The Role of Inflammatory Cytokines in Endothelial Dysfunction in Hypertension

Malvika Sharma

MTech, BTech

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School of Medicine, Dentistry and Nursing

College of Medical, Veterinary, and Life Sciences

University of Glasgow

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Abstract

Hypertension is a pervasive cardiovascular disorder characterized by increased blood pressure levels. It is a global cause of morbidity and mortality and poses a heightened risk of serious cardiovascular complications. Although the role of kidney, vasculature, heart, and brain has been defined before, recent research has illuminated the pivotal role of immune mechanisms and inflammation in the development and progression of hypertension.

To delve into the intricate play of immune mechanisms in the development of hypertension, this thesis aims to identify the role of inflammatory cytokines in endothelial dysfunction in hypertension. Through a multifaceted approach, we pursued three primary aims of identifying association between inflammatory cytokines and incident hypertension in patient populations, assessing their direct vascular effects in mouse blood vessels and probing potential mechanisms of endothelial dysfunction.

To identify inflammatory cytokines potentially contributing to the incidence of hypertension, we ran systematic searches in Medline, Embase, Web of Science and Cochrane CENTRAL. After screening for inclusion and exclusion criteria, we performed meta-analysis of the relationships between the levels of identified cytokines from six records and incident hypertension. We have then undertaken mechanistic studies of the endothelium dependent and independent effects of selected cytokines on vasomotor functions in mouse aortas. Thoracic aortas from 3-month-old C57BL6/J mice were treated with selected cytokine concentrations overnight and endothelium dependent and independent vasoconstriction and relaxation impairment was observed. We further selected two cytokines to study the potential mechanism of endothelial dysfunction by looking at eNOS and ERK phosphorylation in human aortic endothelial cells. The cells were treated with varying cytokine concentrations for different time periods and the differences in phosphorylation were observed in the cell homogenate using western blotting.

Through our systematic searches we selected fifteen records after screening and identified IL-6, IL-1β, TNF-α, IL-17a, and IFN-γ among other cytokines and meta-analysed data available from six records. We found that IL-6 is significantly associated with the risk of developing hypertension in crude models with high

heterogeneity. In organ bath studies significant impairment was observed in vessels treated with selected cytokines. Endothelium-dependent relaxation observed using acetylcholine was significantly altered by IL-1β (1 ng/ml) after pre-constricting the vessels with phenylephrine and serotonin ($P<0.001$ and $P=0.04$, respectively). Similar alteration of vasorelaxations was observed with IFN-γ (10 ng/ml) after constricting the vessels with phenylephrine ($P=0.0099$) and with IL-6 (5 ng/ml) after constricting the vessels with serotonin ($P=0.014$). Furthermore, western blot results demonstrated differences in eNOS and ERK phosphorylation.

We found that IL-6 is positively associated with incidence of hypertension, but this association weakens with adjusting for BMI implying a relationship between inflammation and adiposity linked hypertension. Significant impairment of endothelial function induced by IL-1β, IFN-γ and IL-6 suggests a direct link between endothelial dysfunction and inflammation. Further studies are needed to substantiate the results in identifying the mechanisms of inflammatory cytokines in endothelial dysfunction and understand their significance for patho-mechanisms of hypertension.

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1. Caiazzo, E., **Sharma, M.**, Rezig, A. O., Morsy, M. I., Czesnikiewicz-Guzik, M., Ialenti, A., ... & Guzik, T. J. (2023). Circulating cytokines and risk of developing hypertension: a systematic review and meta-analysis. Pharmacological Research, 107050.

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Author's Declaration

I declare that, except where reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Name: Malvika Sharma

Signature:

Date: 14.03.2024

Definitions/Abbreviations

5-HT	5-hydroxytryptamine (Serotonin)
α	Alpha
ß	Beta
γ	Gamma
δ	Delta
κ	Kappa
ACE	Angiotensin converting enzyme
ACEIs	Angiotensin-converting enzyme inhibitors
ACh	Acetylcholine
Ang	Angiotensin
APS	Ammonium persulphate
ARB	Angiotensin receptor blocker
ART	Antiretroviral therapy
BH4	Tetrahydrobiopterin
BMI	Body mass index
CENTRAL	Cochrane Central Register of Controlled Trials
CD	Cluster of differentiation
CI	Confidence interval

CKD	Chronic kidney disease
CRP	C reactive protein
Ct	Cycle threshold
CTLA	Cytotoxic T lymphocyte antigen
CVD	Cardiovascular disease
DPBS	Dulbecco’s phosphate buffer saline
DUOX	Dual oxidase
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
ESRD	End stage renal disease
ET-1	Endothelin-1
FBS	Fetal bovine serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association studies
HAoEC	Human aortic endothelial cells
HIV	Human Immunodeficiency Virus
HK	Hartung and Knapp
HMOD	Hypertension modulated organ damage

HR	Hazard ratio
hsCRP	High sensitivity C reactive protein
HUVEC	Human umbilical vein endothelial cells
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
iNK	Invariant natural killer
IQR	Interquartile range
JAK	Janus kinase
KPSS	Potassium physiological saline solution
M1	Macrophage 1
M2	Macrophage 2
MAP	Mitogen activated protein
MDSC	Myeloid derived suppressor cells
MESA	Multi-Ethnic Study of Atherosclerosis
MeSH	Medical Subject Headings
mTOR	Mechanistic target of Rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate

NF-κB	Nuclear factor kappa B
NK	Natural killer
NO	Nitric oxide
NOX	NADPH oxidase
$O2^-$	superoxide ion
OR	Odds ratio
p-38	Protein-38
Phe	Phenylephrine
RAAS	Renin angiotensin aldosterone system
RIPA	Radioimmunoprecipitation assay
ROBINS-E	Risk of bias in non-randomized studies of exposures
ROS	Reactive oxygen species
RPMI	Rosewell Park Memorial Institute
RR	Risk ratio
S1177	Serine 1177
SDS	Sodium dodecyl sulphate
STAT	Signal transducers and activators of transcription
SNP	Sodium nitroprusside
SNP	Single nucleotide polymorphism

SNS	Sympathetic nervous system
START	Strategic Timing of AntiRetroviral Treatment
T495	Threonine 495
TBP	TATA binding protein
TEMED	N,N,N′,N′ -Tetramethyl ethylenediamine
TGF	Transforming growth factor
Th	T helper
TNF	Tumour necrosis factor
TNFR	TNF receptor
Treg	T regulatory
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
WHI-OS	Women's Health Initiative-Observational Study
WESDR	Wisconsin Epidemiological Study of Diabetic Retinopathy

Chapter 1
Introduction

1.1 Hypertension

Initially known as the hard pulse disease (Esunge, 1991), hypertension is characterised by consistently elevated blood pressure in the vasculature. According to international guidelines (Mancia Chairperson et al., 2023, Unger et al., 2020), when repeated office measurements of systolic blood pressure and diastolic blood pressure is chronically over 140/90 mmHg, a person is termed to be hypertensive. Hypertension is classified into stages based on blood pressure levels as well as on stages based on the development of complications - such as chronic kidney disease (CKD), cardiovascular complications and hypertension modulated organ damage (HMOD).

The normal range for blood pressure is when systolic blood pressure is 120-129 mmHg and diastolic pressure is from 80-84 mmHg. A person is said to be pre-hypertensive when the systolic and diastolic blood pressure ranges from 130-139 mmHg and 84-90 mmHg, respectively. In Grade I hypertension, the systolic blood pressure ranges from 140-159 mmHg and diastolic pressure ranges from 90-99 mmHg. Grade II is when systolic pressure is 160-179 mmHg and diastolic blood pressure is 100-109 mmHg. Grade III hypertension is classified as blood pressure ≥180/110 mmHg (Mancia Chairperson et al., 2023).

1.1.1 Epidemiology of hypertension

The current world population is more than 8 billion, out of which at least 1.28 billion people between the ages of 30 and 80 suffer from hypertension (World Health Organisation, 2023). According to the World Health Organisation, around 46% of patients are unaware of their hypertensive condition, nearly 42% have controlled hypertension and approximately 66% live in low and middle income countries (World Health Organisation, 2023). This disparity also indicated the difference in level of awareness and available treatment in low and middle income countries when compared with higher income counties (Chow et al., 2013). Age and sex also play a part in the prevalence of hypertension with more biological males suffering from the disease than biological females worldwide (Zhou et al., 2021). More biological males develop hypertension in younger ages than biological females (Gillis and Sullivan, 2016), and the prevalence in biological females increases with increasing age, menopause being a milestone

in the development of hypertension over the age of 50 (Ji et al., 2020). Age is also a factor in increasing the pulse pressure in hypertensive patients (Benetos et al., 2019). In a 135-population based study, out of 0.97 million adults in ninety countries, it was estimated that women had lower prevalence of hypertension in higher income countries and highest in the Sub-Saharan region of Africa. The men, however, had lower prevalence in South Asia, and highest in Eastern Europe and Central Asia (Mills et al., 2016). In another study conducted in 2013, when comparing the rural and urban populations in different countries, rural residents in high income countries had higher prevalence of hypertension and lower prevalence in low income countries (Chow et al., 2013). These differences might likely occur due to and be influenced by risk factors for hypertension like diet, lifestyle, comorbidities and physical activity levels in different regions of the world (Ibrahim and Damasceno, 2012). While comparing socio-economic status and its association with the prevalence of hypertension, a study published in 2022 found that there was nearly 50% more prevalence of hypertension in unemployed or retired subjects indicating the need for focus on socio-economic statuses while developing strategies to prevent or manage hypertension (Qin et al., 2022).

Elevated blood pressure leading to hypertension is also associated with cardiovascular events and a serious cause of premature death worldwide. Increase in systolic and/or diastolic blood pressure leads to increase in development of cardiovascular diseases like ischemic heart disease, ischemic stroke, arrythmia, heart failure and result in hypertension induced cardiovascular event related deaths (Bundy et al., 2017, Bundy et al., 2018). Hypertension is also a risk factor for CKD and end stage renal disease (ESRD) (Anderson et al., 2015). Increase in systolic blood pressure levels has been associated with increase in CKD and ESRD over the years in thousands of patients. During the Multiple Risk Factor Intervention Trial, deaths occurring because of cardiovascular events were observed in men over a 11.6 year follow-up period (Stamler et al., 1993). Over 16 years, men who were free of ESRD had developed the disease, which was associated with increase in systolic blood pressure (Klag et al., 1996). Mills and colleagues addressed the prevalence of hypertension in the world based on economic status of the countries, lifestyle differences and varied eating habits, and how elevated blood pressure levels are

linearly associated with cardiovascular diseases (CVD) and CKD. They also discussed hypertension as a public health challenge and a fast-rising global cause of morbidity and mortality (Mills et al., 2020).

1.1.2 Aetiology of hypertension

Primary hypertension, widely known as essential hypertension, is when a healthy person experiences elevated blood pressure and its specific underlying cause is unidentified. Secondary hypertension is when patients develop hypertension but the cause is identifiable, potentially reversible, and mostly a consequence of another disease or disorder (2022). A majority of hypertensive patients suffer from essential hypertension and around 10-15% suffer from secondary hypertension. There are many clinical indicators that might suggest the development of secondary hypertension. The most common cause of secondary hypertension are renal parenchymal diseases like chronic nephritis, diabetic nephropathy, polycystic kidney disease and renal artery stenosis. Endocrine causes include adrenal disorders such as Cushing's syndrome and primary aldosteronism. Other endocrine causes are said to be hyperthyroidism and hypothyroidism. Cardiovascular disorders like coarctation of aorta, neurological issues such as increased intracranial pressure, other causes like obstructed sleep apnoea, pre-eclampsia can also be a cause of secondary hypertension (2022).

Various risk factors contribute to the development of hypertension. As mentioned earlier, age affect the prevalence of hypertension in both men and women (Ji et al., 2020, Zhou et al., 2021). Race and ethnicity also play a factor in posing a higher risk for the development of hypertension. People of African-American origin have the highest prevalence of hypertension in the world (Al Kibria, 2019) whereas in the United States of America, Hispanics have the lowest prevalence, with non-Hispanic Asians and non-Hispanic Whites having higher prevalence than the Hispanic community (Deere and Ferdinand, 2020).While another study found that non-Hispanic Black people developed hypertension earlier in life than non-Hispanic Whites (Aggarwal et al., 2021).

Lifestyle factors such as sodium and potassium intake, alcohol consumption, smoking, eating habits and obesity can be contributing risk factors in the development of hypertension. A study published in 2014 suggests the effects of

high sodium and low potassium diet influences blood pressure levels in both normotensive as well as hypertensive individuals of all ages, with the effects being profound in older subjects. They found a positive association between sodium excretion and systolic as well as diastolic blood pressure, and the increase per 1g of excreted sodium was higher in hypertensive patients. An inverse relationship was observed for potassium excretion with the increase in blood pressure levels (Mente et al., 2014). Hence a diet rich in potassium and low in sodium is being recommended in recent years. (Staruschenko, 2018)

Some other risk factors include obesity especially in adolescents and young adults (Litwin and Kulaga, 2021), unhealthy diet, smoking, alcohol consumption, air pollution, noise pollution, chronic stress and sleep deprivation. A study published in 2017 meta-analysed nearly 3000 participants, and found that reducing the amount of alcohol consumed in moderation did not bring about a reduction in BP levels but reducing high alcohol consumption reduced blood pressure levels significantly (Roerecke et al., 2017). Lack of physical activity is another risk factor in the progression of hypertension. Many studies have found that physical exercise, even walking in moderation can be effective in reducing BP levels and reduce the incidence of hypertension (Mills et al., 2020). Eating saturated fats and meat in moderation and increased intake of fruits and vegetables has proven to be effective in lowering blood pressure levels (Yokoyama et al., 2014). Bringing about lifestyle and environmental changes can support in lowering blood pressure levels and subsequently help in lowering the risk of hypertension.

1.1.3 Pathophysiology of hypertension

Regulation of blood pressure in our bodies is maintained by mainly three organ systems: the heart, the kidneys, and the vasculature. These are aided by various physiological systems to modulate their functions. Blood is effectively pumped by the heart, carried around to each and every part of the body by blood vessels while the kidneys control blood volume (Drummond et al., 2019). Any alterations in the physiological system results in disturbing the working equilibrium of the organ systems and subsequently causes variations in blood pressure. Another mechanism of pathogenesis of the disease in the recent decades has been found to be the immune system. From discovering the accumulation of immune cells in

the kidneys in early 1960s (White and Grollman, 1964) to finding that activation of NADPH oxidase (NOX) 2 from regulatory T (Treg) cells can stimulate cardiovascular remodelling by angiotensin II induction (Emmerson et al., 2018), research exploring the role of immune system in the progression of hypertension has come a long way but the mechanism through which the immune system acts in promoting hypertension still remains understudied.

1.1.3.1 Classical mechanisms of hypertension pathophysiology

The classical mechanisms of hypertension pathogenesis involve the renin angiotensin aldosterone system (RAAS) and the sympathetic nervous system (SNS), which impact arterial stiffness, water levels and hormone levels in the blood. RAAS is crucial in maintaining vascular tone by controlling blood volume, systemic vascular resistance, and the balance of electrolytes in the body. As the name suggest, RAAS comprises of renin, angiotensinogen, and aldosterone along with angiotensin converting enzymes and angiotensin II receptors (Wu et al., 2018).

Renin, an enzyme produced in the juxtaglomerular cells of the kidney by cleaving prorenin, is released in the blood stream when arterial pressure drops. Renin circulates throughout the body and acts upon angiotensinogen to produce angiotensin (Ang) I which has limited vasoconstrictor properties and is cleaved by angiotensin converting enzyme (ACE) to form an eight amino acid long peptide called angiotensin II. Ang II has strong vasoconstricting properties and has two primary effects in increasing arterial pressure. It results in the constriction of arterioles, which increases the total peripheral resistance, subsequently increasing arterial pressure, but this effect lasts for a shorter duration than salt and water retention in the kidneys (Hall and Hall, 2020). Elevated salt and water levels in the kidney eventually increase the blood volume, thereby increasing the arterial pressure for hours and, sometime, days. Ang II acts on the kidneys directly to cause the salt and water retention by increasing sodium and water reabsorption. Another way in which Ang II increases salt and water retention is by stimulating aldosterone. Aldosterone is a hormone produced by the adrenal glands. The main function of this mineralocorticoid is increasing reabsorption of sodium, increased secretion of potassium in the renal tubular epithelial cells and increasing arterial pressure in the kidneys.

Reabsorption of sodium and potassium secretion is also aided by aldosterone in the salivary and sweat glands (Williams, 2005, Hall and Hall, 2020). When the renin-angiotensin system gets activated, it results in the increased production of aldosterone and consequently increasing sodium reabsorption.

The SNS is a part of the autonomic nervous system which unconsciously works to regulate various functions of the body. The sympathetic nerves originate mainly from thoracic spinal nerves and these innervate the heart and the vasculature except the capillaries (Hall and Hall, 2020). SNS governs the flight or fight response in the body by producing epinephrine and nor epinephrine which modulate constriction of the vasculature and affect arterial pressure. The nerve supply to the arteries and arterioles cause constriction and lead to higher arterial pressure, and the nerve supply to the veins strongly constrict them resulting in increased pressure on the heart to pump blood (Touyz, 2014). The heart is also innervated with the SNS and is stimulated to increase the heart rate. In the kidneys, SNS increases renin secretion to increase the blood volume resulting in increased arterial pressure (Hall and Hall, 2020). This leads to the development of hypertension as well as causing atherosclerosis and insulin resistance (DeLalio et al., 2020). Some causes of overactivity of the SNS is unhealthy diet, unhealthy lifestyle, psychological stress and lack of sleep (Touyz, 2014).

1.1.3.2 Inflammatory mechanisms of hypertension

Along with the organ and physiological systems, immune system of the body is said to play a significant role in the pathogenesis and progression of hypertension. Recent studies have indicated that immune and inflammatory mechanisms can significantly contribute to the disease (Murray et al., 2021). However, the mechanisms of their involvement in hypertension remain poorly defined. Importantly, both genome-wide association studies (GWAS) and gene expression signatures of hypertension strongly point towards the role of the immune system and inflammation (Levy et al., 2009).

From initial days of GWAS looking at quantitative blood pressure phenotypes, multiple loci playing a role in blood pressure regulation with more than 1400 common SNPs have been identified (Lip and Padmanabhan, 2020). A recent

Mendelian randomised study has established a positive association between leukocytes and increased systolic and diastolic blood pressure as well as pulse pressure (Siedlinski et al., 2020). Some SNPs found in immunoregulatory genes of immune cells such as T cell have been found to play a role in hypertension. One such example is the polymorphisms found in the SH2B3 gene which encodes for an adapter protein. Its effect on blood pressure has been demonstrated in murine studies where a knockout model of the gene showed increased blood pressure levels upon Ang II treatment (Huan et al., 2015). Karhunen and colleagues designed a bi-directional Mendelian randomised study to analyse the association between cytokines and cardiometabolic traits to find potential therapeutic target molecules. They found positive associations between BMI and 10 circulating biomarkers including cytokines amongst other bi-directional positive associations between cardiometabolic markers and cardiovascular events (Karhunen et al., 2023) indicating that immune system might play a role in associating genetic variability with hypertension and cardiovascular diseases. Cytokines such as IL-6, IL-1β, and TNF-α have been studied using Mendelian randomisations for their association with disorders such as diabetes and obesity (Meeks et al., 2021), and ESRD (Prakash et al., 2016), subsequently leading to cardiovascular events.

1.2 Immune system and hypertension

Innate and adaptive immune cells play a pivotal role in the production of cytokines and chemokines which regulate the inflammatory levels in the blood and influence the functioning of the vasculature. These inflammatory cytokines and chemokines are said to relay signals by interacting with the vascular cells and might potentially be responsible for the onset of hypertension via the immune pathways.

1.2.1 Innate immune cells and hypertension

Recent studies suggest that the innate immune system may be the first step in the pathogenesis of hypertension. While some cells protect, the others are said to play a part in the promotion of hypertension.

Macrophages are monocytes responsible for conducting phagocytosis. These cells secrete chemokines and cytokines and thus play a key role in the reactions and mechanisms of innate immunity (Drummond et al., 2019). Upon activation by haemodynamic injury, monocytes infiltrate the vasculature and organs (kidney and heart) where they differentiate into macrophage 1 (M1)- and macrophage 2 (M2)-like subtypes and produce cytokines and cause endothelial dysfunction in the vasculature and epithelial dysfunction in the organs (Justin Rucker and Crowley, 2017). M1-like macrophages are responsible for producing pro-inflammatory cytokines and inducing reactive oxygen species (ROS) production. The cytokines produced by M1-like macrophages are interleukin (IL)-1β and tumour necrosis factor alpha (TNF-α). M2-like macrophages, on the other hand, produced anti-inflammatory cytokines IL-10 and TGF-β (Justin Rucker and Crowley, 2017). Monocytes have also been found responsible in Ang II mediated hypertension and vascular function (Wenzel et al., 2011).

Dendritic cells are antigen presenting cells which are produced by the bone marrow and play a role in modulating various inflammatory responses. Their main role is to identify antigens in the bloodstream and act on the removal of foreign molecules. These cells activate the innate immune system by acting as the first defence against an infection by recruiting Natural Killer cells, modulate receptor mediated phagocytosis, process and present antigens, play a role in T-

cell differentiation, by stimulating and controlling the maturation (Liu, 2016). They have multiple subsets and form a dense network in human tissues such as arteries and kidneys. During hypertension dendritic cells become activated and induce the activation of T-lymphocytes leading to increased inflammation (Lu et al., 2020).

Natural killer cells are a form of lymphocytes present as a part of the innate immune system. They are cytotoxic cells responsible for the managing tumour cells and infections by eliminating them. These cells also act as regulatory cells and influence the activity of other immune cells such as B cells, T cells and dendritic cells by secreting inflammatory cytokines(Vivier et al., 2008). These cells produce interferon gamma (IFN-γ) in vessel walls which activates the macrophages and monocytes inducing inflammation and create hypertensive conditions (Li et al., 2021). Natural killer cells have also been found to be associated with systolic blood pressure (Delaney et al., 2021).

Initially found in cancer patients, myeloid derived suppressor cells (MDSC) are a set of immature myeloid cells and myeloid progenitor cells which are immunosuppressive by function (Gabrilovich and Nagaraj, 2009). They are precursors for dendritic cells and macrophages and regulate the production of inducible nitric oxide synthase (iNOS) consequently resulting in the production of reactive oxygen species. These cells get activated by inflammatory cytokines like IFN-γ, TGF-ß, IL-13 and IL-4 via the nuclear factor kappa B (NF-κB) pathway or the Janus kinase (JAK) / signal transducers and activators of transcription (STAT) pathways (Gabrilovich and Nagaraj, 2009). MDSCs produce cytokines like IL-4, IL-10, TGF, and IL-13, all of which are anti-inflammatory cytokines. It has been reported that the levels of MDSC have found to be increased in a variety of hypertensive mouse models (Chiasson et al., 2018).

1.2.2 Adaptive immune cells and hypertension

T helper (Th) cells are the subtype of T cells and the cells of adaptive immune system which activate effector cells through antigen specificity. They lead to the maturation of B cells into plasma and memory B cells. They are also responsible for recruiting eosinophils and basophils to an inflamed or an infected area. They can differentiate into different subtypes depending on the kinds of

cytokines each subtype produces, and the differentiation is dependent on signalling patterns from the antigen presenting cells. Th1 cells produce IFN-γ, TNF and IL-2. Th2 cells produce IL-4, IL-5, IL-13, IL-10 and TGF-ß. Th17 cells are proinflammatory in nature, increase tissue damage and inflammation. These cells produce IL-17, IL-21 and IL-22 (Burrell et al., 2016, Nash et al., 2015, Nussenblatt, 2010, Pahl and Vaziri, 2020).

Other T cell subsets are also found to be actively involved in the development of hypertension. Immunosenescent $CD8^+$ T cells are characterised by short telomeres, are $CD28^-$ and $CD57^+$, and increase production of inflammatory cytokines and chemokines (Huff et al., 2019). They increase in number with ageing and chronic inflammatory disorders and are highly toxic. They are set to play a role in the pathogenesis of hypertension (Youn et al., 2013). Cytotoxic T-cells majorly produce TNF-α, IFN-γ and mediate hypertension. They are activated when the $CD8^+$ receptor recognises an antigen and these cells then produce granzymes and perforins to get rid of them, thereby activating these cells to produce inflammatory cytokines which are said to modulate blood pressure levels (Ren and Crowley, 2019). Gamma-delta (γδ) T cells are also known to produce proinflammatory cytokines like IL-17, TNF-α and IFN-γ (Ren and Crowley, 2019).

Invariant natural killer (iNK) T cells, share features of adaptive and innate immune cells and have been demonstrated to potentially modulate hypertension (Drummond et al., 2019). They are a set of T cells specialized to identify self and non-self-lipid antigens. These cells are activated by CD1 presented lipids by T cell receptors as well as inflammatory cytokines. Upon activation, iNK T cells rapidly produce a variety of cytokines including IFN-γ, TNF, IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, IL-17, IL-21 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Brennan et al., 2013).

T regulatory (Treg) cells as the name suggests, are a type of cluster of differentiation (CD) 4+ T cells which regulate the expression of other immune cells. The main functions of Treg cells are to suppress the inimical activities of the T helper (Th) cells, protect against autoimmune diseases by maintaining immune self-tolerance, tolerance to foetus during pregnancy, suppressing allergies, inducing oral tolerance against antigens ingested through diet, and

regulate the effector cells. These cells, upon activation, produce cytokines like IL-10 and TGF-β. Some Treg cells have found to produce IL-17, when inflammatory conditions were responsible for the cells' activation (Corthay, 2009).

Though their effect in hypertension is not as profound as T cells, B cells mediate humoral immune response by producing antibodies and are present in blood, spleen, and lymph nodes. They develop from hematopoietic stem cells and differentiate into plasma cells and memory cells upon activation. Cytokines produced by various subsets of B cells include IL-2, IL-17, IFN γ, IL-6 and TNF (Shen and Fillatreau, 2015).

1.3 Cytokines produced by immune cells in hypertension

Cytokines are produced by immune cells as well as endothelial cells and they can be pro-inflammatory or anti-inflammatory in action and nature. The balance between the levels of pro- and anti- inflammatory cytokines is estimated to regulate the development of hypertension and cardiovascular diseases. Cytokine levels such as TNF-α and IL-6 are said to consistently increase in hypertension and may convey risk of developing the disease. Such increases in cytokines are also observed in relation to effector T cell specific cytokines such as IL-17 or IFN-γ. Recent attention has been focused on exploring the pathways and inflammatory mediators that immune cells use to drive high blood pressure and end-organ damage. When immune cells become activated and/or are recruited to a target organ, they produce cytokines that determine the local inflammatory response. Its detailed effector contributions to vascular dysfunction and hypertension will be further discussed in subchapter 1.5.

1.3.1 Pro-inflammatory cytokines in hypertension

IL-6 is a cytokine which has both anti and pro-inflammatory effects. Nearly one third of the circulatory IL-6 generates from the adipose tissue and results in T cell activation and B cell differentiation (Krüttgen and Rose-John, 2012). It is said to affect vascular inflammation and endothelial dysfunction. It is also responsible for the development of end organ damage due to hypertension. A study which looked at the role of epigenetics in the relationship between IL-6 and hypertension found that hypomethylation of IL-6 was correlated with the onset of hypertension (Mao et al., 2017a). It is a crucial element in the development of Th17 cells and C reactive protein (CRP)'s hepatic synthesis. IL-6 is essentially produced by the overproduction of ROS due to the effects of TNF-α, IL-1 receptor b, and Ang II (Tanase et al., 2019).

The chief mediator of adaptive and innate immunity, IFN-γ is produced by natural killer cells, $CD4^+$ and $CD8^+$ T cells primarily. It upregulates pro-inflammatory cytokines like TNF-α, IL-12, IL-15, and also iNOS along with NF-κB. IFN-γ is also responsible for the inhibition of IL-1 and IL-8 production, enhancing caspase dependent apoptosis, induction of suppressor of cytokine signalling protein and induction of IL-1Ra (Mühl and Pfeilschifter, 2003).

IL-23 is activated by dendritic cells and macrophages, and it regulates the secretion of various cytokines like IL-6, IL17, IL-1 and TNF. IL-17 stimulates the production of pro inflammatory cytokines regulating endothelial dysfunction, renal dysfunction and, consequently, hypertension (Tanase et al., 2019).

TNF-α is a pro-inflammatory cytokine produced by T cells, B cells, macrophages, natural killer cells, mast cells, smooth muscle cells, endothelial cells, cardiomyocytes, adipocytes and fibroblasts, including others (Bradley, 2008). It can be stimulated by bacterial lipopolysaccharide, immune complexes, IFN, and by autocrine mechanisms. It gets stimulated under the conditions like inflammation, infection, trauma, infarction and heart failure to name a few (Zelová and Hošek, 2013). The two main functions of TNF are to induce apoptosis and to stimulate cell expression of pro-inflammatory genes. It also controls its expression by negative feedback mechanism and by stimulating the production of anti-inflammatory cytokines. TNF α increases the vasodilation by increasing the production of prostanoids by the stimulation of cyclo-oxygenase 2 (Page et al., 2018). It also stimulates endothelial cells to produce ROS which activates some mitogen activated protein (MAP) kinases and increase intercellular adhesion molecule (ICAM)-1 synthesis.

1.3.2 Anti-inflammatory cytokines in hypertension

IL-4, a pleiotropic cytokine, induces differentiation of naïve T helper cells to Th2 cells, which in turn produce more IL-4. Produced primarily by mast cells, Th2 cells, basophils and eosinophils, IL-4 stimulates B cells differentiation and T cell proliferation (Yagi et al., 2002). IL-4 is found to decrease the production of IFN-γ. The presence of IL-4 in extravascular tissues promotes alternative activation of macrophages into M2 cells and inhibits classical activation of macrophages into M1 cells (Kassem et al., 2020).

IL-10 is an anti-inflammatory cytokine which downregulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages (Von der Thüsen et al., 2003). It also enhances B cell survival, proliferation, and antibody production. IL-10 can block NF-κB activity and participates in the regulation of the JAK-STAT signalling pathway. IL-10 predominantly inhibits lipopolysaccharide and bacterial product mediated

induction of the pro-inflammatory cytokines TNFα, IL-1β, IL-12 and IFN-γ (Tanase et al., 2019).

1.3.3 Inflammatory cytokines affect the vascular function

Both pro-inflammatory and anti-inflammatory cytokines are produced by an array of immune cells and even though they have distinct roles and actions, cytokines majorly use the same signalling pathways and mechanisms of action. Most cytokines use the JAK/STAT pathway to mediate their functions where dimerization and phosphorylation of the JAK and STAT subunits take place to effectively mediate the signalling (Ihle, 2001). Some inflammatory cytokines use the NF-κB and the MAP kinase pathway to relay their effects. The signals relayed by these molecules, often act through, and effect the vascular function by interacting with the endothelial cell or the vascular smooth muscle cells. These interactions can result in the production of ROS and the overproduction of which can lead to dysfunction (Sprague and Khalil, 2009). To have a better outlook on how cytokines may influence the vascular function, understanding the endothelium, its components and its functions is important.

1.4 Endothelium and vascular function

The vascular system spans the whole body and circulates blood and plasma. These fluids function as carriers for all the soluble and insoluble molecules produced by cells in different organs. Each vessel has a layer of cells which interact with the fluids and regulate the movement of these molecules through the vessels to the cells of the organs. Endothelium is a single celled innermost lining of the vessels. It acts as a barrier between the blood and vessel walls. Endothelium is made up of squamous cells which originate from the mesoderm (Da Luz et al., 2018).

