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MICROCIRCULATION IN CHRONIC KIDNEY DISEASE: FROM INJURY TARGETS TO POTENTIAL THERAPEUTICS

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Karolinska Institutet

Stockholm 2023

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Printed by Universitetsservice US-AB, 2023

ISBN 978-91-8017-032-1
Cover illustration: Created by Samsul Arefin. Modifications by Karolina Kublickiene

MICROCIRCULATION IN CHRONIC KIDNEY DISEASE: FROM INJURY TARGETS TO POTENTIAL THERAPEUTICS

Thesis for Doctoral Degree (Ph.D.)

By

Samsul Arefin

The thesis will be defended in public at GENE, NEO, Hälsovägen 7, Flemingsberg, Karolinska Institutet, Tuesday, May 30, 2023 at 2.00 PM.

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POPULAR SCIENCE SUMMARY OF THE THESIS

Chronic kidney disease (CKD) is a major public health problem worldwide, and patients with end-stage kidney disease (ESKD) are at increased risk of developing cardiovascular complications. Small artery dysfunction is a common complication of CKD, which contributes to the development of early vascular ageing (EVA) and cardiovascular complications. The exact mechanisms of how small artery dysfunction contributes to these complications are not fully understood. This PhD thesis aims to investigate the functional and structural properties of small resistance arteries in ESKD patients and non-CKD controls and to identify potential therapeutic targets for improving vascular function in this CKD population. The methodological approach involved both in vivo and ex vivo techniques principally based on biochemical assays, immune staining as well as wire-myography technique. The results of this study demonstrate that the microvasculature in ESKD patients is characterized by the presence of EVA phenotype, which may contribute to the development of cardiovascular complications. The uremic environment influences vascular function by altering the contribution of endothelium-derived factors (i.e. reduced nitric oxide and increased endothelium derived hyperpolarization factor) and increasing vascular stiffness, which are further modulated by inflammation, uremic toxins, senescence, and extracellular vesicles. The thesis also investigated the importance of angiotensin-converting enzyme 2 and transmembrane protease serine 2 receptors, prostaglandin system, different amino acids and their metabolites, as well as potential of novel pharmacological compounds in patients with CKD. In summary, the conducted studies present insights into the mechanisms underlying small artery dysfunction in ESKD patients and identify potential therapeutic targets for improving vascular function in this patient population.

POPULÄRVETENSKAPLIG SAMMANFATTNING AV AVHANDLINGEN

Kronisk njursjukdom (CKD) är ett stort globalt hälsoproblem, och patienter med terminal njursjukdom (ESKD) löper ökad risk för att utveckla kardiovaskulära komplikationer. Småkärlsdysfunktion är en vanlig komplikation vid CKD, vilket bidrar till utvecklingen av tidigt vaskulärt åldrande och kardiovaskulära komplikationer. De bakomliggande mekanismerna är dock ofullständigt kända. Denna doktorsavhandling syftar till att undersöka de funktionella och strukturella egenskaperna hos små motståndskärl i ESKD-patienter och icke-CKD-kontroller samt att identifiera nya behandlingsalternativ för att förbättra den vaskulära funktionen hos njursjuka. Resultaten av studien visar att den uremiska miljön påverkar vaskulär funktion genom att rubba utsöndringen av faktorer från endotelceller på kärlens insida och öka kärlstelheten, vilket ytterligare moduleras av inflammation, åldrande och extracellulära vesiklar. Studien undersöker även betydelsen av ACE2- och TMPRSS2-receptorer, prostaglandinsystemet samt olika aminosyror och dess metaboliter i små motståndskärl. Sammantaget ger resultaten värdefulla insikter i mekanismerna bakom småkärlsdysfunktion hos ESKD-patienter och belyser potentiella behandlingsmål för att förbättra kärlfunktionen i denna population.

ABSTRACT

Chronic kidney disease (CKD) is a major public health problem worldwide, and patients with end-stage kidney disease (ESKD) are at increased risk of developing cardiovascular complications. Small artery dysfunction is a common feature of CKD, which contributes to the development of early vascular ageing (EVA) and cardiovascular complications. The exact mechanisms of how small artery dysfunction contributes to these complications are not fully understood. The ultimate goal of this PhD thesis is to gain a better understanding on how small artery dysfunction contributes to EVA and cardiovascular risk in the uremic environment, and to define specific targets for potential therapeutic benefit. The methodological approaches involve both ex vivo and in vivo investigations, including biochemical marker measurements, immuno staining, as well as isolated small artery bioassays and wire myography technique together with EndoPAT to assess peripheral arterial tone.

In **Paper I** we investigated both in vivo and ex vivo functional properties (reactive hyperemia index [RHI], contractility, vasodilatory, stiffness) of small resistance arteries from ESKD patients and non-CKD controls. We also investigated ex vivo effects of trimethylamine N-oxide (TMAO), phenylacetyl glutamine (PAG) and extracellular vesicles (EVs) from CKD-5 patients, as well as pharmacological interventions using senolytics. We assessed markers of senescence, calcification, endothelial function, and oxidative stress; these data were also correlated with functional and structural properties of resistance arteries. We observed that the uremic environment influences vascular function by changing the contribution of endothelium-derived factors (i.e. reduced nitric oxide and increased endothelium derived hyperpolarization factor) and increasing vascular stiffness in patients with ESKD; these events were further modulated by inflammation, TMAO, PAG and EVs. Moreover, the vasculature of ESKD patients was characterized by hallmarks of EVA –presence of the senescence signature, microcalcification, reduced anti-oxidant control, and decreased contractile markers which might confer the development of cardiovascular complications in this specific patient group. We also showed that senolytics could be used to target senescent cells. As **Paper I** comprehensively phenotypes the microcirculation from ESKD patients, this study serves as a backbone for the overall thesis.

Paper II, a complementary study of **Paper I,** adds more insight into endothelial function and vascular structure biology in respect to different amino acids (AA) and their metabolites. New findings include impaired AA metabolism with decreased biopterin BH4/BH2 ratio in CKD, as well as elevated asymmetric dimethylarginine levels that were associated with higher vascular stiffness and reduced NO contribution.

In **Paper III,** we investigated differences in the expression of angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) receptors in resistance arteries and subcutaneous adipose tissue, alongside circulating soluble ACE2 levels in female and male ESKD patients versus non-CKD controls. Our results demonstrated that soluble ACE2 levels

were higher in ESKD patients. In addition, ACE2 tissue expression was higher in ESKD patients with a higher prevalence in male subjects and was present in both the endothelium and VSMCs from arteries in peripheral microcirculation.

The aim of **Paper IV** was to better understand the role of prostaglandin contribution to vasoactive properties and characterize the effects of microsomal prostaglandin E synthase-1 (mPGES-1) inhibition in the microvasculature of CKD patients. A significant reduction in adrenergic vasoconstriction and improvement in relaxation was observed following mPGES-1 inhibition. Based on our findings, it can be inferred that mPGES-1 inhibition has additional vasoactive effects in the human microcirculation beyond the shunting to prostacyclin (PGI_2) pathway, i.e. a reduction in the levels of local prostaglandin E_2 (PGE_2), as well as influencing other vascular factors. This indicates the interaction of several pathways after mPGES-1 inhibition.

The findings of this thesis provide valuable insights into the mechanisms underlying small artery maintenance and dysfunction in ESKD patients and identify potential therapeutic targets for improving vascular function in this patient population.

LIST OF SCIENTIFIC PAPERS

Throughout this thesis, the following papers will be referenced using their corresponding Roman numerals, all of which were reprinted with permission from the publishers. *Authors contributed equally.

I. **Early vascular aging in microvasculature of chronic kidney disease: focus on function, structure and senescence signature.**
Samsul Arefin, Neja Mudrovcic, Sam Hobson, Federico Pietrocola, Thomas Ebert, Liam Ward, Anna Witasp, Lars Wennberg, Torbjörn Lundgren, Julia Steinmetz-Späh, Karin Larsson, Anders Thorell, Stefania Bruno, Marita Marengo, Vincenzo Cantaluppi, Peter Stenvinkel*, Karolina Kublickiene*. *Submitted Manuscript*

II. **Associations of Biopterins and ADMA with Vascular Function in Peripheral Microcirculation from Patients with Chronic Kidney Disease**.
Samsul Arefin, Lars Löfgren, Peter Stenvinkel, Anna B. Granqvist and Karolina Kublickiene. *International Journal of Molecular Science. 2023,24,5582.*

III. **Angiotensin-converting enzyme 2 and transmembrane protease serine 2 in female and male patients with end- stage kidney disease.**
Samsul Arefin, Leah Hernandez, Liam J. Ward, Angelina Schwarz, GOING-FWD Collaborators, Peter Barany, Peter Stenvinkel, Karolina Kublickiene. *European Journal of Clinical Investigation. 2022;52: e13786.*

IV. **Effects of microsomal prostaglandin E synthase-1 (MPGES-1) inhibition on resistance artery tone in patients with end stage kidney disease.**
Julia Steinmetz-Späh*, **Samsul Arefin*,** Karin Larsson, Jabin Jahan, Neja Mudrovcic, Lars Wennberg, Peter Stenvinkel, Marina Korotkova, Karolina Kublickiene*, Per- Johan Jakobsson*. *British Journal of Pharmacology, 2022;179(7):1433-1449; *Shared authorship.*

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. **Senescent Cells in Early Vascular Ageing and Bone Disease of Chronic Kidney Disease-A Novel Target for Treatment.**
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III. **Nrf2 in early vascular ageing: Calcification, senescence and therapy.**
Samsul Arefin, Sarah Buchanan, Sam Hobson, Julia Steinmetz, Shno Alsalhi, Paul G Shiels, Karolina Kublickiene, Peter Stenvinkel. *Clin Chim Acta. 2020;505:108-18.*

IV. **Inflammation and Premature Ageing in Chronic Kidney Disease.**
Thomas Ebert, Sven-Christian Pawelzik, Anna Witasp, **Samsul Arefin,** Sam Hobson, Karolina Kublickiene, Paul G Shiels, Magnus Bäck, Peter Stenvinkel. Toxins (Basel). 2020;12(4).

V. **Cellular mechanisms of aging and their impact on the aortic/arterial wall.**
Samsul Arefin*, Agne Laucyte-Cibulskiene*, Sam Hobson, Angelina Schwarz, Lu Dai, Karolina Kublickiene, Peter Stenvinkel. *Textbook of ''Arterial Stiffness and Pulsatile Hemodynamics in Health and Disease'' 2022, Chapter 25; Pages 391-405; doi.org/10.1016/B978-0-323-91391-1.00025-X*

VI. **Blood-brain barrier and gut barrier dysfunction in chronic kidney disease with a focus on circulating biomarkers and tight junction proteins.**
Leah Hernandez, Liam J Ward, **Samsul Arefin,** Thomas Ebert, Agne Laucyte-Cibulskiene,GOING-FWD Collaborators, Olof Heimbürger, Peter Barany, Lars Wennberg, Peter Stenvinkel, Karolina Kublickiene. *Sci Rep. 2022;12(1):4414.*

VII. **Indoxyl Sulphate Retention Is Associated with Microvascular Endothelial Dysfunction after Kidney Transplantation.**
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VIII. **Effects of a Synbiotic on Plasma Immune Activity Markers and Short-Chain Fatty Acids in Children and Adults with ADHD-A Randomized Controlled Trial.**

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Sam Hobson*, **Samsul Arefin*,** Anna Witasp, Leah. Hernandez, Karolina Kublickiene, PaulG. Shiels, Peter Stenvinkel. *Circulation Research. 2023;132:00–00. DOI: 10.1161/CIRCRESAHA.122.321751*

X. **Blood–Brain Barrier Biomarkers before and after Kidney Transplantation.**

Leah Hernandez, Liam J. Ward, **Samsul Arefin,** Peter Barany, Lars Wennberg, Magnus Söderberg, Stefania Bruno,Vincenzo Cantaluppi, Peter Stenvinkel and Karolina Kublickiene. *Int. J. Mol. Sci. 2023, 24(7), 6628; doi.org/10.3390/ijms24076628*

XI. **Calprotectin is a contributor and potential therapeutic target of vascular calcification in chronic kidney disease.**

Ana Amaya-Garrido, Manon Brunet, Bénédicte Buffin-Meyer, Alexis Piedrafita, Lucile Grzesiak, Ezechiel Agbegbo, Arnaud Del Bello, Inés Ferrandiz, Serban Ardeleanu, Marcelino Bermudez-Lopez, Camille Fedou, Mylène Camus, Odile Burlet-Schiltz, Jean Massines, Marie Buléon, Guylène Feuillet, Melinda Alves, Eric Neau, Audrey Casemayou, Benjamin Breuil, Jean-Sébastien Saulnier-Blache, Colette Denis, Jakob Voelkl, Griet Glorieux, Sam Hobson, **Samsul Arefin**, Awahan Rahman, Karolina Kublickiene, Peter Stenvinkel, Jean-Loup Bascands, Stanislas Faguer, José M. Valdivielso, Joost P. Schanstra, Julie Klein. *Under review from the Journal ''Science Translational Medicine''.*

CONTENTS

LIST OF ABBREVIATIONS

AAs	Amino Acids
AC	Adenylyl cyclase
ACE2	Angiotensin-converting enzyme 2
ACh	Acetylcholine
ADAM-17	A Disintegrin and Metalloproteinase 17
ADMA	Asymmetric dimethylarginine
AI@75	Augmentation index
ALP	Alkaline phosphatase
Ang	Angiotensin
ARBs	ACE inhibitors and angiotensin receptor blockers'
AT1& 2	ACE-Ang II-type 1 & 2 receptor
BH2	7,8-Di-hydrobiopterin
BH4	Tetrahydrobiopterin
BK	Bradykinin
CAC	Coronary artery calcification
cAMP	Cyclic adenosine monophosphate
CBFA1	Core-binding factor α-1
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CKD	Chronic kidney disease
COVID-19	Coronavirus disease 2019
COX	Cyclooxygenase
COXIBs	COX-2 inhibitors
cPGES	Cytosolic prostaglandin E synthase
CVD	Cardiovascular disease
ECM	Extracellular matrix
EDHF	Endothelium derived hyperpolarization factor
eGFR	Estimated glomerular filtration rate
eNOS	Endothelial nitric oxide synthase
ESKD	End-stage kidney disease
ET-1	Endothelin-1
ETs	Endothelins
EVA	Early vascular ageing
EVs	Extracellular vesicles
GC	Guanylyl cyclase
GPCRs	G protein-coupled receptors
ICAM-1	Intercellular adhesion molecule 1
iNOS	Inducible nitric oxide synthase
IP	Prostaglandin I2 receptor
IS	Indoxyl sulfate
L-NAME	L-NG-Nitro arginine methyl ester
LOX	Lipoxygenase
MGP	Matrix Gla protein

MMPs	Matrix metalloproteinases
mPGES-1	Microsomal prostaglandin E synthase 1
mPGES-2	Microsomal prostaglandin E synthase 2
MSX2	Msh homeobox 2
MVs	Microvesicles
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Norepinephrine
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
Nrf2	Nuclear factor-erythroid factor 2-related factor 2
NSAIDs	Nonsteroidal anti-inflammatory drugs
ONOO-	Peroxynitrite
PAG	Phenylacetyl glutamine
PG	Prostaglandin
PGH2	Prostaglandin H2
PGI2	Prostacyclin
PTX-3	Pentraxin-3
RAAS	Renin-angiotensin-aldosterone system
RAS	Renin-angiotensin system
RHI	Reactive hyperemia index
ROS	Reactive oxygen species
RUNX2	Runt-related transcription factor 2
SASP	Senescence-associated secretory phenotype
SCAPs	Senescence cell anti-apoptotic pathways
SIRT1	Sirtuin1
SNP	Sodium nitroprusside
TAFs	Telomere-associated DNA damage foci
TMAO	Trimethylamine N-oxide
TMPRSS2	Transmembrane protease serine 2
TP	Thromboxane receptor
TX	Thromboxane
TXA2	Thromboxane A2
VC	Vascular calcification
VSMCs	Vascular smooth muscle cells

1 INTRODUCTION

Chronic kidney disease (CKD) is a health concern worldwide, with an estimated global prevalence of >10%. Ultimately, this condition can progress to end-stage kidney disease (ESKD), which further exacerbates health risks [1]. ESKD is the final, permanent stage of CKD, where kidney function has declined to the point that the kidney cannot function on its own; dialysis or kidney transplantation is necessary as life-prolonging treatment. Diabetes and hypertension are the most common causes of CKD; however, there are additional risk factors that are also involved in the development of CKD, including but not limited to heart disease, smoking, obesity, prolonged use of certain medications e.g. nonsteroidal anti-inflammatory drugs (NSAIDs), blockages in the urinary tract, family history of polycystic syndrome, inflammation, older age and some genetic disorders [2].

