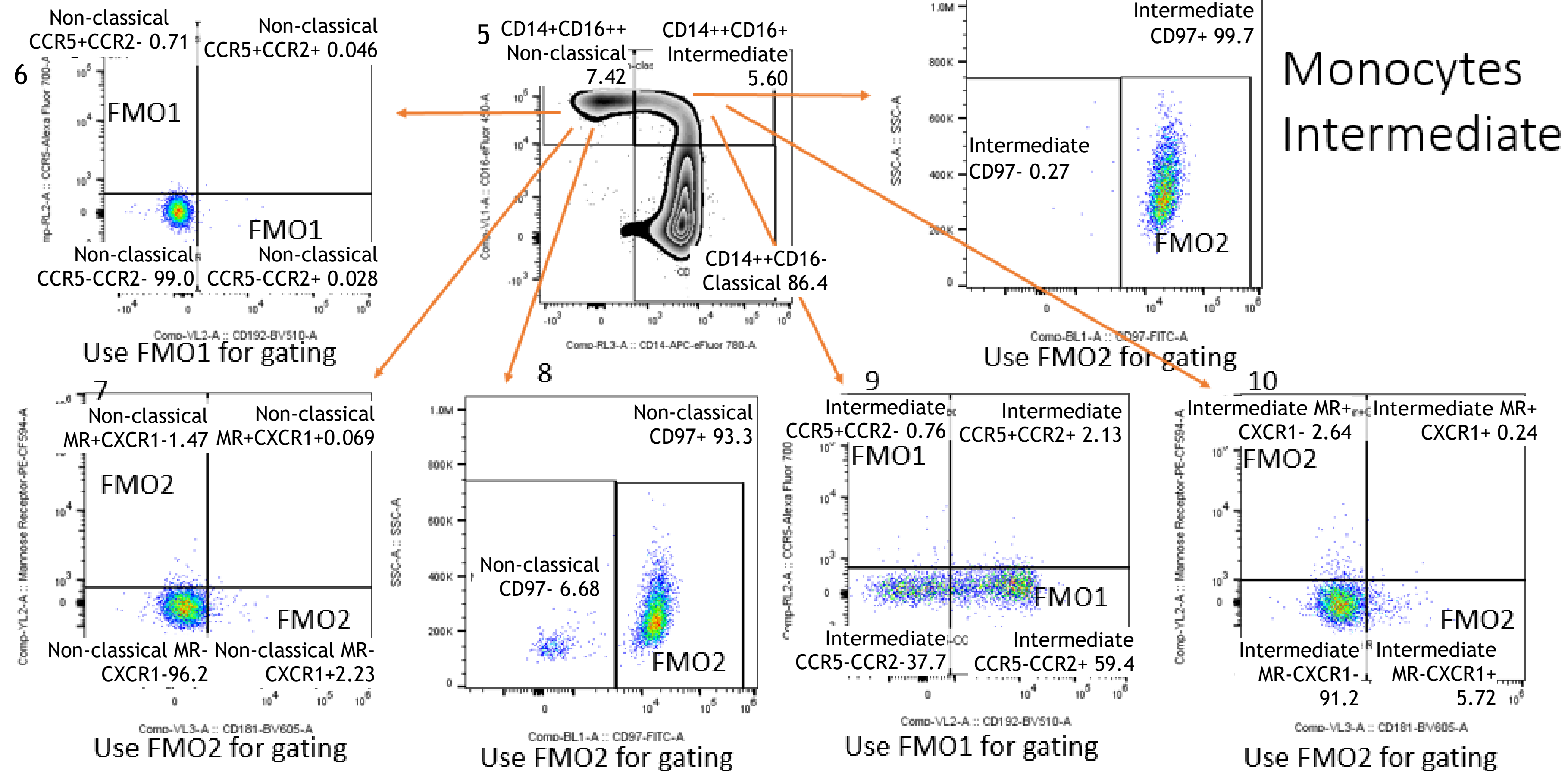


Lineage markers
CD3 T cells
CD56 NK cells
CD15 Granulocytes
CD19 B cells

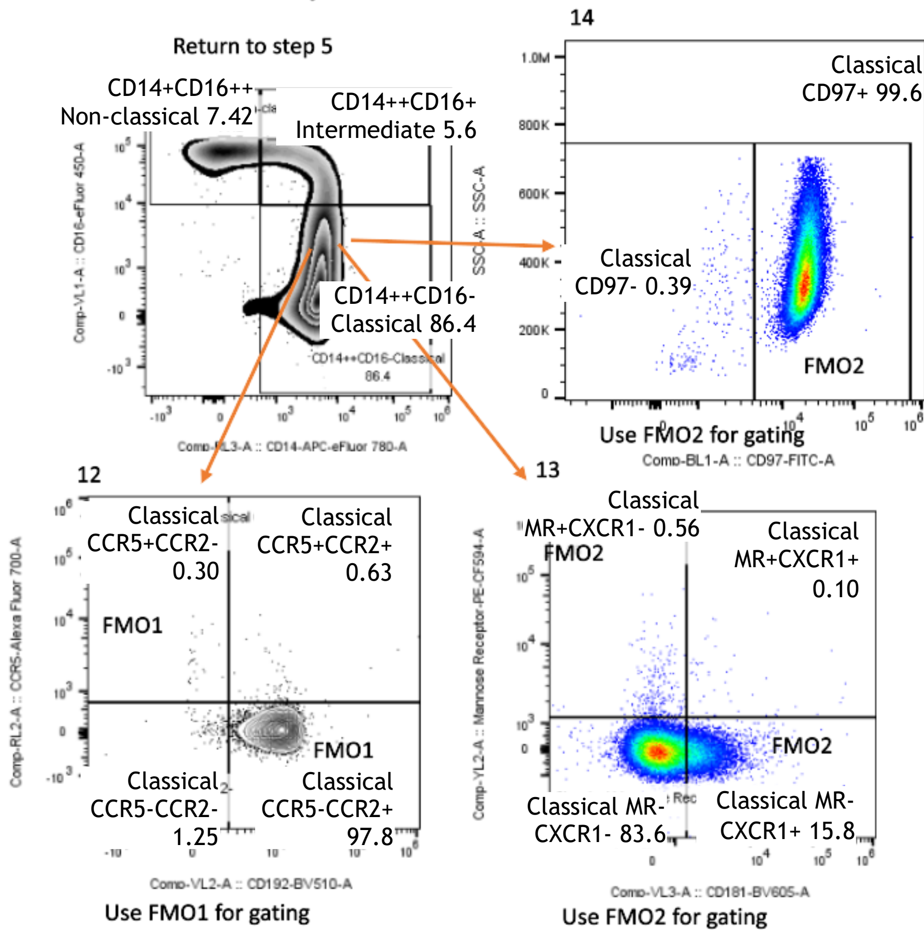
HLA-DR: MHC class II, professional antigen presenting