1.4.1 Function of endothelium

Earlier thought to be a mere interface for facilitating circulation of fluids across its membrane (Da Luz et al., 2018), endothelium has been now found to have a variety of functions. The endothelium forms an interface between circulating blood or lymph in the lumen and the rest of the vessel wall. This forms a barrier between vessels and tissues and control the flow of substances and fluid into and out of a tissue. This controls the passage of materials and the transit of white blood cells into and out of the bloodstream. Molecules such as glucose and amino acids are transported through the endothelial walls. Excessive or prolonged increases in permeability of the endothelium, as in cases of chronic inflammation, may lead to tissue inflammation (Galley and Webster, 2004).

Endothelial cells are involved in many other aspects of vessel function, including thrombosis, angiogenesis, regulating the vascular tone by vasoconstriction and vasodilation (Galley and Webster, 2004, Boulanger, 2016). Angiogenesis takes place to either form new blood vessels or replace old, damaged ones. This process is mediated by vascular endothelial growth factor (VEGF), which is produced by the endothelial cells and acts in an autocrine manner. To maintain coagulation, thrombin binds to thrombomodulin, which is produced by the endothelial cells, to maintains thrombosis and thrombolysis. The vascular tone is maintained by a variety of vasoconstrictors and vasodilators which are produced by the endothelial cells. These are nitric oxide (NO) and endothelin. NO is the major contributor in maintaining endothelial cell functions and the molecule is produced by endothelial nitric oxide synthase (eNOS) (Zhao et al., 2015).

1.4.2 eNOS, NO and ROS

Endothelial nitric oxide synthase (eNOS) is an enzyme of the endothelium which is responsible for maintaining endothelial function by producing NO. Endothelial function is maintained by NO which acts as a signalling molecule and relays vasodilation, stimulate hormones (Kiani et al., 2022). eNOS is activated by phosphorylation of amino acid residues such as threonine, serine, and tyrosine. eNOS is phosphorylated at serine and threonine in a calcium dependent manner whereas the enzyme phosphorylation on tyrosine residues in completely calcium independent (Fleming and Busse, 1999). Coupled eNOS uses L-arginine as a substrate and converts it to L-citrulline by using Nicotinamide adenine dinucleotide phosphate (NADPH) to form nitric oxide (NO). When eNOS is activated by calcium, in a calcium/calmodulin manner, it reduces NADPH with the help of tetrahydrobiopterin (BH4). Under normal circumstances, BH4 is reduced for NO production, but when eNOS is activated under inflammatory conditions, BH4 gets partially reduced and remains partially oxidised. Under such conditions, uncoupled eNOS produces superoxide ions ($O2^-$) a form of ROS, instead of NO.

1.4.3 NADPH oxidases

NADPH is a cofactor that is a reducing agent and is responsible for the production of ROS by NADPH oxidases in immune cells as well as in the endothelium. NADPH oxidase (NOX) is a multimeric molecule with seven homologues in humans, namely NOX1, NOX2, NOX3, NOX4, NOX5, dual oxidase (DUOX)1 and DUOX2. They are transmembrane proteins which oxidise the NADPH molecule and generate ROS. It is found on the membranes of the cell, nucleus, endoplasmic reticulum, and mitochondria. Some of its functions include cellular proliferation, endothelial signalling, and eliciting immune response against infection. NOX1, NOX2, NOX3 and NOX4 show homology to the $gp91^{phox}$ subunit and include $p22^{phox}$ subunit in the active complex. NOX4, DUOX1 and DUOX2 are known to mainly produce hydrogen peroxide (H_2O_2) but all the other homologues of the NADPH oxidase are found to produce superoxide anion (Skonieczna et al., 2017).

1.4.4 Role of NO and NOX in endothelial dysfunction

Endothelial dysfunction, as the name suggests, is the dysregulation of the functioning of endothelium, resulting in the imbalanced production of various vasoconstrictors and dilaters maintaining the vascular tone, anti- and procoagulants for maintaining thrombosis and other molecules which play a role in maintaining endothelial function. NO bioavailability plays a critical role in the development of endothelial dysfunction, as it is responsible for majority of functions of endothelium. Endothelial dysfunction can be categorized by aetiology, function, and prognosis. Aetiological classification involves genotypic and phenotypic dysfunction, such as dysfunction observed in normotensive individuals with a history of hypertension, and dysfunction observed in all cardiovascular disorders, respectively. Functional classification may include vaso-tonic changes indicating dysregulation of vasomotor functions and vaso-plegic changes may refer to dysfunction caused by cytokines which induces increased NO production. The third classification depends on reversible or irreversible effects caused due to cardiovascular events. Under stressful conditions, ROS is mainly produced intracellularly by uncoupled eNOS, xanthine oxidase and cyclooxygenase, and extracellularly by NOXs. Increase in the concentration of ROS results in the rapid reaction of coupling of the molecule with NO to reduce active NO availability and endothelial function. Increased ROS formation also results in uncoupling of eNOS, a condition in which monomeric form of eNOS produces superoxide rather than nitric oxide, subsequently resulting in endothelial dysfunction. ROS is also have been found to activate various signalling pathways such as MAP kinase pathway, activates Rho-kinase and inadvertently induces vasoconstriction (Da Luz et al., 2018).

1.5 Inflammatory cytokines in hypertension and their effect on endothelial dysfunction

1.5.1 IL-1β

IL-1ß is a pro-inflammatory cytokine mostly produced by cells of the innate immune system like monocytes, macrophages, and dendritic cells in an inactive form called pro-IL-1ß. This inactive form is then cleaved by caspase 1 to form the active IL-1ß molecule which is around 17 kilo Dalton (kDa) in molecular weight (Thornberry et al., 1992). IL-1ß is made up of 12 anti-parallel ß strands (Veerapandian et al., 1992).

IL-1ß might be linked to hypertension but not a lot of work has been done to study the effects of the cytokine on the disease, though IL-1ß levels have found to be associated with blood pressure in hypertensive populations. In a prospective cohort of healthy individuals, baseline IL-1ß levels were significantly higher in subjects that developed hypertension over the follow-up period and a linear relationship was recorded between systolic blood pressure and quartiles of the cytokine after adjustments indicating that IL-1ß can be used as a potential marker to predict hypertension (Mauno et al., 2008). In contrast, when black and white women were enrolled in a nested case control study, the risk of developing hypertension was not significantly associated with baseline levels of IL-1ß (Wang et al., 2011). Another study looked at serum levels of the cytokine in patients with essential hypertension and found significantly increased IL-1ß levels when compared with the control group and also found a positive correlation with blood pressure (Dalekos et al., 1997).

IL-1ß has been found to impair endothelium dependent relaxation in diabetic mice. When the vessels were treated with varying concentrations of the cytokine for 2 hours, impairment was observed for all concentrations (Vallejo et al., 2014). The role of IL-1ß in endothelial dysfunction has been studied for diseases like rheumatoid arthritis (Yang et al., 2016, Garg et al., 2017), periodontitis (Bergandi et al., 2019), pulmonary hypertension (Voelkel et al., 1994), and more work is required to comprehend its actions and mechanisms in the development of hypertension and its potential role in endothelial dysfunction.

1.5.2 IL-6

IL-6 was first discovered between 1986 and 1987 when several groups purified the same molecule (Van Snick, 1990). IL-6 is a pleiotropic cytokine and a myokine which is around 21-28 kDa in molecular weight, depending on its source of origin, with four α-helix bundles in its structure (Scheller et al., 2011). IL-6 is produced by macrophages, monocytes, T cells, mast cells, fibroblasts, and its production is stimulated by a viral infection or by lipopolysaccharides (Scheller et al., 2011, Van Snick, 1990, Feng et al., 2022).

The cytokine signals through its receptor which occurs in a transmembrane as well as a soluble form. The cytokine receptor complex then activates the JAK/STAT signalling and MAPK signalling to modulate its functions (Tanaka et al., 2018). Among many functions of IL-6, it is responsible for the differentiation of T cells into Treg, cytotoxic T cells, Th17 cell, proliferation and differentiation of B cells, stimulates the production of acute phase proteins such as CRP and inhibits the production of albumin in the liver, activate vascular endothelial cells to produce cytokines, and increase endothelial permeability (Tanaka et al., 2018, Cronstein, 2007). High levels of the cytokine have pathological effects such as induced inflammation in lymph nodes in Castleman disease, and increased angiogenesis by stimulating VEGFs in rheumatoid diseases (Tanaka et al., 2018).

Increased levels of IL-6 have also found to impact the pathogenesis of hypertension. In a cross-sectional study with obese and hypertensive diabetic patients, while studying the effect of insulin resistance, the researchers found that significant increases in serum levels of IL-6 were found in the group with obese hypertensive patients (Lukic et al., 2014). Blood pressure levels are also associated with IL-6 levels. In a study looking at the cross-sectional relationship of the cytokine with blood pressure, IL-6 was found to be significantly associated with increased blood pressure (Bautista et al., 2005b). Another study found that lowering of blood pressure in young untreated hypertensive patients resulted in lower serum IL-6 values (Vázquez-Oliva et al., 2005). IL-6 is also found to affect the renal function in hypertensive patients. A study observed changes in the cytokine levels and its associations in urinary albumin excretion rate in hypertensive individuals and found positive correlation between the two (Yu et al., 2010). Higher serum levels of IL-6 are also found to be associated with

hypertension prevalent in women but not men, as well as in incident hypertension however genetic polymorphism of the cytokine did not associate with hypertension (Cheung et al., 2011b). Epigenetic changes in IL-6 have also been found to affect the risk of essential hypertension. When hypomethylation of IL-6 was studied in human blood samples from hypertensive and normotensive individuals, correlation between hypomethylated IL-6 and risk of hypertension was found (Mao et al., 2017b).

Studies in animal models also support the involvement of the cytokine in hypertension development. When Ang II induced and high salt diet hypertension was studied in wild type and IL-6 knockout mice, blood pressure levels were similar in both wild type and knockout mice with high salt diet whereas a significant increase in blood pressure levels was observed in wild type mice upon Ang II infusion indicating the role of IL-6 in the development of Ang II induced hypertension (Lee et al., 2006a). IL-6 has also been found to play a role in the JAK/STAT pathway activation in hypertension. When Ang II infusion was carried out in wild type and IL-6 knockout mice, increased arterial pressure was observed in wild type mice along with phosphorylation of JAK2 and STAT3 in the cortex of kidneys whereas no JAK/STAT phosphorylation was observed in knockout mice (Brands et al., 2010). IL-6 also mediates hypertension in pregnant rats by inhibiting eNOS activity resulting in reduced NO production and increased vascular pressure (Orshal and Khalil, 2004).

The mechanism by which IL-6 interferes with the development of hypertension can be its action on endothelium dysregulation. Research on non-diabetic men found negative association of circulating IL-6 levels with endothelium dependent vasodilation but no association was observed with endothelium-independent vasorelaxation (Esteve et al., 2007). In mouse models, when the effect of IL-6 was studied on Ang II induced hypertension in wild type, IL-6 knockout and NOX2 knockout mice, endothelium dependent relaxation with ACh was impaired in wild type Ang II infused mice (Schrader et al., 2007a). While looking for predictors of endothelial dysfunction in coronary arteries, a group of researchers found that coronary vascular resistance was higher in hypertensive patients when compared with normotensive patients and a univariate analysis correlated the increased vascular resistance with increased serum IL-6 levels and

multivariate analysis resulted in IL-6 being an independent predictor of endothelial dysfunction in coronary arteries (Naya et al., 2007b).

1.5.3 TNF-α

TNF-α is a cytokine which was discovered in 1968 in mouse sera and showed tumor cell death (Carswell et al., 1975). It is 233 amino acid long and is first produced as a transmembrane protein, which is then cleaved by TNF-α converting enzyme to produce a soluble form, which is 157 amino acid long,17 kDa in weight, made up of ß sheets, and forms a trimer to become a more stable and active form (Moss et al., 1997, Balkwill, 2006). TNF is highly expressed in the lungs, kidneys, spleen, colon, and thymus in humans. Low levels of the cytokines are found in the brain, liver, and heart. Upon increased stress and inflammation signals by pathogens, the cytokine is produced by the cells of the immune system such as mast cells, monocytes, macrophages, dendritic cells, T cells, NK cells, as well as endothelial cells and fibroblasts (Falvo et al., 2010).

TNF-α has two receptor types, TNF receptor (TNFR) 1 and 2, both of which are transmembrane proteins. TNFR1 is activated by soluble forms of the cytokine whereas TNFR2 requires a membrane bound form if TNF-α (Balkwill, 2006). The cytokine signals via the activation of MAP kinase pathway, NF-κB, by both receptors and AKT pathway by TNFR2 (Jang et al., 2021). TNF-α demonstrates a plethora of functions in cells or tissues. It is responsible for the initiation of apoptosis in almost all the cells in the body, deactivates T cell receptors on both $CD4^+$ and $CD8^+$ T cells, activation of B cells, proliferation of Treg cells, stimulation of myeloid-derived suppressor cells, activation and proliferation of macrophages, monocytes and natural killer cells, induces dysfunction in adipocytes and cytokine production in endothelial cells (Yamada and Takaoka, 2021).

Apart from its apoptosis inducing properties, TNF-α is also a critical factor in the development of hypertension, though the mechanism through which the cytokine acts is understudied. Higher levels of the cytokine are found in hypertensive patients when compared to normotensive patients where increased blood pressure in hypertensive individuals is found to increase oxidative stress (Verma et al., 2019). Whereas, when TNF-α levels were measured in individuals enrolled

in the Multi-Ethnic Study of Atherosclerosis (MESA) study, the cytokine levels were not significantly associated with any blood pressure measurements or hypertension (Allison et al., 2013). In mice, when hypertension was induced by Ang II infusion, TNF-α production was increased in T cells and blocking its production averted the development of hypertension (Guzik et al., 2007). In another study, levels of TNF-α along with other inflammatory cytokines were significantly increased in liver, heart, brain and kidneys of spontaneously hypertensive rats (Sun et al., 2006). TNF-α has been found to play a part in the progression of pulmonary hypertension (Liu et al., 2020, Hurst et al., 2017, Sutendra et al., 2011) and preeclampsia (Davis et al., 2002, LaMarca et al., 2007). Increased plasma levels of TNF-α induces high levels of blood pressure in pregnant rats, suggesting that TNF-α may be responsible for preeclampsia but the increased blood pressure levels due to the cytokine can be countered with endothelin receptor A treatment (LaMarca et al., 2005b). In another study, infusion of TNF-α resulted in increased plasma levels of the cytokine, increased arterial pressure and increased renal vascular resistance in the treated pregnant rats indicating a role of the cytokine in mediating renal alterations and inducing arterial pressure variations (LaMarca et al., 2005a).

TNF-α is known to increase the production of NOXs in the endothelium which results in higher levels of ROS subsequently causing endothelial dysfunction (Gao et al., 2007a). TNF-α has also been found to act on the eNOS promoter and inhibit its action resulting in reduced eNOS activity and low NO levels (Neumann et al., 2004). Intravenous treatment with TNF-α in the 1990s showed significant impairment of endothelium dependent relaxation by ACh which was also supported by in-vitro treatment of vessels demonstrating similar results (Wang et al., 1994). When administered to healthy humans intravenously, plasma TNF-α levels were increased in both administered and non-administered arms but no changes in blood pressure were observed whereas a decrease in relaxation was observed with Ach indicating endothelial dependent impairment (Chia et al., 2003). Another study looking at the effect of TNF-α on endothelium in prediabetic metabolic rat model demonstrated endothelial dependent relaxation was impaired but restored upon treatment with anti-TNF, ROS production was increased and TNF levels were increased in endothelial cells of the heart (Picchi et al., 2006). TNF-α is also associated with endothelial

dysregulation in cardiovascular events like ischemia and reperfusion injuries (Gao et al., 2008, Gao et al., 2007b, Zhang et al., 2010).

1.5.4 IFN-γ

IFN-γ was first discovered in 1965 as an interferon-like virus inhibitor (Wheelock, 1965), and it was designated at it is known now, in early 1970s (Billiau and Matthys, 2009). IFN-γ is a soluble monomer which is composed of six α-helices and occurs as a dimer in its active form (Gray and Goeddel, 1982). It is made up of 146 amino acids (Gray and Goeddel, 1982) and is primarily produced immune cells such as Th1 cells, natural killer cells, and cytotoxic $CD8^+$ T cells and its secretion is activated by increased inflammatory cytokines such as IL-1β, IL-12 and IL-18 (Pereiro et al., 2019, Gocher et al., 2022).

IFN-γ is known for its anti-tumor and pro-inflammatory properties. The cytokine activates macrophages, induces major histocompatibility complex class II and amplifies antigen presentation, increases ROS production and increases phagocytosis (Kak et al., 2018, Ng et al., 2023). IFN-γ is also known to enhance iNOS production by myeloid cells (Gocher et al., 2022). The receptor for IFN-γ is a heterodimer and the cytokine majorly signals through the JAK/STAT pathway. Other signaling pathways activated by IFN-γ are MAP kinase pathway, mechanistic target of Rapamycin (mTOR) signaling pathway (Schroder et al., 2003).

IFN-γ has been studied to have a role in the pathogenesis of hypertension. Higher serum levels of the cytokine have been found in hypertensive patients when compared with normotensive patients in a study conducted by Ji and colleagues (Ji et al., 2017). A study published in 2019 measured serum levels of inflammatory cytokines in diabetic, obese and hypertensive men and found that serum levels of IFN-γ were significantly higher in subjects with both diabetes and hypertension (Asadikaram et al., 2019). In a study on IFN-γ knockout mice, Ang II infusion resulted in blunted blood pressure increase but the diuretic responses were maintained suggesting that IFN-γ might be necessary for sodium reabsorption in the kidneys (Kamat et al., 2015). A study on brain endothelial cells of hypertensive and normotensive rats demonstrated that IFN-γ stimulation increased the level of intercellular adhesion molecule 1 significantly more in

hypertensive rats than in normotensive rats (McCarron et al., 1994), indicating a possible mechanism of hypertension leading to the development of cardiovascular diseases. The role of epigenetics also affects IFN-γ induced hypertension, as studied by Bao and colleagues (Bao et al., 2018). They found that hypomethylation of IFN-γ promoter gene has a positive link with essential hypertension in human subjects.

The pleiotropic effects of IFN-γ are well known and its part in vascular dysfunction is widely studied. IFN-γ has been found to act on endothelial cells but its mechanism of action is still under question. When HUVECs were stimulated with different concentrations of the cytokine [10, 100 and 1000 microgram per milliliter (μg/ml)] for 24 hours, NO production was decreased but the difference was only significant at the highest concentration (Javanmard and Dana, 2012). A significant role played by IFN-γ in modulating endothelial function is interfering with its permeability. In one study, HUVECs were stimulated with various concentrations of IFN-γ for multiple time points and the permeability of the endothelium was studied. A biphasic increase in permeability via actin rearrangement was observed (Ng et al., 2015). IFN-γ is also known to alter eNOS transcription by stimulating class II trans activator which acts on eNOS promoter (Weng et al., 2019).

1.5.5 IL-17A

First discovered as cytotoxic T lymphocyte antigen (CTLA) 8 in 1993, IL-17 gene was cloned from a murine T-cell hybridoma formed by combining mouse cytotoxic T cell clone and rat T cell lymphoma (Rouvier et al., 1993). IL-17A is a 155 amino acid long protein with molecular weight of 35 kDA with disulfide bridges in its conserved C terminus (Onishi and Gaffen, 2010). The IL-17 family has six isoforms IL-17 A-F. IL-17A is interchangeably known as IL-17, and has the highest homology with IL-17F, and both can either be found as homodimers or IL-17 A/F heterodimer (McGeachy et al., 2019).

Majorly produced by the Th17 cells, IL-17A is also produced by γδ T cells, natural killer T cells, neutrophils, innate lymphoid cells, mast cells, cytotoxic $CD8^+$ T cells and myeloid cells (Ge et al., 2020). The cytokine signals via the IL-17 receptors (IL-17R A-E) which are present on various cells such as epithelial

cells and fibroblasts along with neutrophils. IL-17A signals through the NF-κB nuclear factor, the MAP kinase pathway and JAK/STAT signaling (Kostareva et al., 2019). IL-17A is known to mediate inflammation by inducing the production of cytokines, antimicrobial peptides, chemokines responsible for activating immune defense mechanisms via the neutrophils, macrophages, DCs and monocytes. It is also known to induce the production of iNOS and other proteins responsible for vascular maintenance. IL-17 has been also known to play a part in vascular remodeling in pulmonary inflammation, in the spleen, and myocardial infarction (Kostareva et al., 2019, Ge et al., 2020).

The role of IL-17A in the development of hypertension is a subject of debate. A study by Madhur and colleagues (Madhur et al., 2010b) found that infusing Ang II in C57BL/6J mice increased the production of IL-17 by T cells, as well as IL-17 was required in the development of Ang II induced hypertension, and IL-17 induced vascular dysfunction in Ang II treated mouse aortas. Furthermore, they observed that IL-17 serum levels were higher in diabetic patients with hypertension. Another study found that IL-17 induced arterial stiffness with Ang II treatment (Wu et al., 2014) which may lead to increased arterial pressure and subsequently hypertension but the mechanism of action still remains understudied. In variance, a study looking at the effect of anti-IL-17 therapy in reducing blood pressure did not show any changes upon investigation. Blocking the effect of IL-17 did not lower blood pressure nor did it affect cardiac hypertrophy (Markó et al., 2012). Another study looked at the role of IL-17 in hypertensive renal damage and found that IL-17 induces renal inflammation and increased blood pressure (Orejudo et al., 2019). So, to have a clearer understanding of the action mechanism of IL-17, further studies are needed.

To understand the role of IL-17 better in the pathogenesis of hypertension, recognizing the cytokine's role in vascular function is important. IL-17 is known to increase ROS production, alter phosphorylation of eNOS, impairs elasticity of the vasculature and promotes fibrosis (Harrison et al., 2021). A study published in 2013 demonstrated that IL-17 significantly induced eNOS phosphorylation at Threonine 495 (T495) in endothelial cells and Rho-kinase inhibitor treatment prevented the increase in IL-17 induced phosphorylation. The study also demonstrated that IL-17 has direct effects on vascular function by significantly impairing endothelium dependent relaxation by acetylcholine (Ach) and infusion

of IL-17 in mice increased serum levels of the cytokine as well as increased blood pressure significantly (Nguyen et al., 2012). Another study showed that IL-17 induces endothelial cell death in the human umbilical vasculature as well as increased serum levels of the cytokine in patients with acute coronary syndrome (Zhu et al., 2011). The mechanisms by which IL-17 induces its vascular dysregulation are not studied in detail but a few studies suggest signaling pathways that may or may not be used in inducing IL-17 effects. Endothelin-1 pathway is not a mechanism for the endothelial dysfunction caused by IL-17 in human umbilical vein endothelial cells (HUVEC) as well as in pregnant rats (Cornelius et al., 2013). Zhang and colleagues demonstrate the effect of IL-17 on endothelial senescence in their recent study (Zhang et al., 2021). This expression was inhibited by blocking the NF-κB signaling confirming the link between IL-17 and endothelial cell death.

1.5.6 IL-15

IL-15 was discovered 29 years ago in 1994 in two separate laboratories simultaneously and was initially classified as a T cell growth factor (Burton et al., 1994, Grabstein et al., 1994). With a molecular weight of 14-15 kDa, IL-15 is a glycoprotein which belongs to the four-α helix cytokine family (Fehniger and Caligiuri, 2001). IL-15 protein is 114 amino acid long in both humans and mice (Sindaco et al., 2023). Alternate splicing of IL-15 pre-mRNA results in different sizes of signaling peptides which decide if the mature IL-15 will remain cytosolic or is secreted by the cells (Perera et al., 2012). IL-15 is produced and expressed by cells such as macrophages, monocytes, dendritic cells, fibroblasts, a variety of tissue epithelial cells, nerve cells, keratinocytes and low levels in T cells (Perera et al., 2012, Steel et al., 2012, Patidar et al., 2016).

IL-15 plays a vital role in the activation, differentiation, and proliferation of various cells of the innate and adaptive immune system. While the cells do not produce or express IL-15, NK cells express the IL-15 receptor α and βγ complex though this receptor presentation does not act in the relaying the functions of IL-15 in the cells' maintenance (Perera et al., 2012). IL-15 is expressed by monocytes and induces the production of chemokines which attracts the first line of defence to the infection site. They act as the autocrine regulator of inflammatory cytokines, when IL-15 is higher, the cells produce pro-

inflammatory cytokines and lower levels of IL-15 induces the cells to produce anti-inflammatory cytokines (Perera et al., 2012). IL-15 acts in an autocrine manner to modulate the production of DCs which control the activities of NK cells, produce inflammatory cytokines and function as the first line of defence in an infectious environment. IL-15 manages the activities of neutrophils and stimulates their phagocytotic behaviour as well as stimulate the cells to produce IL-8 (Verri Jr et al., 2007). IL-15 is also known to stimulate mast cells via a non-classical receptor unlike NK cells and T cells. IL-15 is responsible for the development of $CD8^+$ T cells and the maintenance of memory $CD8^+$ T cells but only limited concentrations are required to maintain $CD4^+$ T cells (Waldmann, 2017). Whereas the role of IL-15 in the functioning of B cells is very limited and sometimes indirect (Gil et al., 2010).

The role of IL-15 is has been studied in various cardiovascular diseases like myocardial infarction, myocarditis, atherosclerosis and arterial fibrillation and a positive effect has been found (Guo et al., 2020), but its role in the progression of hypertension is not very well studied. Kaibe and colleagues (Kaibe et al., 2005) studied around 400 patients with essential hypertension in Osaka, Japan and found significantly elevated levels of IL-15 in patients with severe organ damage. So, to decipher the role of IL-15 in the pathogenesis of hypertension cannot be strongly supported by this study due to its limitations but exploring the mechanisms of oxidative stress caused by varying levels of IL-15 could be a considerable factor. IL-15 activates various immune cells via JAK/STAT pathways by binding to its receptor domains α and βγ complex (Schluns et al., 2005). It has been found that in brain endothelium, IL-15 signals via tha NF-κB pathway (Stone et al., 2011). Not only the JAK/STAT pathway, IL-15 also activates the MAP kinase pathway (Perera et al., 2012). The interplay between the stimulation of signalling pathways and the activation of immune cells results in the increase of inflammatory cytokines which affect the equilibrium of reactive oxygen species maintaining the functions of the endothelium and may result in endothelial dysfunction.

1.6 Aims and objectives

The main objective of this thesis has been to identify vascular effects of inflammatory cytokines relevant in the development of hypertension and endothelial dysfunction.

To achieve this, three aims were set:

1. Identifying and investigating the association of inflammatory cytokine levels and the development of hypertension in humans.

2. Investigating the direct effects of the identified inflammatory cytokines on vascular function in mouse blood vessels by studying endothelium-dependent and independent vasomotor effects.

3. Investigate mechanisms of endothelial dysfunction caused by the selected inflammatory cytokines by studying.

    a) eNOS phosphorylation in human endothelial cells and expression in mouse vessels

    b) NADPH oxidase expression in mouse vessels

Chapter 2

Inflammatory cytokines and risk of developing hypertension: a systematic review and meta-analysis

2.1 Introduction

Hypertension is a major modifiable risk factor for CVDs but is frequently undiagnosed and poorly controlled (Collaboration, 2017, Liberale et al., 2021, Cheema et al., 2022). The pathophysiology of hypertension is complex (Nosalski et al., 2017, Griendling et al., 2021, Brandes, 2014, Murray et al., 2021, Siedlinski et al., 2020, Mikolajczyk et al., 2021).

Experimental and clinical studies suggest a positive association between pro-inflammatory cytokines and systolic blood pressure (Chae et al., 2001, Nguyen et al., 2013a, Dalekos et al., 1997, Chamarthi et al., 2011, Elmarakby et al., 2008, Madhur et al., 2010a, Saleh et al., 2015, Crosswhite and Sun, 2010, Lee et al., 2006b, Gonzalez-Amor et al., 2022). Consistent with this notion, inhibitors of TNF-α and IL-6 have been shown to reduce blood pressure in experimental models (Abdelrahman et al., 2018, Murray et al., 2021), but findings were contradictory in humans (Faria et al., 2021, Rothman et al., 2020, Provan et al., 2015). The use of angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) might suppress the production of IL-6, IL-1ß, and TNF-α by various cells, perhaps as a result of their antihypertensive properties (Schindler et al., 1995, Peeters et al., 1998, Vazquez-Oliva et al., 2005). The activation of the renin-angiotensin system and sympathetic nervous system stimulates the synthesis of proinflammatory cytokines (Zhang et al., 2003, Funakoshi et al., 1999, Han et al., 1999, Ruiz-Ortega et al., 2002, Sanz-Rosa et al., 2005), which further supports a potential role for cytokines in the regulation of blood pressure. An imbalance between pro-inflammatory cytokines, for instance, TNF-α, IL-6, and IL-1ß (Kany et al., 2019), that initiate inflammation in response to tissue injury, and anti-inflammatory cytokines like IL-10, that inhibit excessive inflammatory reactions (Opal and DePalo, 2000), might lead to the development of hypertension and other severe cardiovascular complications, including coronary artery disease and heart failure (Caiazzo et al., 2022). Importantly, recently pharmacological targeting of IL-1ß was shown to significantly reduce the rate of secondary cardiovascular events in atherosclerotic patients (Ridker et al., 2017a). Similarly, the effect of IL-6 inhibition in patients at high atherosclerotic risk is currently under investigation (Ridker, 2021).

However, whether pro-inflammatory cytokines are associated with the development of hypertension is unclear (Bautista et al., 2005a, Ghazi et al., 2020, Lakoski et al., 2011, Sesso et al., 2007); thus, we performed this systematic review of the literature and a meta-analysis to investigate this association.

2.2 Methods

This study was reported based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (http://www.prisma-statement.org/). The review protocol was registered in PROSPERO, the International Prospective Register of Systematic Reviews (crd.york.ac.uk/prospero/index.asp, identifier: CRD42022378211). This systematic review and meta-analysis is published in Pharmacol Res. 2023, Dec 29;200:107050. DOI: 10.1016/j.phrs.2023.107050.

2.2.1 Data sources

We searched Medline (Pubmed), Web of Science, the Cochrane Central Register of Controlled Trials (CENTRAL) and Embase 1947-present (Ovid) databases for relevant studies published from database inception to the 23rd of August 2022, using the Medical Subject Headings (MeSH) terms/Emtree and keywords related to the topic of interest limited to title and/or abstract. The search was reviewed by an experienced librarian of the University of Glasgow, Paul Cannon.