CKD is a major risk factor for cardiovascular disease (CVD). Patients with CKD are at an increased risk of experiencing adverse cardiovascular events, such as heart attack, stroke, and heart failure. This increased risk is partly due to traditional CVD risk factors, including hypertension, dyslipidemia, and diabetes. However, CKD also contributes to CVD risk through non-traditional mechanisms such as inflammation, oxidative stress, and endothelial dysfunction. CVD is 30-40 fold more prevalent in CKD patients than in the general population and contributes to reduced life expectancy [3]. Functional and structural abnormalities of the vasculature seem to play an important role in the development of CVD and its complications in CKD [4]. Although the precise mechanisms remain elusive, it is clear that both the endothelium and vascular smooth muscle cells (VSMCs) are affected in vascular disease, with common pathways contributing to the development of arteriosclerosis and atherosclerosis [5]. Patients with CKD display an early vascular ageing (EVA) phenotype, and one main hallmark of EVA in CKD is the development of calcification of the arterial tunica media with increased arterial stiffening as a functional consequence [6]. Patients display additional features related to the EVA phenotype, linked to apoptosis and cellular senescence. Senescence is characterized as a state of irreversible cellular growth arrest, while still presenting a proinflammatory secretome [7].

Convincing evidence suggests that the arterial wall of elastic, muscular and small arteries undergo profound functional and structural changes as renal function declines [8]. This process is complex and modulated by a delicate change in inflammatory, oxidative and metabolic milieu where VSMCs can switch from a contractile to a synthetic phenotype [9]. Several markers and pathways are involved in the remodeling process; the switch is characterized by a loss of contractile proteins and upregulation of synthetic proteins. This shift in phenotype alters the morphology and function of VSMCs, leading to the production of extracellular matrix components and the migration and proliferation of VSMCs, which contribute to the development of vascular fibrosis and arteriosclerosis [10].

Uremic toxins also play a crucial role in this change mentioned in the earlier paragraph; recent studies demonstrate the importance of trimethylamine N-oxide (TMAO) and phenylacetyl

glutamine (PAG) in this matter. In vitro studies have shown that extracellular vesicles (EVs) may induce endothelial dysfunction in several pathological states, e.g. acute lung injury, during cholesterol loading or exposure to endotoxins. At the cellular level, vascular ageing shows characteristics of endothelial malfunction by means of impaired endothelium dependent dilatation or changes in the contribution of endothelium-derived factors, inflammation, VSMC proliferation, change of extracellular matrix composition, and/or calcification [11]. Functionally, vascular maintenance is transformed during vascular ageing, determined primarily via impaired contractility, which in turn could also be affected and/or modulated by altered endothelial function.

The focus of this introduction is to provide an overall overview and current updates of the topics of this PhD thesis project, which includes: an insight into the anatomy of the vasculature as well as the underlying endothelial cell physiology and its association with CKD. The brief summary about EVA and the role of calcification, senescence, EVs, uremic toxins and how those events are associated with EVA and vascular dysfunction of microcirculation in CKD. In addition, the introduction also includes an overview about prostaglandins with particular focus on microsomal prostaglandin E synthase-1 (mPGES-1) and angiotensin-converting enzyme 2 (ACE2) in CKD.

1.1 Vascular anatomy and regulation of vascular tone

The primary function of the cardiovascular system is to maintain tissue homeostasis by circulating blood carrying oxygen, immune cells, nutrients, and hormones through blood vessels to target organs. The vascular wall of blood vessels comprises of three layers: the tunica intima, comprising endothelial cells; the tunica media, consisting of smooth muscle cells; and the tunica externa, consisting predominantly connective tissues supporting the vessel (**Fig.1**) [12]. Based on their function and location, blood vessels can be classified into arteries, veins, or capillaries [13]. Arteries can be classified into elastic, muscular or resistance arteries depending on their location in the arterial tree. The largest arteries, including the aorta, pulmonary, and carotid artery, are classified as elastic arteries due to their abundance of elastic fibers and collagen. The elastic arteries further branch into muscular arteries as for example the brachial, femoral and radial arteries, which supply blood to the peripheral tissue. The muscular arteries in turn branch into resistance arteries (100-400μm), further into arterioles (<100μm), and then capillaries, which form the microcirculation[14]. Resistance arteries are actively involved in crucial physiological functions[15].

VSMCs in resistance sized arteries form a layer that allow them to contract and expand in response to sympathetic regulation, flow induced shear stress, changes in intraluminal pressure and other vasoactive stimuli, making them a key effector organ of the cardiovascular system [16]. By managing mechanical activities of VSMCs, the vascular endothelium plays a critical role in maintaining vascular homeostasis and avoiding the onset and progression of CVD. The endothelium is essential for maintaining vascular equilibrium, mediated through endothelium-derived factors. Nitric oxide (NO), prostacyclin (PGI_2) and endothelium derived

hyperpolarization factor (EDHF) are three major vasodilators for endothelium mediated relaxation [17].

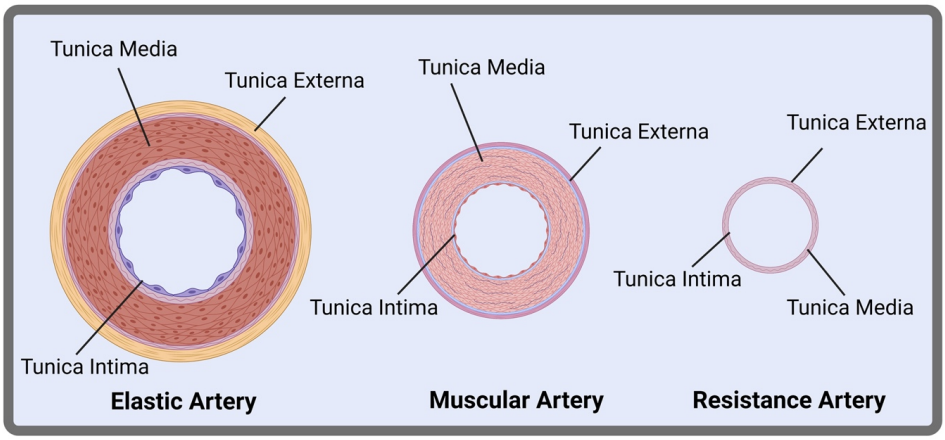

Figure 1: Structure of arteries. The wall of an artery consists of three layers. Large elastic arteries (the aorta and its branches), are rich in elastin. The cellular compartment of elastic arteries is mainly dominated by vascular smooth muscle cells (VSMCs). The muscular arteries contain more VSMCs in tunica media and have fewer elastic fibers in tunica intima. Resistant arteries are mainly made of a grid of VSMCs.

The endothelial mediators (NO, EDHF and PGI_2) are capable of compensating the actions of counterpart mediators [17]. The maintenance of vascular tone is the best example to characterize this relationship, which take place in two steps: first regulating vascular adjustment of vessel diameter and later as a compensatory system that is applied when the expression or action of the alternative mediator is impaired. Even though NO is the major vasodilator but while its contribution/function decreases the effects of EDHF may increase, to maintain the vascular tone [18]. In such manner complete vasodilatory scope of the arterial system is sustained.

1.1.1 Role of endothelial mediators in microcirculation

One of the key mechanisms by which endothelial cells regulate blood flow is through the production of NO. Endothelial NO is synthesized from the amino acid L-arginine by the enzyme nitric oxide synthase (NOS). It is an important mediator of various physiological processes in the human body, including vascular function, immune response, and neurotransmission [19]. NO produced by endothelial cells acts as a potent vasodilator that relaxes the VSMC in the walls of blood vessels and leading to an increase in blood flow. This helps to regulate blood pressure and promote adequate tissue perfusion. In addition, NO inhibits platelet aggregation and adhesion, preventing the formation of blood clots that can lead to cardiovascular events such as heart attack and stroke. There are three isoforms of NOS that are responsible for the production of NO in the human body: neuronal NOS (nNOS or NOS1); inducible NOS (iNOS or NOS2), which is expressed in response to inflammatory mediators or other stimulation; and endothelial NOS (eNOS or NOS3), which produces NO in the vasculature [13]. To generate NO, eNOS, iNOS and nNOS require co-factors, including tetrahydrobiopterin (BH4), nicotinamide adenine dinucleotide phosphate (NADPH), flavins, and flavin mononucleotide, in addition to the substrate L-arginine. Upon an increase in Ca^{2+}

levels for instance induced by agonists like bradykinin, acetylcholine inactive eNOS is converted to active eNOS with the help of Ca^{2+} dependent activation of calmodulin/caveolin system (eNOS detaches from caveolin and becomes activated) and converts L-arginine to generate NO. In tissues, NO can also be produced non-enzymatically through direct disproportionation or nitrite reduction under acidic and highly reduced circumstances such as in ischemic conditions [20]. Upon diffusion from endothelial cell, NO drives the formation of cGMP in the VSMC, which subsequently leads to a reduction in Ca^{2+} release and subsequent relaxation (**Fig. 2**) [21]. It is evident from clinical studies that endothelial dysfunction arises early in the progression of kidney failure and poses the risk for further negative changes in the upstream circulation [22] [23]. The majority of current experimental evidence of endothelial dysfunction in CKD comes from animal models of uremic environment. NO bioavailability is reduced in patients with CKD, which is thought to play a role in vascular disease progression [24].

The EDHF type response is the pathway of endothelium-dependent relaxation, which involves hyperpolarization as a mechanism. This pathway is considered as a signal generated and released by hyperpolarized endothelial cells. The EDHF-mediated response can be divided into two stages. The first stage involves an increase in Ca^{2+} by activation of Ca^{2+} dependent K^+-channels (Kca), K^+ efflux, and hyperpolarization. This leads to the synthesis of substances or signals that can diffuse through membranes or myoendothelial gap junctions to VSMC. The second stage reflects the transfer of endothelial hyperpolarization to VSMC. At the VSMC level, EDHF activates K^+ channels and causes hyperpolarization accompanied by the closure of voltage gated Ca^{2+} channels, resulting in relaxation [25]. The current understanding of EDHF origin and/or mechanisms is gradually unrevealed, involving several pathways. Different factors, including CYP450, products of arachidonic acid [26], hydrogen peroxide (H_2O_2) [27], C-type natriuretic peptide [28] and potassium ions released by hyperpolarized endothelial cells, have been proposed as potential candidates for EDHF. Endothelial cell hyperpolarization can also be transmitted to VSMC through myoendothelial gap junctions. The contribution of EDHF to endothelium-dependent dilation increases as the vessel size decreases [29]. EDHF may act as a backup endothelium-derived vasodilator when NO production is compromised. Therefore, changes in synthesis and/or release of EDHF are critical for the regulation of organ blood flow, peripheral vascular resistance, and blood pressure, especially when NO production is compromised. Our lab, previously demonstrated that ESKD patients undergoing peritoneal dialysis have impaired EDHF contribution, compared with healthy subjects [30].

PGI_2 is a metabolite of arachidonic acid and synthesized by the cyclooxygenase (COX) isozymes. COX1 enzyme is expressed in the endothelium where it helps maintaining vascular homeostasis. COX2 is an inducible isozyme and predominantly found in immune cells and the cardiovascular system. Evidence indicates that COX2 isoforms are constitutively expressed in human endothelial cells, but only during pathogenic episodes [31]. PGI_2 causes smooth muscle relaxation by binding to the specific cell surface receptors prostaglandin I_2 receptor (IPs) thereby activating G protein mediated adenylate cyclase, that increase cyclic

adenosine monophosphate levels [32]. When the above-mentioned process occurs, protein kinase A becomes phosphorylated, which causes a decrease in Ca^{2+} levels within VSMCs and ultimately leads to vasodilation. Additional information regarding PGI_2 is provided in the prostaglandins section.

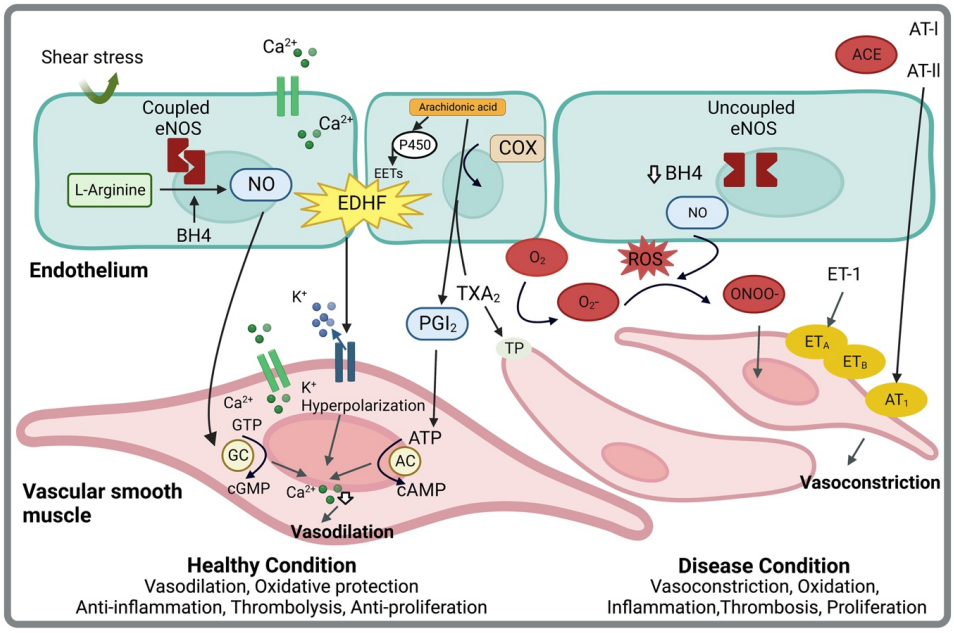

Figure 2: Role of endothelial mediators in healthy and disease conditions.
The vasodilation process, mediated by the endothelium in a **healthy state**, involves various factors. One of the key factors is nitric oxide (NO), which is produced by the endothelial NO synthase (eNOS) from L-arginine. NO causes the relaxation of vascular smooth muscle cells (VSMCs) by activating the soluble guanylyl cyclase receptor (GC). There are several physiological agonists and physical forces, such as shear stress, that trigger the production of endothelium-derived factors. Prostacyclin (PGI_2) induces dilatation through the adenylyl cyclase (AC)/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signal transduction pathway. Another endothelium-derived factor is Endothelium Derived Hyperpolarizing Factor (EDHF), which can induce relaxation through various factors and pathways, leading to changes in the membrane potential of VSMCs.

When endothelial signaling is disrupted in a **diseased state,** NO bioavailability decreases due to increased activation of enzymes that generate reactive oxygen species (ROS). ROS include superoxide (O2−), which reacts with NO to produce peroxynitrite (ONOO-), a cytotoxic molecule that reduces NO bioavailability even further. As a result, platelet aggregation increases, and vasoconstriction is induced. Additionally, ONOO- oxidizes tetrahydrobiopterin (BH4), leads to reduced BH4 and eNOS uncoupling. Abbreviations: ACE: Angiotensin-converting enzyme; EETs: Epoxyeicosatrienoic acids; ET: Endothelin receptor, AT-I and II: Angiotensin II receptor type 1 and 2; TP: thromboxane receptor.