cells (Monocyte/Macrophages, DC, B cells), Activated T cells

Lineage-HLA-DR+: Monocytes/DCs
Monocytes: CD14, CD16

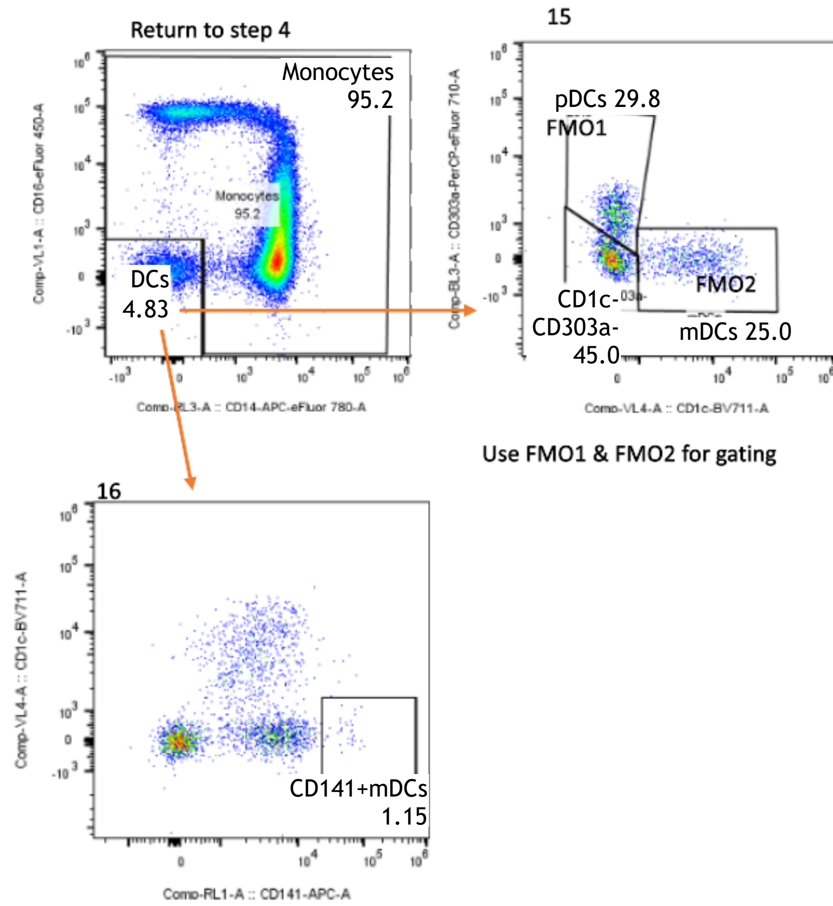
Lineage- HLA-DR+CD14-CD16-: DCs
CD303a: pDCs
CD1c: mDC