2.2.2 Search Strategy

2.2.2.1 Cytokine keywords

Cytokine OR cytokines OR Interleukin OR Interleukins

IL-1 OR IL1 OR interleukin 1 OR interleukin-1 OR interleukin1 OR interleukin 1 alpha OR Interleukin1alpha OR Interleukin 1alpha OR IL-1alpha OR IL-1 alpha OR IL-1A OR IL1A OR Hematopoietin-1 OR Hematopoietin 1 OR interleukin 1 beta OR Interleukin 1beta OR Interleukin-1 beta OR IL-1beta OR IL1 beta OR IL-1B OR IL1B OR IL-1Gamma OR interleukin-1 gamma OR interleukin 1 gamma OR interleukin 1gamma OR Lymphocyte-Activating Factor OR Lymphocyte Activating Factor OR Epidermal Cell Derived Thymocyte-Activating Factor OR Epidermal Cell Derived Thymocyte Activating Factor

IL-2 OR IL2 OR interleukin 2 OR interleukin-2 OR interleukin2 OR Interleukin II OR T Cell Stimulating Factor OR Thymocyte Stimulating Factor OR T-Cell Stimulating Factor OR Mitogenic Factor OR T-Cell Growth Factor OR T Cell Growth Factor

IL-3 OR IL3 OR interleukin 3 OR interleukin-3 OR interleukin3 OR colony-stimulating factor OR multi-CSF OR Multipotential Colony Stimulating Factor OR Multipotential Colony-Stimulating Factor OR Eosinophil-Mast Cell Growth-Factor OR Eosinophil Mast Cell Growth Factor OR Mast Cell Colony-Stimulating Factor OR Mast-Cell Colony-Stimulating Factor OR mast cell growth factor OR Hematopoietin-2 OR Hematopoietin 2 OR Colony-Stimulating Factor 2 Alpha OR hematopoietic growth factor

IL-4 OR IL4 OR interleukin 4 OR interleukin-4 OR interleukin4 OR B Cell Proliferating Factor OR B-Cell Proliferating Factor OR B-Cell Stimulatory Factor-1 OR B-Cell Stimulatory Factor 1 OR B-Cell Stimulating Factor-1 OR B Cell Stimulating Factor 1 OR B-Cell Growth Factor-1 OR B-Cell Growth Factor-I OR B Cell Growth Factor I OR B Cell Growth Factor 1 OR Mast Cell Growth Factor 2 OR Mast Cell Growth Factor-2 OR lymphocyte stimulatory factor 1

IL-5 OR IL5 OR interleukin 5 OR interleukin-5 OR interleukin5 OR B-Cell Growth Factor-II OR B Cell Growth Factor II OR Eosinophil Differentiation Factor OR B-cell differentiation factor 1 OR B-cell differentiation factor-1

IL-6 OR IL6 OR interleukin 6 OR Interleukin-6 OR interleukin6 OR B Cell Differentiation Factor 2 OR B Cell Differentiation Factor OR B-Cell Differentiation Factor-2 OR B-Cell Stimulatory Factor 2 OR B Cell Stimulatory Factor-2 OR B Cell Stimulatory Factor 2 OR IFN-beta 2 OR beta-2 Interferon OR Interferon beta 2 OR Interferon beta-2 OR Myeloid Differentiation-Inducing Protein OR Myeloid Differentiation Inducing Protein

IL-7 OR IL7 OR interleukin 7 OR interleukin-7 OR interleukin7 OR Lymphopoietin 1 OR Lymphopoietin-1

IL-8 OR IL8 OR interleukin 8 OR interleukin-8 OR interleukin8 OR chemokine C-X-C motif ligand 8 OR CXCL8 OR CXCL8 Chemokine OR CXCL8 Chemokines OR Chemokine CXCL8 OR neutrophil chemotactic factor OR Anionic Neutrophil-Activating Peptide OR Anionic Neutrophil Activating Peptide OR Alveolar Macrophage Chemotactic Factor-I OR Alveolar Macrophage Chemotactic Factor I OR Granulocyte Chemotactic Peptide Interleukin 8 OR Granulocyte Chemotactic Peptide-Interleukin-8 OR Monocyte-Derived Neutrophil Chemotactic Factor OR

Monocyte-Derived Neutrophil-Activating Peptide OR Macrophage-Derived Chemotactic Factor OR Lymphocyte-Derived Neutrophil-Activating Peptide OR Granulocyte chemotactic protein 1 OR Neutrophil-activating protein 1 OR T-cell chemotactic factor

IL-9 OR IL9 OR interleukin 9 OR interleukin-9 OR interleukin9 OR T-Cell Growth Factor P40 OR P40 TCell Growth Factor OR T Cell Growth Factor P40 OR P40 T Cell Growth Factor OR Cytokine P40

IL-10 OR IL10 OR interleukin 10 OR interleukin-10 OR interleukin10 OR human cytokine synthesis inhibitory factor OR cytokine synthesis inhibitory factor

IL-11 OR IL11 OR interleukin 11 OR interleukin-11 OR interleukin11

IL-12 OR IL12 OR interleukin 12 OR interleukin-12 OR interleukin12 OR IL-12 p70 OR Interleukin-12 p70 OR Interleukin 12 p70 OR Cytotoxic Lymphocyte Maturation Factor OR Natural Killer Cell Stimulatory Factor OR IL-12 alpha OR IL-12A OR IL 12A OR IL12A OR IL-12a OR IL-12 subunit p35 OR NK cell stimulatory factor chain 1 OR IL-12 beta OR IL-12B OR IL12B OR IL-12 subunit p40 OR NK cell stimulatory factor chain 2

IL-13 OR IL13 OR interleukin 13 OR interleukin-13 OR interleukin13

IL-14 OR IL14 OR interleukin 14 OR interleukin-14 OR interleukin14 OR alpha-taxilin OR alpha taxilin

IL-15 OR IL15 OR interleukin 15 OR interleukin-15 OR interleukin15

IL-16 OR IL16 OR interleukin 16 OR interleukin-16 OR interleukin16 OR Lymphocyte Chemoattractant Factor

IL-17 OR IL17 OR interleukin 17 OR interleukin-17 OR interleukin17 OR interleukin 17A OR interleukin-17A OR IL-17A OR IL 17A OR CTLA-8 OR CTLA8 OR Cytotoxic T lymphocyte Associated Antigen 8 OR Cytotoxic T lymphocyte-Associated Antigen 8 OR Interleukin-17B OR Interleukin 17B OR IL-17B OR IL 17B OR IL17B OR Interleukin-17C OR Interleukin 17C OR IL-17C OR IL17C OR Cytokine

CX2 OR interleukin 17F OR Interleukin-17F OR IL-17F OR IL17F OR Cytokine ML-1 OR Cytokine ML 1

IL-18 OR IL18 OR interleukin 18 OR interleukin-18 OR interleukin18 OR interferon-gamma inducing factor OR Interferon-gamma-Inducing Factor OR Interferon gamma Inducing Factor OR IFN-gamma Inducing Factor OR IFN gamma Inducing Factor

IL-19 OR IL19 OR interleukin 19 OR interleukin-19 OR interleukin19

IL-20 OR IL20 OR interleukin 20 OR interleukin-20 OR interleukin20

IL-21 OR IL21 OR interleukin 21 OR interleukin-21 OR interleukin21

IL-22 OR IL22 OR interleukin 22 OR interleukin-22 OR interleukin22 OR IL-10-related T-cell-derived inducible factor OR IL-TIF

IL-23 OR IL23 OR interleukin 23 OR interleukin-23 OR interleukin23 OR IL-23A OR IL-23alpha

IL-24 OR IL24 OR interleukin 24 OR interleukin-24 OR interleukin24 OR Melanoma differentiation associated gene 7 protein OR MDA-7 OR Suppression of tumorigenicity 16 protein

IL-25 OR IL25 OR interleukin 25 OR Interleukin-25 OR Interleukin25 OR IL-17E OR IL17E OR interleukin-17E OR Interleukin 17E

IL-26 OR IL26 OR interleukin 26 OR interleukin-26 OR interleukin26 OR Protein AK155

IL-27 OR IL27 OR interleukin 27 OR Interleukin-27 OR interleukin27 OR Interleukin-17D OR Interleukin 17D OR IL-17D OR IL17D OR IL-27A OR IL-27 alpha OR Epstein-Barr virus-induced gene 3 protein OR EBV-induced gene 3 protein OR interleukin 27beta

IL-28 OR IL28 OR interleukin 28 OR interleukin-28 OR interleukin28 OR IL-28A OR IL28A OR interleukin 28A OR interleukin-28A OR IL-28B OR IL28B OR interleukin 28B OR interleukin-28B

IL-29 OR IL29 OR interleukin 29 OR interleukin-29 OR interleukin29 OR interferon lambda OR IFN lambda

IL-30 OR IL30 OR interleukin 30 OR interleukin-30 OR interleukin30 OR IL27-p28 OR IL27p28 OR Interleukin-30 IL27p28

IL-31 OR IL31 OR interleukin 31 OR interleukin-31 OR interleukin31

IL-32 OR IL32 OR interleukin 32 OR interleukin-32 OR interleukin32 OR IL-32alpha OR IL-32beta OR IL-32delta OR IL-32gamma OR natural killer cell transcript 4 OR NK4

IL-33 OR IL33 OR interleukin 33 OR interleukin-33 OR interleukin33 OR Interleukin-1 family member 11 OR IL-1F11 OR Nuclear factor from high endothelial venules OR NF-HEV

IL-34 OR IL34 OR interleukin 34 OR interleukin-34 OR interleukin34

IL-35 OR IL35 OR interleukin 35 OR interleukin-35 OR interleukin35

IL-36 OR IL36 OR interleukin 36 OR interleukin-36 OR interleukin36 OR FIL1 epsilon OR Interleukin-1 epsilon OR IL-1 epsilon OR Interleukin-1 family member 6 OR IL-1F6 OR FIL1 eta OR Interleukin-1 eta OR IL-1 eta OR Interleukin-1 family member 8 OR IL-1F8 OR Interleukin-1 homolog 2 OR IL-1H2 OR IL1-related protein 2 OR IL-1RP2 OR Interleukin-1 family member 9 OR IL-1F9 OR Interleukin-1 homolog 1 OR IL-1H1

IL-37 OR IL37 OR interleukin 37 OR interleukin-37 OR interleukin37 OR IL-1 family member 7 OR IL-1F7 OR FIL1 zeta OR IL-1X OR Interleukin-1 family member 7 OR Interleukin-1 homolog 4 OR IL-1H OR IL-1H4 OR Interleukin-1 zeta OR IL-1 zeta OR Interleukin-1-related protein OR IL-1RP1

IL-38 OR IL38 OR interleukin 38 OR interleukin-38 OR interleukin38

IL-39 OR IL39 OR interleukin 39 OR interleukin-39 OR interleukin39

IL-40 OR IL40 OR interleukin 40 OR interleukin-40 OR interleukin40

TNF OR tumor necrosis factor OR tumour necrosis factor OR tumor necrosis factor alpha OR tumour necrosis factor alpha OR Tumor Necrosis Factor-alpha OR Tumour Necrosis Factor-alpha OR TNFalpha OR TNF-alpha OR cachexin OR cachectin OR Cachectin-Tumor Necrosis Factor OR Cachectin Tumor Necrosis Factor OR Tumor Necrosis Factor Ligand Superfamily Member 2 OR Tumour Necrosis Factor Ligand Superfamily Member 2

TNFb OR TNFB OR Tumor Necrosis Factor-beta OR Tumour Necrosis Factor-beta OR Tumor Necrosis Factor beta OR Tumour Necrosis Factor beta OR TNF-beta OR lymphotoxin-alpha OR Lymphotoxin alpha OR alpha-Lymphotoxin OR alpha Lymphotoxin OR Lymphotoxin-alpha3 OR Soluble Lymphotoxin alpha OR Soluble Lymphotoxin-alpha OR Lymphotoxin alpha3 OR Lymphotoxin OR Tumor Necrosis Factor Ligand Superfamily Member 1 OR Tumour Necrosis Factor Ligand Superfamily Member 1 OR LT-alpha

Tumor Necrosis Factor C OR TNF-C OR TNFC OR Tumour Necrosis Factor C OR Lymphotoxin beta OR LT-beta OR Tumor Necrosis Factor Ligand 1C OR Tumour Necrosis Factor Ligand 1C OR TNF Superfamily Member 3

IFN OR IFN a OR interferon alpha OR alpha Interferon OR Interferon alpha 1 OR Interferon alpha-1 OR IFNalpha1 OR IFN-alpha2 OR IFN alpha D OR IFN-alpha D OR Interferon alpha 4 OR Interferon alpha4 OR Interferon alpha-4 OR Interferon alpha-J OR Interferon alpha J OR Interferon alpha 7 OR Interferon alpha-7 OR Lymphoblastoid Interferon OR Leukocyte Interferon OR Interferon Alfa OR Lymphoblast Interferon OR Interferon alpha-17 OR Interferon alpha 17 OR Interferon alpha-88 OR Interferon alpha 88 OR Interferon alpha-T OR Interferon alpha T OR Interferon alpha 5 OR IFN-alpha5 OR Interferon alpha-5 OR IFN alpha5 OR Interferon alpha5

IFN b OR IFNb OR interferon beta OR Interferon-beta OR beta-1 Interferon OR Interferon beta1 OR Interferon-beta1 OR beta 1 Interferon OR Fibroblast Interferon OR beta Interferon OR betaInterferon

IFN g OR IFNg OR interferon gamma OR Interferon-gamma OR gamma-Interferon OR Interferon Type II OR Type II Interferon OR Immune Interferon OR INF-gamma OR INFG

TGF OR TGF a OR TGFa OR TGF-alpha OR TGFalpha OR TGF alpha OR transforming growth factor alpha OR Epidermal Growth Factor Related Transforming Growth Factor OR Epidermal Growth Factor-Related Transforming Growth Factor OR ETGF OR TGF type 1

TGF b OR TGFb OR TGF-beta OR TGFbeta OR TGF beta OR transforming growth factor beta OR transforming growth factor b OR Bone Derived Transforming Growth Factor OR Bone-Derived Transforming Growth Factor OR Platelet Transforming Growth Factor

M-CSF OR macrophage colony stimulating factor OR Macrophage Colony-Stimulating Factor OR colony stimulating factor 1 OR Colony-Stimulating Factor 1 OR CSF-1 OR CSF1

GM-CSF OR Granulocyte macrophage colony stimulating factor OR Granulocyte-Macrophage ColonyStimulating Factor OR colony-stimulating factor 2 OR Histamine-Producing Cell-Stimulating Factor OR Histamine Producing Cell Stimulating Factor OR CSF2 OR CSF-2

G-CSF OR granulocyte colony stimulating factor OR Granulocyte Colony-Stimulating Factor OR Pluripoietin

Growth Differentiation Factor 15 OR Macrophage Inhibitory Cytokine 1 OR GDF-15 OR GDF15 OR Prostate Differentiation Factor OR MIC-11 OR NSAID-activated gene 1 protein OR NAG-1 OR NSAID regulated gene 1 protein OR NRG-1 OR Placental TGF-beta OR Placental bone morphogenetic protein

2.2.2.2 Condition keywords

Incident hypertension OR incident HTN OR incident HT OR incidence hypertension OR incidence HTN OR incidence HT OR incidence of hypertension OR incidence of HTN OR incidence of HT OR incident hypertension cases OR incident HTN cases OR incident HT cases OR incident hypertensive OR incident

hypertension comparing OR incident HTN comparing OR incident HT comparing OR incident hypertension risk OR incident HTN risk OR incident HT risk OR new onset hypertension OR new onset HTN OR new onset HT OR new onset hypertensive OR new onset hypertensive patients OR newly diagnosed hypertension OR newly diagnosed HTN OR newly diagnosed HT OR new HTN OR new hypertension OR new HT OR newly developed hypertension OR newly developed HTN OR newly developed HT OR future hypertension OR future HTN OR future HT OR hypertension occurrence OR HTN occurrence OR HT occurrence OR development of hypertension OR development of HTN OR development of HT OR hypertension development OR HTN development OR HT development OR risk of hypertension OR risk of HTN OR risk of HT OR hypertension risk OR HTN risk OR HT risk

2.2.2.3 Study design keywords

Clinical trials OR clinical trial OR randomized clinical trials OR randomised clinical trials OR randomized clinical trial OR randomised clinical trial OR randomized controlled trials OR randomised controlled trials OR randomized controlled trial OR randomised controlled trial OR controlled clinical trial OR controlled clinical trials OR trial OR randomization OR randomisation OR controlled study OR controlled trial OR single blind method OR single blind OR single blind methods OR double blind method OR double blind methods OR double blind OR placebo OR case control studies OR case controlled studies OR case control study OR case controlled study OR case control OR case controlled OR retrospective OR retrospective studies OR retrospective study OR prospective OR prospective studies OR prospective study OR observational OR observational study OR observational studies OR cohort study OR cohort studies OR cohort OR cross sectional OR cross sectional study OR cross sectional studies OR population studies OR population study OR population OR follow up studies OR follow up study OR follow up OR cohort analysis OR cohort analyses OR longitudinal study OR longitudinal studies OR longitudinal OR nested study OR nested studies OR nested case study OR nested case studies OR nested control study OR nested control studies OR nested case control OR nested case controlled OR nested case control study OR nested case control studies OR nested case controlled study OR nested case controlled studies OR proportional hazards models OR hazard ratio OR hazards ratio OR HR OR risk ratio OR relative risk OR rate ratio OR RR

2.2.3 Eligibility

We selected studies that reported associations for circulating cytokines with incident hypertension in the general population with no restrictions on age, gender, or ethnicity. We included randomized trials and observational studies (cohort studies, nested case–control studies). We excluded studies that enrolled pregnant women with preeclampsia and articles not written in English. Conference abstracts, editorials, letters, comments, cellular and animal model experiments, narrative or systematic reviews and meta-analyses, and ongoing studies were also excluded.

2.2.4 Definition of primary and secondary outcomes

The primary outcome was the risk for incident hypertension, defined as SBP ≥140mmHg or diastolic blood pressure ≥90mmHg and/or the use of antihypertensive medications. The secondary outcomes were the identification of cytokines associated with incident hypertension, and differences in baseline and longitudinal changes in cytokine levels in patients who developed hypertension compared to those who did not.

2.2.5 Study selection

Articles were initially screened by title and abstract according to the inclusion and exclusion criteria, independently by four investigators. For secondary screening, full-text articles and supplemental materials were independently reviewed by four investigators. Reasons for study exclusion were recorded and cross-checked. Any inconsistencies were resolved through consultation with supervisors. A flow diagram summarizes the process of study selection (Figure 2.1).

2.2.6 Data extraction and quality assessment

For eligible studies, the following information were independently extracted by three reviewers: publication data including the first author's name, country, study design, population type, total number of participants, patients with events, follow-up time, incident hypertension definition, baseline and follow-up cytokine levels, change in blood pressure by cytokine levels, participants’

baseline demographics [age, gender, ethnicity, body mass index (BMI), glucose, blood lipid levels, blood pressure status, alcohol and tobacco consumption, baseline comorbidities]. Three reviewers independently assessed the risk of bias using the risk of bias in non-randomized studies of exposures (ROBINS-E).

2.2.7 Statistical analysis

Pooled estimates of hazard ratios (HRs) and risk ratios (RRs) with 95% confidence intervals (CIs) for incident hypertension were calculated using the DerSimonian and Laird method in a random-effects model for the highest quartile compared with the lowest quartile (reference group) before and after adjustment for potential confounding factors including age, gender, and BMI. When data were available only in figures, HRs and RRs were extracted from figures with Digitizelt (version 2.5) software. To account for the small number of studies included, the Hartung and Knapp (HK) correction was used. Cytokine concentrations were reported using the median (interquartile range, IQR) or mean differences and 95% CI. Heterogeneity among the studies included in the meta-analysis was assessed through visual inspection of the forest plots and using the I^2 statistic. All analysis was performed using the R package meta, Version 5.2 with two-sided $p < 0.05$ set for statistical significance.

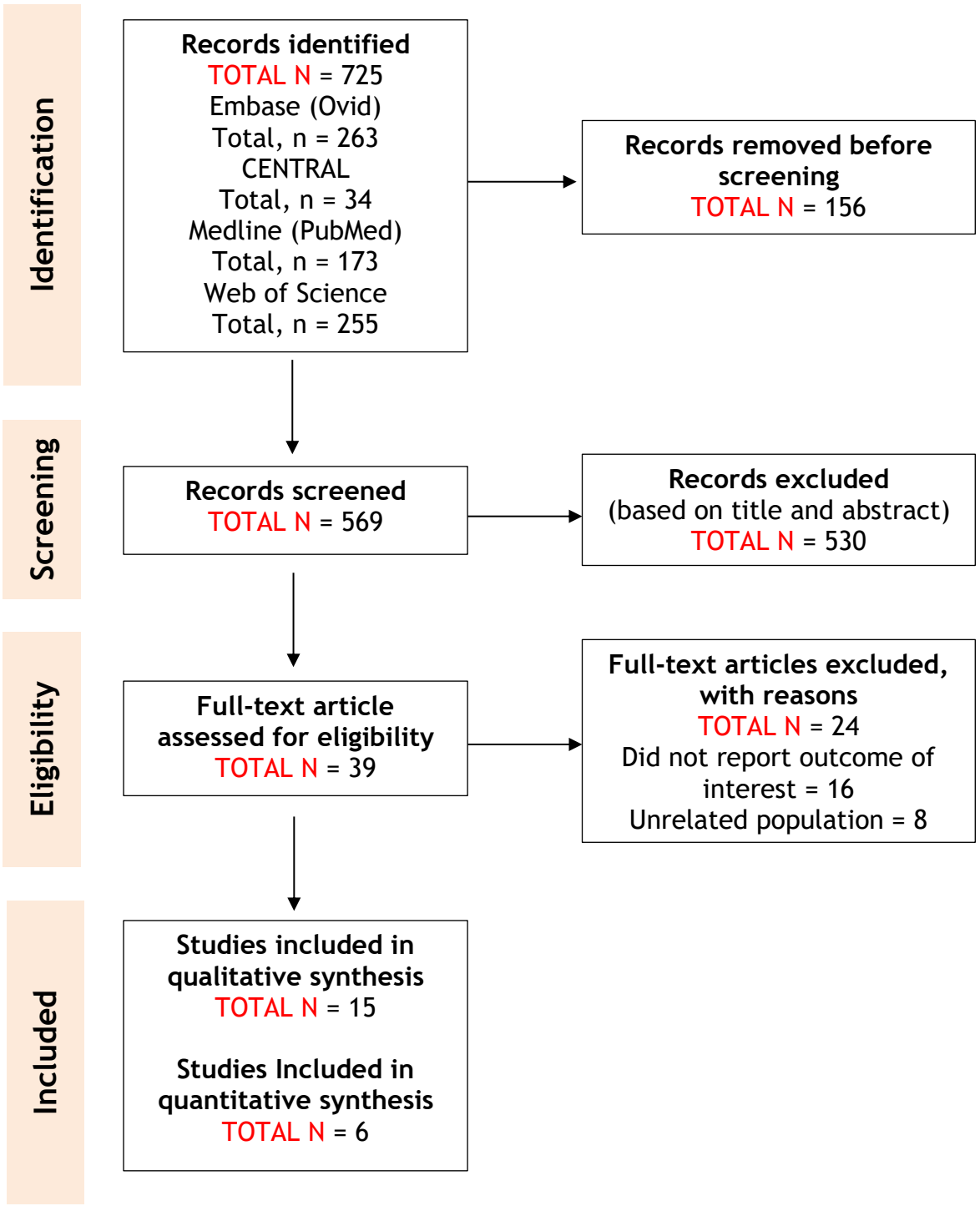


Figure 2.1 Flow chart reporting the search strategy according to PRISMA.

2.3 Results

A total of 725 studies were identified through the initial online search of the databases. Of these, 156 studies were duplicates. Title and abstract screening identified 39 studies for final full-text review using the inclusion/exclusion criteria. Of the 39 potentially relevant articles, after reading the full text of all articles, 24 were excluded because they did not report the outcome or the population of interest. Finally, the search yielded 15 studies for the qualitative analysis and of these, 6 studies were included in the quantitative analysis. The process of literature selection is shown in Figure 2.1. Among the searched cytokines, a pooled estimate of HRs and RRs for incident hypertension could only be produced for IL-6 and IL-1β that were thus meta-analysed.

2.3.1 Characteristics of studies and quality assessment

Of the six studies included in the meta-analysis, three of them were based on populations originally enrolled in randomized trials. The first study by Sesso (2007) (Sesso et al., 2007) was a prospective nested case-control analysis from the Women's Health Study, a randomized trial assessing the role of low-dose aspirin and vitamin E in the primary prevention of CVD and cancer, the other (2015) (Sesso et al., 2015) was a prospective nested case-control analysis of the Physicians' Health Study, a randomized trial of low-dose aspirin and beta carotene in the primary prevention of CVD and cancer. The study by Ghazi and colleagues (Ghazi et al., 2020) was a prospective cohort analysis from the Strategic Timing of AntiRetroviral Treatment (START) trial assessing immediate vs. deferred antiretroviral therapy (ART) in Human Immunodeficiency Virus (HIV)-positive participants. Wang and colleagues (Wang et al., 2011) reported findings from a nested-control analysis of the Women's Health Initiative-Observational Study (WHI-OS); the remaining two were observational, prospective cohort studies (Crouch et al., 2020a, Lakoski et al., 2011). Four studies were conducted only in the USA (Lakoski et al., 2011, Sesso et al., 2015, Sesso et al., 2007, Wang et al., 2011), one in South Africa (Crouch et al., 2020a), and one was from USA, Asia, Africa, Australia, Europe, Israel and Latin America (Ghazi et al., 2020).

Table 2.1 Main characteristics of the studies included in the meta-analysis.

Study ID	Country	Study design	Population	Total participants	Participants with events	Follow up	Incident Hypertension Definition	Cofounders that were controlled
Sesso 2007(Sesso et al., 2007)	USA	Nested Case-Control	Women aged ≥45 years	800	400	10 years	Self-reports of a physician diagnosis of hypertension at years 1, 3, and all annual questionnaires thereafter; self-reports of antihypertensive treatment at years 1, 3, and 4; self-reports of systolic BP 140 mm Hg; or self-reports of diastolic BP 90 mm Hg	Age, smoking status, alcohol use, exercise, BMI, age, follow-up time, post-menopausal hormone use, parental history of myocardial infarction, personal history of hypercholesterolemia, history of diabetes
Lakoski 2011(Lakoski et al., 2011)	USA	Prospective Cohort	General population aged 45–84 years	3543	714	5 years	Participants with a blood pressure ≥140/90 mm Hg or a history of	Age, gender, ethnicity, smoking, diabetes, statin use, aspirin use, alcohol use, Multi-Ethnic

							hypertension and use of blood pressure medications at Exam 2 or Exam 3	Study of Atherosclerosis (MESA) study site, BMI
Wang 2011 (a)(Wang et al., 2011)	USA	Nested Case-Control	Post-menopausal black women aged < 70 years	800	400	5.9 years (Median)	Initiation of medication specifically for increased BP and/or measured BP at the year-3 follow-up visit of either SBP ≥140 mmHg or DBP ≥90 mmHg	Age, clinical centre, time of enrolment, BMI, smoking, alcohol intake, physical activity, hormone replacement therapy
Wang 2011 (b)(Wang et al., 2011)	USA	Nested Case-Control	Post-menopausal white women aged < 70 years	800	400	5.9 years (Median)	Initiation of medication specifically for elevated BP and/or measured BP at the year 3 follow-up visit of either SBP ≥140 mmHg or DBP≥90 mmHg	Age, clinical centre, time of enrolment, BMI, smoking, alcohol intake, physical activity, hormone replacement therapy
Sesso 2015(Sesso et al., 2015)	USA	Nested Case-Control	Men aged 40-84 years	792	396	14 years	Self-reported initiation of antihypertensive	Age, follow-up time, date of hypertension, randomized

							treatment, systolic blood pressure ≥140 mm Hg, or diastolic blood pressure ≥90 mm Hg during follow-up	treatments, smoking status, physical activity, alcohol consumption, parental history of myocardial infarction, BMI, high cholesterol, diabetes mellitus
Crouch 2020(Crouch et al., 2020a)	South Africa	Prospective Cohort	20-30-year-old general population	358	68	4.45 years (Median)	4 repeated clinic blood pressure measurements, i.e., SBP>=140 mmHg and/or DBP >=90 mmHg.	Glucose, Socio-economic Status, Activity Energy Expenditure, Total Cholesterol, Estimated glomerular filtration rate, Cotinine, Gamma glutamyl transferase, Sex, Ethnicity, Age, BMI
Ghazi 2020(Ghazi et al., 2020)	Asia, Africa, Australia Europe, Israel, Latin America, USA	Prospective Cohort	HIV infected adults	3313	554	3 years (Median)	Systolic blood pressure ≥140 mm Hg, or diastolic blood pressure ≥90 mm Hg, or use of blood pressure lowering therapy among participants who	Age, gender, race, BMI, diabetes mellitus, smoking, treatment group, HIV RNA at baseline, $CD4^+$ T cell

							were not hypertensive at baseline	

Abbreviations: BP, blood pressure; BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure

The population included in the meta-analysis varied among the six studies. One study reported results for middle-aged and older women (Sesso et al., 2007), one reported separate analyses for black and white postmenopausal women (Wang et al., 2011), and one reported results for middle-aged and older men (Sesso et al., 2015). Crouch and colleagues reported separate results for young black and white men and women (Crouch et al., 2020a); upon request, we obtained the original data (Schutte et al., 2019) of this study and calculated the hazard ratio for the highest quartile compared with the lowest quartile of IL-6 and IL-1ß, before and after adjustment for age, sex and BMI. Ghazi et al. reported results for HIV-positive persons (Ghazi et al., 2020). Lakoski and colleagues (Lakoski et al., 2011) reported results from a longitudinal analysis of the Multi-Ethnic Study of Atherosclerosis (MESA) study including the adult general population.

Table 2.2 Baseline Characteristics of patients included in the meta-analysis.