The regulation of vascular tone is dependent on vasodilation as well as vasoconstriction. The three endothelium-derived factors mentioned above act in a coordinated way to preserve regular endothelial activity and serve as backup system if one pathway is impaired **(Fig. 2)**. Major endothelium-dependent vasoconstrictors are Endothelin-1 and thromboxane A_2 (TXA_2). Endothelins (ETs), which include three distinct isoforms (ET-1, ET-2, and ET-3), play an important role in regulating vascular tone. ET-1 is a potent vasoconstrictive peptide that is mainly produced by endothelial cells but is also synthesized to a lesser extent by VSMCs[33]. Various stimuli, such as hypoxia, ischemia, thrombin, insulin, inflammatory

cytokines, or shear stress, can induce the transcription of ET-1 mRNA and the rapid secretion of ET-1 [34]. The biological effects of ET-1 are mediated by two receptor subtypes, ETA and ETB. When ET-1 binds to ETA receptors, it leads to vasoconstriction, cell proliferation, cell growth, and cell adhesion [35]. In contrast, binding to ETB receptors on endothelial cells results in the release of NO and vasodilation [36]. In addition to maintaining vascular tone in healthy individuals, ET-1 may also indirectly induce vasoconstrictor effects through the generation of endothelium-derived TXA_2[37]. The thromboxane receptor (TP), can be activated by TXA_2, resulting in vasoconstriction and platelet aggregation [38]. The regulation of vascular tone is also dependent on the interplay of the autonomic nervous system combining the parasympathetic and sympathetic nervous system and the blood vessels. Neurotransmitters like norepinephrine (NE), ATP and neuropeptide released from innervating nerve ends are potent vasoconstrictors. In CKD, endothelial dysfunction is widely recognized, and a persistent loss of endothelial homeostasis leads to organ damage. Microvascular rarefaction, defined as a loss of perfused microvessels resulting in a considerable reduction in microvascular density, has been observed in numerous studies conducted both in CKD animal models and patients with CKD [39].

The microcirculation is responsible for a large part of the endothelial surface area and plays a critical role in the development and progression of CVD associated with CKD. Microvascular disease in CKD is the base of the iceberg in cardiovascular comorbidity [15]. Despite the prevalence of microvascular complications in CKD, most research on CKD-related cardiovascular disease has focused on macrovascular consequences. Thus, there is a pressing need for more extensive research specifically in human microcirculation in the context of CKD. Understanding the role of microcirculation in CKD is crucial for developing new treatments and interventions to prevent or slow down the progression of CVD in these patients.

1.1.2 Role of amino acids in vascular function

Amino acids (AAs) are essential components of proteins and play a crucial role in the regulation of vascular function in the peripheral microcirculation. AAs and their metabolites have been shown to modulate vascular tone, blood flow, and endothelial function in the microcirculation. As previously stated, NO is a major factor in relaxing the endothelium, and BH4 is a crucial cofactor that works with other AAs like L-arginine, L-phenylalanine, citrulline, or ornithine to produce NO (**Fig. 3**). When BH4 is limited, it causes uncoupling of NOS, which produces superoxide at the cost of NO, exacerbating oxidative stress. The reduced availability of NO is a prevalent issue in CKD, and several factors contribute to low NO bioavailability in CKD [24], including the oxidation of BH4 by peroxynitrite ($ONOO^-$) forming 7,8-dihydrobiopterin (BH2), that promotes eNOS uncoupling. This condition may lead to cardiovascular complications, a primary cause of morbidity and mortality among CKD patients. Furthermore, AAs metabolites such as homocysteine and asymmetric dimethylarginine (ADMA) may impair endothelial function and promote inflammation; ADMA is an endogenous inhibitor of NOS that reduces NO production and increases reactive

oxygen species (ROS) generation [40]. As the ratio of BH4:BH2 is critical for eNOS coupling and uncoupling, strategies to increase BH4 levels have been suggested to enhance vascular hemodynamics.

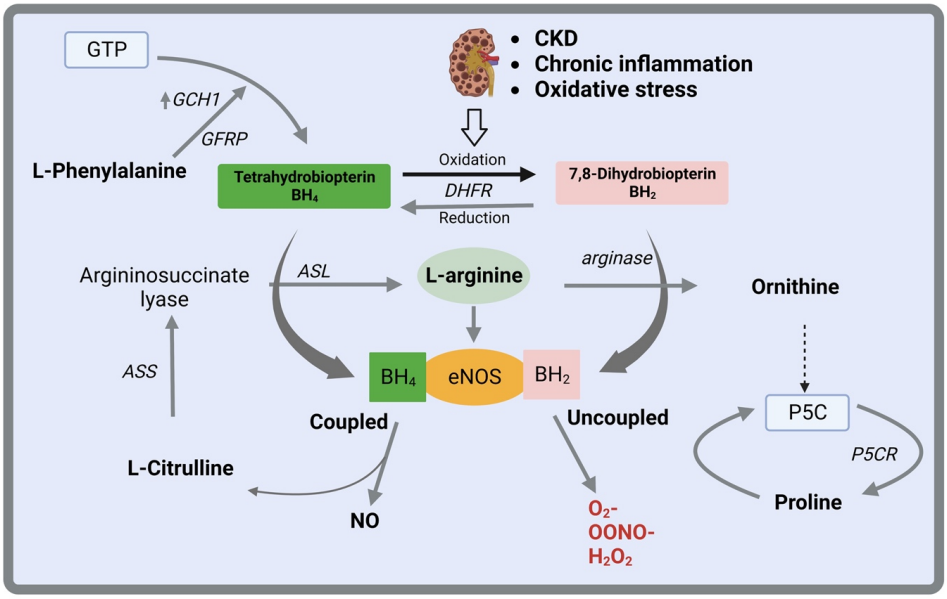

Figure 3: The figure illustrates the metabolic pathways of amino acids (AAs) in endothelial cells and their impact on nitric oxide (NO) generation. Under normal physiological conditions, L-arginine is metabolized to produce NO and L-citrulline through the coupled action of tetrahydrobiopterin (BH4) and endothelial nitric oxide synthase (eNOS). Citrulline can converted back to L-arginine with the help of argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) to maintain NO balance. However, in pathological conditions like chronic kidney disease (CKD), BH2 can uncouple eNOS, leading to the generation of reactive free radicals. Furthermore, L-phenylalanine facilitates the production of BH4 through various enzymes such as guanosine triphosphate cyclohydrolase 1 (GCH1) and GTP cyclohydrolase 1 feedback regulatory protein (GFRP). Excessive L-arginine can also be converted to ornithine and proline via the action of arginase and pyrroline-5-carboxylate reductase (P5CR), respectively. **Abbreviations:** NO: nitric oxide; NOS: nitric oxide synthase; ASS: argininosuccinate synthase; ASL: argininosuccinate lyase; P5C:1-pyrroline-5-carboxylate; DHFR: dihydrofolate reductase; GCH1: GTP cyclohydrolase-1; GFRP: GTP cyclohydrolase 1 feedback regu-latory protein; BH2: 7,8-dihydrobiopterin; BH4: Tetrahydrobiopterin.

L-arginine is synthesized from glutamic acid through a row of enzymes. In the body, L-arginine is broken down into urea and ornithine by arginase or into citrulline and NO by NOS. Citrulline can be transformed into L-argininosuccinate and further back into L-arginine, contributing to the increase in levels of L-arginine in plasma and tissue. L-phenylalanine is another AA that regulates endothelial function by promoting BH4 synthesis and increasing nitrite levels via the guanosine triphosphate cyclase-hydrolase-1 (GCH1) pathway, leading to the attenuation of ROS and the increase of NO levels [41].

BH4 is created from GTP through a reaction in which GCH1 is the rate-limiting step. The modulation of GCH1 expression regulates BH4, NO, and cardiovascular function [42]. L-phenylalanine controls GCH1 through feed-forward regulation and by communicating with the GCH1 feedback regulatory protein (GFRP). Proline is an essential component of collagen [43] involved in the regulation of processes such as dehydration stress, redox, cell proliferation and

differentiation among others [44]. Thus, the role of AAs and their metabolites in vascular function in the peripheral microcirculation is complex and multifactorial, and further research is needed to fully understand their mechanisms of action and clinical implications.

1.2 Early vascular ageing in CKD

One of the major contributors to CVD risk in CKD is early vascular ageing, which refers to the premature ageing of blood vessels. As scientists are becoming increasingly convinced that biological vascular ageing is a more reliable indicator of CVD than chronological age, the concept of EVA has emerged, encompassing structural and functional changes in the arterial wall, such as increased arterial stiffness, endothelial dysfunction, and impaired vasodilation which are associated with adverse cardiovascular outcomes [6,45]. Compared to the typical vascular ageing, EVA involves alterations in the smaller arteries of the peripheral circulation. This results in an improved cross-talk with large elastic arteries and arteriosclerosis [46]. Emerging evidence suggests that media vascular calcification (VC) plays a key role in the development of EVA in CKD [6]. Chronic inflammation is the probable cause of EVA signs in the arterial wall among patients with CKD [47]. These changes appear to reflect early adaptive responses to persistent cellular damage, imbalances between pro-ageing and anti-ageing mechanisms, and an increase in allostatic load. Recent studies have highlighted the importance of early detection and management of EVA in CKD, including the use of lifestyle interventions, pharmacological therapies, and management of traditional cardiovascular risk factors [48]. Further research is needed to elucidate the mechanisms underlying EVA in CKD and to develop more effective strategies for its prevention and treatment [46].

We are still lacking detailed knowledge on the underlying mechanisms that give rise to EVA in CKD. However, recent literature suggests a pathogenic model which includes inflammation, oxidative stress, endothelial dysfunction, senescence, calcification, uremic toxins accumulation, genomic instability, telomere attrition, metabolic disturbances, mitochondrial dysfunction, and extracellular matrix dysfunction **(Fig. 4)**. These factors, which may occur independently in either macro- or microvasculature or in both, are strongly interrelated. Among others, in the upcoming paragraphs, we will discuss more in detail about the relationship between EVA and senescence, calcification, uremic toxins, and EVs as these topics will be further explored in the results section to provide a comprehensive understanding of their association with EVA.

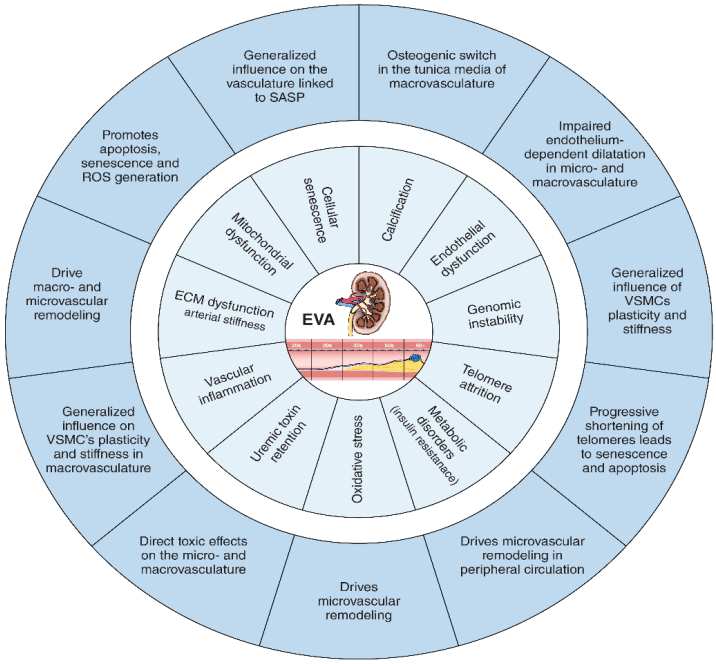

Figure 4: According to the model, the development of early vascular aging (EVA) in patients with chronic kidney disease is influenced by a range of factors including inflammation, oxidative stress, endothelial dysfunction, senescence, calcification, uremic toxins accumulation, genomic instability, telomere attrition, metabolic disturbances, mitochondrial dysfunction, and extracellular matrix (ECM) dysfunction. These may occur in either micro- or macrovasculature or in both, acting individually or interconnected, further exacerbating the development of EVA and ultimately contributing to the increased risk of cardiovascular complications. Adopted from Hobson S, **Arefin S** et al 2023.

1.2.1 Role of vascular calcification

Vascular calcification (VC) has been identified as a key factor that contributes to the development of EVA and vascular dysfunction in individuals with CKD [49]. VC is a complex process that involves the deposition of calcium and other minerals in the walls of blood vessels, leading to a loss of vessel elasticity and function. The presence of VC in individuals with CKD is linked to a substantial increase in both morbidity and mortality [50,51]. The prevalence of vascular calcification arises as estimated glomerular filtration rate (eGFR) deteriorates [52]. Although the VC processes in patients with CKD are diverse and poorly understood, experimental and clinical investigations show that abnormal mineral metabolism, such as calcium and phosphate imbalance, promotes VC [53]. While calcified arteries and osteoporosis are both commonly observed in elderly populations, the incidence of these conditions is notably amplified in those with CKD. Increasing evidence suggests that VC is a coordinated event that resembles embryonic endochondral osteogenesis and involves osteoblastic differentiation of VSMCs [53]. Calcified vessel walls express markers of osteoblast-like differentiation, such as transcription factors Runt-related transcription factor 2 (RUNX2) and Sox9, and the mineralization regulating proteins alkaline phosphatase (ALP), osteocalcin and bone sialoprotein, amongst others [54]. While VC displays

resemblances to osteogenesis and chondrogenesis during development, it is a pathological change that is actively induced rather than a physiological process **(Fig. 5)** [55].

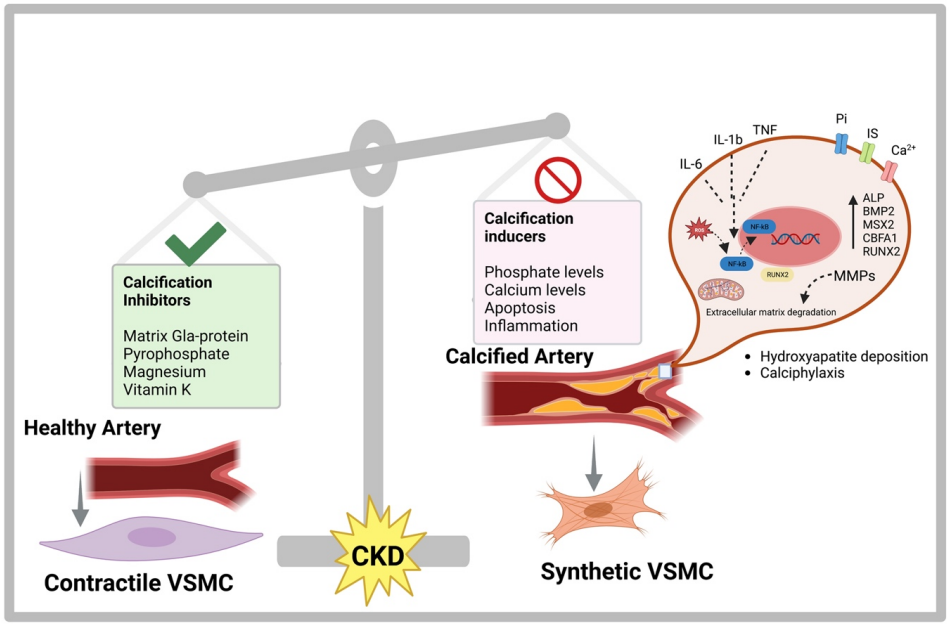

Figure 5: Simple illustration showing vascular calcification in the context of CKD. The imbalance between calcification inhibitors and inducers are considered a key driver of vascular calcification in CKD, a hallmark of early vascular ageing. The key mechanisms associated with vascular calcification include increased inflammation, apoptosis, phosphate and calcium levels and reduced matrix Gla protein, pyrophosphate, poor Vitamin K among others. Vascular calcification promotes trans-differentiation of VSMCs from a contractile to synthetic osteoblast-like phenotype. **Abbreviations:** ALP: Alkaline Phosphatase, CKD: chronic kidney disease, BMP2: Bone morphogenetic protein 2, RUNX2: Runt-related transcription factor 2, MSX2: Msh homeobox 2, CBFA1: core-binding factor α-1, MMPs: matrix metalloproteinases, VSMCs: vascular smooth muscle cells.