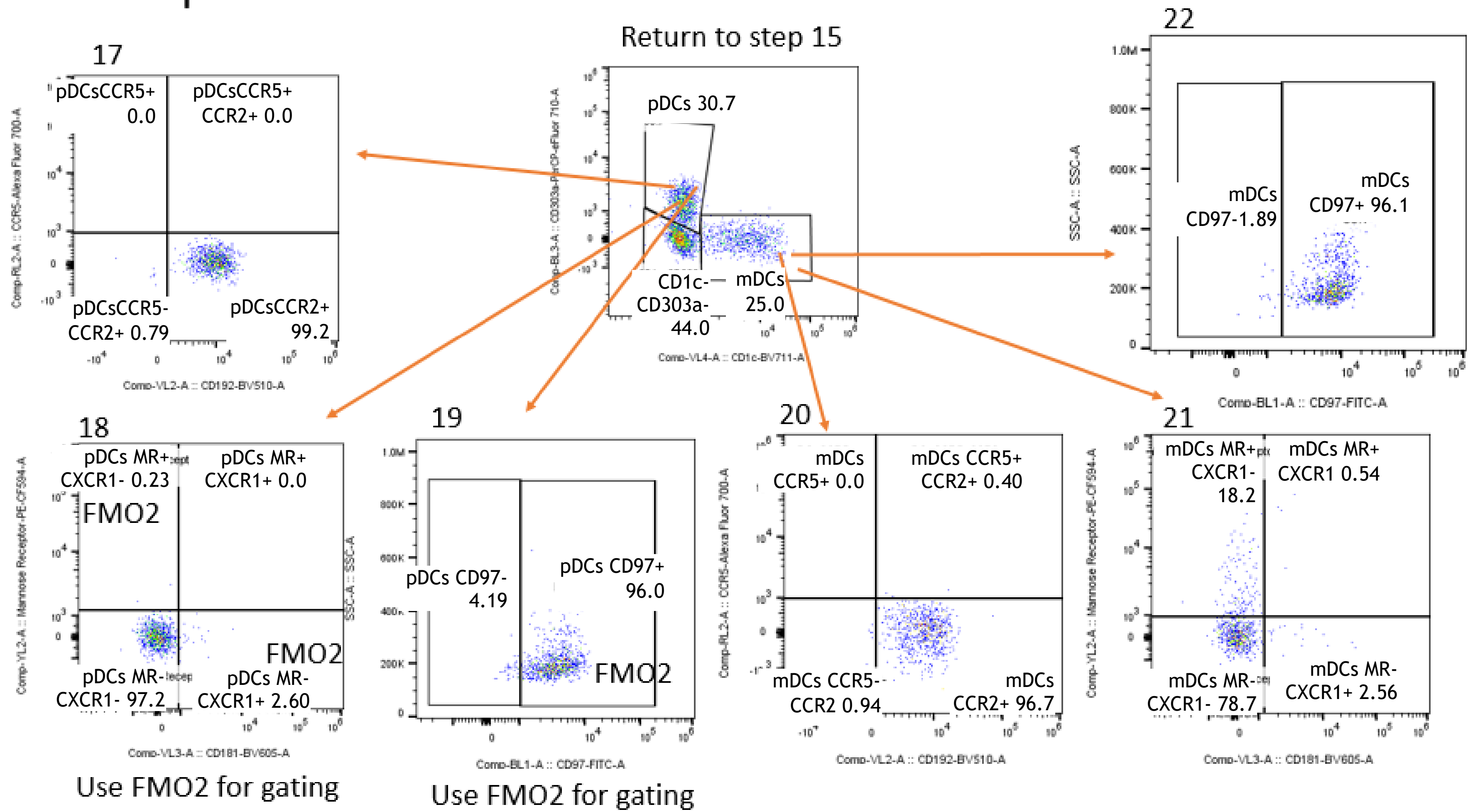

Monocytes Non-Classical
Monocytes Intermediate
5 CD14+CD16++ Non-classical 7.42
CD14++CD16+ Intermediate 5.60
CD14++CD16- Classical 86.4
6
Non-classical CCR5+CCR2- 0.71
Non-classical CCR5+CCR2+ 0.046
FMO1
Non-classical CCR5-CCR2- 99.0
Non-classical CCR5-CCR2+ 0.028
Use FMO1 for gating
7
Non-classical MR+CXCR1-1.47
Non-classical MR+CXCR1+0.069
FMO2
Non-classical MR- CXCR1-96.2
Non-classical MR- CXCR1+2.23
Use FMO2 for gating
8
Non-classical CD97+ 93.3
Non-classical CD97- 6.68
FMO2
Use FMO2 for gating
9
Intermediate CCR5+CCR2- 0.76
Intermediate CCR5+CCR2+ 2.13
FMO1
Intermediate CCR5-CCR2-37.7
Intermediate CCR5-CCR2+ 59.4
Use FMO1 for gating
10
Intermediate MR+ CXCR1- 2.64
Intermediate MR+ CXCR1+ 0.24
FMO2
Intermediate MR-CXCR1- 91.2
Intermediate MR-CXCR1+ 5.72
Use FMO2 for gating
11
Intermediate CD97+ 99.7
Intermediate CD97- 0.27
FMO2
Use FMO2 for gating