	Sesso 2007(Sesso et al., 2007)		**Lakoski 2011(Lakoski et al., 2011)**		**Wang (a) 2011(Wang et al., 2011)**		**Wang (b) 2011(Wang et al., 2011)**		**Sesso 2015(Sesso et al., 2015)**		**Crouch 2020(Crouch et al., 2020a)**		**Ghazi 2020(Ghazi et al., 2020)**	
	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control
No. of Participants	400	400	714	2829	400	400	400	400	396	396	36	320	554	2759
Age (years)	54.5 ± 6.7	54.5 ± 6.6	63	58	58.2 ¥	58.1 ¥	60.8 ¥	60.8 ¥	47.5 ± 6.1	47.3 ± 6.1	26.0 ± 2.6	25.1 ± 3.2	38 [31, 45]*	34 [27, 41]*
Gender (% female)	100	100	53	51	100	100	100	100	0	0	36.1	59.4	21.1	26.8
Ethnicity (% White)	-	-	38	45	0	0	100	100	-	-	63.9	41.6	57.2	42.4
SBP, mmHg(%)			-	-	121.1 ¥	115.3 ¥	120.9 ¥	113.3 ¥	122.1 ± 8.1	118.9 ± 8.0	133 ± 6.45	115 ± 8.0	125 [118, 131]*	116 [110, 124]*
<110 (%)	6.9	24.1												
110-119 (%)	25.3	44.1												
120-129 (%)	45.4	25.6												
130-139 (%)	22.4	6.3												
DBP, mmHg (%)			-	-	74.7 ¥	71.9 ¥	74.1 ¥	70.2 ¥	77.3 ± 5.9	75.0 ± 6.7	78 ± 6.23	68 ± 5.24	80 [74, 84]*	73 [67, 80]*
<65 (%)	3.6	13.8												
65-74 (%)	29.8	48.4												
75-84 (%)	50.6	34.3												

85-89 (%)	16.0	3.5												
BMI (kg/m^2)	26.8 ± 4.8	24.4 ± 3.9	29 ± 6	27 ± 5	30.1 ¥	28.2 ¥	26.8 ¥	25.2 ¥	24.6 ± 2.4	24.1 ± 2.4	29.07 §	24.39 §	25.2 [22.8, 29.2]*	23.7 [21.5, 26.5]*
Waist circumference (cm)	-	-	100 (14)$	94 (14)$	88.8 ¥	84.6 ¥	83.6 ¥	79.0 ¥	-	-	91.53 §	77.75 §	-	-
HDL [(mg/dl)/ (mmol/l)]	52.0 ± 12.9	56.2 ± 15.5	50 ± 15	52 ± 15	-	-	-	-	-	-	1.26 ± 0.38	1.36 ± 0.40	-	-
LDL [(mg/dl)/ (mmol/l)]	123.8 ± 33.5	120.6 ± 31.8	119 ± 33	119 ± 30	-	-	-	-	-	-	3.03 §	2.51 §	-	-
Triglycerides (mg/dl)	-	-	116 (82)$	105 (79)$	-	-	-	-	-	-	1.05 §	0.81 §	-	-
History of HCL (%)	30.0	22.1	-	-	-	-	-	-	9.6	13.2			-	-
Diabetes (%)	2.3	1.0	13	7	9.8	4.0	2.5	1.0	0.5	1.0			3.1	1.5
Parental history of MI, <60y (%)	12.2	12.1	-	-	-	-	-	-	10.2	11.3			-	-
Smoking (%)													38.3	32.2
Rarely/ Never	53.5	50.3	48	51	46.5	51.4	49.1	50.9	57	61	61.1	67.2		
Former	33.5	37.7	38	34	40.2	38.2	45.1	43.0	36.9	33.7	13.9	9.4		
Current	13	12	14	15	13.3	10.4	5.8	6.1			25.0	23.4		
Current <15cigarettes /day	-	-	-	-	-	-	-	-	1.8	2.0				

Current >15 cigarettes/ day	-	-	-	-	-	-	-	-	4.3	3.3				
Alcohol use (%)											66.7	62.2	-	-
Rarely/ Never	43.5	34.8	19	18	13.9	17.3	8.8	8.3	12.9	14.4				
Former	-	-	-	-	30.1	26.0	16.8	15.0	-	-				
Current	-	-	-	-	-	-	-	-	-	-				
1-3 drinks/ month	13.7	14.8	-	-	-	-	-	-	-	-				
<1 drink/ week	-	-	-	-	36.5	30.8	34.8	28.3						
<2 drinks / week	-	-	-	-	-	-	-	-	28.4	29.1				
1-6 drinks / week	33.8	39.2	-	-	14.7	20.4	27.0	35.6	36.8	36.7				
≥ 7 drinks/ week					4.8	5.6	12.8	12.8						
≥ 1 drink/ day	9.0	11.2	-	-	-	-	-	-	21.8	19.8				
Physical activity			-	-	10.8 MET-h/wk ¥	12.9 MET-h/wk ¥	15.0 MET-h/wk ¥	16.9 MET-h/wk ¥			472.2 ± 234.7 AEE kcal/day	412.9 ± 191.3 AEE kcal/day	-	-
Rarely/ Never (%)	38.3	30.8							13.4	8.4				

< 1 time/week (%)	20.0	17.5							-	-				
< 2 times/week (%)	-	-							28.1	29.9				
≥ 2 times/week (%)	-	-							58.5	61.8				
1-3 times/wee (%)	30.0	41.0							-	-				
≥ 4 times/week (%)	11.7	10.7							-	-				
Statins use (%)	-	-	10	12	-	-	-	-	-	-	-	-	-	-
Asprin use (%)	-	-	24	20	-	-	-	-	-	-	-	-	-	-
Current hormone therapy (%)	44.6	44.5	-	-	39.4	34.5	60.5	53.8	-	-	-	-	-	-
IL-6	1.08 (µg/dL) §	0.93 (µg/dL) §	1.24 (1.2) pg/mL $	0.99 (0.9) pg/mL $	1.99 (ng/L) §	1.71 (ng/L) §	1.50 (ng/L) §	1.29 (ng/L) §	1.51 (pg/mL) §	1.07 (pg/mL) §	1.71 (pg/mL) §	2.08 (pg/mL) §	1.35 [0.93, 2.11] pg/mL *	1.33 [0.94, 2.08] pg/mL *
IL-1ß	-	-	-	-	0.27 (ng/L) §	0.25 (ng/L) §	0.25 (ng/L) §	0.24 (ng/L) §	-	-	1.01 (pg/mL) §	0.99 (pg/mL) §	-	-

*Median [25th, 75th]; $Median (IQR); §Geometric Mean; ¥Mean; All other values as Mean ± S.D. if not mentioned

Abbreviations: AEE, activity energy expenditure; BMI, body mass index; DBP, diastolic blood pressure; HCL, hypercholesterolemia; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MET, metabolic equivalent of task; MI, myocardial infarction; SBP, systolic blood pressure

A total of six studies with 10,406 participants and 2,932 incident cases of hypertension were included in the meta-analysis. The age of patients enrolled ranged from 20 to 84 years and the total follow-up times ranged from 3 to 14 years. The confounder factors more commonly used for adjustment were age, BMI, smoking status and alcohol use; specifically, all the studies controlled for age, smoking, BMI and alcohol intake, and three controlled for gender and ethnicity (Crouch et al., 2020a, Ghazi et al., 2020, Lakoski et al., 2011). Circulating cytokine levels of IL-6 were measured by ultrasensitive enzyme-linked immunosorbent assay (ELISA) (Ghazi et al., 2020, Lakoski et al., 2011, Sesso et al., 2015, Sesso et al., 2007, Wang et al., 2011) or Luminex xMAP technology (Crouch et al., 2020a) in serum or plasma samples. All studies defined incident hypertension as new-onset hypertension (SBP/DBP ≥140/90 mm Hg) or use of antihypertensive medications. The main characteristics of studies included in the current meta-analysis and patients' characteristics of studies included in the meta-analysis are reported in Table 2.1 and Table 2.2.

Most of the studies were assessed at low risk of bias, some concerns were reported about two studies due to self-reports of BMI values (Sesso et al., 2015, Sesso et al., 2007) and about one study for 11% missing data on IL-1β (Wang et al., 2011); all studies were included in the meta-analysis. The result of the risk of bias assessment through ROBINS-E is shown in Table 2.3.

Table 2.3 ROBINS-E risk of bias assessment. A summary table reporting each risk of bias item for each study is shown.

Study	Confounding	Measurement of the exposure	Selection of participants	Post-exposure interventions	Missing data	Measurement of the outcome	Selection of the reported result	Overall
Sesso 2007(Sesso et al., 2007)	Some concerns	Low	Low	Low	Low	Low	Low	Some concerns
	Participants provided self-reports of BMI							
Lakoski 2011(Lakoski et al., 2011)	Low	Low	Low	Low	Low	Low	Low	Low
Wang 2011(Wang et al., 2011)	Low	Low	Low	Low	Low	Low	Low	Low for IL-6
	For IL-6 low risk of bias about missing data and some concerns about IL-1β for the same domain due to 11% missing data.							
Sesso 2015(Sesso et al., 2015)	Some concerns	Low	Low	Low	Low	Low	Low	Some concerns
	Participants provided self-reports of BMI							
Crouch 2020(Crouch et al., 2020a)	Low	Low	Low	Low	Low	Low	Low	Low
Ghazi 2020(Ghazi et al., 2020)	Low	Low	Low	Low	Low	Low	Low	Low

Green: Low risk of bias, Yellow: Some concerns.

2.3.2 IL-6

All studies included in the quantitative analysis reported effect estimates per quartile of IL-6 levels. In crude models, the highest quartile of IL-6 levels was associated with a significantly higher risk of developing hypertension than the lowest quartile, with substantial heterogeneity across studies (Figure 2.2A; HR/RR: 1.61; [95% CI, 1.00 to 2.60]; I2=87%; n= 6]. In multivariable models not adjusted for BMI, HR/RR for the highest versus the lowest quartile of IL-6 was still associated, significantly greater with the risk of hypertension (Figure 2.2B; HR/RR: 1.71; [95% CI, 1.22 to 2.39]; I2=39%; n= 5). There was no significant association after adjustment including BMI (Figure 2.2C; HR/RR: 1.24; 95% CI, 0.96 to 1.61; I2= 56%, n= 6).

Hypertension is a growing problem in HIV-infected adults, especially in HIV-infected adults on ART (Gazzaruso et al., 2003, Peck et al., 2014, Xu et al., 2017, Nduka et al., 2016, Onen et al., 2010, Fahme et al., 2018). Pathophysiologic mechanisms for hypertension in HIV-infected adults may include chronic inflammation. In HIV-infected adults who participated in a trial of early versus delayed initiation of ART in Port-au-Prince, Haiti, Batavia et al. showed that higher plasma levels of IL-6 were independent predictors of incident hypertension only after adjustment for age, BMI and SBP (aHR 1.24 per log2 increase [95% CI, 1.12 to 1.38], p-value <0.001). In crude models, IL-6 did not predict incident hypertension (HR 1.09 per log2 increase [95% CI, 0.98 to 1.21], p-value 0.123)(Batavia et al., 2018). In contrast, in the nested case-control study performed by Okello and colleagues involving HIV-infected subjects taking ART in southwestern Uganda, the risk of incident hypertension was associated with age, male gender, and BMI, but not with IL-6 level in both univariate (OR 1.12 per log10 unit increase [95% CI, 0.81 to 1.56], p-value 0.483) and multivariate analyses (aOR 1.12 per log10 unit increase [95% CI, 0.73 to 1.74], p-value 0.600) (Okello et al., 2016) suggesting that traditional risk factors and not inflammation are predictive of hypertension in HIV-infected individuals (Fourie et al., 2015, Phalane et al., 2019, Phalane et al., 2020). Interestingly, in our sensitivity analysis excluding Ghazi 2020 (Ghazi et al., 2020) that enrolled HIV infected adults, baseline levels of IL-6 were associated with future hypertension in both crude and adjusted models after adjustment for BMI (Figure 2.3; respectively A, HR/RR: 1.78 [95% CI, 1.04 to 3.04], I2=79%, n= 6; B, HR/RR:

1.41 [95% CI, 1.19 to 1.67], I2=0%, n= 6), suggesting IL-6 as independent predictor of incident hypertension in HIV population. Therefore, conflicting data highlights the need for a better understanding of the relationship between circulating IL-6 levels and risk of developing hypertension in HIV-infected that may help identify underlying mechanisms of cardiovascular diseases in this population.

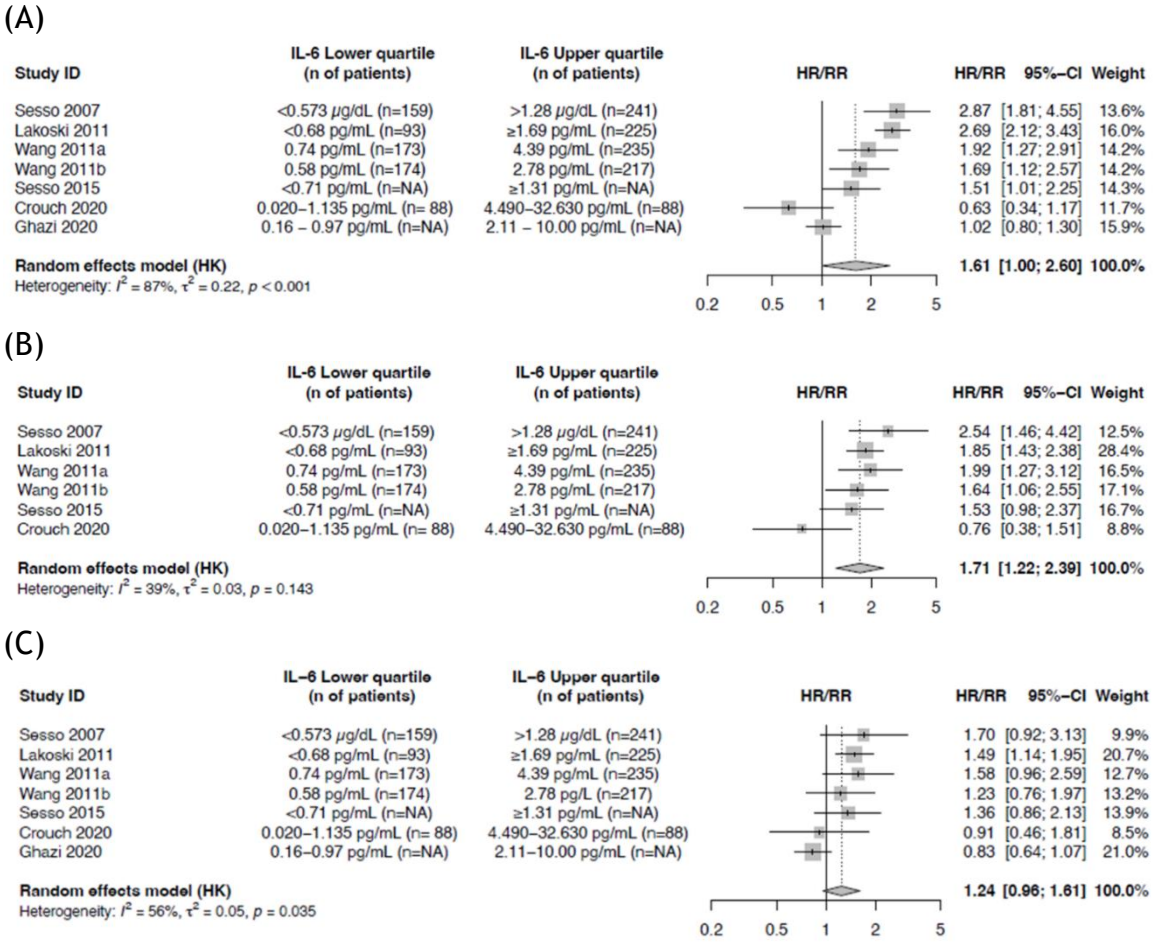


Figure 2.2 Forest plots of pooled HR/RR of hypertension for the highest versus lowest quartile of IL-6.Hazard ratio (HR)/Risk ratio (RR) and 95% confidence interval (CI) from the eligible studies of the association between IL-6 circulating levels and incident hypertension estimates in a random effects model with crude RR/HR pooled in (A), adjusted RR/HR pooled in (B) without BMI and adjusted RR/HR pooled in (C) including BMI.

Hypertension is more common in black populations with an earlier onset of the disease (Lackland, 2014) mainly attributed to several factors including differences in salt sensitivity, ionic transport mechanisms, obesity, renin-angiotensin-aldosterone system (Gafane et al., 2016, Lindhorst et al., 2007,

Sowers et al., 1988). Interestingly, a different inflammatory profile has been reported between blacks and whites but with contradictory findings (Mwantembe et al., 2001, Albandar et al., 2002, Crouch et al., 2020b). Crouch et al. reported a significant increase in blood pressure over 4-5 years only in young white populations (Crouch et al., 2020a) than young black populations. The authors have reported association between a large panel of pro- and anti-inflammatory mediators (individually and in clusters) and ΔBP only in white participants suggesting that in young age, new onset of hypertension may be driven by changes in inflammation, in this population. Wang et al. (Wang et al., 2011) reported a significant positive association for IL-6 and risk of developing hypertension in both black and white postmenopausal women that was lost after controlling for BMI suggesting adiposity as link to explain the association between inflammation and hypertension in this population. Thus, the association of cytokines with risk of developing hypertension in black and white individuals requires further investigation.

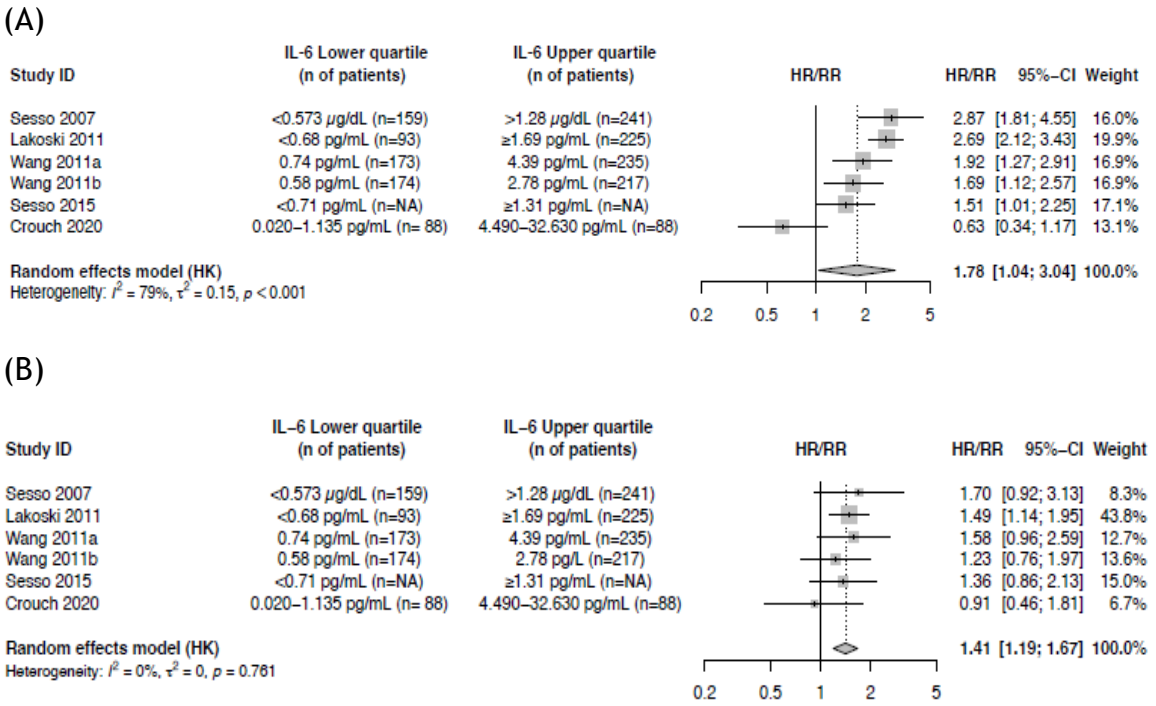


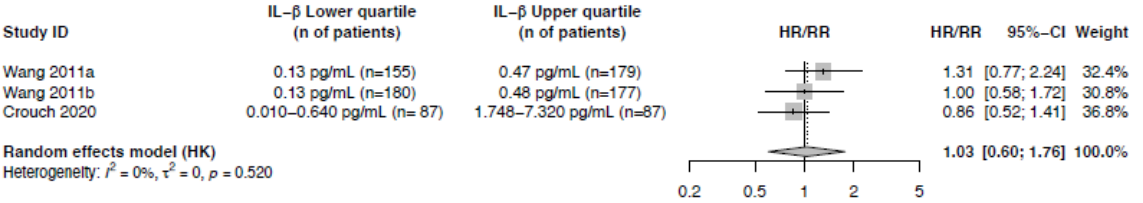
Figure 2.3 Forest plots showing sensitivity analysis performed by removing Ghazi 2020. Hazard ratio (HR)/Risk ratio (RR) and 95% confidence interval (CI) from studies of the association between IL-6 circulating levels and incident hypertension estimates in a random effects model with crude RR/HR pooled in (A), adjusted RR/HR pooled in (B) including BMI.

In addition, genetic variants in the gene encoding IL-6 may predispose to hypertension. Cheung and colleagues showed no association of plasma IL-6 and three single nucleotide polymorphisms (SNPs) in IL-6 (rs17147230, rs1800796, rs2069837) with incident hypertension. However, they did not investigate SNPs in genes encoding other inflammatory markers that may affect SNPs in IL-6 and included a small sample size of Hong Kong Chinese (Cheung et al., 2011a).

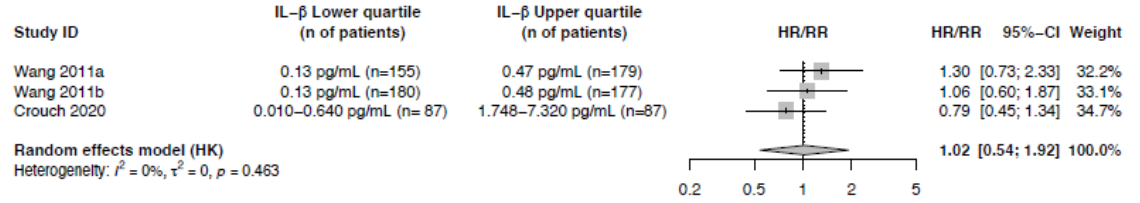
2.3.3 Interleukin-1β

Two studies assessed the risk of new onset hypertension associated with higher quartiles for IL-1ß (Crouch et al., 2020a, Wang et al., 2011). In crude models, HR/RR comparing the highest versus the lowest quartile of circulating IL-1ß was 1.03 [95% CI, 0.60 to 1.76]; I2= 0%, n=2 (Figure 2.4A). Similarly, there was non-significant difference in multivariable models with and without adjustment for BMI (Figure 2.4, respectively B, HR/RR: 1.02 [95% CI, 0.54 to 1.92], I2= 0%, n= 2; C, HR/RR: 0.97 [0.60 to 1.56], I2= 0%, n= 2). In contrast, Mauno et al. in their longitudinal study of 396 apparently healthy middle/aged men and women showed a statistically significant linear association between the quartiles of IL-1ß and change of SBP in a 6.5-year follow-up confirmed after adjustments for age, follow-up time, sex, baseline SBP, and BMI (P = 0.036) (Mauno et al., 2008).

(A)



(B)



(C)

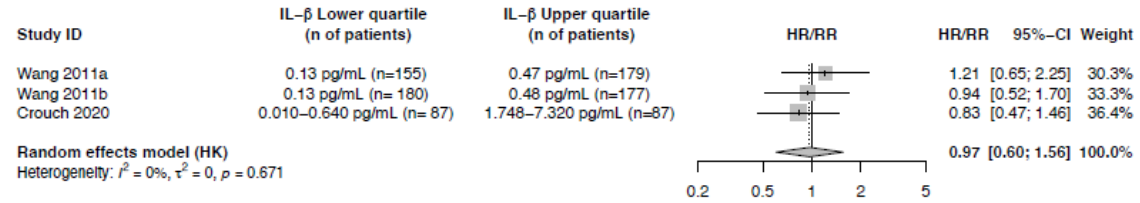


Figure 2.4 Forest plots of pooled HR/RR of hypertension for the highest versus lowest quartile of IL-1β. Hazard ratio (HR)/Risk ratio (RR) and 95% confidence interval (CI) from the eligible studies of the association between IL-6 circulating levels and incident hypertension estimates in a random effects model with crude RR/HR pooled in (A), adjusted RR/HR pooled in (B) without BMI and adjusted RR/HR pooled in (C) including BMI.

2.3.4 Other cytokines

It has been shown that high levels of plasma transforming growth factor ß1 (TGF-ß1) predict the development of hypertension (Nakao et al., 2017). Specifically, TGF-ß1 was significantly related to higher odds of developing incident hypertension in a Japanese normotensive population (OR 1.948; [95% CI, 1.015 to 3.740]; P=0.045).

In a study that enrolled patients with type 1 diabetes mellitus (Wisconsin Epidemiological Study of Diabetic Retinopathy; WESDR) (Sahakyan et al., 2010) followed up for over 15 years, serum IL-6 and TNF-α were associated with the development of hypertension in models adjusted for age and sex; however, when BMI was added to the model only soluble intercellular adhesion molecule-1 (sICAM-1) was associated with greater risk.

In a cohort of 471 postmenopausal women, of whom 195 developed hypertension, no statistically significant associations were found amongst several markers, including IL-8, IL-10, TNF-α, IL-2, IL-4 and IL-6 with greater risk (Gordon et al., 2021). Interestingly, statistically significant interactions were observed for SBP with IL-10 (P = 0.07). Baseline SBP of 120-139 mmHg was associated with higher hypertension risk for IL-10 (HR 1.11, 95% CI 0.91-1.34) in comparison with SBP <120 mmHg (0.85; 95% CI 0.68-1.05). These studies did not report RRs/HRs according to the highest quartile compared with the lowest quartile of the cytokines, therefore were not included in the quantitative analysis.

2.3.5 Baseline and follow-up plasma and serum cytokine levels

In a prospective cohort of 1,500 heathy subjects aged 20 to 69 years, among TNF-α, IL-1ß and IL-6, Yoo et al. found higher significant levels of IL-1ß, at baseline, in subjects who developed hypertension than those who did not develop hypertension (P = 0.028, adjusted for BMI at baseline) (Yoo et al., 2020). In addition, they found higher significant IL-1ß levels in new-onset hypertension group after the 2-year follow-up (P = 0. 003, adjusted for BMI at follow-up). Similarly, baseline plasma IL-1ß levels were statistically higher in newly diagnosed hypertensive patients compared with normotensive patients at the end of study performed by Mauno et al (Mauno et al., 2008).

These findings contrast those from the Anniston Community Health Survey study that showed higher, but not statistical significant geometric means (adjusted for age, sex, race, gender, and BMI) of IL-1 ß together with TNF-α, IL-6, IL-8 and IL-17 in the incidence of hypertension (Pavuk et al., 2019) and trials reported in other studies(Pavuk et al., 2019, Wang et al., 2011). Similarly, median IL-8, TNF-α and IL-10 concentrations were higher but not statistically significant in postmenopausal women in the Buffalo OsteoPerio Study who developed incident hypertension compared with those who did not (Gordon et al., 2021).

In the prospective study conducted in Japan on 133 normotensive participants by Nakao and colleagues, subject with baseline plasma TGF-ß1 levels ≥9.0 ng/ml were shown to have a 3.58-fold higher risk of developing hypertension in 14 years than those with TGF-ß1 levels ≤8.9 ng/ml (Nakao et al., 2017).

Baseline plasma IL-6 levels were not statistically different between 100 subjects with and 415 without incident hypertension in Hong Kong Chinese cohort over 5 years follow-up after adjusting for age, sex and follow-up duration (P = 0.355) (Cheung et al., 2011a). Similarly, other studies showed no statistically significant difference in baseline plasma IL-6 levels among subjects who developed hypertension compared with subjects without new-onset hypertension at follow-up (Ghazi et al., 2020, Sesso et al., 2015, Cheung et al., 2011a, Gordon et al., 2021, Pavuk et al., 2019, Yoo et al., 2020). In contrast, other studies showed higher significant baseline IL-6 levels among participants who developed incident hypertension compared with those who did not (Lakoski et al., 2011, Sesso et al., 2007, Wang et al., 2011). Baseline cytokine levels in plasma or serum together with follow-up changes in qualitative and quantitative studies are shown in Table 2.4.

Table 2.4 Baseline cytokine levels in plasma or serum together with follow-up changes in qualitative and quantitative studies.

Study ID	Group (Incident Hypertension)	Cytokine	Baseline level	P value	Follow-up level	P value	SBP at Baseline (mmHg)	P value	DBP at Baseline (mmHg)	P value	Follow-up time
Sesso 2007(Sesso et al., 2007)	Yes	IL-6 (µg/dL)	1.08¥	0.0007	-	-	-	-	-	-	10 years
	No		0.93¥		-	-	-	-	-	-	
Mauno 2008(Mauno et al., 2008)	Yes	IL-1ß (pg/mL)	0.67 ± 0.62	0.020	-	-	-	-	-	-	2 years
	No		0.56 ± 0.32		-	-	-	-	-	-	
Cheung 2011(Cheung et al., 2011a)	Yes	IL-6 (ng/l)	0.51 (0.45 – 0.59)*	0.355	-	-	124.8 ± 8.7	<0.001	77.0 ± 7.4	<0.001	5 years (median)
	No		0.44 (0.41 – 0.48)*		-	-	112.4 ± 10.2		71.9 ± 8.1		
Lakoski 2011(Lakoski et al., 2011)	Yes	IL-6 (pg/ml)	1.24 (1.2)$	<0.001	-	-	-	-	-	-	5 years
	No		0.99 (0.9)$		-	-	-	-	-	-	
Wang (a) 2011(Wang et al., 2011)	Yes	IL-6 (ng/L)	1.99¥	0.007	-	-	Yes: 121.1 No: 115.3	<0.001	Yes: 74.7 No: 71.9	<0.001	5.9 years
	No		1.71¥		-	-					
	Yes	IL-1ß (ng/L)	0.27¥	0.36	-	-					
	No		0.25¥		-	-					
	Yes	IL-6	1.50¥	0.006	-	-	Yes:	<0.001	Yes:	<0.001	5.9 years

Wang (b) 2011(Wang et al., 2011)	No	(ng/L)	1.29¥		-	-	121.1 No: 115.3		74.7 No: 71.9		
	Yes	IL-1β (ng/L)	0.25¥	0.37	-	-					
	No		0.24¥		-	-					
Sesso 2015(Sesso et al., 2015)	Yes	IL-6 (pg/mL)	1.51¥	0.12	-	-	122.1 ± 8.1	<0.001	77.3 ± 5.9	<0.001	14 years
	No		1.07¥		-	-	118.9 ± 8.0		75.0 ± 6.7		
Nakao 2017(Nakao et al., 2017)	Yes	TGF-β1 (ng/mL)	5.05 (3.78 – 8.93)$	0.030	-	-	-	-	-	-	14 years
	No		4.70 (3.45 – 6.43)$		-	-	-	-	-	-	
Pavuk 2019(Pavuk et al., 2019)	Yes	TNF-α (pg/mL)	3.02¥	0.71	-	-	-	-	-	-	8 years
	No		2.94¥		-	-	-	-	-	-	
	Yes	IL-1β (pg/mL)	0.35¥	0.31	-	-	-	-	-	-	
	No		0.30¥		-	-	-	-	-	-	
	Yes	IL-6 (pg/mL)	0.50¥	0.07	-	-	-	-	-	-	
	No		0.40¥		-	-	-	-	-	-	
	Yes	IL-8 (pg/mL)	4.43¥	0.44	-	-	-	-	-	-	
	No		3.99¥		-	-	-	-	-	-	
	Yes	IL-17 (pg/mL)	2.60¥	0.70	-	-	-	-	-	-	
	No		2.47¥		-	-	-	-	-	-	
	Yes	IFN-γ	6.94 ±2.11€	-	-	-	-	-	-	-	

Crouch 2020(Crouch et al., 2020a)	No	(pg/mL)	6.91±2.29€	-	-	-	-	-	-	-	4.45 years (Median)
	Yes	IL-1β	1.01±2.52€	-	-	-	-	-	-	-	
	No	(pg/mL)	0.99±2.36€	-	-	-	-	-	-	-	
	Yes	IL-2	0.68±3.26€	-	-	-	-	-	-	-	
	No	(pg/mL)	0.83±2.49€	-	-	-	-	-	-	-	
	Yes	IL-7	5.31±2.06€	-	-	-	-	-	-	-	
	No	(pg/mL)	5.58±2.40€	-	-	-	-	-	-	-	
	Yes	IL-8	1.64±2.21€	-	-	-	-	-	-	-	
	No	(pg/mL)	1.81±2.24€	-	-	-	-	-	-	-	
	Yes	IL-12	1.69±2.19€	-	-	-	-	-	-	-	
	No	(pg/mL)	1.82±2.47€	-	-	-	-	-	-	-	
	Yes	IL-17a	3.19±2.32€	-	-	-	-	-	-	-	
	No	(pg/mL)	3.23±2.64€	-	-	-	-	-	-	-	
	Yes	IL-23 (pg/mL)	121.48±3.43 €	-	-	-	-	-	-	-	
	No		119.98±3.26 €	-	-	-	-	-	-	-	
	Yes	TNF-α	1.54±2.11€	-	-	-	-	-	-	-	
	No	(pg/mL)	1.69±2.15€	-	-	-	-	-	-	-	
	Yes	IL-4	43.47±2.51€	-	-	-	-	-	-	-	
	No	(pg/mL)	43.19±2.4€	-	-	-	-	-	-	-	
	Yes	IL-5	0.83±2.65€	-	-	-	-	-	-	-	
	No	(pg/mL)	0.99±2.35€	-	-	-	-	-	-	-	
	Yes	IL-10	4.43±2.63€	-	-	-	-	-	-	-	
	No	(pg/mL)	4.89±2.54€	-	-	-	-	-	-	-	
	Yes	IL-13	4.03±3.27€	-	-	-	-	-	-	-	
	No	(pg/mL)	4.41±3.00€	-	-	-	-	-	-	-	
	Yes	GM-CSF	7.67±2.45€	-	-	-	-	-	-	-	
	No	(pg/mL)	7.36±2.92€	-	-	-	-	-	-	-	
	Yes	IL-6	1.71±3.33€	-	-	-	-	-	-	-	

	No	(pg/mL)	2.08±3.02€	-	-	-	-	-	-	-	
	Yes	IL-21	1.31±2.98€	-	-	-	-	-	-	-	
	No	(pg/mL)	1.38±2.53€	-	-	-	-	-	-	-	
Ghazi 2020(Ghazi et al., 2020)	Yes	IL-6 (pg/mL)	1.35 [0.93, 2.11]&	0.60	-	-	125 [118, 131] &	<0.0001	80 [74, 84] &	<0.0001	3 years (median)
	No		1.33 [0.94, 2.08]&		-	-	116 [110, 124] &		73 [67, 80] &		
Yoo 2020(Yoo et al., 2020)	Yes	TNF-α (pg/mL)	11.5 ± 1.37	0.487	10.7 ± 0.92	0.067 Adjusted for BMI at follow-up; 0.726. Adjusted for baseline values at the changed value	Yes: 126.0 ± 0.86 No: 116.8 ± 0.71	<0.001	Yes: 79.9 ± 0.66 No: 73.8 ± 0.54	<0.001	2 years
	No		9.83 ± 0.74		10.2 ± 1.22						
	Yes	IL-1β (pg/mL)	0.91 ± 0.10	0.028	0.86 ± 0.06	0.003 Adjusted for BMI at follow-up; 0.969.					
	No		0.73 ± 0.06		0.74 ± 0.07						

						Adjusted for baseline values at the changed value					
	Yes	IL-6 (pg/mL)	4.10 ± 0.34	0.086	3.92 ± 0.36	0.420 Adjusted for BMI at follow-up; 0.806 Adjusted for baseline values at the changed value					
	No		3.64 ± 0.25		3.61 ± 0.23						
Gordon 2021(Gordon et al., 2021)	Yes	IL-2 (pg/mL)	0.75 (0.43-1.53)&	-	-	-	Yes: 118.4 ± 11.0 No: 109.0 ± 11.4	<0.001	Yes: 70.5 ± 7.7 No: 66.5 ± 6.9	<0.001	10.2 years (average)
	No		0.76 (0.49-1.44)&	-	-	-					
	Yes	IL-4 (pg/mL)	1.7 (1.2-3.2)&	-	-	-					
	No		1.8 (1.3-3.6)&	-	-	-					
	Yes	IL-6 (pg/mL)	0.88 (0.48-2.21)&	-	-	-					

	No		0.92 (0.50-2.18)&	-	-	-					
	Yes	IL-8 (pg/mL)	9.9 (6.9-15.4)&	-	-	-					
	No		9.0 (6.4-13.4)&	-	-	-					
	Yes	IL-10 (pg/mL)	0.85 (0.40-2.35)&	-	-	-					
	No		0.83 (0.49-2.32)&	-	-	-					
	Yes	TNF-α (pg/mL)	1.6 (1.2-2.0)&	-	-	-					
	No		1.5 (1.2-1.9)&	-	-	-					

*Geometric mean (95% CI); $ Median (IQR); ¥ Geometric mean; & Median (25th–75th percentile); Otherwise, values Mean ± S.D.; € Geometric mean ± S.D

2.4 Discussion

In this systematic review and meta-analysis, we analysed associations for circulating cytokines with incident hypertension. Fifteen studies met the inclusion criteria; however, although many cytokines were reported they could not be combined because of heterogeneity of statistical methodology for reporting effect estimates and variation in unit change; therefore, pooled estimates could only be combined for two cytokines (IL-6 and IL-1β) from six studies.