Medial calcification is linked to ageing, CKD, hypertension, diabetes mellitus and osteoporosis [56]. Medial vascular calcification leads to arterial stiffness and thus cardiovascular complications [57]. Healthy individuals have a potent defense mechanism that protects VSMCs against phenotypic differentiation and ectopic calcification. However, in patients with CKD, this protective system is significantly diminished due to a heightened allostatic load [58]. Among the various inducers of calcification in CKD, hyperphosphatemia is considered the most potent and is strongly associated with VC [59]. The sustained increase in serum phosphate, oxidative stress, inflammation, and retention of toxins within the uremic environment diminishes VSMCs capacity to secrete adequate amounts of VC inhibitors, such as Matrix Gla protein (MGP), which renders them susceptible to phenotypic differentiation from a contractile to a synthetic VSMC phenotype. As a result, VSMCs in the arterial walls become susceptible to phenotypic transformation towards synthetic VSMCs and calcification, leading to the acceleration of EVA [60]. Another culprit is a deficient nuclear factor-erythroid factor 2-related factor 2 (Nrf2) signaling, which has been found to play a vital role in the phenotypic remodeling of VSMCs in response to different inducers [61,62].

Commonly, VC can be divided into two forms, depending on where the calcium is deposited - in intimal or medial calcification. It can also form valvular calcification and calciphylaxis. Microcalcification is the small calcium deposit size < 5 μm started by apoptotic or necrotic cell death within lipid core. Rupture of the resulting plaque can lead to macrocalcification (>5 μm) and subsequent plaque stabilization [63]. Newly formed microcalcification plays pathological role for inception and advancement of vascular disease.

Majority of the research in the field of VC in patients with CKD are about coronary artery calcification (CAC) or in bigger vessels. Our group has previously demonstrated that there is a significant association between medial calcification and three times increased risk of cardiovascular events even after adjusting for traditional risk factors [64]. A multitude of pathophysiologic pathways have been identified that contribute to CAC in CKD however, little is known about the pathophysiology of VC in microcirculation. Further investigation is needed to better understand these unique aspects of VC in microcirculation in patients with CKD. Understanding the factors that disrupt the natural defense systems in CKD and identifying strategies to mitigate their impact may hold promise in reducing the burden of vascular disease in this population.

1.2.2 Pathophysiological links between senescence and EVA in CKD

Senescence, or the loss of cellular capacity for proliferation and renewal, is a fundamental hallmark of ageing. In CKD, the process of senescence is accelerated, with a pathophysiological link between senescence and EVA [65]. Senescent cells accumulate in the arterial wall, where they release inflammatory cytokines, growth factors, and enzymes that can damage the extracellular matrix. The accumulation of senescent cells can promote phenotypic changes in VSMCs that lead to the development of VC, a key feature of EVA in CKD [66]. However, it is still debated whether senescence triggers calcification, or vice versa, or if the mechanism is bidirectional. Metabolically senescent cells are active but according to the current evidence they do not make a beneficial contribution to the tissue or organ in which they exist [67]. Various external factors, such as ROS, DNA damage, inflammation, oncogenes, and proteotoxic environments, can act as triggers, causing healthy cells to transition into a senescent state (**Fig. 6**) [7]. Senescent cells release a senescence-associated secretory phenotype (SASP), which is rich in pro-inflammatory, pro-fibrotic, and matrix-degrading factors such as chemokines, cytokines, proteases, and growth factors. This SASP causes damage to the surrounding tissue, leading to organ dysfunction. Senescence cell anti-apoptotic pathways (SCAPs) are termed the ‘‘Achilles heel’ of senescent cells’’ because they prevent an immune response from removing senescent cells. The combination of SCAPs and SASP leads to age-related diseases, which has created interest in developing novel treatments to target this hallmark of EVA. Additional information regarding SASP and SCAPS are provided in sections below.

As a result of chronological ageing, exclusively after middle age, senescent cells resign up in numerous tissues central to the pathogenesis of chronic diseases. Replicative senescence,

stress-induced EVA and epigenetic alterations have all been linked to the onset and progression of CKD [60]. There is a strong correlation between the accumulation of senescent cells in various tissues and age-related metabolic disorders, as well as the early development of chronic diseases [68]. This excess accumulation of senescent cells is a significant contributor to the ageing process, and may play a key role in the development of age-related pathologies [69]. The underlying source(s) of chronic inflammation in EVA processes have yet to be identified, although senescent cells that develop SASP may be implicated.

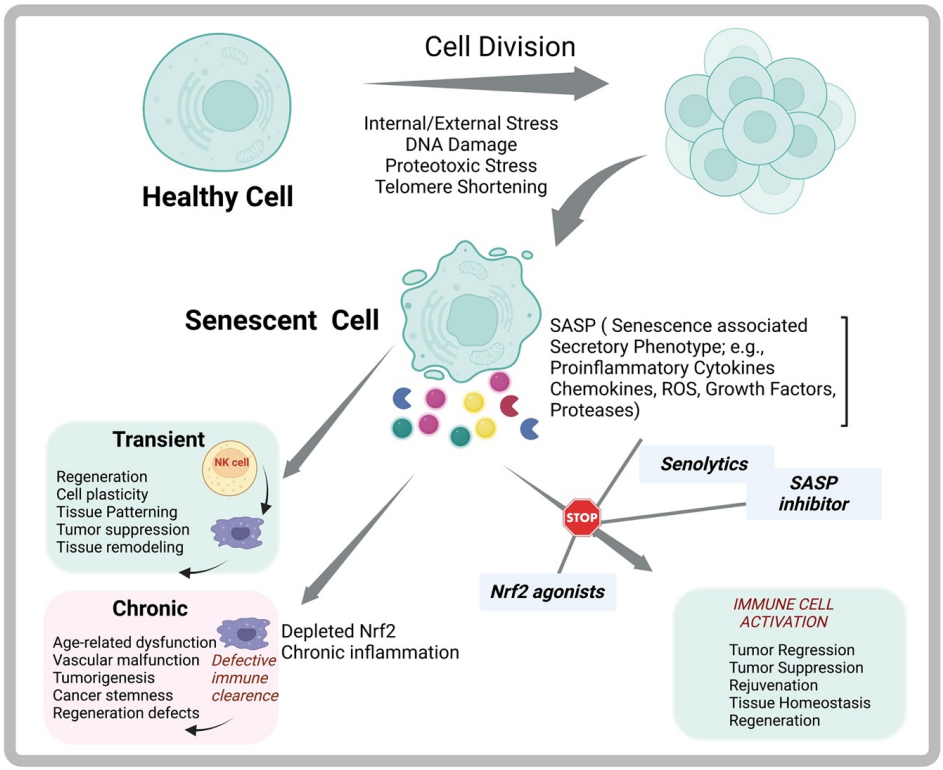

Figure 6: Simplified illustration of senescence. Senescence, a state of irreversible cell cycle arrest, can occur transiently as a physiological response to stress or in a chronic manner, leading to tissue dysfunction and ageing-related diseases. Different types of novel treatment options e.g. senolytics, SASP inhibitor, Nrf2 agonists can be used to ameliorate the deteriorating effects of senescence.

There are several markers considered as senescence markers, the common markers are tumor suppressor protein cyclin-dependent kinase inhibitor 2A (p16INK4A/CDKN2A), p21CIP1, SA-βgal activity, SASP factors like IL-6, IL-8, MCP-1, PAI- 1, and many others. H2A histone family member X(γH2AX) and Telomere-associated DNA damage foci (TAFs) are also commonly used markers of senescence [70]. However, a single marker in general cannot demonstrate if a cell is senescent or not. Scientists often rely on a combination of several markers to draw conclusions. Recently, cellular senescence has become a progressively attractive and broadly studied field because of its role in the manifestation and progression of chronic diseases like CKD.

The arterial mRNA expression of p16Ink4a /CDKN2A was shown increased in rats with adenine-induced uremia, indicating a potential involvement in the development of arterial calcification [71]. Moreover, the presence of p16Ink4a and RUNX2 proteins in and around calcified areas of the aorta suggests their potential role in this process as well [71]. These findings shed light on the mechanisms underlying arterial calcification in adenine-induced uremia and could pave the way for novel therapeutic interventions, while also indicating that CKD may accelerate the ageing process not only in specific tissues, but also at the cellular level. Previously, we have demonstrated that epigastric arteries from patients with ESKD exhibit higher senescence markers and positively associated with VC [72]. Nevertheless, little is known about the presence of senescence footprint in resistance vessels with the physiological role related to regulation of peripheral resistance and blood pressure control. The molecular mechanisms that underlie this phenomenon require further investigation. Additionally, investigations into potential interventions that could mitigate or even reverse the adverse effects of CKD on vascular ageing are of interest.

1.2.3 Role of uremic toxins

Uremic toxins are a group of molecules that accumulate in the blood due to impaired kidney function. These toxins have been implicated in the pathogenesis of EVA and vascular dysfunction in CKD patients[73]. Uremic toxins can be classified into three subgroups based on their molecular characteristics: small water-soluble compounds, middle molecules, and protein-bound molecules. Small water-soluble compounds, such as urea and creatinine, are typically removed from the body via dialysis and are not considered to have significant toxic effects. Middle molecules, such as beta 2-microglobulin and parathyroid hormone, are too large to be effectively cleared by dialysis and can accumulate in the blood of CKD patients, contributing to the development of complications such as bone disease and cardiovascular disease. Protein-bound molecules, such as indoxyl sulfate and p-cresol sulfate, are tightly bound to plasma proteins and are not effectively removed by conventional dialysis techniques [74].

The most common uremic toxins include indoxyl sulfate (IS), p-cresyl sulfate, hippuric acid, trimethylamine N-oxide (TMAO), ADMA, phenylacetyl glutamine (PAG) among others. The accumulation of these toxins also contributes to an overall increase in the allostatic load, which can accelerate the ageing process and lead to EVA [75]. Even though many uremic toxins have been identified to date, and many of them are thought to have a role in the progression of CKD and CVD, only a handful have been thoroughly investigated. Most studies fail to include the potential synergistic effects of multiple toxins on organs in their experimental design, even though it is likely that various toxins combined contribute to the development of complications in CKD patients. Although many studies have reported on the systemic effects of uremic toxins, in this thesis we will concentrate solely on their harmful effects on the vasculature. Among other uremic toxins TMAO and PAG have drawn our attention as growing evidence illustrates their involvement in the pathogenesis of endothelial dysfunction, vascular calcification and ultimately CVD [76,77]. By understanding how these

toxins affect the vasculature, more targeted therapies could be developed to alleviate their effects and enhance the overall well-being of CKD patients.

The function of gut microbiota in common diseases has been extensively investigated in recent years because of advances in bioinformatics technology. Growing data suggests that the imbalance of intestinal microbiota is linked to coronary artery disease and cardiovascular risk factors such as diabetes, dyslipidemia, obesity, and CKD [78-80]. TMAO a small amine plasma molecule with a molecular weight of 75.1 Da that is produced by intestinal microbial metabolism. It is a pro-inflammatory metabolite which can be produced in the large intestine by bacterial digestion of choline-rich meals including red meats and eggs and is rapidly oxidized in the liver by flavin-containing monooxygenase. Stubbs et al. reported a significant negative correlation between serum TMAO concentration and eGFR [81]. Increased levels of TMAO are also observed in patients with severe CVD [82], and several studies have linked TMAO with vascular inflammation [83], platelet hyperactivity, as well as atherosclerosis [84]. This strengthens its pro-atherogenic role to act as an independent predictor of negative cardiovascular outcomes both in the general population [85] and CKD patients [86]. TMAO has also been linked to endothelial dysfunction, as demonstrated in HUVECs [83]. Correlation between circulating TMAO and vascular ageing further indicates that TMAO might jeopardize endothelial function by elevating oxidative stress [87]. In an animal model, it has been shown that TMAO disrupts EDHF type relaxation in a time-dependent manner in rat superior mesenteric artery [88]. At this stage, clinical evidence on the effects of TMAO on endothelial function is unclear and requires further research. The majority of work showing a link between TMAO and endothelial dysfunction have been conducted on rodents [89] or cells [83].

Another important toxin that receives increased attention is a colonic microbial product from amino acid fermentation PAG. It is derived by the glutamine conjugation of phenylacetic acid, which is entirely produced from phenylalanine conversion by microbes, and a commonly found metabolite in human urine. PAG may also be formed in larger amounts in the body as a result of the metabolic breakdown of the more hazardous pharmaceutical chemicals sodium phenylbutyrate, glycerol phenylbutyrate, and sodium phenylacetate, which are used to treat physiological urea cycle disorders [90]. Poesen et al., has reported that PAG is associated with overall mortality and CVD in CKD patients [91]. PAG has been shown to stimulate platelet activation-related phenotypes in whole blood and animal model of arterial injury [92]. Recently, Nemet et al., showed that PAG can trigger adrenergic receptor to transmit cellular responses via the α2A, α2B, and β2 adrenergic receptors [77]. They have also suggested that PAG may contribute to cardiac disease. The researchers indicated that PAG may control or modulate the epinephrine receptors. They proposed that this may be explained by its close association to distinct CVD phenotypes. Of note, stimulation of epinephrine receptor causes vasoconstriction of the arteries. In CKD, PAG levels in serum are utilized as a predictor of mortality [91]. However, very little information is available regarding PAG and its association with small resistance arteries from microcirculation in patients with CKD. Therefore,

intensive research is needed for PAG to investigate how it affects the microcirculations in patients with CKD.

1.2.4 Extracellular vesicles (EVs)

EVs are a diverse group of membrane- defined vesicles emerging from the endosome or plasma membrane. EVs were first described by Pan and Johnstone in 1983 [93]. It was first believed that EVs were released as a waste to eliminate unwanted components from the cells, but it later emerged that EVs play a crucial role in intercellular communication, both in normal physiological as well as in pathological conditions. Currently, EVs are classified either based on their size or on their mode of release. Exosomes are smaller in size, whereas microvesicles (MVs) are bigger than exosomes [94]. In general, EVs compose a broad array of vesicles from 30 to 1000 nm in diameter along with mixture of cargos. Upon release, EVs are taken up by recipient cells either through endocytosis or membrane fusion, leading to the discharge of their cargo[95]. Several researchers have shown that EVs consists of discrete lipids, proteins, sugars, and a broad range of genetic components including mRNA, DNA and non- coding (nc)RNAs [96,97] and can have both, pathogenic as well as protective effects, depending on origin, composition and context **(Fig. 7).**

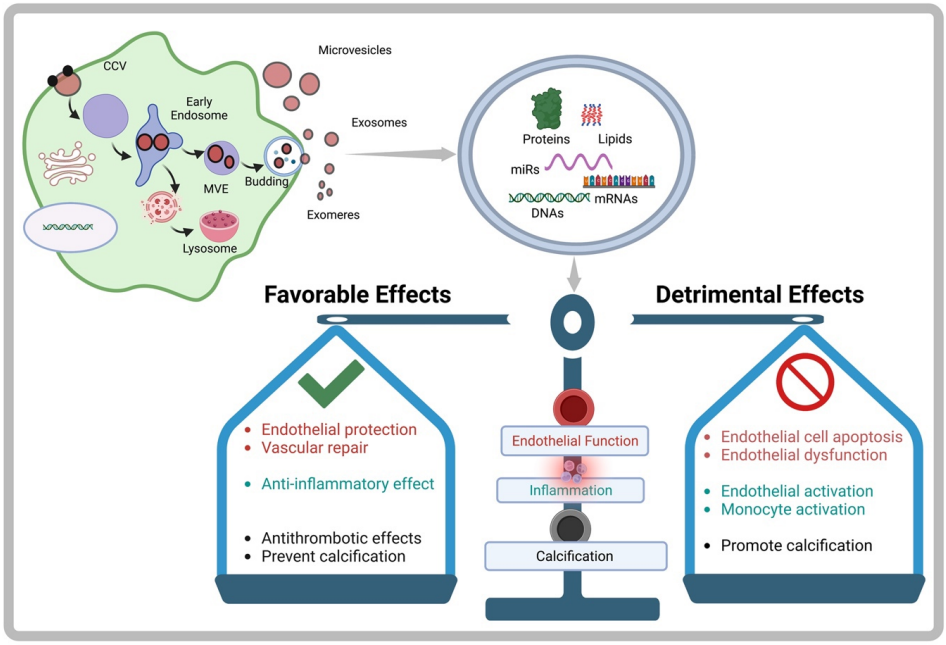

Figure 7: Simplified illustration of extracellular vesicles (EVs) showing both their beneficial and detrimental effects. EVs enable intercellular signaling and provide a mechanism for cell-to-cell communication. EVs can deliver biological messages to specific recipient cells through the transportation of cytokines, proteins, nucleic acids, and lipids. While some EVs can play a significant role in various stages of disease development e.g. endothelial dysfunction, calcification and unstable plaque progression, others can have beneficial effects on vascular function and endothelial regeneration. The effects of EVs are complex and depend on several factors such as the cellular origin, functional state of the releasing cells and the biological content. Abbreviations: CCV: Clathrin-coated vesicles; MVE: Multivesicular endosome.