Monocytes Classical



DCs

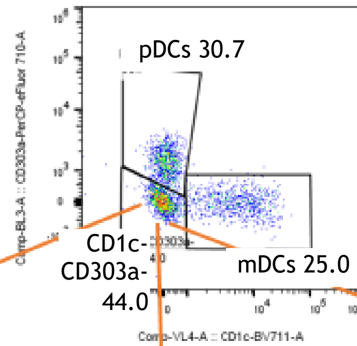




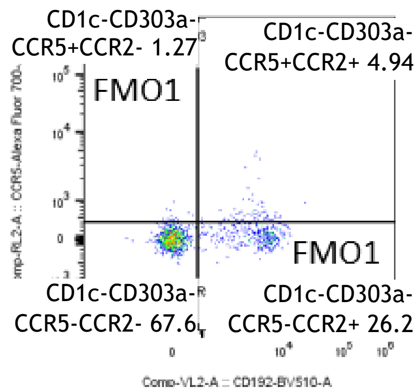
pDCs
Return to step 15
pDCs 30.7
CD1c-
CD303a-
44.0
mDCs
25.0
17
pDCsCCR5+
0.0
pDCsCCR5+
CCR2+ 0.0
pDCsCCR5-
CCR2+ 0.79
pDCsCCR2+
99.2
18
pDCs MR+
CXCR1- 0.23
pDCs MR+
CXCR1+ 0.0
FMO2
pDCs MR-
CXCR1- 97.2
pDCs MR-
CXCR1+ 2.60
FMO2
19
pDCs CD97-
4.19
pDCs CD97+
96.0
FMO2
20
mDCs
CCR5+ 0.0
mDCs CCR5+
CCR2+ 0.40
mDCs CCR5-
CCR2 0.94
mDCs
CCR2+ 96.7
21
mDCs MR+
CXCR1-
18.2
mDCs MR+
CXCR1 0.54
mDCs MR-
CXCR1- 78.7
mDCs MR-
CXCR1+ 2.56
22
mDCs
CD97-1.89
mDCs
CD97+ 96.1
Use FMO2 for gating
Use FMO2 for gating