We found that higher levels of circulating IL-6 were significantly associated with 61% greater risk of developing hypertension. In contrast, higher levels of IL-1β were not associated with the risk of incident hypertension, but few studies were available for the quantitative analysis. Thus, additional studies are needed to define the role of IL-1β more clearly in the development of the hypertensive phenotype.

IL-6 is a pleiotropic cytokine secreted by various cells including macrophages, monocytes, T lymphocytes, endothelial cells and vascular smooth muscle cells, typically regarded as a pro-inflammatory cytokine and an anti-inflammatory myokine (Barton, 1996). Preclinical evidence suggests that IL-6 might be involved in the initiation, as well as in the progression and maintenance of hypertension through a reduction in nitric oxide bioavailability, increase in vascular superoxide, regulation of angiotensin II expression and alterations in vascular function and structure (Schrader et al., 2007b, Gomolak and Didion, 2014). IL-6 is the major proinflammatory cytokine that has been described to be linked with hypertension; however, preclinical and clinical studies associate increased circulating levels of IL-6 with hypertension without demonstrating causality (Gibas-Dorna et al., 2015, Naya et al., 2007a, Fernandez-Real et al., 2001). Thus, more research is required to identify the specific contribution of IL-6 in hypertension development.

In line with a previous meta-analysis (Jayedi et al., 2019) that included a smaller number of studies, we found that the positive association between IL-6 and risk of hypertension became insignificant after adjustment for BMI. This finding suggests a link between IL-6 and obesity driving hypertension risk. Obesity is a

well-known risk factor for hypertension, and weight loss is an important treatment strategy to prevent and control hypertension (Kuwabara et al., 2018, Neter et al., 2003, Xu et al., 2022). Obesity, inflammation and hypertension share common underlying cellular and humoral mechanisms involving oxidative stress (Camargo et al., 2022, Sagris et al., 2022), activation of cytokines and activation of the renin–angiotensin system (Yudkin et al., 2000), and alteration of immune function. A positive relationship between BMI and circulating IL-6 has been shown in postmenopausal women (Straub et al., 2000) as well as in healthy subjects with obesity (Werida et al., 2021). On the other hand, Sesso and colleagues also reported high association between IL-6 and risk of developing hypertension except for women with a BMI <25 kg/m^2 with no significant interactions (P= 0.51) (Sesso et al., 2007). Similarly, Wang et al. reported no significant associations between IL-6 and risk of developing hypertension in any category of baseline BMI in a cohort of white and black postmenopausal women, suggesting an independent association between obesity and IL-6 in the risk of future hypertension (Wang et al., 2011). Interestingly, IL-6 level is a well-known predictor of cardiovascular mortality also when adjusting for BMI (Botha et al., 2015). It has been estimated that around 25% of circulating IL-6 is released by subcutaneous adipose tissue (Mohamed-Ali et al., 1997, Schmidt et al., 2015); thus, adipose tissue-derived IL-6 may be involved in hypertension development.

In our sensitivity analysis, removing patients with HIV (Ghazi et al., 2020), high baseline IL-6 levels were significantly related to a higher risk of developing incident hypertension in both crude and adjusted models after adjustment for BMI suggesting IL-6 as independent from BMI in predicting hypertension incidence in individuals without HIV. However, there is no consensus about the association between high levels of IL-6 as a predictor of incident hypertension in HIV-infected adults (Batavia et al., 2018, Okello et al., 2016), highlighting the need for a better understanding of this relationship in HIV population. On the other hand, IL-6 has been shown to predict cardiovascular mortality in black South Africans also when adjusting for BMI (Botha et al., 2015), therefore may be interesting investigating more in-depth the association of cytokines with the risk of developing hypertension and adiposity in black and white individuals.

Our meta-analysis has several strengths. First, we performed a broad and systematic search to identify cytokines associated with incident hypertension. Secondly, our study shows that the association between IL-6 and the risk of hypertension is weakened by BMI, highlighting the important relationship between inflammation and adiposity in the risk of developing hypertension. Third, we have updated the existing evidence of the role of elevated IL-6 on the risk of hypertension with a robust sample size, inclusion of modern cohorts and unpublished data.

There are also several limitations in this study. First, cytokines were only measured at enrolment. Second, the number of studies for IL-1β analysis was limited to two. Third, even if we reported multivariate analysis after adjustment for known risk factors, residual confounding may occur. Fourth, we were unable to perform subgroup analysis based on characteristics, such as race/ethnicity or genetic polymorphisms, which may predispose to hypertension.

2.5 Conclusion

The mechanisms underlying the involvement of cytokines in hypertension range from classical immune-regulatory functions (Bullenkamp et al., 2021) to direct modulation of vascular and renal functions (Alexander et al., 2021). The importance of these observations extends beyond our understanding of the pathophysiology of hypertension and provides important clues for common mechanisms between hypertension, atherosclerosis (Libby, 2021, Welsh et al., 2017) and heart failure (Pugliese et al., 2023, Bertero et al., 2022). It is also important to consider inflammatory cytokines as biomarkers for identifying patients presenting with the immune and inflammatory mechanisms of hypertension (Evans et al., 2022) and atherosclerosis (Hettwer et al., 2022). We observed that higher levels of circulating IL-6, but not of IL-1β, were significantly associated with risk of incident hypertension. The positive association observed for IL-6 was no longer statistically significant after adjusting for BMI. Our results highlight the important link between inflammation and adiposity in the risk of developing hypertension. Further research is required to better identify their links to incident hypertension.

Chapter 3

Investigating the effects and mechanisms of endothelial dysfunction induced by inflammatory cytokines

3.1 Introduction

Hypertension is characterised as a disorder where the blood pressure is chronically over 140/90 mmHg. It is a global cause of morbidity and mortality affecting over a billion people worldwide with the primary cause of the disease being unknown after years of research (Drummond et al., 2019). A variety of treatment options targeting the renal, cardiac, vascular, and central nervous system are available but hypertension still remains untreated and increases cardiovascular risks like myocardial infarction, heart failure and stroke in a majority of patients undergoing treatment (Drummond et al., 2019, Lackland and Weber, 2015).

The regulation of blood flow and volume involves interactions between the heart, kidneys, and the vascular system by means of physiological systems such as RAAS and SNS. These physiological systems play a crucial role on blood pressure regulations and are found to be upregulated in hypertensive conditions. Recent studies have indicated that immune and inflammatory mechanisms can significantly contribute to the disease. However, the mechanisms of their involvement in hypertension remain poorly defined.

The immune system can protect and promote hypertension by producing cytokines which are pro – or anti – inflammatory in nature. A number of cytokines such as: IFN-γ, TNF, TGFß, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17, IL-21, and IL-22 are produced by innate and adaptive immune cells such as T regulatory cells, invariant natural killer T cells, macrophages, myeloid derived suppressor cells, and dendritic cells (Nussenblatt, 2010, Nash et al., 2015, Burrell et al., 2016, Pahl and Vaziri, 2020, Corthay, 2009, Brennan et al., 2013, Justin Rucker and Crowley, 2017, Shen and Fillatreau, 2015, Chiasson et al., 2018). These inflammatory cytokines along with others may possibly modulate vascular mechanisms involved in increasing blood pressure and cardiovascular risk including oxidative stress and NADPH oxidase activity, and consequently endothelial dysfunction (Rucker et al., 2018).

Endothelium is a single celled, innermost lining of the circulatory system. It is semi-permeable and acts as a barrier to the exchange of material in the vasculature. Functions of endothelium include maintaining vascular tone by

vasoconstriction and vasodilation, maintaining cardiac homeostasis by regulating thrombosis and thrombolysis, cell proliferation and angiogenesis among other functions (Tousoulis et al., 2012). These functions maintain the bioavailability of NO, which is produced by eNOS.

eNOS converts L-arginine to L-citrulline by reducing NADPH in a calcium/calmodulin dependent manner and produces NO with the help of BH4, a redox labile cofactor (Drummond and Sobey, 2014). Under inflammatory conditions caused by diabetes or hypertension, ROS production causes oxidation of BH4, resulting in the uncoupling of eNOS to produce ROS, and hence causing an imbalance in the bioavailability of NO to maintain endothelial function (Montezano and Touyz, 2012a, Chen et al., 2008, Landmesser et al., 2003). NO production is also regulated by phosphorylation of amino acid residues on eNOS. Most phosphorylation sites studied before are Serine 1177 (S1177) and Threonine 475 (T495) in humans. When eNOS is phosphorylated at S1177 NO production is enhanced and when phosphorylated at T495, NO production is inhibited (Guzik et al., 2000).

Even though eNOS is responsible for the production, it is said to be the secondary producer or ROS, while specific NOX isoforms are the primary producers in the endothelium. There are seven isoforms of NOXs named NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2. Four of these isoforms are found in the endothelium. NOX1, NOX2 and NOX5 produce superoxide anions resulting in reduced NO bioavailability and subsequently endothelial function. NOX4 is highly expressed in the endothelium and produces hydrogen peroxide (Drummond and Sobey, 2014) which is not found to react with NO. Hence NOX4 can be considered to act as a vaso-protective entity and its overproduction has found to be related with overexpression of eNOS (Craige et al., 2011).

To study and comprehend the effect of inflammatory cytokines like IL-1β and IL-6, which have been associated with the incidence of hypertension, and TNF-α, IFN-γ, IL-17a and IL-15, which are otherwise said to affect endothelial function, the effects of the selected cytokines on vascular function, gene expression and their mechanisms of action was investigated.

3.2 Materials and methods

3.2.1 Vasomotor studies

3-month-old C57BL/6 male mice were obtained from Charles River, and their thoracic aortas were used to study the effect of IL-1β, IFN-γ, IL-6, IL-17A, IL-15 and TNF-α on endothelium-dependent and endothelium-independent function of the vessel. 610DMT multi-wire myograph system, PowerLab data acquisition system, and LabChart software were used to record and analyse the changes in vasomotor function. Contraction and relaxation of mouse vessels were conducted using high potassium physiological saline solution (KPSS), phenylephrine (Phe), serotonin (5-hydroxytryptamine, 5HT), acetylcholine (ACh), and sodium nitroprusside (SNP).

3.2.1.1 Vessel harvesting

A mouse was carefully transferred to a carbon dioxide chamber using a cardboard tube. After securing the lid, carbon dioxide was let inside the chamber at an appropriate flow rate to allow a slow increase of gas. Once the animals were unconscious, the flow of carbon dioxide was rapidly increased and maintained for 2 minutes or until respiratory arrest was observed. Death was confirmed by dislocation of upper cervical spinal column or by severing the femoral artery. The mouse was then pinned down on a surgical board with ventral side exposed. After sterilizing the site of incision, a vertical cut was made in the abdomen using sterile forceps and scissors. While holding the skin in place, the skin was cut until the diaphragm in the thoracic cavity, and lungs, along with rib cage were removed. The heart was then perfused with cold sterile Dulbecco's phosphate buffer saline [DPBS (Gibco™ Thermo Scientific, cat. no. 14190144)] using a syringe and extra fluid was removed using a sterile gauze. The abdominal cavity was cleared of all the gastro-intestinal organs, and the aorta was carefully separated from the spine and collected in cold sterile DPBS.

3.2.1.2 Cytokine induced media preparation

In a sterile environment, Rosewell Park Memorial Institute (RPMI) 1640 media (Thermo Scientific, cat. no.11875093) with antibiotics was used to prepare cytokine rich media. Cytokine concentrations were selected from previous

experimental values available in literature and has been discussed in detail in Section 3.4 and Table 3.3. The selected cytokine concentrations are as follows:

i) Mouse IL-1β: 1 ng/ml

ii) Mouse IFN-γ: 10 ng/ml

iii) Mouse IL-6: 5 ng/ml

iv) Mouse TNF-α: 1 ng/ml

v) Mouse IL-17a: 1 ng/ml

vi) Mouse IL-15: 10 ng/ml

3.2.1.3 Vessel treatment

Upon harvesting, the perivascular adipose tissue was carefully removed from the vessels in cold conditions. The vessels were then cut into 2 mm rings and were put in media containing cytokines or a vehicle. Then the vessels were cultured for 18 hours in 95% oxygen - 5% carbon dioxide at 37°C.

3.2.1.4 Mounting of vessels

The myograph chambers were first cleaned with double distilled water and cold PSS (118.99 mmol/l NaCl, 4.69 mmol/l KCl, 1.17 mmol/l MgSO4-7H2O, 1,18 mmol/l KH_2PO_4, 2,5 mmol/l $CaCl_2$-$2H_2O$, 25 mmol/l $NaHCO_3$, 11.1 mmol/l glucose, 0.03 mmol/l EDTA) thoroughly, and were then filled with cold PSS. Around 2.2 cm of 40 μm was placed between the jaws of the myograph and one end was wrapped around a fixing screw in clockwise manner. The rings were removed from the incubator and were mounted on the wire through the unscrewed end. The wire was wound at both ends with the screws and another piece of wire was passed through the lumen and wound clockwise onto the other jaw of the myograph chamber with the jaws always aligned together.

3.2.1.5 Myography protocol

Once the rings were mounted, they were let to acclimatise in the chambers until the chamber temperature rose to 37°C. Once the optimum temperature was attained, the rings were washed with warm oxygenated PSS and let to equilibrate for 15 minutes. The rings were normalised to 15 mN slowly but steadily increasing no more than 2 mN tension at once. After the rings normalised and equilibrated for 10-15 minutes without any changes in tension, the rings were pre-constricted with 5 ml KPSS (64.86 mmol/l NaCl, 58.82 mmol/l KCl, 1.17 mmol/l $MgSO_4$-$7H_2O$, 1,18 mmol/l KH_2PO_4) twice to determine maximum constriction. Then the rings were constricted with increasing doses (10^{-6}M – 10^{-2}M) of Phe or 5-HT. The endothelium-dependent vasorelaxation of rings were observed using increasing doses of ACh (10^{-6}M – 10^{-2}M). Endothelium-independent vasodilation was assessed using increasing doses of SNP (10^{-6}M – 10^{-2}M) as a vasorelaxant. Figure 1 illustrates the sequential flow of the experiment and Table 1 presents the quantities of concentrated stock solutions added to the organ bath. The vessels were washed three times with warm oxygenated PSS after each relaxation until they reached baseline tension of 15 mN.

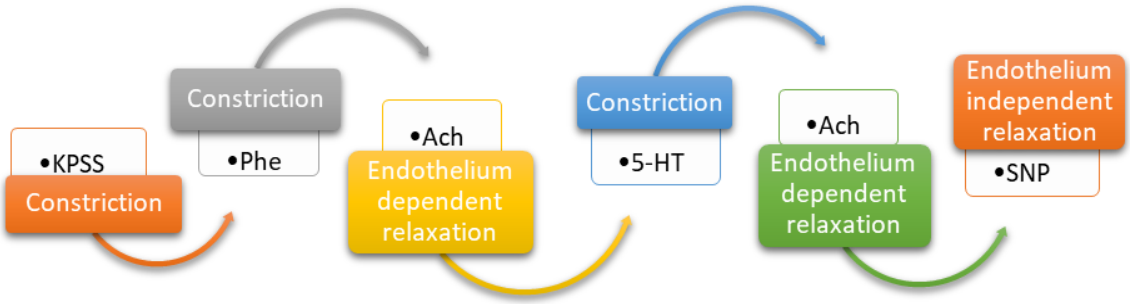


Figure 3.1 Sequential flow of organ bath experiment.

3.2.1.6 Data analyses and statistics

The curves were analysed using the LabChart software and data was analysed in Microsoft Excel. Graphs were generated in GraphPad Prism and repeated measure two-way ANOVA with Geisser-Greenhouse correction was also performed using GraphPad Prism.

Table 3.1 Volume of concentrated stock solutions to add in the bath to increases doses of Phe, Ach, 5-HT, and SNP

Volume of bath = 5 ml		
Stock solution (Molar)	**Volume to add (µl)**	**Concentration in bath (M)**
$1x10^{-6}$	5	$1x10^{-9}$
$1x10^{-6}$	10	$3x10^{-9}$
$1x10^{-6}$	35	$1x10^{-8}$
$1x10^{-5}$	10	$3x10^{-8}$
$1x10^{-5}$	35	$1x10^{-7}$
$1x10^{-4}$	10	$3x10^{-7}$
$1x10^{-4}$	35	$1x10^{-6}$
$1x10^{-3}$	10	$3x10^{-6}$
$1x10^{-3}$	35	$1x10^{-5}$
$1x10^{-2}$	10	$3x10^{-5}$

3.2.2 Gene profiling

For RNA extraction and qRT-PCR, qiazol, chloroform, metal beads, elusion columns, RNAse free tubes, centrifuge, RNAse free water, homogeniser, 100% ethanol, Qiagen RNeasy Mini Kit (Cat. No. 74106), cDNA kit (reverse transcription buffer, dNTP mix, reverse transcription primers, multi-scribe reverse transcriptase, RNAse inhibitor), TaqMan gene expression master mix, TaqMan probes (NOS3, NOX1, CYBB, NOX4 and TBP), PCR thermal cyclers and TaqMan PCR machine were used.

3.2.2.1 RNA extraction

For RNA extraction, to an RNAse free tube, a metal bead and 700 µl of qiazol was added along with the mouse vessel. The tissue was homogenised for 6 minutes at 30 Hz. The homogenised mix was rested at room temperature for 5

minutes after which 140 µl of chloroform was added to the mix, vortexed for 15 seconds and incubated for 3 minutes at room temperature. The mix was then centrifuged at 12000 g for 15 minutes at 4°C, and the aqueous phase was collected in a separate RNAse free tube. Equal volume of 100% ethanol was added, vortexed, and then transferred to a column. The column was then centrifuged at 15000 g for 30 seconds at room temperature. The column was then washed with 400 µl of wash buffer and centrifuged again at 15000 g for 30 seconds at room temperature. The column was then incubated with 80 µl DNAse-I solution mix for 15 minutes at room temperature. Later the column was washed with 400 µl pre-wash buffer twice followed by centrifugation at 15000 g for 30 seconds at room temperature. 700 µl wash buffer was added and the column was centrifuged for 2 minutes at 15000 g, followed by another 30 seconds centrifugation. The column was then transferred to a new RNAse free tube and RNAse free water was added and incubated for 3 minutes after which the columns were centrifuged and at 15000 g for 1 minute at room temperature and the eluted RNA was quantified using nanodrop ND1000 (ThermoFisher Scientific) by assessing the A260/280 ratio.

3.2.2.2 RT-PCR

For cDNA preparation, 13.2 µl RNA sample was added to Eppendorf tubes with 6.8 µl 2X reverse transcription master mix (2 µl 10x reverse transcription buffer, 0.8 µl 25X dNTP mix, 2 µl 10X reverse transcription primers, 1 µl RNAse inhibitor and 1 µl multi scribe reverse transcriptase). The Eppendorf tubes were sealed, centrifuged to remove air bubbles, and settle the mix. The tubes were then placed in a thermal cycler. The different steps were defined for temperature and time, and the cycler was run for 20 µl sample volume.

Table 3.2: PCR annealing, polymerisation and enzyme denaturation steps defining time and temperature per step

	Step 1 – primer annealing	Step 2 – polymerisation	Step 3 – enzyme denaturation	Step 4 – stabilisation and storage
Temperature (°C)	25	37	85	4
Time	10 minutes	120 minutes	5 minutes	∞

3.2.2.3 qPCR

After the cDNA was obtained, qPCR mix was prepared using 4.5 µl cDNA template and 5.5 µl PCR reaction mix [5 µl 2xTaqMan gene expression master mix (ThermoFisher, cat. No. 4444556) and 0.5 µl 20xTaqMan probe] and added to each well on a 96 well plate. The plate was sealed and centrifuged to remove any air bubbles. The plate was then run on Quant Studio 5 real-time PCR system (ThermoFisher Scientific) and PCR was conducted. The probes were run for eNOS, NOX1, NOX2, NOX4 and housekeeping gene TATA-binding protein (TBP).

3.2.2.4 Data analyses and statistics

Samples were run in technical replicates and Cycle threshold (Ct) values were obtained after the qPCR. Relative quantification of genes of interest was carried out in Excel. The results were compiled, and two tailed unpaired t-test was performed, and graphs were generated using GraphPad Prism (version 9.5.1).

3.2.3 Cell culture

Human aortic endothelial cells (HAoEC) were obtained from PromoCell (Cat. No. C-12271) along with human endothelial cell growth media (Cat. No. C-22010). For cell stimulation, recombinant human IL-15 (10 µg, Cat. No. 200-15) and recombinant human IFN-γ (100 µg, Cat. No.300-02) were ordered from PeproTech. DPBS (Gibco™) was used in the cell cultures and radioimmunoprecipitation assay (RIPA) buffer from Thermo Scientific (Cat. No. 89901) was used to lyse the harvested cells. Trypsin, fetal bovine serum (FBS), penicillin and streptomycin, cell culture dishes, 15 ml tubes and flasks were used from the laboratory stock.

3.2.3.1 Media preparation

Human endothelial cell growth media, human endothelial cell growth supplement and antibiotics (Penicillin and Streptomycin) were thawed and warmed up to 37°C in a water bath. Upon reaching the required temperature, all vials and bottles were opened in sterile conditions, and 10 ml growth supplement and 10 ml antibiotic mixture were added to 500 ml growth media. This growth media mix was then used to seed the cells.

3.2.3.2 HAoEC seeding

Cryopreserved HAoECs were thawed at 37°C in a water bath. Under sterile conditions, 1 ml of warm media mix was added to the cell vial and gently mixed. The contents of the vial were then added to a 15 ml tube along with 8 ml media mix. The tube was then centrifuged at 1200 revolution per minute for 3 minutes at room temperature. The supernatant was discarded, and the cell pellet was then resuspended in 1 ml media mix and mixed gently. A T-75 flask was prepared with 9 ml media mix and the resuspended cells were added to the flask. The flask was then lightly tapped to spread the cells evenly and then incubated at 37°C with 95% oxygenation.

3.2.3.3 Media change and cell passaging

After 24 hours of seeding the cells, the flask was observed under a microscope to check for cell adherence, cell growth and acidity of media. Media mix was warmed up to 37°C, and the pre-existing media from the T-flask was aspirated and replaced with the warm media mix under a laminar flow hood to maintain sterility.

Upon reaching nearly 90% confluence, the cells were prepared to split into more than 1 T-150 flask. Media mix was warmed up in a water bath to 37°C and 12 ml of warm media was added to each sterile T-150 flask. The cells were then removed from incubation and the media was aspirated. 2 ml of warm trypsin was added to the cells and the flask was tapped lightly to detach the cells from the surface. The flask was then placed in the incubator for 3 minutes for detachment of the cells and the same was observed under the microscope afterwards. If the cells were not detached, the flask was kept in the microscope

for two addition minutes and then 10 ml warm media mix was added to the flask to stop the trypsinisation process. The media was then mixed around on the attachment surface to remove all the loosely attached cells and the media was then centrifuged in a 15 ml conical centrifuge tube at 1200 revolutions per minute for 3 minutes at room temperature. The cell pellet was resuspended in 1 ml media mix after removing the supernatant and equal volumes of the resuspended cells were then added to each T-150 flask. The flasks were tapped gently to evenly distribute the cells on the adherent surface and then were placed in an incubator with 95% oxygenation at 37°C. The cells were observed for adherence and cell growth after 24 hours and fresh media was changed to aid cell growth.

3.2.3.4 Cytokine induced media preparation

Varying concentrations of cytokines was added to growth media mix to prepare cytokines induced media. 10 ng/ml and 50 ng/ml IL-15 was added growth media mix to prepare two different concentrations of IL-15 stimulation media. 10 ng/ml and 100 ng/ml IFN-γ was added growth media mix to prepare two different concentrations of IFN-γ stimulation media. The control media consisted of growth media mix without any additions.

3.2.3.5 Cell stimulation

After reaching nearly 90% confluence upon the 7th passage, the cells were ready to be stimulated with desired cytokine concentrations. Starving media was prepared by mixing 0.5% FBS with endothelial cell growth media and antibiotics. The cells were then starved for 2.5 hours, in an incubator at 37°C, by replacing the growth media mix with starving media mix. After starvation, the cells were then induced with desired cytokine media mix for varying time points (15 minutes, 30 minutes, 4 hours, and 24 hours). Upon stimulation with the cytokines for the required amounts of time, the process was stopped by washing the cells with DPBS after removing the media and then immediately freezing the cells using dry ice. The cell plates were then moved to -20°C to be harvested at a later stage.

3.2.3.6 Cell extraction and homogenate preparation

Cell lysis mixture was prepared in cold conditions using RIPA buffer (Thermo Scientific, cat. No. 89901), Halt Protease Inhibitor cocktail (Thermo Scientific; cat. No. 78430) and Halt Phosphatase Inhibitor cocktail (Thermo Scientific; cat. No. 78420) along with EDTA. For every 100 µl of RIPA, 1 µl of 100x phosphatase inhibitor, 1 µl protease inhibitor and 1 µl 0.5M EDTA were added. The cells were transferred from –20°C and thawed on ice by placing the dishes in a vertical manner to collect the excess DPBS that was not removed after stopping the stimulation. Upon complete thawing of cells, the excess liquid (if present) was aspirated and 100ul of cell lysis mix was added to the dishes. Using a sterile scraper, the cells were scraped from the dishes for 30 seconds while moving the dish around to keep the cells covered with the liquid and efficient scraping. The liquid containing scraped cells was then collected from the dishes and was stored in well labelled 1 ml Eppendorf tubes.

To prepare the cell homogenate, the tubes containing cells were vortexed, centrifuged at 10000 g for 1 minute and then sonicated at 50% amplitude for 10 seconds twice. The probe was washed between each set of samples and the sonicated samples were then centrifuged at 14000 g for 10 minutes at 4°C. The supernatant was then collected and stored for further estimations and investigations.

3.2.4 Protein quantification using BCA assay

Protein concentrations were estimated in cell homogenate samples using the Pierce BCA Protein Assay kit obtained from Thermo Scientific (Cat. No. 23227). Standards were prepared using BSA ampule provided in the kit by diluting it in RIPA buffer and working reagent was prepared by mixing 50:1 Reagent A: B. 10 µl of each standard and 5 µl of each sample diluted with 5 µl water was added to a 96 well plate. After 200ul working reagent was added to all the wells, the plate was covered and kept on a shaker for 30 seconds to mix the solutions. The plate was then incubated at 37°C for 30 minutes and the absorbance was measured after cooling the plate at 562nm using plate reader Victor X3 (PerkinElmer). The standard curve was prepared using the absorbance and

concentrations of the standards and the unknown protein concentrations were estimated.

3.2.5 Protein profiling: Western blotting from cell homogenate

For primary antibodies, BD Biosciences' mouse anti – eNOS [pT495] (Cat. No. BD – 612707), and mouse anti-eNOS [pS1177] (Cat. No. BD – 612393), Abcam's rabbit anti – eNOS (Cat. No. Ab – 66127), and Sigma Aldrich's rabbit anti-NOX1 (Cat. No. SAB2108601) were used. Secondary antibodies were IRDye 800CW and IRDye 680RD (LI-COR) for both goat anti–mouse and goat anti–rabbit. Pipettes, Eppendorf and falcon tubes and all chemicals and required solutions were used from the laboratory stock.