The release of EVs is triggered by various stressors including oxidative and mechanical stresses, chronic inflammation, and deregulation of calcium and phosphate homeostasis [98]. Studies have shown that EVs derived from senescent cells and inflammatory cells contribute to EVA by inducing vascular calcification, endothelial dysfunction, and VSMC phenotypic switching [66]. Furthermore, EVs derived from endothelial cells and VSMCs can promote the progression of EVA by spreading oxidative stress and inflammation to neighboring cells [99]. Patients with CKD show elevated EVs in the body fluids [97] and specific EV subpopulations have been associated with clinical complications in CKD [100]. Several cross-talking factors seem to play a role for elevated EVs in CKD, of which uremic toxins seem to be central [101]. In addition, there is an inverse association of EVs with shear stress in patients with ESKD [102]. When cells are exposed to uremic compounds, released EVs can cause endothelial progenitor cells to experience functional loss, while also promoting the proliferation of VSMC. Circulating EVs of endothelial origin from ESKD patients are tightly associated with endothelial dysfunction and arterial dysfunction [103]. Xie et al. reported endothelial dysfunction and fibrosis caused by EV-derived miRNA transfer mechanisms in patients with CKD [104]. On the contrary, in vitro studies have shown that EVs released from endothelial cells containing caspase 3 could potentially protect the endothelium from apoptosis and detachment [105]. Furthermore, certain types of EVs are capable of inducing anti-inflammatory responses in the cells that receive them. As mentioned before, depending on the origin and pathological conditions, EVs could be damaging or protective for the vasculature. However, to determine the validity of this assumption, it is necessary to conduct a comprehensive analysis of EVs under various clinical and treatment conditions to fully understand their phenotypic characteristics.

1.3 Identify injury targets

The thesis title "Microcirculation in chronic kidney disease: from injury targets to potential therapeutics" focuses on exploring the various injury targets associated with CKD and their potential for therapeutic intervention. The injury targets identified in the thesis include senescence, calcification, uremic toxins, EVs and endothelial dysfunction, which all play critical roles in the pathogenesis of CKD. Calcification and VSMC senescence contribute to vascular stiffness and impaired microvascular blood flow whereas uremic toxins can cause endothelial dysfunction, oxidative stress, and inflammation, leading to damage of the vasculature. Understanding these injury targets and the mechanisms by which they contribute to EVA in CKD can help identify potential therapeutic strategies to alleviate their effects and improve patient outcomes.

1.4 Angiotensin-converting enzyme 2 in microcirculation in the context of COVID-19

Angiotensin-converting enzyme 2 (ACE2) is an enzyme that plays a crucial role in the renin-angiotensin system (RAS) and is responsible for the conversion of angiotensin II (Ang II) to angiotensin 1-7 (Ang 1-7) thereby counteracting the effects of the ACE-Ang II-type 1 (AT1) receptor axis [106]. ACE2 is a homologue of ACE and plays a crucial role in regulating blood

pressure, fluid volume, sodium retention, regulation of vascular tone and the prevention of vasoconstriction, inflammation, and oxidative stress (**Fig 8**) [107]. Several studies have suggested that ACE2 deficiency may result in microvascular dysfunction, including impaired capillary density, reduced dilation, and increased permeability [108,109]. Decreased ACE2 expression has been reported in patients with CKD [110], which could lead to impaired regulation of the renin-angiotensin-aldosterone system (RAAS) and contribute to hypertension and other cardiovascular complications. Recently, ACE2 has drawn our attention due to its involvement with coronavirus disease 2019 (COVID-19) as it has been shown that the SARS-CoV-2 virus uses ACE2 as an entry receptor, which has led to interest in the role of ACE2 in cardiovascular maintenance.

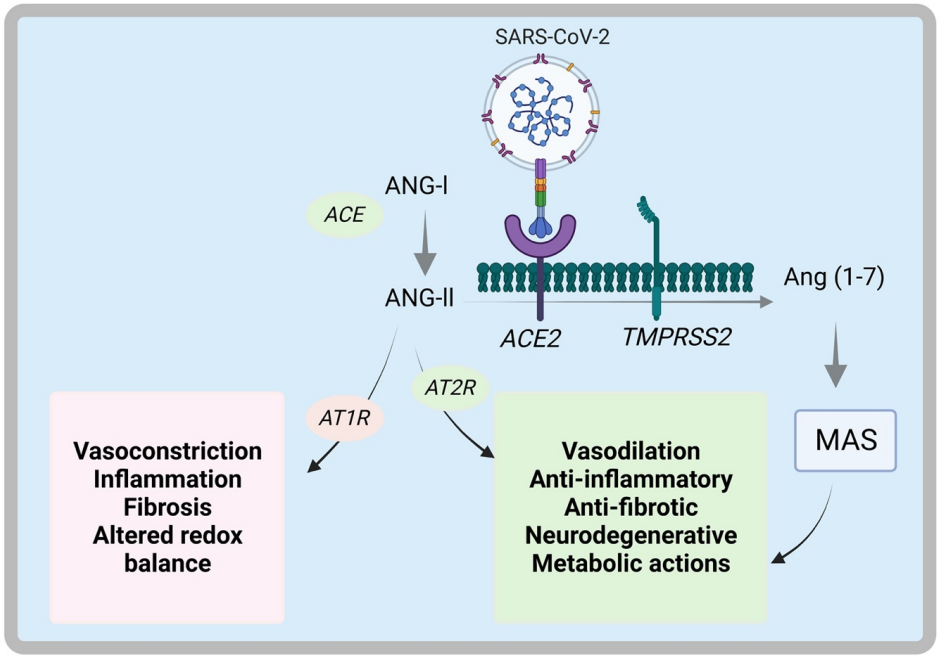

Figure 8: Angiotensin-converting enzyme 2 (ACE2) is a type I integral membrane protein that plays a crucial role in the regulation of the renin-angiotensin system (RAS) by cleaving angiotensin II (Ang II) into Ang (1-7). Ang II is a potent vasoconstrictor, while Ang (1-7) promotes vasodilation via Mas receptor, and the balance between these two peptides is critical for maintaining normal blood pressure and vascular function. SARS-CoV-2, the virus responsible for the COVID-19 pandemic, uses ACE2 as a cellular receptor to enter host cells. The transmembrane protease serine 2 (TMPRSS2) is also required for viral entry and infection by cleaving the viral spike protein, which facilitates fusion with the host cell membrane. Abbreviation: AT1 and 2: ACE-Ang II-type 1and 2 receptors.

CKD as a comorbidity in patients with COVID-19 is associated with worse outcomes, including higher rates of hospitalization, need for mechanical ventilation, and mortality. SARS-CoV-2 can directly infect and damage kidney cells, leading to acute kidney injury and exacerbating underlying CKD [111]. Recent studies suggest that patients with CKD may have altered ACE2 expression and activity, which could affect their susceptibility to SARS-CoV-2 infection and the severity of COVID-19 disease [112]. Others have argued that the administration of ACE inhibitors and angiotensin receptor blockers (ARBs), frequently

prescribed to CKD patients, may augment ACE2 expression [113], thereby potentially enhancing their vulnerability to SARS-CoV-2 infection.

The SARS-CoV-2 virus enters cells by binding to ACE2, which is then primed by transmembrane protease serine 2 (TMPRSS2) (**Fig. 8**). This leads to downregulation of ACE2 expression, which disrupts the balance of the RAAS system and promotes vasoconstriction, inflammation, and oxidative stress [114]. These changes can contribute to the development of severe respiratory symptoms and multiorgan dysfunction in COVID-19 patients. ACE2 is widely expressed in various tissues throughout the body, including the lungs, heart, kidneys, liver, and gastrointestinal tract [115]. The widespread expression of ACE2 in different tissues highlights its importance in regulating multiple physiological functions throughout the body. ACE2 can exist in different forms due to alternative splicing and post-translational modifications. The full-length form of ACE2 is a type I transmembrane protein that consists of an extracellular domain, a transmembrane domain, and a cytoplasmic tail. This form of ACE2 is the most well-characterized and serves as the receptor for SARS-CoV-2 entry into host cells [116]. In addition to the full-length form, there are also soluble forms of ACE2 that lack the transmembrane domain and can circulate in the bloodstream. These soluble forms of ACE2 can act as receptors and bind to the SARS-CoV-2 virus, potentially reducing viral load and decreasing the severity of COVID-19 disease [117]. Finally, ACE2 can also undergo post-translational modifications such as glycosylation and cleavage by proteases, which can modulate its activity and affect its interactions with other proteins. ACE2 undergoes proteolysis by A Disintegrin and Metalloproteinase 17 (ADAM-17), which can both promote and inhibit ACE2 shedding [118].

On the one hand, ADAM-17 can cleave ACE2 and release its extracellular domain into the bloodstream, potentially reducing ACE2 expression on the cell surface and increasing susceptibility to SARS-CoV-2 infection. In addition, ACE2 shedding by ADAM-17 can also generate soluble ACE2, which can bind to the SARS-CoV-2 virus and inhibit its entry into host cells [119]. Understanding the different forms of ACE2 and their roles in various physiological processes is important for developing targeted therapies for COVID-19 and other diseases associated with dysregulation of the renin-angiotensin-aldosterone system, such as CKD.

Circulating levels of the soluble ACE2 primarily indicate the constitutive shedding. However, the presence of ACE2 in the vasculature raises the question of whether the expression levels affect the susceptibility of secondary viral infection in the blood vessels and other body parts. Very little is known about the expression of ACE2, particularly in microcirculation in patients with CKD. Overall, the relationship between ACE2, CKD, and COVID-19 is a complex and evolving area of research and further studies are needed to gain more insight and develop targeted therapies that can improve outcomes for this vulnerable patient population.

1.5 Prostaglandins as therapeutic target to treat inflammation

Prostaglandins are a group of lipid mediators that play an important role in inflammation. They are produced by various cells in response to a range of stimuli, including injury and infection,

and can cause pain, fever, and swelling. However, prostaglandins can also have a protective role in the body, for example by promoting blood flow to damaged tissues and enhancing the immune response to infection. As such, prostaglandins have been the subject of many research regarding their potential as therapeutic targets for treating inflammation.

Prostaglandins are derived from the polyunsaturated fatty acid precursor arachidonic acid. These metabolites play crucial roles in the pathogenesis of several human chronic inflammatory diseases including CKD [120]. When cells are damaged or exposed to inflammatory stimuli, such as bacterial or viral infections, arachidonic acid is released from cell membranes and metabolized by enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX) to produce a variety of pro-inflammatory mediators, including prostaglandins, thromboxanes (TX), and leukotrienes [121]. These mediators can cause local vasodilation, increased vascular permeability, and the recruitment of immune cells to the site of inflammation, resulting in pain and swelling. For prostaglandins and TX synthesis, arachidonic acid is first converted by COX-1 and COX-2 into prostaglandin H_2 (PGH_2). Downstream synthases utilize PGH_2 as a substrate to produce a variety of prostaglandins including PGE_2, PGD_2, PGI_2, $PGF_2\alpha$, and TXA_2 [122]. COX-1 and COX-2 are two isozymes that differ in their expression pattern and tissue localization. COX-1 is constitutively expressed, while COX-2 is inducible and plays a role in the production of prostaglandins during inflammatory responses [123]. These prostaglandins exert their effects through the activation of G protein-coupled receptors (GPCRs) that are similar to rhodopsin and possess seven transmembrane-spanning domains. Depending on the tissue distribution of their receptors and the pathological conditions, prostaglandins may exert diverse effects.

Prostaglandins are also produced in the kidney where they trigger an inflammatory response. However, their occurrence and contribution differ in different stages of kidney disease [124]. Given its central role in pain and inflammation, arachidonic acid and its metabolites have been the target of numerous pharmacological interventions aimed at reducing inflammation and relieving pain. One approach has been to develop drugs that inhibit the production of prostaglandins, such as non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin and ibuprofen which inhibit both COX1 and COX2, while COX2 inhibitors (COXIBs) (e.g. celecoxib, etorocoxib) show enhanced COX2 selectivity [125]. In addition, current attempts to develop drugs that target specific prostaglandin receptors may make it possible to selectively modulate the inflammatory response, leading to more effective and less toxic treatments for a range of inflammatory conditions.

1.5.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs and COXIBs are two classes of medications commonly used to manage inflammation and pain. Both classes of drugs work by inhibiting the production of prostaglandins, which are responsible for causing inflammation, pain, and fever [126]. While NSAIDs target PGE_2 synthesis by inhibiting both COX-1 and COX-2 enzymes, COXIBs specifically target the COX-2 enzyme (**Fig. 9**) [127]. Some commonly prescribed NSAIDs include diclofenac and meloxicam. NSAIDs are commonly used to treat a variety of conditions such as arthritis, menstrual cramps,

headaches, and back pain. While these drugs are generally safe and effective when used as directed but they can have side effects, such as stomach pain, nausea, and indigestion [128]. For many inflammatory conditions NSAIDs are the first-choice treatment due to their effective anti- inflammatory and analgesic effects. Nevertheless, prolonged use of COX-1 and COX-2 inhibitors is linked to a higher incidence of gastrointestinal ulceration, whereas inhibitors selectively targeting COX-2 are associated with cardiovascular side-effects such as myocardial infarction, pulmonary hypertension and heart failure [125,129].

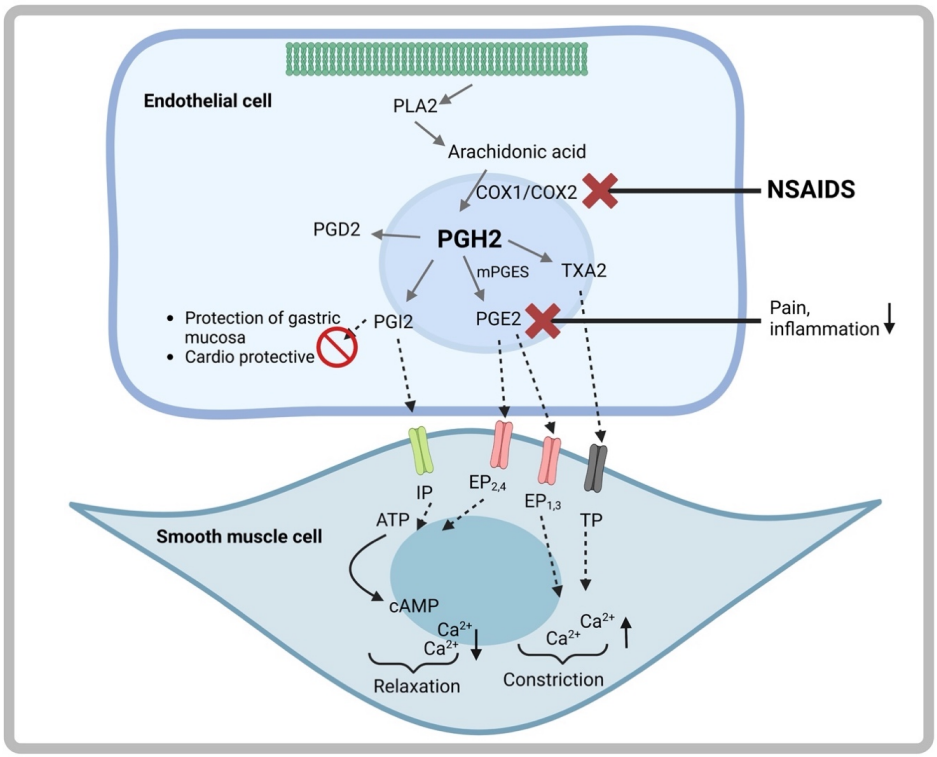

Figure 9: Simplified illustration of cyclooxygenase (COX-1 and 2) inhibition. The COX system plays a key role in the production of prostaglandins from arachidonic acid, involved in a variety of physiological and pathological processes. Traditional non-steroidal anti-inflammatory drugs (NSAIDs) inhibit both COX-1 and COX-2, while selective COX-2 inhibitors target only COX-2 to inhibit PGE_2. COX inhibition by NSAIDs further leads to the inhibition of downstream prostaglandin production, including cardioprotective prostacyclin (PGI_2). The use of COX-2 inhibitors has been associated with an increased risk of cardiovascular events.