CD1c-CD303a-DCs

Return to step 15

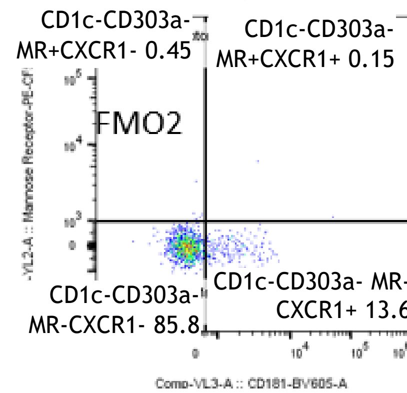


23



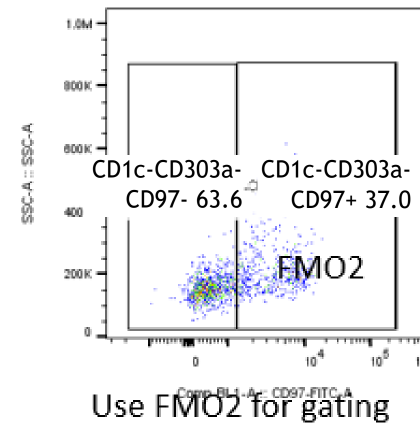
Use FMO1 for gating

24



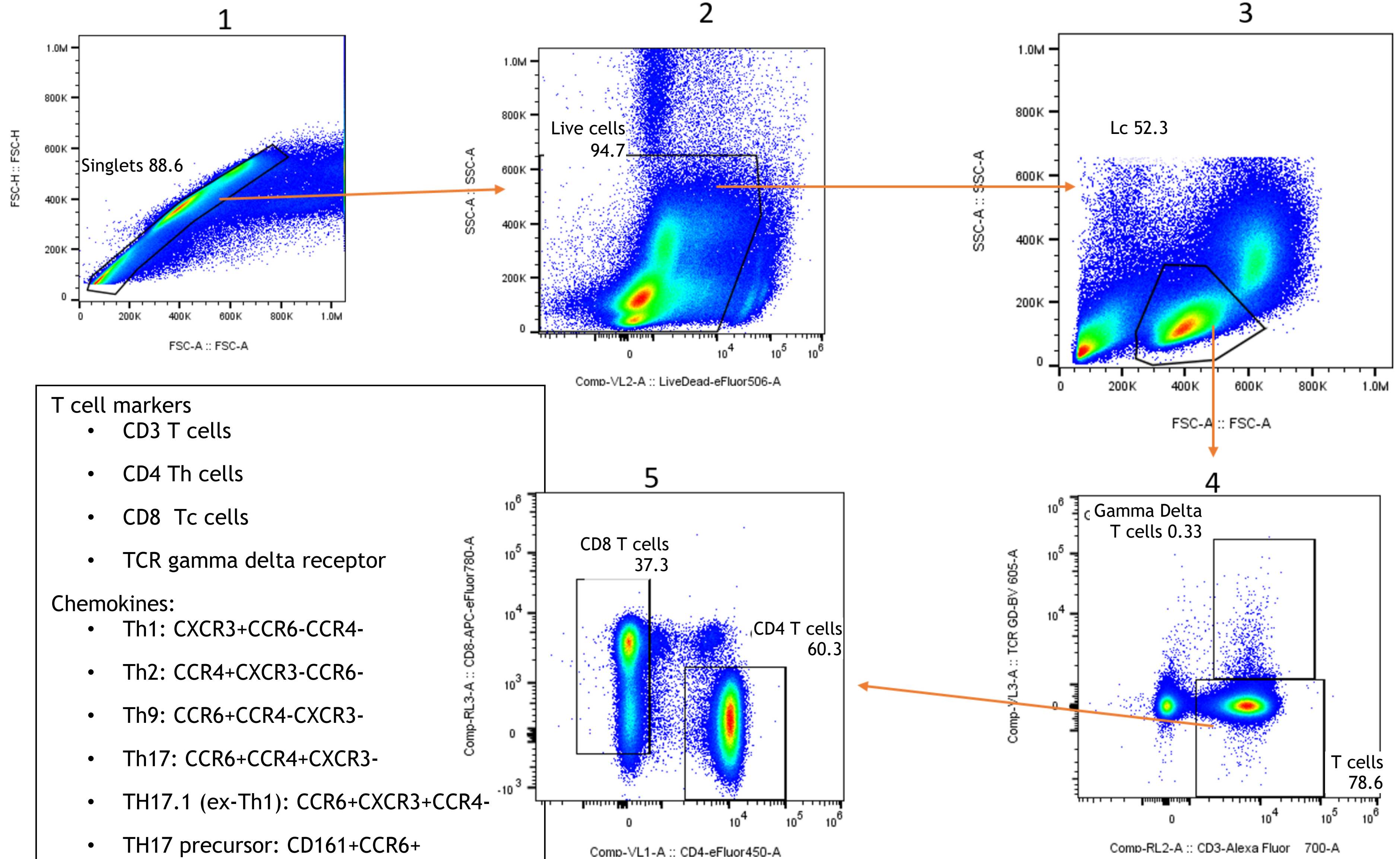