3.2.5.1 Sample preparation

After estimating the concentration of protein in the cell homogenate, protein samples were prepared to run western blots. Cell homogenate was diluted with double distilled water to prepare samples with 15 µg protein in 25 µl volume. 5 µl loading buffer (6X) was added and a total volume of 30 µl was prepared. The samples with the loading dye were heated in 95°C for 5 minutes. The samples were then removed from the thermoblock and were vortexed, followed by a short 30 second spin in a centrifuge to collect the samples at the bottom.

3.2.5.2 Gel preparation

To prepare a 7.5% resolving gel, the apparatus was first cleaned with 70% ethanol and dried completely. The plates were assembled in a casket, mounted on the gel solidification apparatus, and were checked for any leaks using double distilled water. The plates were then dried completely with filter paper and were ready to be poured with the gels. In two separate 50 ml tubes, the stacking and separating gel solutions were prepared.

To prepare 7.5% resolving gel, 1.5M Tris pH 8.8, 40% acrylamide, 10% SDS, 10% APS, and TEMED were required. 10% SDS was prepared by dissolving 10 g SDS in 100 ml water.1.5M Tris buffer was prepared by dissolving 18.165 g Tris base in 80 ml water and topping up with water to prepare 100 ml solution. The pH was adjusted to 8.8. In a falcon tube, 5.475 ml water, 2.5 ml 1.5M Tris pH 8.8, 1.875

ml 40% acrylamide, 0.1 ml 10% SDS, 90 µl 10% APS and 5 µl TEMED were added bringing the total volume to 10 ml for preparing 1 gel.

For stacking gel, 0.5M Tris pH 6.8 along with 40% acrylamide, 10% SDS, 10% APS, and TEMED were required. 0.5M Tris buffer was prepared by dissolving 6.055 g in 80 ml water and a final volume of 100 ml was prepared by adding water. The pH was adjusted to 6.8. In a falcon tube, 2.54 ml water, 1 ml 0.5M Tris pH 6.8, 0.4 ml 40% acrylamide, 40 µl 10% SDS, 40 µl 10% APS and 4µl TEMED were added bringing the total volume to 4 ml for preparing 1 gel.

After adding TEMED and APS, the tubes were quickly vortexed to mix the solution well. The separating gel was added to the chamber first and isopropanol was added over it to remove any air gaps or bubbles. Once the gel solidified, the alcohol was removed using a filter paper. The stacking gel was prepared using and immediately after adding TEMED and APS, the tubes were quickly vortexed, and the gel was added over the separating gel in the gel casting chambers. A 15 well comb was introduced to the gel to create the desired number of sample loading chambers. The gel was then allowed to solidify.

3.2.5.3 Gel loading and running

10x Tris-glycine buffer was prepared by mixing 30g Tris base and 145g glycine in IL water. Running buffer was prepared by adding 100 ml of 10x Tris-glycine buffer with 890 ml water and 10 ml 10% SDS. Once the gel was solidified, the gel caskets were removed from the gel solidification apparatus and were placed in the running chamber. The chamber was then filled with running buffer halfway. The gels were then loaded with 5 µl ladder and 30 µl protein samples in desired chambers in a careful manner. The running chamber was then filled with more running buffer until the required levels with the gels submerged in the buffer. The probes were connected to a power supply and the gels were run at 150 V, at room temperature, for 1 hour 15 minutes or until the samples and ladder run to the bottom of the gel.

3.2.5.4 Transfer of protein

1L of transfer buffer was prepared by mixing 200 ml methanol, 700 ml water and 100 ml 10x Tris-glycine and was cooled down to 4°C. After the gels were run,

the caskets were removed from the electrophoresis apparatus and the gels were then removed from the plates and placed in a transfer buffer. The transfer apparatus including the filter papers, transfer membranes and sponges were soaked in transfer buffer to activate them for the protein transfer. The gels and membranes were arranged in the transfer cassettes in a transfer sandwich order. The air bubbles between each layer were removed using a rolling pin and once set, the transfer cassette was placed in a transfer tank. The tank was then filled with cold transfer buffer and an ice block was placed inside the tank to maintain the cold temperature. The transfer tank was then placed in an ice bath. The probes were then connected, and the protein transfer was run at 100 V, at 4°C, for 1 hour 55 minutes.

3.2.5.5 Blocking of membrane

After the proteins were transferred to the membranes, the membranes were placed in Ponceau S to confirm success and the membranes were cut and labelled as desired. 10x TBS was prepared by mixing 24.2g Tris base with 8g sodium chloride in 1L double distilled water. The pH was adjusted to 7.6 and the solution was diluted to 1x for use. The membranes were then washed in TBS 3 times for 5 minutes each while placed on a shaker. After washing the membranes, they were blocked in 5% BSA prepared in TBS for 1 hour at room temperature, while placed on a shaker.

3.2.5.6 Antibody incubation

TBS-T was prepared by adding 1 ml Tween-20 and 100 ml 10x TBS to 900 ml double distilled water. Once the membranes were successfully blocked, they were then washed with TBS-T quickly. The membranes were then incubated with primary antibodies at 4°C on a shaker. After an overnight incubation, the membranes were removed from the primary antibodies and again washed with TBS-T 3 times for 5 minutes each. Secondary antibodies were added to the membranes at room temperature and the membranes were incubated for 1 hour on a shaker. After the incubation, the membranes were again washed with TBS-T and analysed for relative protein quantification using Licor Odyssey Clx infrared imaging system.

3.2.5.7 Stripping the membrane for housekeeping proteins

Once the membranes were analysed, they were quickly washed with double distilled water for a maximum of 10 seconds. Restore Plus stripping buffer from ThermoFisher Scientific (cat. No. 46430) was added to the membranes for 5 minutes. The membranes were again washed with TBS-T 3 times for 5 minutes each. The membranes were blocked again with 5% BSA prepared in TBS for 1 hour at room temperature while shaking. Once the membranes were blocked, they were quickly rinsed with TBS-T and then were incubated with housekeeping protein antibodies overnight at 4°C. The membranes were washed with TBS-T 3 times on a shaker for 5 minutes each. The membranes were then incubated with the secondary antibodies for 1 hour at room temperature. The membranes were then washed in TBS-T and analysed again.

3.3 Results

3.3.1 The effect of inflammatory cytokines on vascular function in mouse aortas.

To have a better understanding of how the vascular function is affected by inflammatory cytokines, vasomotor studies were conducted. Mouse vessels were co-cultured for 18 hours with pro-inflammatory cytokines: IL-6, TNF-α, IL-15, IFN-γ, IL-1ß, IL-17a, and then vasoconstriction was studied using Phe and 5-HT, and relaxation was studied using ACh and SNP in an organ bath.

3.3.1.1 The impact of IL-1β on vascular function ex vivo

The vascular constriction mediated by Phe was not significantly different when compared IL-1ß (1 ng/ml) treated with the control vessels (Fig.3.2A) In contrast, endothelium-dependent relaxation was significantly impaired upon IL-1ß treatment ($P < 0.001$, Fig.3.2B) with 35.88±6.79% relaxation of vessels in response to Ach ($3x10^{-5}$), whereas the control vessels relaxed to 90.05± 5.35% (Mean±SEM). Similarly, to Phe, the 5HT–mediated constriction of the vessels was the same in both studied groups ($P= 0.9354$, Fig.3.2C). Vascular relaxation, upon 5-HT pre-constriction, showed significant differences in relaxation in comparison to the untreated vessels ($P= 0.004$). The stimulated vessels reached 80.36±7.60% relaxation while the unstimulated vessels reached 103.98±1.86 % relaxation with $3x10^{-5}$ ACh (Fig.3.2D). The endothelium-independent relaxation in response to SNP did not show any significant differences between the studied groups (Fig 3.2E).

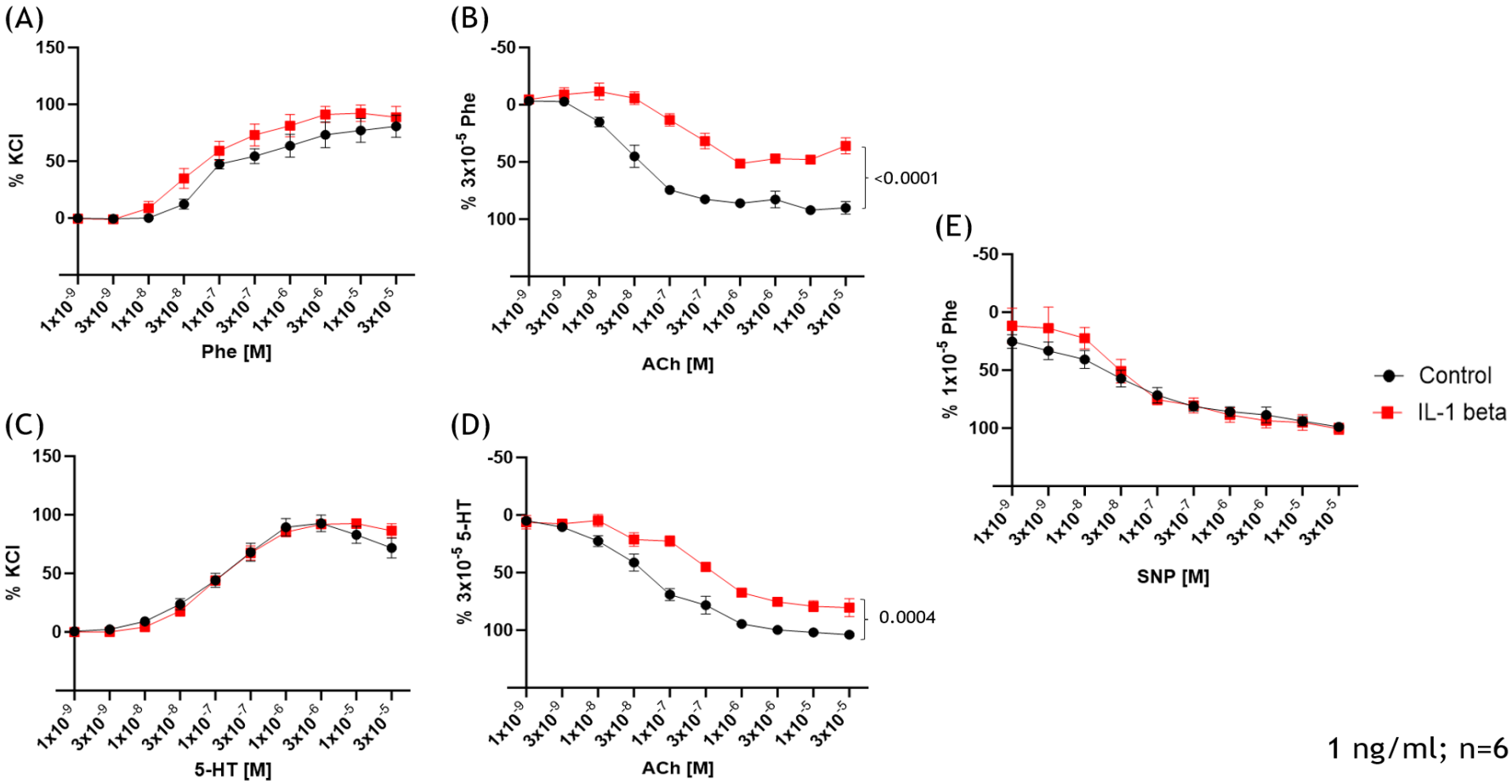


Figure 3.2: Effect of IL-1β (1 ng/ml) on vasoconstriction and relaxation on mouse thoracic aortas. (A) Effect of IL-1β (1 ng/ml) on constriction with increasing doses of Phe. (B) Effect of IL-1β (1 ng/ml) on endothelium-dependent relaxation with increasing doses of ACh after pre-constriction with Phe. (C) Effect of IL-1β (1 ng/ml) on vascular constriction with increasing doses of 5HT. (D) Effect of IL-1β (1 ng/ml) on endothelium-dependent relaxation with increasing doses of ACh after pre-constriction with 5HT. (E) Effect of IL-1β (1 ng/ml) on endothelium-independent relaxation induced by SNP. The data points are represented as Mean ± SEM, n=6.

3.3.1.2 The impact of IFN-γ on vascular function ex vivo

The aortic constriction attained with Phe was not significantly different between the IFN-γ-treated and control vessel (Fig.3.3A). The vascular relaxation after pre-constriction with Phe showed a trend toward impairment of endothelial function upon IFN-γ incubation, however, these changes were not significantly different when compared with the control group (P=0.0924, Fig.3.3B). The 5HT mediated constriction was not affected in both studied groups (Figure.3.3C). After pre-constricting, the vessels with 5HT, IFN-γ-treated aortic rings displayed significant impairment of endothelial function in compassion to the control group (P=0.0099). The control vessels reached a relaxation of 99.30± 3.12% whereas the incubated vessels reached an 83.46±5.81% relaxation with $3x10^{-5}$M ACh (Fig 3.3D). Endothelium-independent relaxation mediated by increasing doses of SNP was not significantly different between stimulated and unstimulated vessels (Fig. 3.3E).

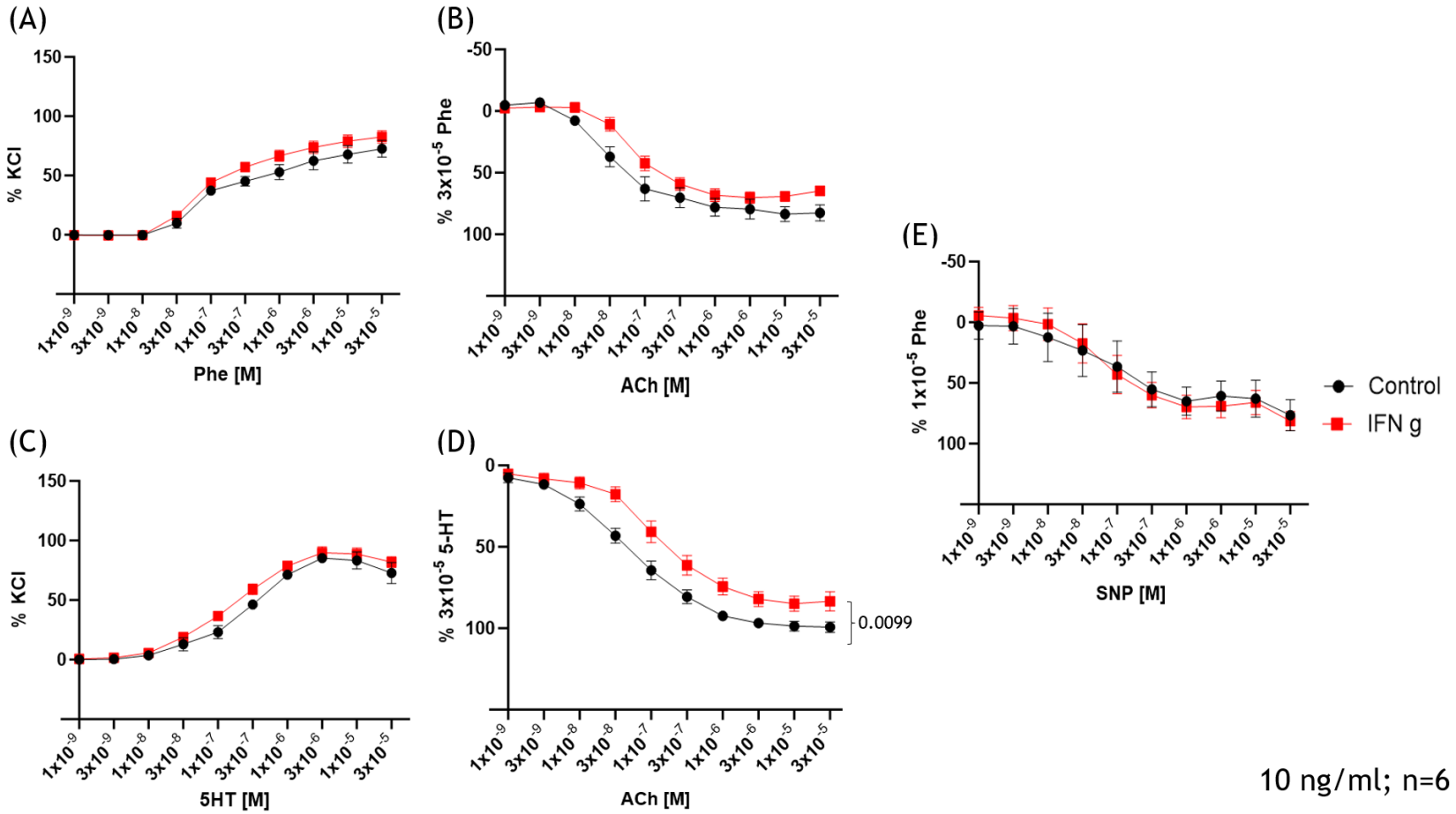


Figure 3.3: Effect of IFN-γ (10 ng/ml) on vasoconstriction and relaxation on mouse thoracic aortas. (A) Effect of IFN-γ (10 ng/ml) on constriction with increasing doses of Phe. (B) Effect of IFN-γ (10 ng/ml) on endothelium-dependent relaxation with increasing doses of ACh after pre-constriction with Phe. (C) Effect of IFN-γ (10 ng/ml) on vascular constriction with increasing doses of 5HT. (D) Effect of IFN-γ (10 ng/ml) on endothelium-dependent relaxation with increasing doses of ACh after pre-constriction with 5HT. (E) Effect of IFN-γ (10 ng/ml) on endothelium-independent relaxation induced by SNP. The data points are represented as Mean ± SEM, n=6.

3.3.1.3 The impact of IL-6 on vascular function ex vivo

Aortic rings incubated with 5 ng/ml of IL-6, showed similar Phe-mediated vasoconstriction to vessels in vehicle treated group (Fig.3.4A). The endothelium-dependent relaxation, after pre-constriction with Phe, was significantly affected in the IL-6-treated when compared with the control group. The maximum relaxation in the IL-6-treated group was 76.78±5.51% with $3x10^{-5}$ ACh, while the control relaxed to 90.06±5.35% (P=0.014, n=6, Fig.3.4B). The 5HT-induced constriction was comparable between groups (Fig.3.4C). Similarly, there was no significant difference in both treated and control groups when the vessels were relaxed with ACh after constriction with 5HT (Fig.3.4D) Endothelium-independent relaxation was also not significantly affected by IL-6 or vehicle treatment (Fig.3.4E).

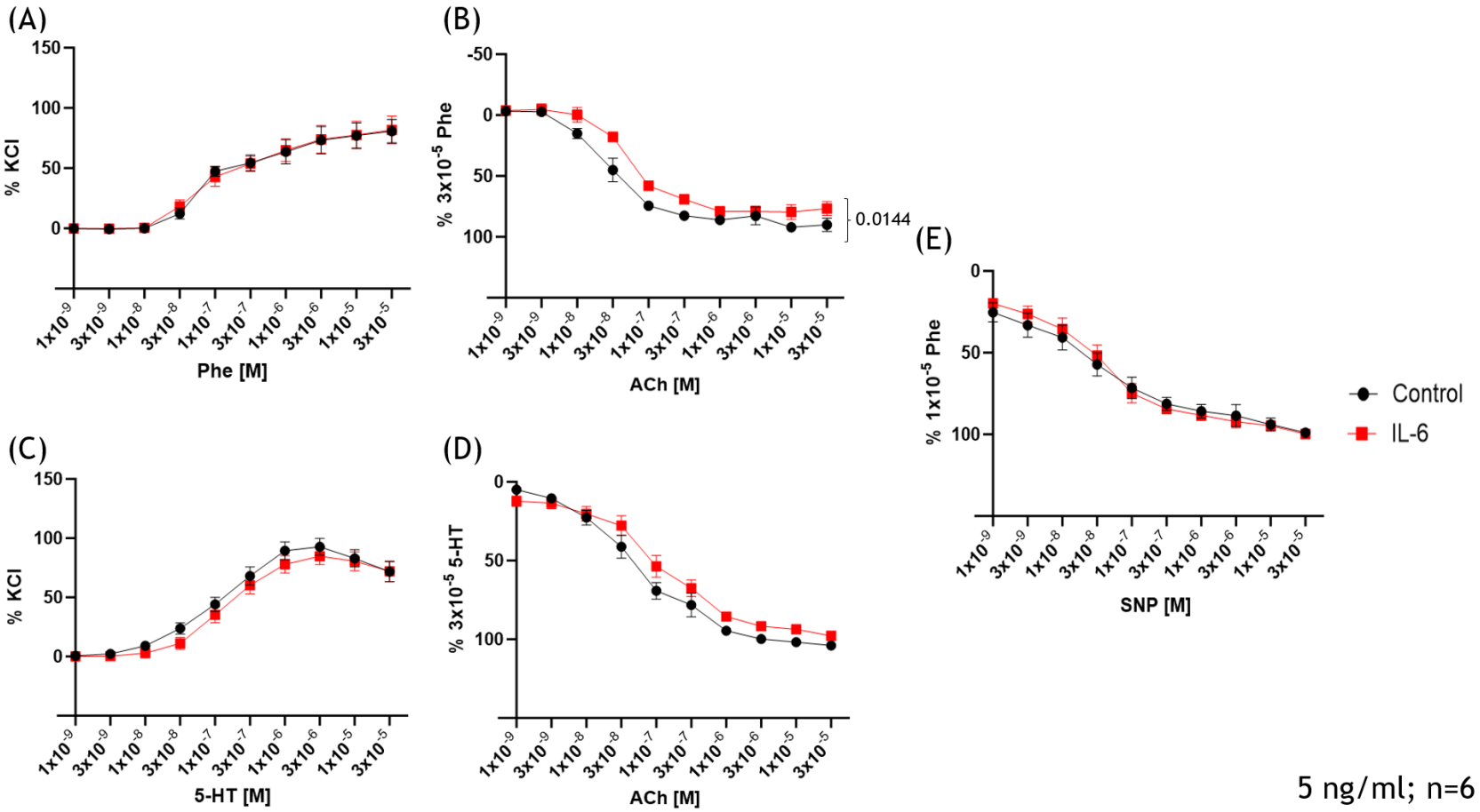


Figure 3.4: Effect of IL-6 (5 ng/ml) on vasoconstriction and relaxation on mouse thoracic aortas. (A) Effect of IL-6 (5 ng/ml) on constriction with increasing doses of Phe. (B) Effect of IL-6 (5 ng/ml) on endothelium-dependent relaxation with increasing doses of ACh after pre-constriction with Phe. (C) Effect of IL-6 (5 ng/ml) on vascular constriction with increasing doses of 5HT. (D) Effect of IL-6 (5 ng/ml) on endothelium-dependent relaxation with increasing doses of ACh after pre-constriction with 5HT. (E) Effect of IL-6 (5 ng/ml) on endothelium-independent relaxation induced by SNP. The data points are represented as Mean ± SEM, n=6.

3.3.1.4 The impact of TNF-α on vascular function ex vivo

Vessels incubated with 1ng/ml TNF-α did not show any significant changes in Phe- and 5-HT- induced vasoconstriction when compared with the control group, with the maximal contraction 79.71±9.02% vs 80.82±9.69% and 79.00±6.93% vs 71.81±8.54%, respectively (Fig. 3.5A and B). Aortic rings treated with TNF-α exhibited comparable vascular relaxation to control vessels upon pre-constriction with Phe (Fig. 3.5B). Similarly, upon initial exposure to 5HT, endothelium-dependent relaxation was comparable between the studied groups with 98.12±2.36% and 103.98±1.86% in TNF-α and the control group, respectively (Fig.3.5D). Endothelium-independent relaxation was also not affected by TNF treatment (Fig. 3.5E).

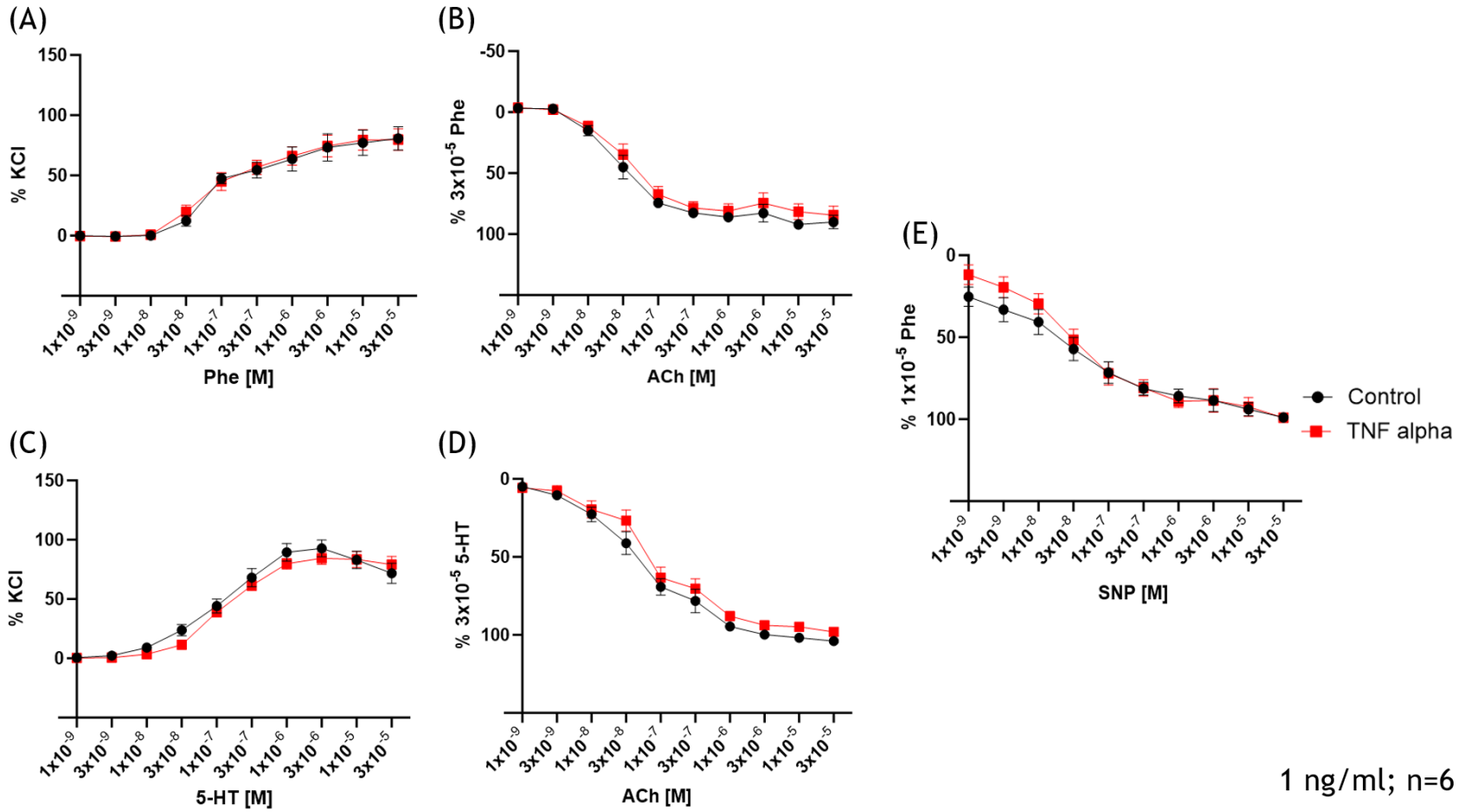


Figure 3.5: Effect of TNF-α (1 ng/ml) on vasoconstriction and relaxation on mouse thoracic aortas. (A) Effect of TNF-α (1 ng/ml) on constriction with increasing doses of Phe. (B) Effect of TNF-α (1 ng/ml) on endothelium-dependent relaxation with increasing doses of ACh after pre-constriction with Phe. (C) Effect of TNF-α (1 ng/ml) on vascular constriction with increasing doses of 5HT. (D) Effect of TNF a (1 ng/ml) on endothelium-dependent relaxation with increasing doses of ACh after pre-constriction with 5HT. (E) Effect of TNF-α (1 ng/ml) on endothelium-independent relaxation induced by SNP. The data points are represented as Mean ± SEM, n=6.

3.3.1.5 The impact of IL-15 on vascular function ex vivo

Vessels incubated with 10 ng/ml IL-15 did not show any significant changes in Phe- and 5-HT- induced vasoconstriction when compared with the control group, with the maximal contraction 82.49±11.55% vs 86.59±12.60% and 97.37±6.26% 96.27±5.84%, respectively (Fig. 3.6A and C). Aortic rings treated with IL-15 exhibited comparable vascular relaxation to control vessels upon pre-constriction with Phe (Fig. 3.6B). Similarly, upon initial exposure to 5HT, endothelium-dependent relaxation was comparable between the studied groups with 86.66±13.36% and 80.96±11.70% in IL-15 and the control group, respectively (Fig. 3.6D). Endothelium-independent relaxation was also not affected by IL-15 treatment (Fig. 3.6E).

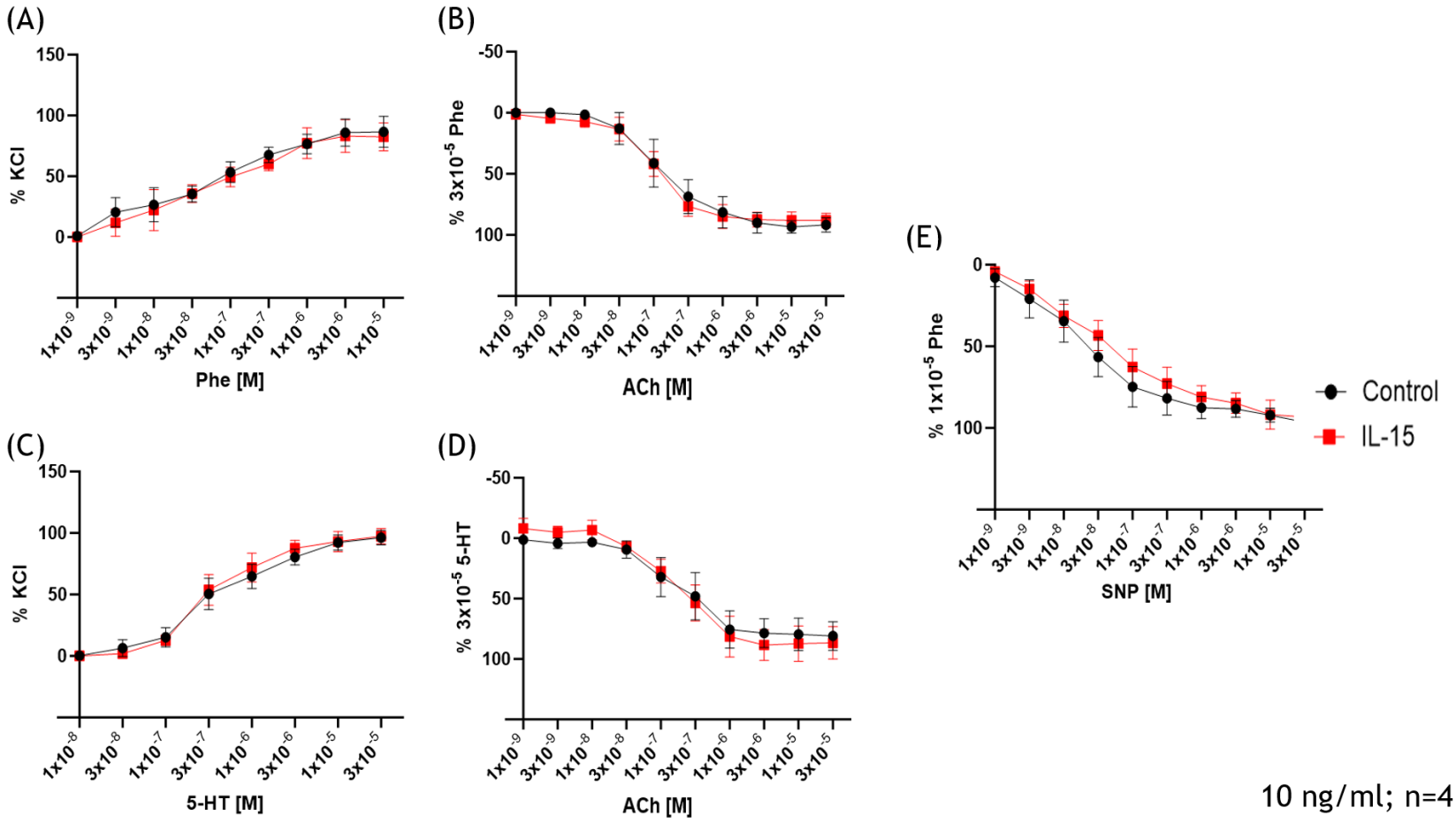


Figure 3.6: Effect of IL-15 (10 ng/ml) on vasoconstriction and relaxation on mouse thoracic aortas. (A) Effect of IL-15 (10 ng/ml) on constriction with increasing doses of Phe. (B) Effect of IL-15 (10 ng/ml) on endothelium-dependent relaxation with increasing doses of ACh after pre-constriction with Phe. (C) Effect of IL-15 (10 ng/ml) on vascular constriction with increasing doses of 5HT. (D) Effect of IL-15 (10 ng/ml) on endothelium-dependent relaxation with increasing doses of ACh after pre-constriction with 5HT. (E) Effect of IL-15 (10 ng/ml) on endothelium-independent relaxation induced by SNP. The data points are represented as Mean ± SEM, n=4.