It has been assumed that selective inhibition of COX-2 results in a safer alternative resulting in less gastrointestinal side effects however, the blockage of COX-2 in endothelial cells turned out to cause severe cardiovascular problems due to the reduction of cardio protective PGI_2 and resulted in the withdrawal of several drugs [130] thus targeting both COX or COX-2 alone can inhibit the constitutive production of prostaglandins which can further disrupt prostaglandin homeostasis and cause alterations in cell integrity [131].

1.5.2 mPGES-1: an alternative anti-inflammatory target

Managing pain in patients with CKD poses several challenges. Altered drug metabolism and excretion make them more vulnerable to adverse drug reactions, and there is a scarcity of safety data for treating this population, despite their significant pain burden. An alternative therapeutic approach to inhibit the production of PGE_2 is through selective inhibition of PGE_2 synthase while sparing other prostaglandins or even support the elevation of other anti-inflammatory prostaglandins such as PGD_2 and PGI_2 (**Fig. 10**) [132].

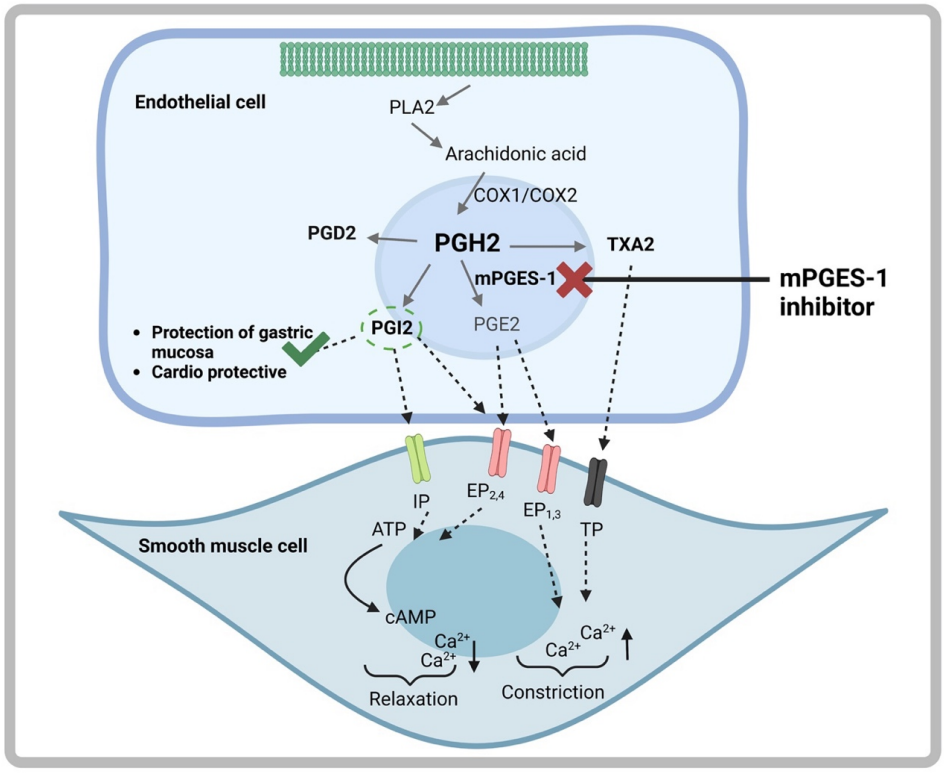

Figure 10: Simplified illustration of microsomal prostaglandin E synthase 1 (mPGES-1) inhibition. Cyclooxygenase (COX) inhibition by non-steroidal anti-inflammatory drugs (NSAIDs) leads to the inhibition of downstream prostaglandin production, including cardioprotective prostacyclin (PGI_2). The use of COX-2 inhibitors has been associated with an increased risk of cardiovascular events. An alternative therapeutic approach to inhibit the production of PGE_2 is through selective inhibition of mPGES-1while sparing other prostaglandins or even support the elevation of other anti-inflammatory prostaglandins such as PGD_2 and PGI_2.

PGE_2 is abundant in our body. Three types of PGE_2 synthases play important role in the regulation of PGE_2 synthesis under different physiological and pathological conditions: microsomal prostaglandin E synthase 1 (mPGES-1), cytosolic prostaglandin E synthase (cPGES), and microsomal prostaglandin E synthase 2 (mPGES-2) [133]. mPGES-1 is an inducible enzyme that is mainly expressed in inflammatory cells, such as macrophages, and is upregulated in response to inflammatory stimuli, thus responsible for the majority of PGE_2 synthesis in inflammation makes it is an attractive therapeutic target for the treatment of inflammatory disorders [134]. cPGES is constitutively expressed in a wide range of tissues and is involved in the basal production of PGE_2; it is important for the regulation of PGE_2 levels in

normal physiological conditions [135]. mPGES-2 is also constitutively expressed in various tissues, including the kidney, lung, and brain. It has been shown to be involved in the regulation of PGE_2 biosynthesis in certain cell types, although its precise role in PGE_2 production is not fully understood [136]. Among these three enzymes, inhibiting mPGES-1 synthase showed promising result in preclinical studies [137]. Several mPGES-1 inhibiting compounds have been developed over the past years including the FLAP inhibitor MK-886 which was also shown to inhibit mPGES-1. Despite their potential benefits, the majority of these compounds were unsuitable for clinical investigations due to drawbacks such as poor selectivity, high lipophilicity, and interspecies differences [138]. So far only two compounds have reached clinical trials: GRC 27864, produced by Glenmark and LY3023703 developed by Eli Lilly, whereby the latter was prematurely stopped due to liver toxicity detected in two study participants [139].

Previous work of our collaborators group has reported the characterization of dual murine/human mPGES-1 inhibitors (CII and CIII) in different models of inflammation in vitro and in vivo [140]. Both inhibitors demonstrated dual activity against human and rodent recombinant mPGES-1 enzymes, resulting in potent inhibition of PGE_2 in various cell types [141]. Also Ding and co-workers recently published novel compounds with the potential of dual inhibition both of human and mouse mPGES-1 enzymes [137]. Nevertheless, mPGES-1 is part of a complicated interplay within the eicosanoid pathways and inhibition of mPGES-1 might be more promising in some disease areas than others [134]. Therefore, careful investigations of the complex effects of mPGES-1 inhibition in vitro and in vivo are needed. In this thesis the shunting towards the PGD_2 and the PGI_2 pathways upon mPGES-1 inhibition are of main interest.

1.6 Senolytics as novel therapeutic alternative

Senolytics represent a promising and novel therapeutic approach to combat ageing-related diseases. Two very important features of senescence are SASP and SCAPs. The SASP is composed of cytokines, chemokines, tissue-damaging proteases, elements which could affect the function of stem and progenitor cell, hemostatic factors, and growth factors [69]. Developmental and oncogene-induced senescent cells may secret various molecules, among others IL-6, IL-1β, MCP-1 and PAI-1, as part of the SASP in various cell types and between cells activated by differential triggers [7]. The secretion of SASP by senescent cells may have significant pathogenic effects both locally and systemically. It is important to stress that senescent cells may trigger the accumulation of inflammatory milieu to the extend, which at present is still unclear and/or warrants extended research. Such kind of communication may be connected to altered Nrf2 activity, a transcriptional factor that activates the expression of genes involved in the antioxidant response that is currently appreciated as a hallmark of chronic 'burden of lifestyle' diseases [142].

When senescence occurs, several pathways prevent apoptotic clearance of cells – these pathways are called SCAPs. These are anti-apoptotic defense pathways which senescent cells use to defend against their own SASP. Using bioinformatics approaches, several SCAPs have been identified including BCL-2/BCL-Xl family, PI3Kδ/ AKT/ ROS-protective/ metabolic,

MDM2/ p53/ p21/ serpine (PAI-1&2), HIF-1α and HSP-90 [143]. Knocking down or suppressing these SCAPs proteins has been shown to cause death of senescent cells while not compromising healthy cells [143]. SCAPs needed for senescent cell survival against apoptosis differ between cell types and this knowledge has been used to identify and develop putative senolytic targets[144]. The targeting of senescent cells has gained significant attention in recent years as they are a hallmark of ageing. Senolytic agents, SASP inhibitors, and immunosurveillance agents are among the approaches used to achieve this and are considered as potential therapeutics. One of the most promising senolytic drugs currently being studied is the combination of quercetin (Q) and dasatinib (D). In preclinical studies, this combination extended the lifespan of mice and reduced the incidence of age-related diseases [145]. The treatment was also effective in reducing age-related tissue damage and promoting tissue regeneration [146].

Other compounds that have shown promising senolytic effects include navitoclax, fisetin, and UBX0101. The promising results of pre-clinical studies targeting senescent cells have prompted the initiation of clinical trials to explore their athero-protective and anti-ageing effects. One such trial is a phase 1 study involving the use of a D+Q senolytic cocktail in diabetic nephropathy patients and the first results indicate a significant amelioration of the senescence cell burden [147]. These clinical trials, along with other positive findings, provide support to target senescent cells as a therapeutic approach [147].

In summary, senolytics represent a novel therapeutic approach that has shown promising results in preclinical studies. Further research is required to comprehensively understand the safety, efficacy and application of senolytics in conditions associated with different clinical settings e.g. CKD.

1.7 Research gap

Although there has been ongoing research linking CKD, endothelial dysfunction, and EVA, there are still many unknowns regarding underlying mechanisms that connect this interplay. The majority of studies concentrate on bigger vessels, and microvascular dysfunction is less appreciated even if it remains the base of the iceberg of CVD in CKD, as recently summarized by Querfeld et al.[15]. More research could help to further clarify the mechanisms of injury and improve our understanding of the pathogenesis. Another potential research gap is the lack of reliable biomarkers that can accurately detect EVA in CKD. Although there are some treatments available for endothelial dysfunction, to delay EVA phenotype in CKD, there is still a need for more effective therapies. Further research could help to identify new therapeutic targets and develop more effective treatments for these conditions. The thesis can provide more insights for a better understanding about microvascular dysfunction to target or delay EVA phenotype in CKD.

2 RESEARCH AIMS

2.1 GENERAL AIM

The overall aim of this PhD project is to increase our understanding on how functional and structural changes of small artery maintenance contribute to EVA and CVD complications under uremic environment or could favor other diseases e.g. COVID 19 and define specific injury targets for potential therapeutic benefit.

2.2 SPECIFIC AIMS OF INDIVIDUAL STUDIES

Study I

• To assess a number of senescence markers, including biochemical markers relevant to EVA and increased CVD risk**.**

• To assess functional and structural properties of the microcirculation in uremia**.**

• To assess if and how certain toxins/EVs affect vascular function**.**

• To test if senolytics may modulate SASP in arteries from ESKD**.**

Study II

• To explore the extent to which levels of AAs and their metabolites are altered in patients with CKD. In addition, to examine the relationship between these changes and the functional and structural properties of the vasculature.

Study III

• To assess if there is a difference in soluble ACE2 levels, ACE2 and TMPRSS2 receptor expression in resistance arteries, adipose tissue, and the circulation in CKD vs controls.

Study IV

• To examine the impact of mPGES-1 inhibition on vascular function ex vivo, both in healthy individuals and those with CKD.

3 MATERIALS AND METHODS

This section outlines the research methodology utilized, which involves a combination of patients cohorts, primary methods, statistical analysis, and ethical considerations. The different techniques used in this thesis are illustrated below in **figure 11**. For a more detailed understanding of the various techniques imployed for this thesis work, it is recommended to follow the corresponding papers with a detailed description of methods involved.

3.1 Study population

All studies only included human participants and did not incorporate any animal studies into the project. Participants were recruited from different CKD cohorts, including patients in CKD stage 3 or those in stage 5 who were either undergoing living donor kidney transplantation (Kärl-tx) or receiving dialysis (MIA). In addition, kidney donors (PC) as well as participants undergoing planned hernia and gallbladder operation (Ersta), were included as non-CKD controls.

The **Kärl-tx** cohort is a group of CKD-5 patients who underwent living donor kidney transplantation. This study cohort was established with the aim of gaining a deeper understanding of the mechanisms underlying bone turnover and VC. Inclusion criteria: age > 18 years, able to provide informed consent and met the criteria for kidney transplantation. Participants were recruited at the Department of Renal Medicine and Transplantation surgery, Karolinska University Hospital, Stockholm, Sweden.

The **MIA** cohort is a group of CKD-5 patients underwent peritoneal dialysis catheter insertion at the Department of Renal Medicine Karolinska University Hospital, Stockholm, Sweden. This study only enrolled patients who were commencing dialysis treatment. However, those with acute infection, vasculitis, or liver disease at the time of assessment were excluded from the study.

The **MIMICK1** (Mapping of Inflammation Markers in Chronic Kidney Disease) cohort is a group of CKD-5 patients underwent haemodialysis at the Department of Renal Medicine Karolinska University Hospital, Stockholm, Sweden. Inclusion criteria was age > 18 years, able to provide informed consent. Exclusion criteria was acute infection at the time of evaluation or unwilling to take part in the study. Only blood sample was utilized in the current study from those patients.

CKD-3 participants were recruited at the Department of Renal Medicine Karolinska University Hospital, Stockholm, Sweden. Inclusion criteria was (eGFR 30–45 mL/min/1.73 m2), age > 18 years and able to provide informed consent. Exclusion criteria was acute infection at the time of evaluation or unwilling to take part in the study. Only blood samples were collected from these patients.

The **PC** Control group pertains to living donors who underwent standard screening and were deemed eligible for kidney donation. During the transplantation procedure subcutaneous fat tissue and blood samples were collected.

The **Ersta** control cohort is a group of non-CKD participants who underwent planned hernia and gallbladder operation at Ersta Hospital, Stockholm, Sweden. The inclusion criteria for this study were individuals over 18 years of age, while exclusion criteria were the presence of inflammatory disease, diabetes mellitus, a history of CVD, or the use of certain medications such as insulin, oral anti-diabetic drugs, glucocorticoids, immunosuppressants, or antibiotics.

The **PRIMA control** group was selected from the general population with age > 18 years at the Stockholm Region in Sweden, in a manner that matched the age and sex distribution of the overall MIMICK-1 cohort. This selection was carried out randomly by the government agency Statistics Bureau of Sweden. The only exclusion criterion used for the selection of control participants was willingness to participate in the study.

Details regarding the different cohorts involved in various papers are mentioned in the **table 1** below

Table 1: Cohorts involved in different papers

Study	Cohorts	
	CKD	**Control**
Paper I	Kärl-tx, MIA	PC, Ersta
Paper II	Kärl-tx, CKD-3	PC
Paper III	Kärl-tx, MIMICK-1, MIA	PRIMA control, PC, Ersta
Paper IV	Kärl-tx, MIA	PC, Ersta

3.2 Baseline laboratory analysis and biochemical measurements

The morning after overnight fasting, blood samples were collected from participants and stored at −80°C if not immediately analysed. Several biomarkers were measured at the Department of Laboratory Medicine at Karolinska University Hospital, Huddinge, Sweden using various techniques, including serum creatinine, albumin, calcium, phosphorus, 25(OH)-vitamin D, triglycerides, cholesterol, HDL-cholesterol, hsCRP, glucose, HbA1c, PTH, FGF-23, Klotho, plasma interleukin IL-6, IL-10, IL-1β and TNF. The baseline demographic data for each participant was obtained from the patient's journal from Karolinska University Hospital. Alkaline phosphatase, Sclerostin, fibronectin, PTX3-3, Copeptin, kallistatin, cathepsin B, cathepsin D, and 8-OHdG were analyzed using the enzyme-linked immunosorbent assay technique.

TMAO was measured using LC-MS/MS, where TMAO-D9 standards were mixed with plasma sample in methanol and water, with proline13C5 as a recovery standard, and then injected onto an Agilent Technologies chromatographic system fitted with an Acquity UPLC Amide column

and a VanGuard pre-column. The detection and processing of the compounds were completed using a mass spectrometer and MassHunter Quantitative Analysis QQQ.