Use FMO2 for gating

25

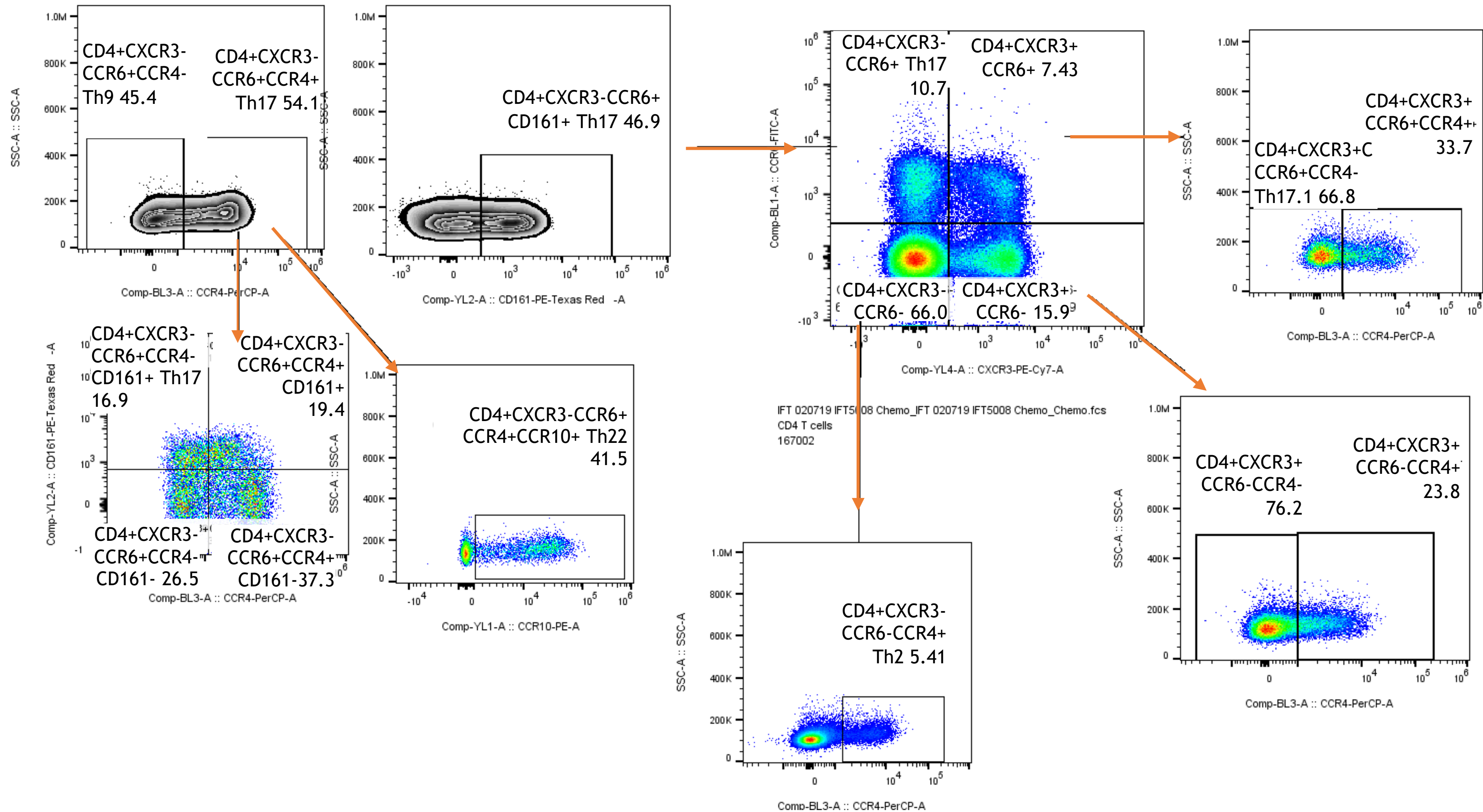


Use FMO2 for gating

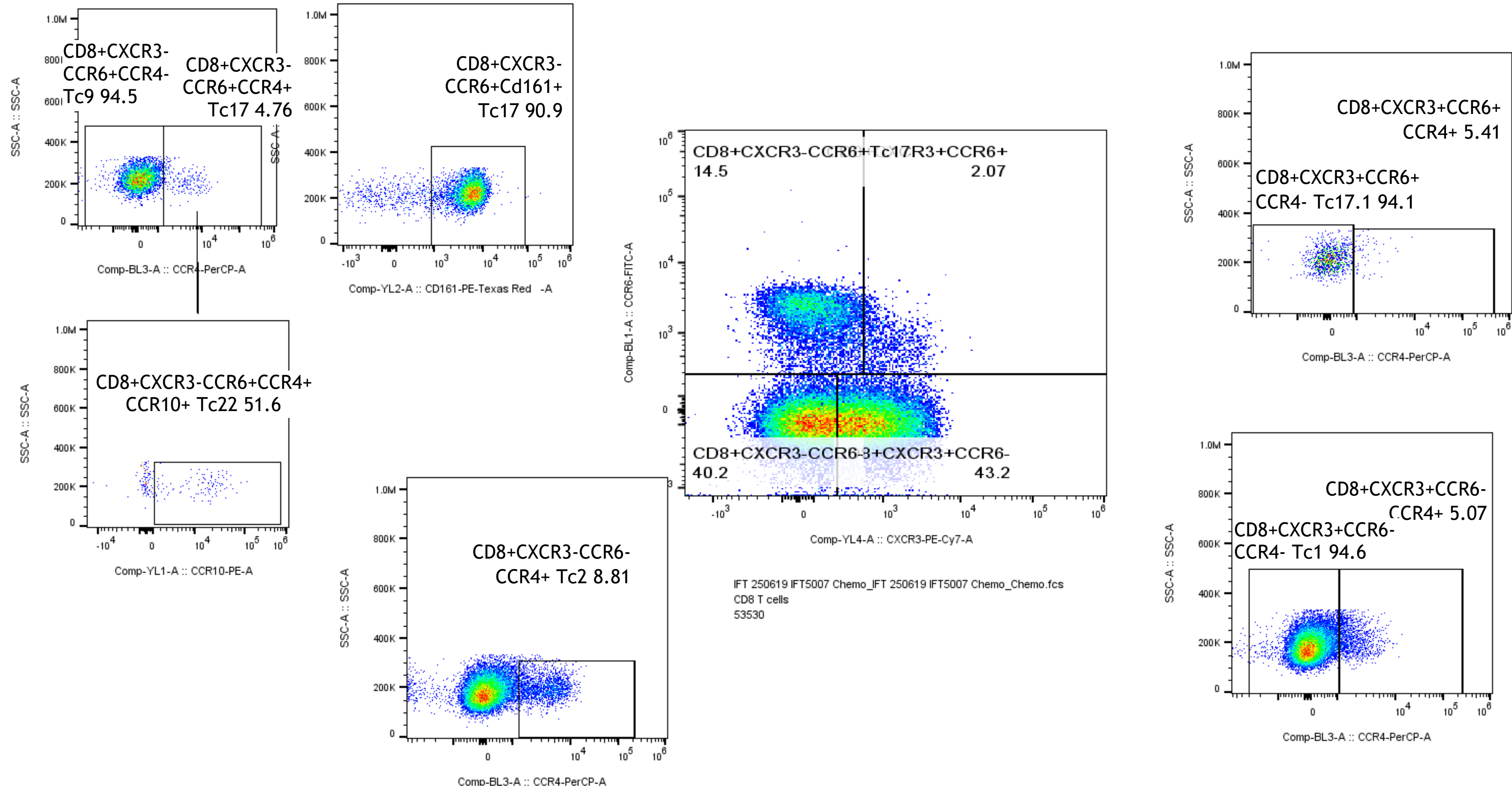
1.4 Chemokine subsets



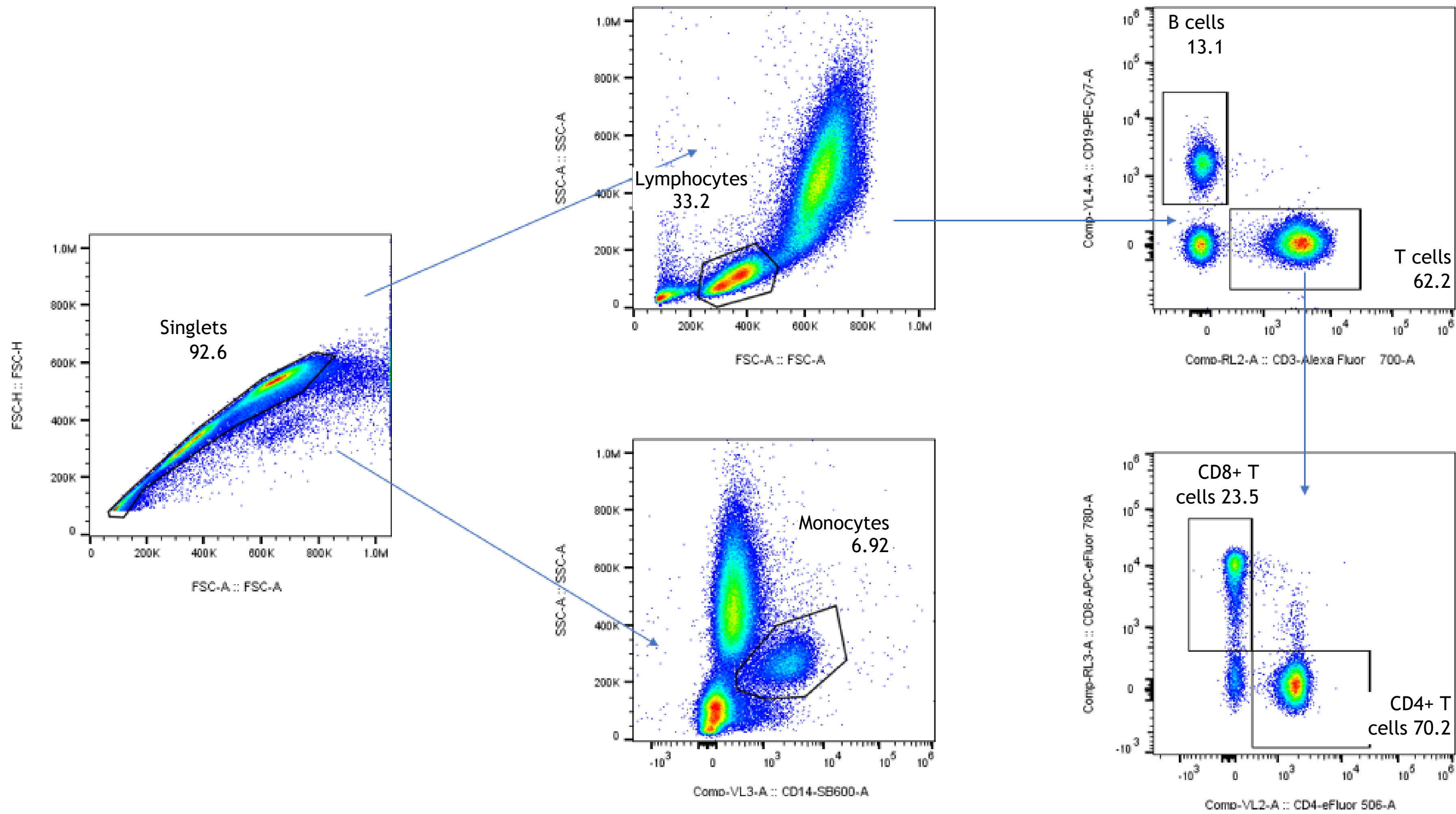
1.4.1 CD4+ T cell chemokine gating



1.4.2 CD8+ T cell chemokine gating



1.5 Lineage (PhosphoFACS)



Appendix 6. Full list of proteins included in the Olink® Inflammation panel, split by cluster group.