3.3.1.6 The impact of IL-17a on vascular function ex vivo

The vascular constriction attained with Phe in the treated vessels showed a trend towards higher contractility, in initial doses of Phe, when compared to the control vessels. However, these changes were not significant, and maximal constriction was similar (87.70±5.94% vs 84.32±6.47%). (Fig. 3.7A) The maximum relaxation of Phe-constricted vessels was also not significantly affected by 1 ng/ml IL-17a treatment (Fig.3.7B). Upon exposure with 5HT, similar constriction was observed in both groups (Fig. 3.7C). Similarly, there were no significant differences between treated and control vessels in endothelium-dependent relaxation after constriction with 5HT (Fig.3.7D) Endothelium-independent relaxation was also not significantly different between studied groups (Fig.3.7E).

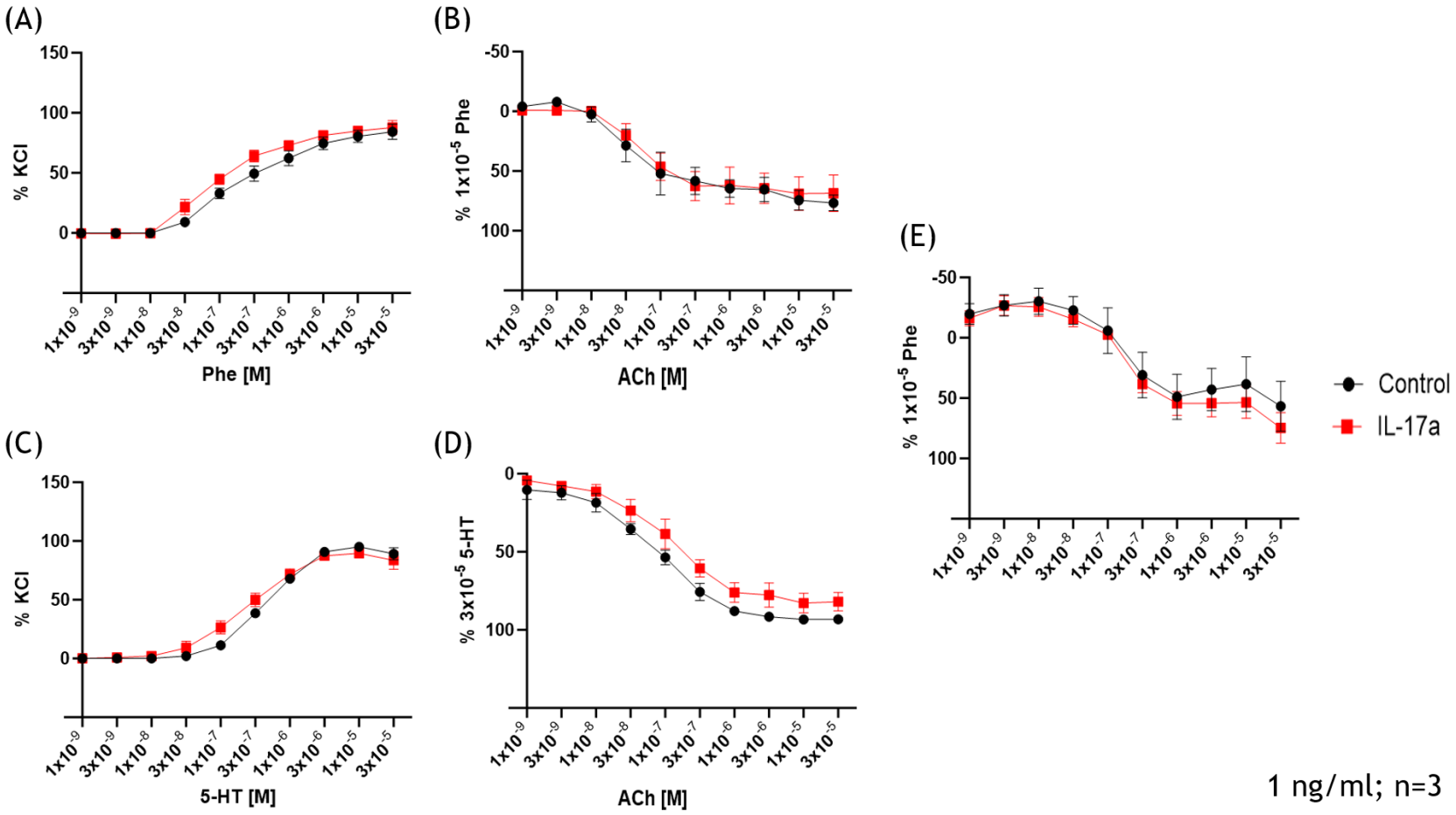


Figure 3.7: Effect of IL-17a (1 ng/ml) on vasoconstriction and relaxation on mouse thoracic aortas. (A) Effect of IL-17a (1 ng/ml) on constriction with increasing doses of Phe. (B) Effect of IL-17a (1 ng/ml) on endothelium-dependent relaxation with increasing doses of ACh after pre-constriction with Phe. (C) Effect of IL-17 (1 ng/ml) on vascular constriction with increasing doses of 5HT. (D) Effect of IL-17a (1 ng/ml) on endothelium-dependent relaxation with increasing doses of ACh after pre-constriction with 5HT. (E) Effect of IL-17a (1 ng/ml) on endothelium-independent relaxation induced by SNP. The data points are represented as Mean ± SEM, n=3.

3.3.2 Changes in NADPH oxidase subunits and eNOS mRNA expression level in mouse vessels treated with IL-1β and IFN-γ

Upon observing significant differences in vascular relaxation impairment after treatment with IL-1ß and IFN-γ, the gene expression of eNOS and NADPH oxidase subunits was studied next. This was done to determine the direct effect of studied cytokines on the transcription changes of genes implicated in NO and ROS production.

3.3.2.1 Changes in gene expression induced by IL-1β

Vessels treated with 1 ng/ml IL-1ß, showed 1.45- fold induction in *nox1* (Fig.3.8A), and 0.71 decrease of NOX2 (Fig. 3.8B) mRNA expression level compared to untreated vessels; however, the differences were not statistically significant. In contrast, IL-1ß treatment significantly reduced NOX4 gene expression (1±0.012 vs 0.59±0.08, p = 0.0082) (Fig.3.8C). A similar trend was observed in eNOS mRNA level (Fig.3.8D) where the treated vessels showed 0.69-decrease compared with untreated vessels (P = 0.0185).

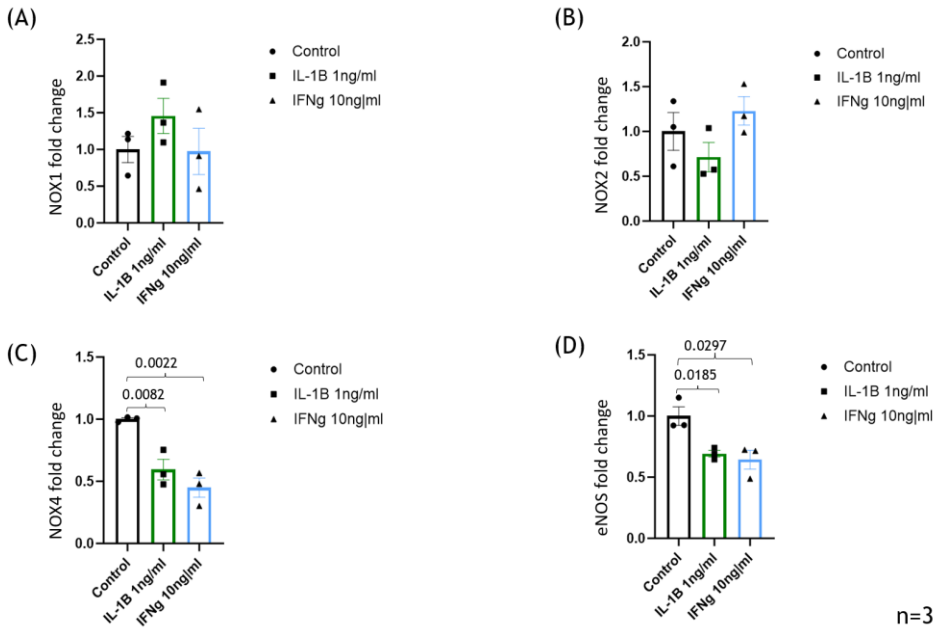


Figure 3.8: Effect of IL-1β and IFN-γ on NADPH oxidase subunits and eNOS gene expression in mouse thoracic aortas. (A) Effect of cytokine treatment on NOX1 gene expression (B) Effect of cytokine treatment on NOX2 gene expression (C) Effect of cytokine treatment on NOX4 gene expression (D) Effect of cytokine treatment on eNOS gene expression. Data presented as Mean ± SEM and analysed using two-tailed unpaired T-test; n=3.

3.3.2.2 Changes in gene expression induced by IFN-γ

IFN-γ treatment at 10 ng/ml showed no significant difference in NOX1 gene expression compared to untreated vessels (1±0.18 vs 0.97±0.32 1, Fig. 3.9A).The induction of NOX2 expression was slightly higher (1.23-fold) in treated vessels compared to untreated vessels, but the difference was not statistically significant (Fig. 3.9B). A significant impact of IFN-γ treatment was observed in NOX4 mRNA level ($p = 0.0022$, Fig 3.9C) with 0.45- fold decrease compared to control. A similar effect was observed for eNOS (Fig.3.9D) where the treated vessels showed a significant reduction in gene expression when compared with the untreated vessels (1±0.08 vs 0.64±0.08, $P = 0.0297$).

3.3.3 Cytokine induced eNOS and ERK phosphorylation in HAoECs in vitro.

To explore the potential mechanism of observed endothelium dysfunction and changes in eNOS and NOX4 expression levels, human aortic endothelial cells were used to study the direct effect of cytokines on eNOS (Threonine 495 and Serine 1177) and ERK phosphorylation. Since IFN-γ showed significant effects on gene expression as well as receptor dependent endothelial function, the cytokine was selected to study the mechanisms of endothelial function. As a comparator, IL-15 was selected due to no significant responses in the receptor dependent endothelial function.

3.3.3.1 Effect of IL-15 stimulation on eNOS and ERK phosphorylation

Upon extracting the cell homogenate after treatment, western blots were run to observe phosphorylation of eNOS at T495 and S1177, and phosphorylation of ERK.

HAoECs stimulated with 10 ng/ml and 50 ng/ml of IL-15 for 15 minutes showed no differences in phosphorylation of eNOS T495 (Fig. 3.9A), but 50 ng/ml IL-15 showed a slight increase in phosphorylation of S1177 (Fig 3.9B) when compared with controls. When the cells were stimulated for 30 minutes, no significant differences in phosphorylation of eNOS at T495 (Fig. 3.9A) as well as at S1177 (Fig 3.9B) were observed for treated and untreated cells, even though phosphorylation at both S1177 and T495 was observed for cells stimulated with 50 ng/ml. Four hours stimulation with 10 ng/ml IL-15 showed a significant decrease ($P=0.0498$) in eNOS phosphorylation at T495 (Fig. 3.9A) and a significant increase ($P=0.0035$) in phosphorylation at S1177 (Fig 3.9B). In contrast, 50 ng/ml IL-15 showed no significant difference in eNOS phosphorylation at T495 (Fig. 3.9A) and at S1177 (Fig 3.9B). Although non-significant, an upward trend was observed in the phosphorylation of eNOS at S1177. After 24 hours of stimulation by IL-15, the phosphorylation of eNOS at T495 was not significantly affected (Fig. 3.9A). There was a significant 0.91-fold decrease ($P=0.0032$) in phosphorylation at S1177 (Fig 3.9B) observed in cells stimulated with 10 ng/ml IL-15, whereas no difference in cells stimulated with 50 ng/ml IL-15 was observed.

A non-significant change with a downward trend in phosphorylation of ERK was observed after 15 minutes treatment with 10 ng/ml IL-15 and 50 ng/ml (Fig 3.9C). Phosphorylation of ERK decreased significantly when 10 ng/ml IL-15 was used (P=0.0011) and increased upon 50 ng/ml stimulation cells for 30 minutes, though the difference was not significant. The longer exposure of HAoECs to both IL-15 concentrations did not result in significant changes in ERK phosphorylation at 4 and 24 hours compared to the control.

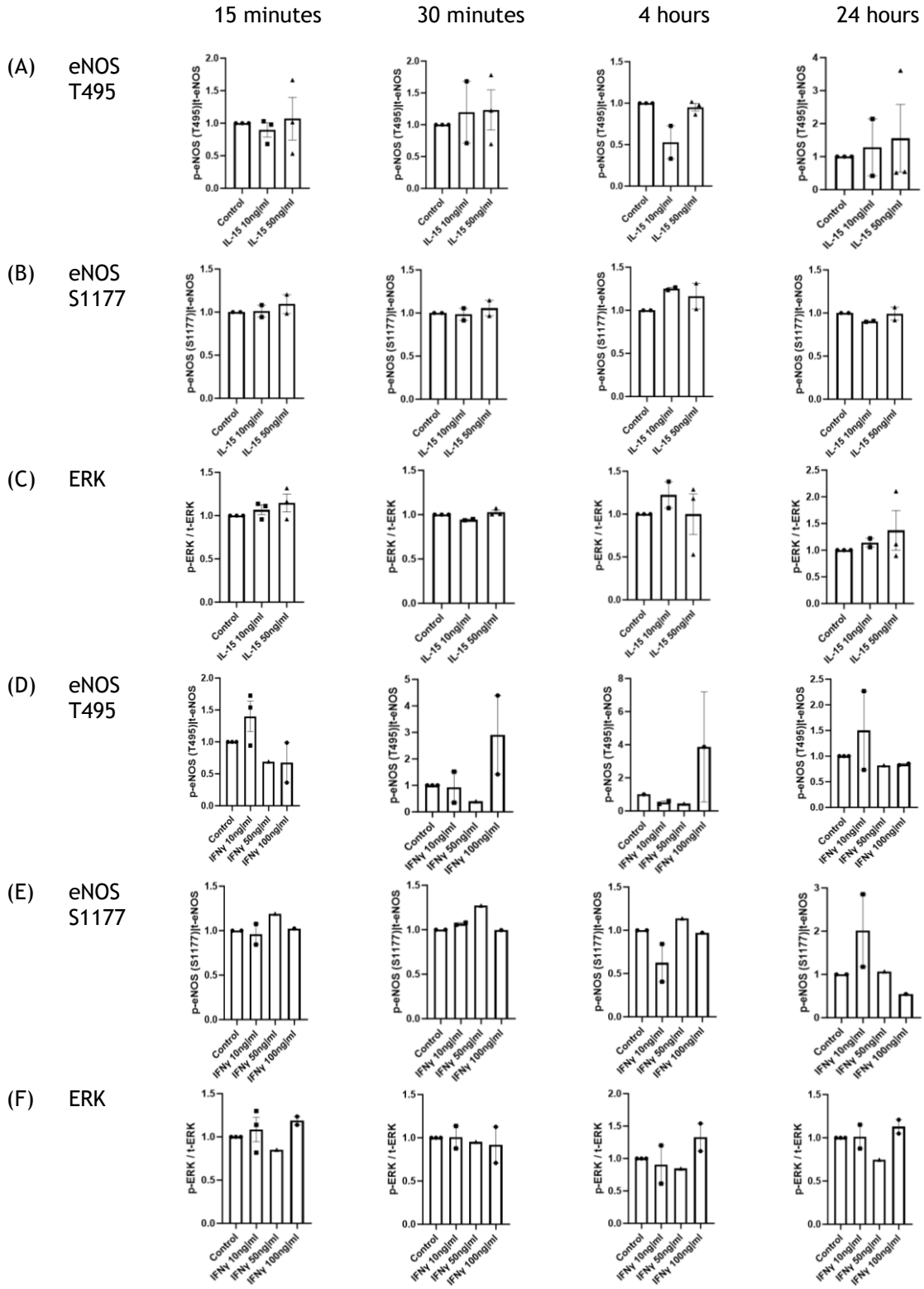


Figure 3.9 Western blot results for HAoEC homogenate for various time points when the cells were stimulated with varying concentrations of IL-15 and IFN-γ. (A) Graphs depicting phosphorylation of eNOS (T495) for various time points when cells were stimulated with 10 ng/ml and 50 ng/ml IL-15. (B) Graphs depicting phosphorylation of eNOS (S1177) for various time points when cells were stimulated with 10 ng/ml and 50 ng/ml IL-15. (C) Graphs depicting phosphorylation of ERK for various time points when cells were stimulated with 10 ng/ml and 50 ng/ml IL-15 (D) Graphs depicting phosphorylation of eNOS (T495) for various time points when cells were stimulated with 10 ng/ml, 50 ng/ml and 100 ng/ml IFN-γ. (E) Graphs depicting phosphorylation of eNOS (S1177) for various time points when cells were stimulated with 10 ng/ml, 50 ng/ml and 100 ng/ml IFN-γ. (F) Graphs depicting phosphorylation of ERK for various time points when cells were stimulated with 10 ng/ml, 50 ng/ml and 100 ng/ml IFN-γ.

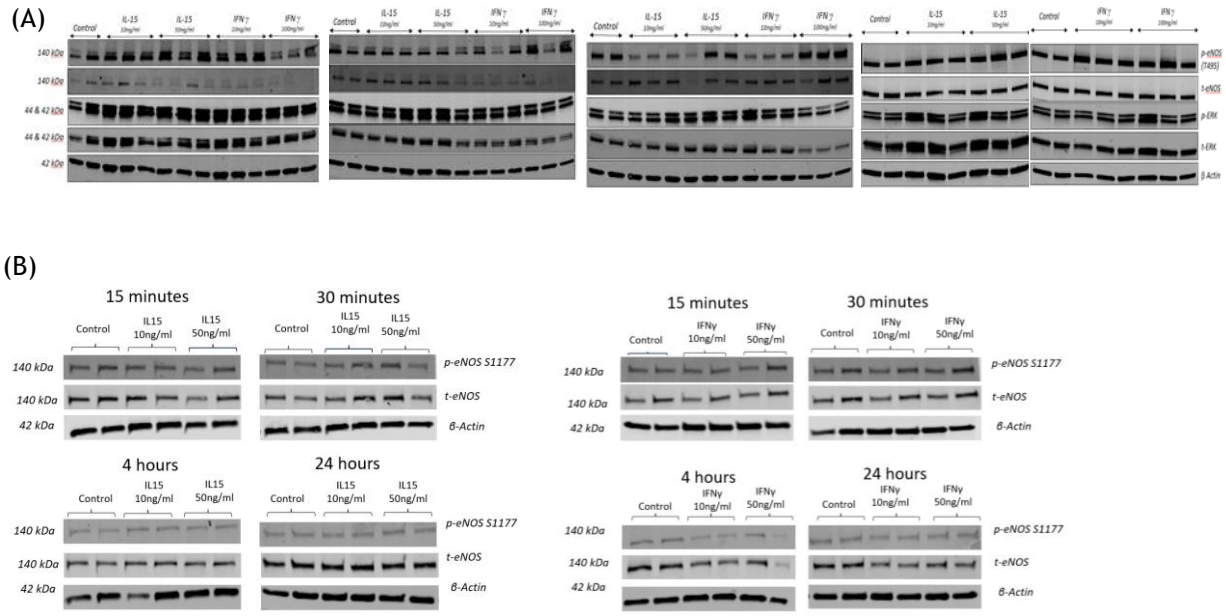


Figure 3.10 Western blot membranes for HAoEC homogenate for various time points when the cells were stimulated varying concentrations of IL-15 and IFN-γ. (A) Western blot membranes showing protein bands for p-eNOS (T495), t-eNOS, p-ERK, t-ERK and housekeeping protein beta actin. (B) Western blot membranes showing protein bands for p-eNOS (S1177), t-eNOS, and housekeeping protein beta actin. Data represented as mean±SEM.

3.3.3.2 Effect of IFN-γ stimulation on eNOS and ERK phosphorylation

HAoECs stimulated with 10 ng/ml IFN-γ for 15 minutes showed increased phosphorylation of eNOS T495 and negligible changes in the phosphorylation of eNOS S1177. 50 ng/ml of IFN-γ decreased phosphorylation of eNOS at T495 but increased at S1177. 100 ng/ml of IFN-γ showed decrease in phosphorylation at eNOS T495 but had no effect on the phosphorylation of eNOS at S1177 (Fig. 3.9D). When the cells were stimulated for 30 minutes, no significant differences in phosphorylation of eNOS at T495 (Fig. 3.9A) were observed. In contrast, 10 ng/ml IFN-γ showed significant difference in the phosphorylation of eNOS at S1177 (Fig 3.9E). Four hours stimulation with 100 ng/ml IFN-γ showed a significant increase (P=0.0007) in eNOS phosphorylation at T495 (Fig. 3.9D), and no phosphorylation of eNOS at S1177 (Fig. 3.9E). When the cells were stimulated for 24 hours, an increase in the phosphorylation of eNOS at both T495 and S1177 was observed but the difference between the treated and untreated groups were not statistically significant (Fig. 3.9D, E).

A significant increase in phosphorylation of ERK was observed after 15 minutes treatment with 100 ng/ml IFN-γ (P=0.0137; Fig 3.9C) and a non-significant downward trend was observed for 50 ng/ml. Negligible changes were observed with all concentrations of the cytokine after 30 minutes of treatment. After four hours of treatment, 100 ng/ml IFN-γ showed increased ERK phosphorylation in comparison to the control group. Prolonged exposure to all concentrations of IFN-γ did not result in significant changes in ERK phosphorylation at 24 hours compared to the control.

3.4 Discussion

Hypertension is a globally recognized disorder of blood pressure affecting more than a billion people worldwide with almost 70% of the patients subsequently developing cardiovascular disorders (Drummond et al., 2019). Endothelial dysfunction has been found to be a major factor in the development of hypertension and other cardiovascular diseases. The bioavailability of NO plays a role in maintaining a proper endothelial function by regulating the vascular tone through constriction and relaxation of vascular smooth muscle cells.

The role of inflammatory cytokines IL-1β, TNF-α, IL-6, IFN-γ, IL-15 and IL-17a was studied on vascular function by conducting vasomotor studies. Thoracic aortas from C57BL/6 male mice were co-cultured with inflammatory cytokines and vascular constriction, endothelium-dependent and endothelium-independent relaxation were studied. The concentration of cytokines was selected based on serum concentrations of the cytokines in mice as well as concentrations selected for previously undertaken work available in the literature. 1 ng/ml IL-1β, 5 ng/ml IL-6, 1 ng/ml TNF-α, 1 ng/ml IL-17a, 10 ng/ml IL-15 and 10 ng/ml IFN-γ were selected for the experiments and vasoconstriction and relaxations were observed for all stimulated and unstimulated vessels.

Table 3.3 Mouse serum concentration of TNF-α, IL-1β, IL-6, IFN γ, IL-17a and cytokine concentrations used in vasomotor experiments in the available literature.

Cytokine	Serum levels in mice	Concentrations used in experiments
TNF-α	3 pg/ml (Cheng et al., 2019)	0.1 ng/ml (Chen et al., 2017), 1 ng/ml (Chen et al., 2017, Giardina et al., 2002), 3 ng/ml (Oike et al., 2018) and 10 ng/ml (Chen et al., 2017, Choi et al., 2018)
IL-1β	1.5 pg/ml (Cheng et al., 2019)	1 ng/ml (Dorrance, 2007, Vallejo et al., 2014), 2.5 ng/ml (Vallejo et al., 2014), 5 ng/ml (Vallejo et al., 2014), 10 ng/ml (Dorrance, 2007, Vallejo et al., 2014), 20 ng/ml (Dorrance, 2007)

IL-6	400 pg/ml (Wang et al., 2018)	5 ng/ml (Suda et al., 2011)
IFN-γ	0.5 pg/ml (Cheng et al., 2019)	10 ng/ml (Kimura et al., 2018)
IL-17a	1 pg/ml (Oike et al., 2018), 23 ug/l (Tang, 2019)	1 ng/ml (Nguyen et al., 2013b)

In the current study, it was observed that even though the vessels demonstrated vasoconstriction and relaxation, significant impairment in relaxation with increasing doses of Ach were only observed after constriction with both Phe and 5HT in IL-1β stimulated vessels indicating endothelium dependent dysfunction. These results are in accordance with the previously published data. Vallejo et.al. (Vallejo et al., 2014) demonstrated through their studies that after incubating mesenteric arteries from diabetic mice with increasing concentrations of IL-1β (1, 2.5, 5 and 10 ng/ml) for 2 hours, endothelium-dependent and independent relaxation impairment was observed in all treatment concentrations when compared with the control group. Though the contractility did not show any impairments, the relaxation after constriction with Phe showed a significant difference in treated vessels. In another study, the effect of IL-1β on endothelium-dependent relaxation was investigated after incubating rat thoracic aortas with 500 pg/ml IL-1β for 2 hours (Loughrey et al., 2003). The authors showed a significant impairment in the endothelium function in the relaxation of Phe pre-constricted vessels. However, not only rodent vessels but also human vessels also showed an adverse effect of IL-1β on vascular function. A study by Foudi et.al. showed that vasorelaxation of internal mammary arteries in humans, stimulated with 100 ng/ml IL-1β, for 24 hours along with lipopolysaccharide was impaired when compared with the control group (Foudi et al., 2017). Chook and colleagues (Chook et al., 2023), while studying the effect of protocatechuic acid on endothelial dysfunction in diabetic mice, also studied the effect of IL-1β on wild type C57BL/6 male mice. The treatment caused significant impairment in vascular dependent relaxation using Ach but endothelium independent relaxation using SNP was not affected. Similar to our results, a recently published research, studying the effect an oxidative stress sensitive transcription factor on IL-1β-induced inflammatory response, found that incubating C57BL/6 mouse aortic rings with 10 ng/ml of IL-1β for 24

hours caused significant impairment in endothelium-dependent relaxation (Gonzalez-Carnicero et al., 2023).

When vessels were treated with 10 ng/ml IFN γ, no significant contractile impairment was observed between treated and untreated vessels except for endothelium-dependent relaxation after constriction with 5-HT. Impairment after constricting with Phe was also absent for both endothelium-dependent and -independent relaxations. In a study conducted by Mikolajczyk and colleagues (Mikolajczyk et al., 2016) to understand chemokines in perivascular inflammation and endothelial dysfunction, mouse aortas from C57BL/6 mice were incubated with 50 ng/ml IFN-γ overnight, and significant impairment in endothelium dependent relaxation was observed.

When studying the effect of IL-6 on endothelium dependent vascular function, the mouse aortas were cocultured with 5 ng/ml IL-6 overnight. The aortic rings showed no contractile impairment upon treatment with Phe and 5HT but showed significant impairment upon relaxation with ACh after pre-constriction with PE. A study published in 2011, looking at the effect of simvastatin on endotoxin induced cardiovascular dysfunction also studied the effect of IL-6(Suda et al., 2011). They incubated aortas harvested from the control group with 5 ng/ml IL-6 for 30 minutes before observing a significant difference in Ach-mediated dilation between the IL-6 treated and untreated vessels. In a study by Lee et.al (Lee et al., 2017), when wild-type mice aortas were incubated with 5 ng/ml of IL-6 and a significant impairment in endothelium-dependent relaxation with ACh was observed amongst the IL-6 treated and untreated vessels. Another study where exercise training in diabetic mice restored endothelial dysfunction, incubation of C57BLK/J (wild type) mouse aortas with 5 ng/ml IL-6 for 30 minutes displayed impairment of endothelium dependent relaxation with ACh (Lee et al., 2011).

In the current study vessels incubated with TNF-α, IL-17a, and IL-15 did not show any significant impairment in vascular function. Contrary to our results, a study by Nyugen et.al., while studying the effect of IL-17a on Rho-kinase mediated endothelial dysfunction, mouse aortas were incubated with 1μg/ml IL-17A for 1 hour and they observed significant impairment of Phe-induced endothelial-dependent relaxation by ACh (Nguyen et al., 2013b). Giardina and colleagues studied the effect of TNF-α on pregnancy by collecting aortic vessels from

pregnant and non-pregnant virgin mice. They found that when vessels were pre-treated with 1000 pg/ml cytokine for 2 hours, the contractile effects of Phe were considerable in both the groups and the differences were significantly different from the non-treated group. Endothelium-dependent relaxation with ACh was also impaired due to TNF-α in both virgin and pregnant mice groups when compared with the control group, though endothelium-independent relaxation due to SNP was not significantly affected (Giardina et al., 2002). In another study, when mesenteric arteries from male Wistar rats were treated with 1 nM TNF-α for 30 minutes, significant differences in endothelium-dependent relaxation with Ach were observed after the vessels were constricted with Phe, but endothelium-independent relaxation with SNP did not show significant differences between the treated and the vehicle group (Wimalasundera et al., 2003). Similar to our findings for TNF-α treated vessels, when aortas from male Sprague-Dawley rats were treated with 100 ng/ml TNF-α for nearly 24 hours, no significant differences were observed in endothelium-dependent relaxation (Blaedel et al., 2016). Not only mouse vessels, but human arteries also show no impairment when treated with TNF-α. A research led by Gillham and colleagues studied the effect of TNF-α treatment (1 nM; 1-2 hours) on human omental arteries and found no effect on endothelium-dependent relaxation (Gillham et al., 2008).

After observing significant impairments amongst aortas treated with IL-1β, IL-6 and IFN-γ, the next step was to identify the effect of these cytokines on gene expression of molecules indicating endothelial dysfunction. IL-1β and IFN-γ were selected as they developed stronger impact on endothelial function impairment than IL-6.