For the measurement of AAs metabolites, blood samples were collected from three groups, including individuals with CKD-3, CKD-5, and control participants. K3-EDTA tubes were utilized for blood collection, and plasma was prepared and stored at -80 °C within 30 minutes of collection. For BH2 and BH4 analysis, a blood collection method described by Fekkes & Voskuilen-Kooijman was followed [148], which involved collecting blood in K3-EDTA tubes containing DTT at a final concentration of 0.1%. Plasma was then prepared by storing it in the dark for 2.5 hours at room temperature and stored immediately at -80 °C. To quantify proline, ornithine, phenylalanine, arginine, citrulline, ADMA, BH2, and BH4 in plasma samples, an internal standard solution containing acetonitrile, methanol, water, and specific isotopically labeled AAs was added to the samples. The mixture was then centrifuged, and a portion of the sample extract was transferred to a new plate for analysis using LC-MS. Chromatographic separation was achieved through hydrophilic interaction chromatography (HILIC), and MRM was utilized in positive electrospray mode to quantify the analytes with specific transitions, cone voltages, and collision energies. The quantification of AAs was achieved by comparing the response area obtained for each analyte to that of the internal standard and using a calibration curve for each analyte; all samples were within the calibration curve range.

3.3 In vivo functional study

In vivo functional studies were performed using EndoPAT device (Itamar Medical Israel) in **Paper I and Paper II**. Participants fasted overnight before measurements were performed in a temperature-controlled room during the morning hours. Participants were advised to refrain from using tobacco products and to temporarily discontinue their blood pressure medication prior to the study. Using the EndoPAT device, the changes in blood volume from the index fingers of the participants were measured. To induce reactive hyperaemia, a sphygmomanometer cuff was applied to the non-dominant arm and inflated to 60 mmHg above systolic BP or a minimum of 200 mmHg. For individuals with arteriovenous fistula, the cuff was placed on the opposite arm. The opposite arm was utilized as a control. The signal was captured over three intervals of 5 minutes each, comprising a 5-minute baseline recording, 5 minutes of interrupted blood flow, and a 5-minute recording of the reactive hyperaemia response. The software automatically computed the reactive hyperaemia index (RHI) and augmentation index (AI@75).

3.4 Subcutaneous artery preparation

During the scheduled surgery, a portion of the subcutaneous fat was excised from the incision site, and the specimens were immediately immersed in iced physiological salt solution (PSS) and stored at 4ºC until dissection. Utilizing stereomicroscopy, resistance arteries measuring approximately 150-400 μm in diameter were extracted from the sample. One to four artery segments, each with a length of roughly 1.6-2.0 mm, were dissected from each biopsy utilizing microsurgery instruments. Ice-cold PSS was continuously used throughout. Microsurgery

instruments were utilized to carefully remove the surrounding tissue, while care was taken to avoid any damage to the endothelial layer during the dissection and vessel mounting process. The overall strategy was to obtain the maximal number of available vessels which could be utilized for functional and structural studies with possibility to fix the vessels to assess the markers of senescence signature. When available, the vessels were used for isolated artery bioassays to further assess effects of prolonged incubations with the compounds of interest (please see below).

3.5 Ex vivo functional study

In brief, the vessels were carefully mounted in an organ bath of a four-vessel wire-Myograph System (model 610M, Danish Myo Technology A/S, Denmark), with two steel wires measuring between 25-40 μm in diameter inserted into the lumen of each artery to prevent damage to the endothelium. The organ baths were perfused with PSS, heated to 37ºC, and continuously aerated with a mixture of 95% O_2 and 5% CO_2. Following a 60-minute equilibration period, a standardized normalization procedure was implemented to establish the optimal circumference. Vessel segments were deemed eligible for inclusion if they met the following criteria: at least 50% endothelium-dependent relaxation with acetylcholine (ACh, 10μM) or bradykinin (BK, 1 μM) after pre-contraction with phenylephrine (10 μM) and force development of at least 1mN/mm of preparation. The wire myography technique was employed in **Paper I, Paper II and Paper IV.**

3.6 Experimental protocol for ex vivo functional study

The experimental procedure commenced with the establishment of concentration-response curves to norepinephrine (NE) (1 nM to 3 μM). Once a stable contraction to NE (3 μM) was achieved, the arteries were subjected to concentration-response curves to the endothelium-dependent vasodilator BK (10 pM to 1 μM). To investigate the role of endothelium-derived factors, the vessels were then treated with nitric oxide synthases (NOS) inhibitor L-NG-Nitro arginine methyl ester (L-NAME) (100 μM) and cyclooxygenase (COX) inhibitor Indomethacin (100 μM, 20 min), pre-constricted once again, and then exposed to concentration-response curve to BK (10 pM to 1 μM). To assess endothelium-independent dilation, concentration-response curves to the endothelium-independent vasodilator sodium nitroprusside (SNP) (NO-donor) (1 nM to 100 μM) were constructed. At the end of each experiment, a stretching procedure was carried out in the presence of 1 mM SNP, 0.2 mM papaverine, and 1 mM EGTA in Ca^{2+} free PSS to obtain the passive-length relationship. The above-mentioned protocol was applied in **Paper I and Paper II.**

In **Paper IV,** NE was used to constrict the arteries to a stable plateau, after which they were returned to their basal tone by washing them with PSS. They were then incubated with either a test compound or a combination of test compounds, such as mPGES-1 inhibitors, COX inhibitors, receptor agonists, or antagonists for 30 minutes. A second NE-concentration-response curve was then performed to determine the impact of the test compounds. The percentage of initial high potassium constriction was used to present the concentration response

curves, and the differences between the first and second NE-constriction curves were analyzed to determine the effect of these compounds. To evaluate the effects of mPGES-1 inhibitors or COX inhibitors on ACh-induced relaxation, arteries were pre-constricted with NE at a single concentration of 3 μM, and ACh was added cumulatively at concentrations ranging from 0.0001 -10 μM. The impact of the test compounds was determined by expressing the concentration response curves before and after treatment as a percentage of maximal relaxation and analyzing the differences between the first and second response curves. Furthermore, PGE_2 or PGI_2 (stable analogue Iloprost) was cumulatively added to NE-pre-constricted arteries to evaluate their effects on vascular tone. Control experiments with the vehicle was performed, and artery viability was evaluated after the experiment with NE at 1 μM.

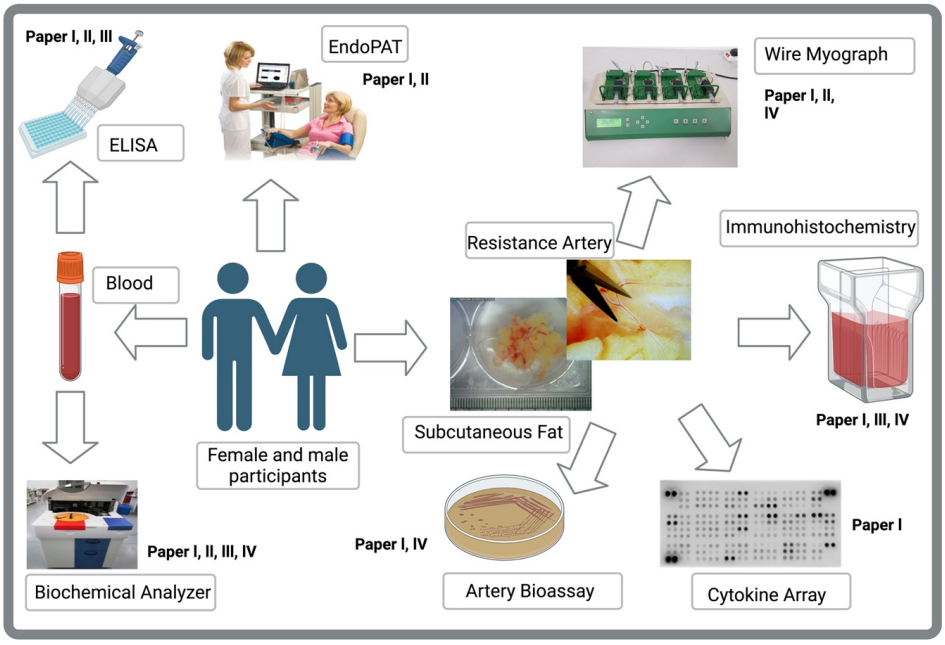

Figure 11: Simplified illustration of different techniques used in this thesis.

The active force generated by K^+ and NE contractions was determined by measuring the baseline before the addition of stimuli. The data was then expressed as a percentage of the K^+ reference contraction. For the relaxation responses to BK and SNP, the baseline prior to addition of the vasodilators was considered as 100%, and the stable level of contraction achieved after the addition was set at 0%. An exponential function ($Y=Aexp(bX)$) was used to fit the data on the passive length-tension relationship, where Y represents passive tension (mN/mm), A represents the first fitted parameter (mN/mm), B represents the second fitted parameter (mm-1), and X represents circumference (mm).

3.7 Immunohistochemistry

Immunohistochemistry was performed in **Paper I, Paper III Paper IV.** Arteries were isolated from fat biopsies, flash-frozen on dry ice and stored at -80°C until transversal sectioning (Thermo cryostat, 7 µm thickness) and mounted onto glass slides (Thermo Fisher Scientific, Germany). Staining was performed for the following markers p16INK4a, p21CIP1, Ki67, RUNX2, NRF2, Sirtuin1(SIRT1), MHY11, Sclerostin, Alizarin red, SA-β-gal (**Paper I);** ACE2 and TMPRSS2 **(Paper III);** and mPGES-1, COX-1, COX-2, PGIS, EP1-EP4, TP, and IP (**Paper IV).** In order to perform immunohistochemical staining, the frozen sections were first fixed with 4% formaldehyde. For markers listed in **Paper I and Paper III** immunofluorescence was performed whereas for the markers for **Paper IV** light microscopy diaminobenzidine (DAB) immunohistochemistry was performed. (Please see respective paper for more details protocols)

For the calcification assay, Alizarin red (Sigma-Aldrich) was used with a dilution of 2g/100ml and the pH was adjusted to 4.1-4.3. For SA-β-gal staining, arteries were fixed with 0.2% glutaraldehyde fixing solution for 10 min at room temperature. After this, the tissues were washed 2x in phosphate buffered saline (PBS) and stained overnight (16-18h) in 200 µL SA-β-gal staining solution (pH=6) at 37°C and 0% CO2.

The fluorescence microscopy (Olympus, Japan) was used to examine and capture images of the slides. The objectives used were x20 and x40. Alizarin red, RUNX2 and SIRT1 staining were scored semi-quantitatively. The staining intensity was evaluated independently by three different observers who were blinded to the experimental conditions and/or case or control. The intensity was rated using a scale of 0-3, where 0 indicated no staining and 3 indicated the highest intensity of staining. The expression levels of ACE2, TMPRSS2, Nrf2, Sclerostin, Ki67 and MHY11 were quantified using Image J software. The software measured the area of positive staining and expressed it as a percentage of the total tissue area. In the case of p21CIP1 and p16INK4a cellular staining, the software Image J was used to count the number of positive cells, which were then expressed as a percentage of the total number of cells.

3.8 Ex vivo organ culture

To investigate the impact of soluble TMAO, PAG, and EVs organ culture was utilized **Paper I**. Isolated arteries were taken from non-CKD individuals who were either living kidney donors or undergoing planned surgeries such as hernia repair, cholecystectomy or bariatric surgery. Two separate wells were used to culture the isolated arteries in DMEM medium for 24 hours at 37°C and 5% CO2. Either 10µM TMAO, 100µM PAG, or EVs were added to wells, while another well served as a control with DMEM. The EVs were obtained from the plasma of CKD-5 patients through ultracentrifugation, with a concentration of 1.5×10^{10}. After 24 hours, the arteries were washed with PSS and mounted onto the organ bath Myograph System for vascular function assessment as described in the section 3.6.

3.9 Isolation and phenotypic characterization of EVs from plasma of ESKD patients

The process for purifying and characterizing plasma EVs in ESKD patients was conducted according to the ISEV 2018 guidelines [149], acquired from Vincenzo Cantaluppi lab, Department of Translational Medicine, Nephrology and Kidney Transplantation Unit, University of Piemonte Orientale (UPO), Italy. The study was approved by the local Ethics Committee, and patients provided written informed consent. Blood was collected using EDTA as anticoagulant, and EVs were isolated by ultracentrifugation. After resuspending the resulting pellet in DMEM, the EVs were stored at -80°C. The size and concentration of the EVs were determined using NanoSight LM10 and transmission electron microscopy. Bead-based multiplex analysis by flow cytometry was also conducted using the MACSPlex Exosome Kit to detect the expression of 39 different surface markers on the EVs. APC-conjugated antibodies were used to detect EVs that had linked to the capture beads, and the fluorescence intensity of each marker was corrected for background and gated accordingly.

3.10 Cytokine array and characterization of EVs after senolytics treatment

To investigate the effects of senolytics on secreted SASP, human resistance arteries were treated with a cocktail of senolytics (D+Q), and a cytokine array assay was performed. Human resistance arteries were isolated from patients with ESKD and cultured in DMEM medium with DMSO and senolytics cocktail [D (1μM) + Q (20μM)] for 24 hours at 37°C in a humidified atmosphere with 5% CO2. After 24 hours, the culture supernatants were collected and hybridized to the antibody-coated array membrane as per the instructions of the manufacturer (Human cytokine Array kit, Bio-techne, USA). Part of the supernatants were collected for EVs isolation, and the size, concentration, and protein profiling of the EVs were characterized using standard protocols (for more details please see paper I).

3.11 Statistical analysis

The comparisons of clinical and biochemical characteristics, in vivo and staining studies, as well as ex vivo experiments between ESKD and control participants were conducted through several types of statistical analyses, as appropriate. For in vivo and ex vivo experiments, the Mann-Whitney U test was utilized for group-wise comparisons, while the two-way ANOVA was used for concentration-dependent experiments. Parametric paired t-test was employed to compare changes in a compound of interest-induced effect before and after treatment. Sex-disaggregated statistical analyses were conducted when feasible (and significant results were presented), while non-significant results were displayed as a group. Spearman's rank correlation method was used for correlation analysis. Continuous variables were expressed as median (interquartile range), and the statistical significance level was set at $p<0.05$. The software used for statistical analysis included GraphPad Prism 6.0, SPSS and STATISTICA 7.0. For preparing the illustrations of this thesis Biorender was used.

3.12 Ethical considerations

The Swedish Ethical Review Authority approved the study protocols and written informed consents was obtained from all patients.

4 RESULTS AND DISCUSSION

The thesis consists of four studies with the goal of increasing our understanding how and why small artery dysfunction under uremic environment contributes to increased EVA and CVD complications and to define specific injury targets for potential therapeutic benefit. In the first study, **Paper I**, comprehensive phenotypic analyses, specifically focused on EVA, including the senescence signature and vascular function and structure of the microcirculation of patients with CKD stage 5 and non-CKD controls, were performed. Additionally, the effects of selected toxins and EVs were assessed as well as senolytics and their effects on vascular outcomes. Subsequently, in **Paper II**, we focused on the associations between various amino acids and their metabolites with vascular function and structure, where we also added an additional patient group of CKD stage 3 patients. In **Paper III** we investigated whether soluble ACE2 levels varied in the circulation, as well as the expression of ACE2 and TMPRSS2 receptors, in resistance arteries and subcutaneous adipose tissue of individuals with CKD compared to healthy controls. Finally, we conducted interventional studies utilizing senolytics in **Paper I** and mPGES-1 inhibitors in **Paper IV**. In the upcoming section, selected results from the four papers will be presented, and an explanation of their significance within the research context of this PhD thesis is presented.