Cluster 0	Cluster 1	Cluster 2	Cluster 3	Cluster 4
AOC1	LAP3	TNFAIP8	LRRN1	CXCL14
IL10	PRKAB1	NBN	SCGN	NFATC3
ARNT	RABGAP1L	SIT1	WNT9A	TNF
LTO1	MYO9B	GBP2	IL3RA	IL12RB1
ACTN4	MVK	ENAH	CXCL12	CCL3
AMBN	FXYD5	RAB37	GALNT3	SULT2A1
PREB	DGKZ	PARP1	TNFRSF13C	CCL7
IL24	PRKCQ	SH2D1A	IL17D	FCRL6
CEP164	TANK	LSP1	MILR1	ISM1
FCRL3	SPRY2	PSIP1	FGF5	SLC39A5
PNPT1	YTHDF3	BTN3A2	ITGB6	MLN
ARTN	WAS	PROK1	IL15RA	CLEC4C
NCLN	PRDX3	PSMG3	LILRB4	CXADR
IL22RA1	ICA1	FCAR	CLEC4D	CD83
IL2RB	IRAK1	CCL28	DPP10	NPPC
IL20	IL1B	EGLN1	HLA-E	LY75
RGS8	EDAR	PIK3AP1	SIGLEC10	CD4
IL11	SRPK2	GZMB	COL9A1	LTA
IL17F	FGF2	RAB6A	CNTNAP2	NTF3
NRTN	BACH1	CCL13	NCR1	CD200R1
PADI2	TRAF2	AGRP	PTX3	PTPRM
IL17A	NUB1	GZMA	PTH1R	SLAMF7
IL5	BCL2L11	CXCL6	CLEC4A	IL32
TPT1	FOXO1	TNFSF12	HSD11B1	HGF
IL17C	VASH1	SERPINB8	IL4R	CTSO
IL1RL2	BID	CXCL8	ITGA11	TNFSF10
IL1A	METAP1D	IL16	IL17RB	IDS
CSF3	TBC1D5	ADA	KRT19	CXCL17
AMN	DAPP1	VEGFA	CD70	BSG
JCHAIN	TRIM5	KYNU	TGFA	KLRB1
IFNG	ITGA6	MMP1	CD200	KLRD1
SLAMF1	STX8	ANGPT1	LIFR	NFASC
IL2	TRIM21	DNPH1	C1QA	CD79B
IL10RA	IL7	CCL17	MERTK	CD22
PAPPA	SAMD9L	LHPP	VEGFD	LAMP3
IL20RA	SCRN1	TPP1	CKAP4	FCRL2
EIF5A	IKBKG	CRELD2	TPSAB1	TNFRSF11A
JUN	NFATC1	CXCL1	CD160	CD6
IL4	GOPC	LGMN	ADAM23	IL18R1
IL33	MAP2K6	CTSC	FASLG	TNFRSF4

Cluster 0	Cluster 1	Cluster 2	Cluster 3	Cluster 4
IL13	NT5C3A	TIMP3	PGF	IL12B
FABP9	ARHGEF12	IL18	COLEC12	CCL11
ITM2A	CASP2	CST7	SIRPB1	LY9
IFNLR1	MAPK9	CCN2	MEGF10	ENPP5
SPINK4	BCR		MEPE	LAIR1
PSPN	CLIP2		ADGRE2	CRHBP
ALDH3A1	IRAK4		AGER	LGALS4
HLA-DRA	DECR1		ESM1	CTRC
EPO	AXIN1		PLAUR	CDON
CCL26	ANXA11		PRELP	IL1R2
CEACAM21	MGMT		LTBR	SPON1
SELPLG	HEXIM1		CHRDL1	PRSS8
IL15	BANK1		CCL24	CCL22
IL6	PLXNA4		OSCAR	MATN2
NCF2	CD84		PON3	LAMA4
OSM	HPCAL1		B4GALT1	FST
ICAM4	CD40LG		WFIKKN2	FABP1
CLEC4G	FKBP1B		TFF2	LGALS9
TNFSF11	DFFA		IFNGR1	FSTL3
MICB_MICA	TGFB1		CD276	REG4
IL5RA	NUDC		CCL21	AGRN
FLT3LG	EIF4G1		MMP10	NME3
TLR3	DNAJA2		SCGB1A1	CXCL9
PCDH1	CD244		CCL23	SMPDL3A
CLEC7A	LAT		CRLF1	EPHA1
CLSTN2	GMPR		CRIM1	ENPP7
CDSN	PPP1R9B		ANGPTL4	SIGLEC1
CCL20	PRDX5		NELL2	CD48
CCL25	HCLS1		LY6D	CCL4
EPCAM	PDGFB		DNER	IL10RB
PNLIPRP2	CRKL		BTN2A1	CXCL10
PKLR	ATP5IF1		CD58	IL1RN
FGF19	SKAP2		SCGB3A2	TNFRSF13B
CKMT1A_CKMT1B	DAG1		OMD	ERBB3
MZB1	MPIG6B		GAL	CSF1
	TNFRSF14		SCG3	ANGPTL2
	CXCL3		TNFSF13	SMOC2
	DBNL		TNFRSF11B	TREM2
	NCK2			ROBO1
	PLA2G4A			
	F2R			
	EGF			
	MANF			
	GLOD4			
	MGLL			
	SHMT1			
	PTPN6			
	HSPA1A			

Cluster 0	Cluster 1	Cluster 2	Cluster 3	Cluster 4
	SPINT2			
	FIS1			
	CD40			
	PDLIM7			