In the current study both IL-1β and IFN-γ cytokines significantly reduced vascular NOX4 and eNOS gene expression in comparison to the untreated vessels. Although no-significant changes were observed, there was an upward trend in NOX1 and a downward trend of NOX2 gene expression by IL-1β. There was no significant difference observed in NOX1 and NOX2 gene expression because of IFN-γ but NOX2 gene expression demonstrated an upward trend in gene expression upon treatment. Few studies have looked at role of IL-1β on eNOS as well as NOXs gene expression. Yamagata et.al showed that treatment with IL-1β for 8 hours significantly reduced eNOS gene expression in human endothelial

cells in vitro (Yamagata et al., 2012). Ellmark and colleagues studied the effect of IL-1β on the mRNA expression of NOX1 and NOX4 by stimulating endothelial cells obtained from aortas of C57BL/6 mice. They observed that IL-1β stimulated cells did not affect the NOX1 expression but reduced the NOX4 expression (Ellmark et al., 2005). Some studies demonstrated outcomes contrary to our results, though they were not based on endothelial cell stimulations. A study conducted 10 years ago found that 25 ng/ml of IL-1β increased the production of NOX1 protein when Caco-2 cells were co-cultured with the cytokine for 24 hours (Tesoriere et al., 2014). Human knee articular chondrocytes showed upregulation of iNOS gene expression upon 24-hour treatment with 10 ng/ml IL-1β (Wu et al., 2020).

Once it was established that IL-1β and IFN-γ altered the vascular function as well as reduced gene expression levels of NOX4 and eNOS, the molecular mechanisms of this interaction were further explored. In the next steps, the IFN-γ and IL15 were chosen to study their effect on eNOS and ERK phosphorylation in human aortic endothelial cells. The first cytokine exerted detrimental effects, while the second one was used as a comparator due to the lack of direct effect on endothelial function ex vivo.

For selecting the concentrations of the cytokines and time points for stimulation of cells 10 ng/ml IFN-γ was selected as it already demonstrated its effects on vasomotor and gene expression. 10 ng/ml IL-15 was selected to compare its effect on the mechanisms of action and contrast the results with its effect on vasomotor studies and gene expression The higher cytokine concentrations were added by considering previously undertaken work on IL-15 and IFN-γ stimulated endothelial cells. Estess et.al (Estess et al., 1999) stimulated human umbilical vein endothelial cells (HUVEC) with 50 ng/ml IL-15 and 10 ng/ml IFN-γ for 4 hours to study the hyaluronan expression. Nilsen et.al. (Nilsen et al., 1998) used 100 ng/ml IFN-γ to study the cytokine profile of HUVEC for 2 to 24 hours. A study by Kou and Michel on murine endothelial cells observed phosphorylation of eNOS when stimulated with epinephrine for 2, 5, 15, 30 and 60 minutes (Kou and Michel, 2007).

After culturing the cells in different concentrations of IL-15 and IFN-γ for varying time points, phosphorylation of ERK and eNOS at T495 and S1177 was studied by

western blotting. In contrast to its effect on vascular function and gene expression, 10 ng/ml IFN-γ showed no significant effect on eNOS phosphorylation at T495, though there were sporadic fluctuations in phosphorylation when compared with the control group throughout the time points studied. A brief period of 10 ng/ml IL-15 treatment resulted in significantly decreased phosphorylation of ERK (P=0.0011, n=3). This result is in contrast to a previous study (Yeghiazarians et al., 2014), where they treated cardiomyocytes with 5 ng/ml of IL-15 resulted in increase of phosphorylation of ERK1/2. The disparity in results from the vasomotor studies and the cell culture studies suggested that even though IFN-γ may directly affect endothelial-dependent vascular relaxation, a few nanograms of the cytokine per millilitre of culture media may not be responsible for affecting NO production through eNOS phosphorylation. Stating that the concentrations used are low would not be superlative as there is a very high variation of serum concentrations observed in untreated groups of various murine models (Cheng et al., 2019, Schloot et al., 2002, Buatois et al., 2017). Furthermore, IFN may be responsible in promoting vascular dysfunction by activating other pathways than directly affecting eNOS phosphorylation. IFN is found to be responsible for the activation of iNOS, which disturbs the NO-ROS equilibrium (Kopitar-Jerala, 2017) subsequently causing endothelial dysfunction.

As observed, IL-15 might not directly affect endothelial function, but it may function as a precursor in increasing NO concentration by downregulating eNOS phosphorylation at T495, and upregulating eNOS phosphorylation at S1177. However, in pathological conditions such as inflammation, NO production in conjunction with superoxide, or other ROS, may contribute to oxidative stress and promote vascular dysfunction (Da Luz et al., 2018).

Finally, it is important to note that the n numbers were low and not consistent throughout the phosphorylation study so a meaningful conclusion cannot be made from the observed results. Furthermore, underpowering study may lead to false positive results and further mechanistic studies are needed.

Chapter 4
Discussion

Hypertension is a main contributor to a global burden of cardiovascular diseases and is responsible for cardiovascular events like myocardial infarction, stroke, renal and other target organ damage, all subsequently leading to cardiovascular event related deaths (Mancia Chairperson et al., 2023). Many different patterns of hypertension are observed such as essential hypertension, masked hypertension, secondary hypertension, as well as hypertensive emergencies in special populations such as pre-eclampsia (Unger et al., 2020). While hypertension is quite clearly defined clinically, its clinically silent initial stages of development make it difficult for physicians to diagnose a person suffering from hypertension before complications develop and a diagnosis is usually made in the advanced stages of chronically high blood pressure. Indeed current awareness of hypertension in general population suffering from high blood pressure is as low as 40.5% (Beaney et al., 2019).

As evident from heterogeneity of clinical presentations and predominating clinical complications, hypertension pathogenesis is complex and includes number of different key changes in the kidney, vasculature, and central nervous system.

The kidneys play an essential role in the regulation of blood pressure and are extensively involved in the progression and pathogenesis of hypertension. The main function of the kidneys in hypertension is to regulate blood volume by adjusting secretion and reabsorption rates of water and sodium(Coffman and Crowley, 2008). The kidneys manage this function by various methods. They are responsible for filtering salt from the blood to manage excessive levels from dietary intake of salt (Grillo et al., 2019). They are also responsible for balancing fluid levels in the blood by maintaining sodium and water concentrations to regulate the blood volume. When the blood pressure drops, the kidneys retain more sodium and water to elevate arterial pressure and conversely release excess sodium and water in high blood pressure conditions to lower arterial pressure by decreasing blood volume (Samue et al., 2018). The kidneys also produce Renin in response to low arterial pressure, which initiates a cascade of events leading to the production of a vasoconstrictor Ang II(Coffman and Crowley, 2008). Ang II increases vascular constriction and increases arterial pressure. Ang II is responsible for the increased production of aldosterone which leads to the reabsorption of water and sodium in the kidneys and increases blood

volume resulting in increased blood pressure. In an event of kidney dysfunction, impaired blood volume regulation, and dysregulated removal of electrolytes and salts may contribute to hypertension (Adamczak et al., 2002). Hence regular monitoring of kidney function is essential in individuals suffering from hypertension as kidney health is imperative in managing hypertension and vice versa.

The vasculature also plays an integral role in the development, progression, and the pathogenesis of hypertension. Blood pressure is essentially the force exerted by blood against the arterial walls as the heart pumps it through the circulatory system (Ma et al., 2023). The vasculature is responsible for maintaining the blood pressure to provide adequate blood flow to all body parts without rupturing or damaging the vessel walls. The blood vessels have the capacity to maintain their elasticity, allowing expansion and contraction at each heartbeat to regulate diastolic and pulse pressure constantly. The structure of the blood vessels can be altered by various signals from the cells of the vessels such as VSMCs, fibroblasts, adipocytes as well as endothelial cells (Martinez-Quinones et al., 2018). The vasculature also maintains vascular tone by constricting and dilating blood vessels to regulate blood pressure under low- or high-pressure levels, respectively. Vascular tone is also mediated by the endothelium. Endothelial cells produce molecules such as nitric oxide and reactive oxygen species which regulate vasodilation (Ambrosino et al., 2022). A change in the NO-ROS equilibrium results in impaired endothelium mediated vascular tone and results in increased blood pressure (Kruger-Genge et al., 2019). The blood vessels are also responsible for managing the heart rate and maintaining homeostasis by signalling the central nervous system in response to changing arterial pressure using baroreceptors and chemoreceptors(Chaudhry et al., 2023). The ability of the vascular system to manage and maintain elasticity, vascular tone among other functions is critical for maintaining arterial and blood pressure. Any dysfunction in these mechanisms can lead to increased blood pressure and can contribute to various cardiovascular issues.

The brain and central nervous system represent another key regulator blood pressure. It is responsible for secreting signalling molecules through the sympathetic and parasympathetic nervous system which have antagonistic functions (Kelly and Rothwell, 2020). Signalling molecules such as epinephrine

and nor-epinephrine are known to modulate the vascular tone by constricting and dilating the blood vessels, altering vascular resistance and maintaining blood pressure (Floras, 1992). The brain is also responsible for influencing cardiac output and these signals are majorly controlled by the medulla oblongata in the brain (Kelly and Rothwell, 2020). In high blood pressure conditions, the regulatory mechanisms of the brain might be dysregulated leading to chronic increase in blood pressure levels.

Interestingly, the common denominator of these pathogenetic changes is linked to inflammation as each of the above mechanisms can be modulated by inflammatory mechanism and local immune/inflammatory milieu. The mainstream available treatment for the disease includes targeting the organ systems and physiological systems such as the renin angiotensin aldosterone system and the sympathetic nervous system. Over the years, the role of immune system has been widely studied in the progression and pathogenesis of hypertension (Zhang et al., 2022) and targeting the immune system to treat hypertension may be instrumental in the future supporting the development of a newer line of treatment for managing and modifying course of the disease (Wenzel et al., 2021). Inflammation induced by cytokines is suspected to have a crucial role in the development of hypertension and to understand the effects and mechanisms of inflammatory cytokines and their role in hypertension, this thesis extensive researched to identify inflammatory cytokines involved in the development of hypertension, the role and potential mechanism of action of these cytokines in endothelial dysfunction.

To identify the possible role of inflammatory cytokines in the development of hypertension, we performed a systematic search of the available literature which reported associations between cytokine levels and incidence of hypertension (crd.york.ac.uk/prospero/index.asp, identifier: CRD42022378211). After searching four databases: Embase, Medline, Web of Science and Cochrane CENTRAL, using a comprehensive search strategy, a total of 725 records were identified and upon screening for inclusion and exclusion criteria, 15 records were selected.

Out of the 15 records, 13 were identified for IL-6; 5 for TNF-α; 4 for IL-1ß; 3 for IL-8; 2 for IL-2, IL-4, IL-10, IL-17A; and single studies were identified for the rest

of the cytokines (Table 6.1). Though pooled estimates based on quartiles could only be studied for IL-6 and IL-1β and hence only six records with seven studies were meta-analysed.

Table 4.1 Summary of cytokines identified in different studies through systematic search

Cytokines	No of studies	Study IDs
IL-6	13	Sesso 2007, Mauno 2008, Sahakyan 2010, Cheung 2011, Lakoski 2011, Wang 2011, Sesso 2015, Okello 2016, Pavuk 2019, Crouch 2020, Ghazi 2020, Yoo 2020, Gordon 2021
TNF-α	5	Sahakyan 2010, Pavuk 2019, Crouch 2020, Yoo 2020, Gordon 2021
IL-1β	4	Mauno 2008, Pavuk 2019, Crouch 2020, Yoo 2020
IL-8	3	Pavuk 2019, Crouch 2020, Gordon 2021
IL-2	2	Crouch 2020, Gordon 2021
IL-4	2	Crouch 2020, Gordon 2021
IL-10	2	Crouch 2020, Gordon 2021
IL-17A	2	Pavuk 2019, Crouch 2020
IL-5	1	Crouch 2020
IL-7	1	Crouch 2020
IL-12	1	Crouch 2020
IL-13	1	Crouch 2020
IL-21	1	Crouch 2020
IL-23	1	Crouch 2020
IFN-γ	1	Crouch 2020
TGF-β	1	Nakao 2017
GM-CSF	1	Crouch 2020

When the highest and the lowest quartiles of IL-6 were compared, our analysis revealed noteworthy associations between the cytokine levels and the likelihood of developing hypertension. Initially a significant association was evident without adjusting any variables, as well as when adjustments for age and gender were incorporated. However, the significance diminished when BMI adjustments were introduced. This observation is suggestive of a potential connection between elevated IL-6 levels and increased risk of hypertension specifically linked to obesity. Although a sensitivity analysis excluding a study with HIV patients maintained a significantly higher risk association in both crude and adjusted models, lack of conclusive and substantial evidence to prove the

relationship between HIV-linked hypertension and elevated IL-6 levels needs more established efforts.

In contrast, our investigation into IL-1β levels for which only two records were available for analysis, yielded no significant impact on the prospective influence of IL-1β levels on the risk of developing hypertension. This was true for crude and multivariate models, regardless of BMI adjustments. The limited data for IL-1β emphasizes the necessity of more studies to clarify its function in hypertension development.

The current meta-analysis raises the question on the relationship between inflammation and obesity linked hypertension by demonstrating weakened association between cytokine levels and risk of hypertension development. The results of our meta-analysis act as an update and extension to a previous study which looked at the association of hsCRP, IL-6 and IL-1β levels with the risk of developing hypertension in a healthy population (Jayedi et al., 2019).

In the meta-analysis conducted by Jayedi and colleagues, they extracted and examined records from two databases for CRP, hsCRP and IL-6, with their IL-6 analysis comprising five studies with effect exposure as HR, OR, and RR. They included prospective and retrospective cohort, case- cohort or nested case-control studies in their systematic search, with adults above the age of 18 or older, and excluded cross-sectional and case-control studies along with patient population-based studies. Their findings, based on tertile classification with a fixed-effects model, indicated a 51% increase in the risk of hypertension development with elevated IL-6 levels demonstrating no heterogeneity. For IL-1β analysis, they included 3 studies from 2 records, and did not find a significant association between elevated IL-1β levels and the risk of hypertension (Jayedi et al., 2019).

In contrast, our systematic search was performed in four databases, excluded inflammatory markers such as CRP and hsCRP and included randomised control trials along with observational studies, and an extensive list of inflammatory cytokines such as interleukins, interferons, tumour necrosis factors, growth differentiation factors, etc. Our analysis, which incorporated data from seven studies for IL-6 with effect exposure as HR and RR, utilized quartile distribution

and revealed a 61% increase in hypertension risk with elevated IL-6 levels, albeit with considerable heterogeneity.

Consistent with the prior meta-analysis, our results also exhibited diminished association when BMI was introduced as an adjustment in IL-6 and hypertension risk association analysis. Our analysis for IL-1β demonstrated no , similar to the previous meta-analysis. Although the study numbers were consistent, the studies included in this analysis were different from the previous meta-analysis.

Even though the meta-analysis comprehensively studied the association between inflammation and hypertension, a deeper understanding of the cytokine levels in hypertension will lead to significantly increasing the pre-existing knowledge and add substantially to the volatile relationship between inflammation, adiposity, and hypertension.

Following the establishment of the potential role of IL-6 and IL-1β in the development of hypertension, we delved into a comprehensive examination of the impact of the identified cytokines on endothelial function. Stimulation of thoracic aortas from wild-type C57BL/6 mice with identified cytokines revealed notable impairment in vasomotor responses. Specifically, vessels treated with 1 ng/ml IL-1β displayed significant impairment in endothelium-dependent relaxation mediated by ACh. In comparison to vehicle treated vessels, IL-1β treated vessels exhibited 54% less relaxation upon pre-treatment with Phe and 23.62% less relaxation upon pre-treatment with 5-HT. Similarly, vessels treated with 10 ng/ml IFN-γ were found to develop significant impairment in endothelium dependent relaxation, with 15.8% reduction in comparison to vehicle treated group when pre-constricted with 5-HT. Furthermore, 5 ng/ml IL-6 treated vessels exhibited impairment in endothelium-dependent vascular relaxation after the vessels were constricted with Phe, with cytokine treated vessels relaxing 13.28% less than vehicle treated vessels.

The effects observed in our study by IL-1β are similar to observations by a few other studies conducted in the past. While we incubated thoracic mouse aortas from C57BL/6J mice for 24 hours, some studies used Sprague Dawley rats to study the effects of IL-1β on thoracic arteries(Loughrey et al., 2003) and mesenteric arteries (Vallejo et al., 2014), while others used thoracic aortas from

C57BL/6J mice (Chook et al., 2023, Gonzalez-Carnicero et al., 2023). One study reported the use of internal mammary artery from humans (Foudi et al., 2017). While 2 of these studies incubated the vessels with IL-1β for 2 hours (Loughrey et al., 2003, Vallejo et al., 2014), the other 3 studies incubated the vessels for 18-24 hours (Chook et al., 2023, Gonzalez-Carnicero et al., 2023, Foudi et al., 2017). It is important to note that all these studies demonstrated a significant contractile impairment upon treating aortas with IL-1β even though the concentrations of the cytokine were diverse (Vallejo et al., 2014, Loughrey et al., 2003, Foudi et al., 2017, Gonzalez-Carnicero et al., 2023, Chook et al., 2023). Limited work had been done to understand the effects of IFN-γ on vasomotor studies and our results are similar to a previous study published in 2021 where 50 ng/ml of the cytokine elicits a similar impairment pattern (Mikolajczyk et al., 2021). While previous studies on the vasomotor effects of IL-1β incorporated different variable cytokine levels, three studies were identified for IL-6 with the same cytokine concentration as used in our study. Two studies used coronary arterioles (Lee et al., 2017, Lee et al., 2011) whereas one study used abdominal aorta for functional assessment of the blood vessels (Suda et al., 2011). All these studies used C57BL mice as wild type models and incubated the vessels for 30 minutes with IL-6 (5 ng/ml). All three studies observed impaired relaxation in vessels treated with the cytokine when compared to vehicle treated vessels.

Following the observation of direct impact of the cytokines on endothelium-dependent vascular function, we proceeded to explore their effects on the gene expressions of NOXs and eNOS. Treatment of mouse thoracic aortas with 1 ng/ml IL-1β and 10 ng/ml IFN-γ significantly downregulated NOX4 and eNOS gene expression compared to the untreated group. However, no significant changes were observed in NOX1 and NOX2 gene expression. These results are in line with some previous studies looking at the effects of IL-1β on gene expression in murine aortas as well as aortic cells (Ellmark et al., 2005, Yamagata et al., 2012).

NOXs are an enzyme family playing a significant role in hypertension development and progression. They are known to be the primary source of ROS in blood vessels (Rodrigo et al., 2011). These enzymes produce superoxide and other ROS, overproduction of which leads to oxidative stress, leading to an

imbalance between ROS production and elimination (Montezano and Touyz, 2012b). Oxidative stress can damage blood vessels, impair endothelial function and vascular tone contributing to elevated arterial pressure and subsequently increased blood pressure. NOXs and ROS contribute to vascular remodelling leading to vascular resistance, subsequently causing hypertension (Garcia-Redondo et al., 2016).

The role of NOXs and H_2O_2 in atherosclerosis has also been researched over the years. A study on atherosclerosis by Ketonen and colleagues demonstrated that high sodium intake combined with a high fat diet induced endothelial dysfunction via elevated superoxide anion production in LDL receptor deficient mice and reduced production of ROS was observed when the researchers inhibited NOXs using apocynin, (Ketonen and Mervaala, 2008). Another study researching the effects of aerobic training on endothelial function in LDL receptor deficient mice found that impaired endothelial function was fully reversed by increasing H_2O_2 production and NO bioavailability in treatment groups (Guizoni et al., 2016).A study published in 2011 looked at the role of H_2O_2 in endothelial function and NOX4 during laminar sheer stress subsequently found that the sheer stress elevated hydrogen peroxide levels leading to the stimulation of eNOS and elevated NO production. Excessive ROS in blood can cause various inflammatory pathways to be activated leading to an increased production of inflammatory cytokines, increased SNS activity, altered renal function and increased fluid retention due to overactive RAAS (Manuela et al., 2017). Their role in ROS production in the vasculature makes NOXs potentially promising target for hypertension management.

The next steps, after establishing that inflammatory cytokines which affect vasomotor function by directly acting on endothelium also regulate gene expressions of NOXs and eNOS, were to identify mechanisms by which these signals were relayed. HAoECs were stimulated for varying time points with varying IFN-γ and IL-15 concentrations to study their effects on phosphorylation of eNOS at two phosphorylation sites along with the phosphorylation of ERK. Even though IFN-γ showed significant effects on vasomotor function and gene expression, 10 ng/ml IFN-γ did not show any significant changes in phosphorylating eNOS at T495, whereas it demonstrated a significant difference at S1177 after 30 minutes of stimulation. ERK was also phosphorylated and the

difference between the stimulated and unstimulated cells were significant after a short 15-minute treatment with 100 ng/ml IFN-γ.

While IL-15 showed no effect on endothelium dependent and independent vasomotor function, 10 ng/ml IL-15 demonstrated significantly decreased eNOS phosphorylation at T495 and significantly increased eNOS phosphorylation at S1177 after 4 hours of stimulation. After 24 hours stimulation, eNOS phosphorylation at S1177 was significantly downregulated with 10 ng/ml IL-15 whereas ERK phosphorylation was only significantly downregulated with 10 ng/ml IL-15 after a short 30 min stimulation. Higher concentrations of IFN-γ have found to have detrimental effects on endothelial cells and may lead to the activation of iNOS which causes an imbalance in the NO and ROS equilibrium and may possibly lead to decreased NO availability to maintain vascular functions (Kopitar-Jerala, 2017, Javanmard and Dana, 2012).

IL-1β is a pro-inflammatory cytokine belonging to the IL-1 superfamily and is primarily produced by the innate immune cells such as monocytes and macrophages (Dinarello, 2018). It is produced as a precursor molecule which activates upon interaction with foreign bodies acting as infections (Werman et al., 2004). While the relationship between the cytokine and hypertension remains an area of research, emerging evidence suggests its potential involvement in the progression and pathogenesis of hypertension and associations with blood pressure levels (Mauno et al., 2008, Dalekos et al., 1997, Crouch et al., 2020a). However, the findings are not entirely consistent with the hypothesis that IL-1β might be associated with the risk of developing hypertension (Wang et al., 2011). IL-1β can impair endothelial function by interfering with the production of vasodilators such as NO and vasoconstrictors such as ET-1. A study conducted to test the effects of NO and ET-1 on hypo- and hypertensive patients undergoing dialysis suggests that these molecules alter vascular tone by interfering with vasoconstriction and dilation (Erkan et al., 2002). IL-1β also causes remodelling leading to increased vascular resistance in diverse kinds of hypertension (Melton and Qiu, 2021). Our study focuses on identifying the relationship between endothelial dysfunction and IL-1β, and successfully identifies the effects on endothelium dependent relaxation along with NOX4 and eNOS gene expression regulation. While the relationship between IL-1β and hypertension is an active area of research, it is increasingly recognized

that IL-1β may play a role in hypertension, not only in the context of blood pressure regulation (Rothman et al., 2020) but also in terms of its influence on endothelial function in various disease states such as autoinflammatory diseases, metabolic syndrome, and cancer (Kaneko et al., 2019). Hence, research identifying mechanisms of IL-1β and its role in hypertension is ongoing, and a better understanding of these mechanisms may lead more targeted therapeutic interventions for hypertension associated with IL-1β.

IFN-γ is a soluble monomeric cytokine known for its anti-tumour and inflammatory properties (Jorgovanovic et al., 2020). It is produced by immune cells such as NK cells, Th1 cells, and cytotoxic $CD8^+$ T cells and IFN-γ production is stimulated by increased levels of inflammatory cytokines such as IL-1β, IL-12 and IL-18 (Griffin, 2008). The role of IFN-γ in hypertension is a complex area of study and IFN-γ has been the subject of investigation regarding its potential role in the pathogenesis of hypertension. Some studies have found higher levels of the cytokine in hypertensive patients and its associations with high blood pressure levels in murine models (Ji et al., 2017, Kamat et al., 2015). The mechanism of action of the cytokine in the progression of the disease remains under study but its role in endothelium function has been demonstrated in the current study. Our results suggest that even though IFN-γ was not found to alter eNOS activation by phosphorylating the molecule at two sites, the cytokine altered endothelium dependent vascular function by significantly interfering with contractile functions of the vasculature. IFN-γ has been implicated in endothelial function and is known to impair vasomotor functions, and alter vascular permeability (Ng et al., 2018). The cytokine may cause an imbalance in ROS production and subsequently damage blood vessels. IFN-γ might also cause dysregulation of RAAS and alter kidney function, over-activate SNS leading to increased heart rate and elevated blood pressure. The cytokine may also contribute to vascular remodelling resulting in increased vascular resistance. The interplay between IFN-γ and hypertension remains a dynamic area of investigation requiring further explorations to fully understand its mechanisms and implications.

IL-6 plays a significant role in the mechanisms contributing to the pathogenesis and progression of hypertension. While precise pathways through which this pleotropic myokine exerts its influence on hypertension are still a subject of

ongoing explorations, several key avenues have been already identified. One prominent effect is the impairment of endothelial function. IL-6 has been found to mediate vascular constriction by acting on VSMCs and increase ROS in both in-vitro and in-vivo murine models leading to endothelial dysfunction (Wassmann et al., 2004) and thereby exacerbating hypertension development. The cytokine may also alter RAAS function and has been found to play a role in Ang II mediated hypertension in animal models (Maranduca et al., 2020) as well as humans (Chamarthi et al., 2011), thereby influencing blood volume and contributing to elevated pressure. The influence of epigenetic factors such as hypomethylation of IL-6 adds another layer to its role in predisposing individuals to the risk of hypertension (Mao et al., 2017b). Underlying health conditions like metabolic disorders have been identified as factors that elevate IL-6 levels, potentially aggravate hypertension (Mohammadi et al., 2017). The present study contributes to the understanding of IL-6 in endothelial dysfunction by revealing its impact on vasorelaxation, further research is imperative to comprehensively elucidate the intricate mechanisms and clinical implications of IL-6 in its relationship with hypertension.

Strengths and Limitations

This thesis has various strengths and weaknesses. We began our thesis by questioning the relationship between inflammatory cytokines and the incidence of hypertension in humans. First strength of this thesis is that our study comprises of a comprehensive list of keywords to effectively look for associations between cytokines and hypertension.

Secondly, the meta-analysis highlights the importance of confounding factors such as BMI which weaken the positive association between IL-6 and the incidence of hypertension. The weakened association links inflammation and adiposity with the risk of hypertension development.

Third strength of this thesis is that the experimental data is consistent with the results from systematic review and meta-analysis. This shows that even though cytokine levels are not a part of regular testing and a means of identifying the progression of hypertension, it could prove to be instrumental in early diagnosis

of the disease with further research connecting the link between serum cytokine levels and elevated blood pressure.

Apart from the strengths, this thesis also has some limitations. Even though our systematic review updates the existing association between IL-6 and incident hypertension, limited records for IL-1β leaves the question of whether increasing levels of this cytokine have an association with incidence of hypertension. In general, robust large-scale data on cytokine levels in hypertension is lacking and studies are extremely focused, with different sets of cytokines studies using different methods making integration of these data difficult.

Secondly, even though we demonstrate that IL-1β has significant effects and is detrimental to vascular function at the endothelium level and regulates gene expression, its mechanism of action was not clear from our mechanistic vascular biology studies. Hence, further investigations are imperative to discover and unravel the precise pathways and molecular interactions through which IL-1β exerts its effect on the endothelium.

Third limitation that this thesis presents is the limited sample size from cell culture experiments which, if substantiated, could result in concrete evidence supporting the trends of vasomotor activity and gene regulation by inflammatory cytokines. Further studies need to focus on a comprehensive assessment of a broad range of cytokines in hypertension as there is an urgent need to establish an immune signature of hypertension in humans given the effects of a broad range of cytokines in animal models of hypertension (Maranduca et al., 2020, Segiet et al., 2019).

While this thesis has established a connection between inflammatory cytokines and its effect on endothelium dependent vascular function, further exploration is essential to strengthen the claims of this thesis. Future experimentation should include quantifying the effects of identified cytokines on NO, ROS, and NOXs at cellular and organ level as well as identifying the mechanisms of action of IL-1β on signalling molecules such JAK/STAT, ERK, p-38 and eNOS, and increasing replicates for IFN-γ. To fortify the results from the systematic review, there is a need to design a clinical study that quantifies baseline and follow-up serum levels of inflammatory cytokines in diverse study groups, including healthy

normotensive individuals, hypertensive patients receiving treatment and hypertensive patients previously on medication, stopping their medication for the follow-up period. Although a recently published study did not find any differences in cytokine levels between normotensive and hypertensive subjects (Dzieża-Grudnik et al., 2023), previously conducted studies looking at serum levels of inflammatory cytokines have successfully established a link between increased cytokine levels and hypertension (Stumpf et al., 2005).

The success of the CANTOS trial is proof that immune mechanisms need to be the new focus of research to identify links between the immune system and diseases leading to higher risk cardiovascular events (Ridker et al., 2017b). Although the secondary analysis did not yield any significant associations between blood pressure or incidence of hypertension with the benefits of canakinumab in atherosclerosis (Rothman et al., 2020), the results of the trial have opened up a new prospect of using anti-inflammatory drugs to target the immune function to manage and treat cardiovascular diseases.

In summary, evidence presented in this thesis in line with data in the literature provide clear view of possible role of selected cytokines in potential development of hypertension with IL-6 and IL-1β being cytokines with most available evidence while we urgently need to better understand the role of other cytokines implicated in hypertension such as IL-17, IFN-γ and others.

Chapter 5
Conclusions

Hypertension is now widely identified as a disorder acting as a precursor to cardiovascular diseases and kidney disorders which subsequently lead to death and even though there are treatments available, hypertension at large remains an uncurable disease. Due to rising incidences of the disease because of lifestyle, genetics, and environmental factors, newer lines of treatment are a necessity to manage, maintain and potentially cure the issue of chronically high blood pressure.

In conclusion, this PhD thesis has undertaken a comprehensive investigation into the role of inflammatory cytokines in hypertension, with a particular focus on their potential mechanisms of action in the development of the disease. The key findings and conclusions are summarized as follows:

1. Through a systematic review of the literature, the thesis identified several inflammatory cytokines, including IL-6, IL-1β, TNF-α, IL-17a, and IFN-γ, as potential contributors to the incidence of hypertension. Systematic search has however shown that sufficient quantitative evidence is only available in relation to IL-6 and to a lesser extent IL-1β.

2. A significant positive association exists between IL-6 levels and the risk of developing hypertension, although this association weakened when adjusted for BMI, suggesting a link between inflammation and adiposity-related hypertension.

3. In animal tissue studies, IL-1β, IFN-γ, and IL-6 were found to significantly impair endothelium-dependent vasorelaxation in mouse aortas. These results suggest a direct connection between inflammatory cytokines and endothelial dysfunction.

4. IL-1β and IFN-γ were shown to reduce the gene expression of NOX4 and eNOS in treated vessels, indicating potential mechanisms through which these cytokines may impact vascular function.

While the thesis provides valuable insights into the direct effects of inflammatory cytokines on endothelial function, it suggests that further research is needed to fully understand the underlying mechanisms and their significance

in the patho-mechanisms of hypertension. Some discrepancies in outcomes were observed compared to previous studies, highlighting the complexity of cytokine interactions in the context of endothelial function. These variations may be attributed to different experimental conditions and concentrations of cytokines.

In summary, the studies described in this thesis contribute to our understanding of the role of inflammatory cytokines in hypertension and their potential impact on endothelial dysfunction. The findings highlight the need for continued research to elucidate the intricate mechanisms involved and their implications for the development and treatment of hypertension.

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