4.1 Phenotyping of early vascular ageing in microcirculation

EVA is a common phenomenon observed in patients with CKD, which is characterized by functional and structural changes of the vasculature. Here, our focus is resistance arteries from the microcirculation. Vascular changes are associated with an increased risk of CVD complications and mortality in CKD patients. In **Paper I** we conducted immunohistochemical staining in resistance arteries of ESKD patients using a selected set of markers of interest, including senescence markers p21CIP1 and p16INK4a, proliferative marker Ki67, calcification marker RUNX2, antioxidant markers Nrf2 and SIRT1, contractile marker MHY1 and structural marker sclerostin to investigate their phenotypic features related to EVA. The results showed that the expression levels of senescence markers p21CIP1 and p16INK4a were significantly higher in ESKD patients compared to controls, while the expression level of Ki67, Nrf2, SIRT1 and MHY11 was significantly lower in ESKD patients (**Fig 12, Table 2**). Additionally, more intense SA-β-gal staining was observed in ESKD patients compared to controls, whereas the expression levels of sclerostin did not differ between ESKD patients and controls (**Fig 12, Table 2**).

The increased expression of p21CIP1 and p16INK4a in resistance arteries from ESKD patients, is supported by our previous observation of increased CDKN2A expression and cellular senescence in mid-sized uremic epigastric arteries [72]. As senescent cells secrete SASP that can damage surrounding tissue and neighboring cells, increased senescence may negatively impact cellular functions and contribute to EVA and organ dysfunction. Therefore, targeting and eliminating senescent cells, specifically p16INK4a positive cells, using senolytic compounds may help to reduce the EVA phenotype not only in CKD but

also in other lifestyle diseases associated with ageing. The underlying cause of increased expression of p21CIP1 and p16INK4a in uremic resistance arteries is not clear. Nevertheless, studies suggest that cellular senescence can be triggered by oxidative stress [150]. Although we did not find a direct association between p21CIP1, p16INK4a, and the biomarkers of oxidative stress, indirect evidence suggests that a decreased expression of Nrf2, known to control the basal and induced expression of antioxidant response element–dependent genes, could be responsible for this increased senescence [151]. Indeed, our study clearly shows reduced expression of Nrf2 in uremic arteries.

Further support for occurrence of senescence footprint comes from our observation of a negative correlation between p21CIP1 expression and MHY11, which suggests that higher prevalence of senescence in these arteries from ESKD can lead to VSMC switching from a contractile to a synthetic phenotype, another feature of EVA with consequences on functional and structural abnormalities [152]. In addition, our observation of decreased expression of the contractile marker MHY11 and proliferative marker Ki67 supports increased stiffness or decreased passive elasticity in resistance arteries from ESKD, as shown in vivo and ex vivo investigations (further elaborated in following chapter).

Arteries from ESKD patients also exhibited presence of calcification and increased RUNX2 expression compared to controls (**Fig 12, Table 2**). Our observation of vascular calcification in resistance arteries in individuals with ESKD reinforces our previous findings of a correlation between senescence and calcification in medium-sized uremic arteries in humans [72]. p16INK4a- associated accumulation of prelamin A in VSMCs promotes calcification and arterial stiffening [153], however, it is uncertain whether calcification triggers senescence or senescence triggers calcification, or if the effect is bidirectional [154]. Even though our study was conducted on a selected group of younger and healthier ESKD patients, it showed presence of calcification in 30 % of investigated patients, as detected by alizarin red, and upregulation of RUNX2 (**Paper I, fig 9 b, c).** We are the first to report calcification in resistance vascular bed under uremic conditions, adding to the scarce literature on this topic.

Our findings of reduced Nrf2 in resistance arteries from patients with ESKD are consistent with previous reports of decreased Nrf2 expression in cerebrovascular tissues and muscle of patients with uremia [155,156]. Furthermore, arteries from ESKD patients with higher RUNX2 expression showed significantly lower Nrf2 expression (**Paper I, fig 9f**). Animal studies has shown that activating the Nrf2/Keap/NQO1 pathway can prevent calcification of VSMCs [157]. Our observation of a negative association between higher RUNX and lower Nrf2 suggests that the calcification-inhibiting effects of Nrf2 may be reduced or lost in arteries from ESKD patients, which support the report by Hinoi et al showing Nrf2 may negatively regulate cellular differentiation through inhibition of the Runx2-dependent transcriptional activity in osteoblasts [158]. The cause of reduced Nrf2 expression in the uremic environment is not yet clear, but it may be related to oxidative stress associated with uremia or SASP secretion from senescent cells, as Nrf2 deficiency in aged mice has been

shown to worsen cellular senescence [159]. Additionally, it has been reported that uremic toxins, such as indoxyl sulfate, can downregulate expression of Nrf2 in the kidney [160], suggesting that the toxic pro-inflammatory and pro-oxidant uremic environment is a likely culprit. In vitro studies showed that mRNA expression of Nrf2 and its downstream targets NQO1 and SOD1 were linked to VSMC calcification in the context of uremia. This observation may indicate the existence of circulating factors in the serum of calcified uremic patients that could deplete Nrf2 signaling, such as the Nrf2 repressor Bach1 [161]. In vitro studies have also shown that hyperphosphatemia-induced calcification is associated with Nrf2 depletion, and that calcium-phosphate deposition can be alleviated by Nrf2 agonists, such as tert-butylhydroquinone and dimethyl fumarate [162,163]. These findings have potential therapeutic implications for treating ESKD-related arterial calcification.

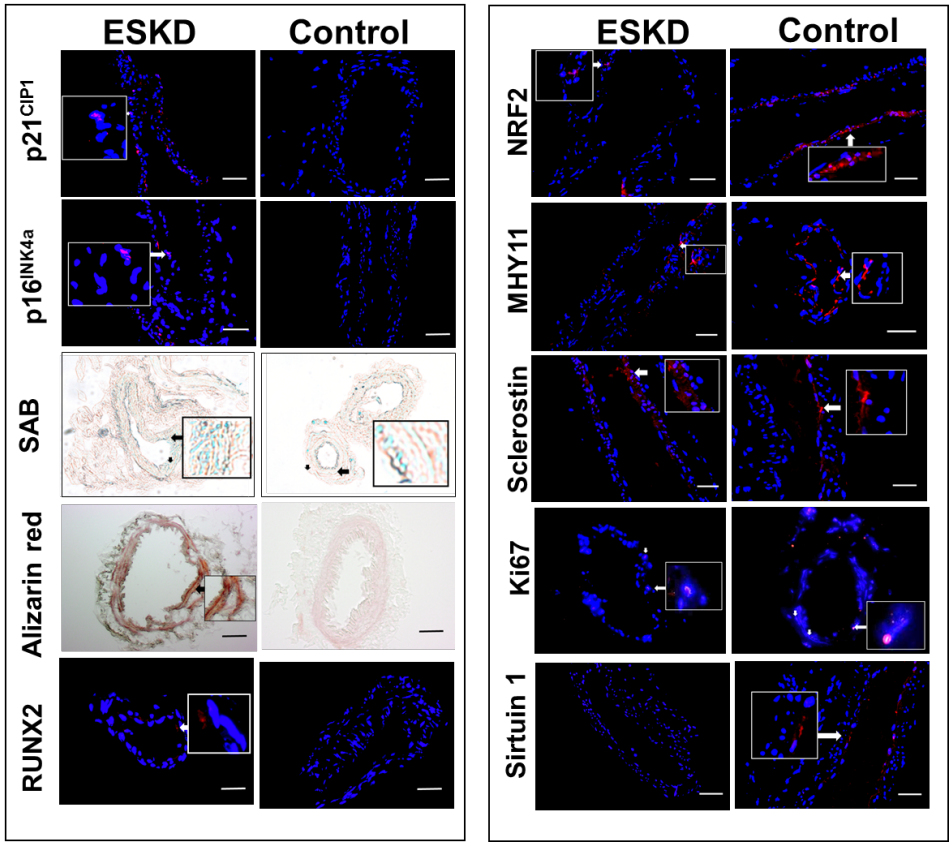

Figure 12: Representative images of senescence markers p21CIP1 and p16INK4a, proliferative marker Ki67, calcification marker staining alizarin red, transcription factor RUNX2, antioxidant markers Nrf2 and SIRT1, contractile marker MHY1 and structural marker sclerostin in resistance arteries of ESKD end stage kidney disease (ESKD) patients and controls.

The reduced SIRT1 expression in arteries from ESKD patients is noteworthy because altered SIRT1 expression has been linked to metabolic syndrome, diabetes, cancer and ageing [164]. Although, little is known about SIRT1 expression in the vascular wall, it has been shown to have cytoprotective effects in the kidney by inhibiting cell apoptosis,

inflammation, and fibrosis [165]. The reduced expression of SIRT1 in our study suggests that the peripheral vasculature in uremia may be more prone to senescence, leading to structural and functional abnormalities in the artery wall, including increased stiffness as determined by both in vivo and ex vivo investigations. In addition, we analyzed the expression of sclerostin in uremic resistance arteries and report no difference to controls. It could be speculated that the resistance vasculature serves as a less important primary target for sclerostin action [166], as several studies have shown the relationship between sclerostin levels, bone density and arterial stiffness in CKD [167-169].

Table 2: Markers of senescence signature.

Markers	Role	ESKD
p16INK4a	Senescence	↑
p21CIP1	Senescence	↑
SA-β-gal	Senescence	↑
Ki67	Proliferation	↓
Alizarin red	Calcification	↑
RUNX2	Calcification	↑
NRF2	Anti-oxidant, vascular maintenance	↓
SIRT1	Vascular maintenance	↓
MHY11	Contractile VSMC phenotype	↓
Sclerostin	Vascular maintenance and structure	-

To sum up, we present changes in the senescence signature in the resistance vasculature of CKD patients, as summarized in **Table 2**. Identifying and phenotyping EVA markers could emerge as a valuable approach to identify individuals at risk of developing CVD. Furthermore, comprehensive phenotyping may lead to the development of novel biomarkers and therapeutic targets for the prevention and treatment of CVD in this high-risk CKD patient group.

4.2 Vascular function and structure

The presence of markers of senescence signature/EVA led us to further investigate vascular function and structure in resistance arteries of microcirculation with the working hypothesis that, this phenotype will be accompanied by alterations of vascular function and structure with a subsequent effect of vascular stiffness. Both in vivo and ex vivo approaches were utilized in **Paper I** for the assessment of vascular function and structure in microcirculation of patients with ESKD. The in vivo measurement of peripheral arterial endothelial function,

represented by RHI, showed no difference between participants with ESKD and non-CKD controls (**Paper I, fig. 1A**). On the other hand, the augmentation index (AI@75), which reflects the stiffness of the circulation, was found to be higher in participants with ESKD compared to the control group (**Paper I, fig. 1B**). In addition, ex vivo studies showed similar magnitude of BK mediated endothelium-dependent relaxation compared to controls (**Fig. 13a**). However, pharmacological inhibition showed differences of the contribution of NO and EDHF between ESKD and control group. The contribution of NO was reduced and the contribution of EDHF was increased in ESKD patients (**Fig. 13b**).

Although patients with ESKD are exposed to a toxic uremic environment, the comparable levels of RHI between ESKD patients and the control group suggest that the maintenance of endothelial function is preserved in ESKD. We speculate that although the overall function of the endothelium appears to be preserved, various factors that contribute to endothelial function, such as NO, PGI_2, and EDHF may have been influenced [170], to which our ex vivo experiments give a support. Although endothelium dependent dilatation is preserved, pharmacological inhibition of NOS/COX pathway showed a difference in the ratio of endothelial mediators (NO versus EDHF) responsible for conferring this dilatation. Of note, NOS/COX inhibition primarily eliminates NO, and to a lesser extent, if any, PGI_2-mediated dilation thus, the remaining dilation is attributed to EDHF-mediated response [171]. Essentially, the reduced contribution of NO observed is compensated by EDHF. However, even if EDHF would contribute to preserved dilatation, it cannot substitute for the function of NO. In addition, the similarity in responses to NO donors between ESKD and control arteries, suggests that the downstream pathway of NO is functioning properly while the upstream NO machinery is impaired.

We argue that the reduced contribution of NO in patients with ESKD may be attributable to ADMA. We support this argument by observing that ADMA levels increased gradually as eGFR decreases and CKD progresses in **Paper II** [172]. The inhibition of endothelial eNOS by ADMA can have deleterious effects on vascular function, ultimately leading to endothelial dysfunction. This is due to the negative impact of ADMA on NO production, which may lead to vasoconstriction, hypertension, and immune dysfunction [173]. Additionally, ADMA can exacerbate oxidative stress, which further promotes endothelial dysfunction and adverse vascular remodeling [172,174]. In our CKD patients, there is a significant inverse correlation between ADMA and NO contribution (**Fig. 14f**), suggesting a link between ADMA and upstream vascular dysfunction that could contribute to impaired blood flow to target organs, arteriosclerosis, cardiovascular events, and mortality [175]. These findings highlight the importance of addressing ADMA levels in CKD management to mitigate the risk of adverse CVD outcomes. Our study has identified a compensatory upregulation of EDHF, which is an underestimated factor in the vascular physiology of CKD. It is possible that the EDHF pathway compensates for the diminished function of NO when NO bioavailability is compromised [176,177]. However, we believe that this equilibrium will become further disturbed in deteriorating uremic conditions with increased pro-inflammatory and pro-oxidant factors, which is supported by our previous observation of

impaired NO and EDHF-mediated dilatation in severe ESKD patients who underwent PD [178].

The fact that ESKD patients showed preserved responses to NO donors implies a sufficient NO signaling in VSMC, which suggests that the functional capacity of the VSMC is tolerant to the uremic environment. However, the inverse correlation between PTX-3, ICAM-1 and SNP (**Paper I, fig. 10c, d**) suggests a vulnerability towards diminished VSMC function. It is again important to stress here that our patients with ESKD are "relatively" healthy as they are getting a living donor kidney transplantation, and hypothetically a robust change in the uremic environment with subtle changes in inflammation and pro-oxidative environment could further impair even VSMCs function. Higher levels of PTX-3 have been linked to abnormal vascular function [179] in kidney failure patients with an increased risk of CVD events [180]. Elevated levels of circulating PTX-3 and ICAM-1 could trigger the inflammation and could further affect VSMC function in addition to the endothelium, as inflammation has been reported to have a deteriorating effect on VSMC function [181,182].

Our study found that arterial stiffness, as defined by the inverse of distensibility measurement in peripheral microcirculation using in vivo measurement, with EndoPAT, and ex vivo, as passive length force measurement, was elevated in ESKD patients (**Paper I, fig. 1B and 7B**). The primary contributors to arterial stiffness are extracellular matrix proteins and VSMC and the increase in arterial stiffness in CKD is, primarily due to the accumulation of uremic toxins, maladaptive metabolic and hormonal processes, calcification, and other EVA features [183]. CKD can promote arterial stiffening by stimulating VSMC proliferation and fibrosis [184]. As increased CVD risk in CKD is directly correlated with the degree of VC and arterial stiffness, our observation of 30% VC is worrisome [185]. The higher arterial stiffness observed in female participants compared to male (**Paper I, fig 1C, D**) suggests that females are more vulnerable to the uremic environment than males. This finding could be due to several factors, such as the slightly higher age of female participants compared to males, which showed a positive correlation with arterial stiffness in controls. Additionally, the majority of the female participants from both groups were either in the perimenopausal or post-menopausal stage, which could also contribute to the higher stiffness among females, as evidence suggested that menopause amplifies the age-dependent increase in arterial stiffness [186].

Vascular function, structure, and stiffness are all closely interconnected and crucial for maintaining a healthy cardiovascular system. Proper vascular function is essential for adequate blood flow to tissues and organs, while the structural integrity of blood vessels helps prevent the development of CVD. Understanding the intricate relationship between these factors are crucial for the development of effective interventions to maintain vascular health and prevent CVD.

4.3 Effects of TMAO, PAG and ESKD plasma-derived EVs on vascular function

In previous paragraphs, features of impaired vascular function and structure in patients with ESKD were discussed. The following paragraph focusses on uremic toxins and EVs derived from ESKD plasma as a potential link for this impaired vascular function and structure, along with known factors - inflammation, oxidative stress, vascular calcification, dyslipidemia among others [187]. To assess the ex vivo impact of uremic toxins and EVs derived from ESKD plasma on isolated artery, we subjected the artery to a 24-hour incubation with soluble TMAO, PAG and EVs. Among others, uremic toxins TMAO and PAG are of special interest as growing evidence illustrate their involvement in the pathogenesis of endothelial dysfunction, vascular calcification and ultimately CVD [76,77].

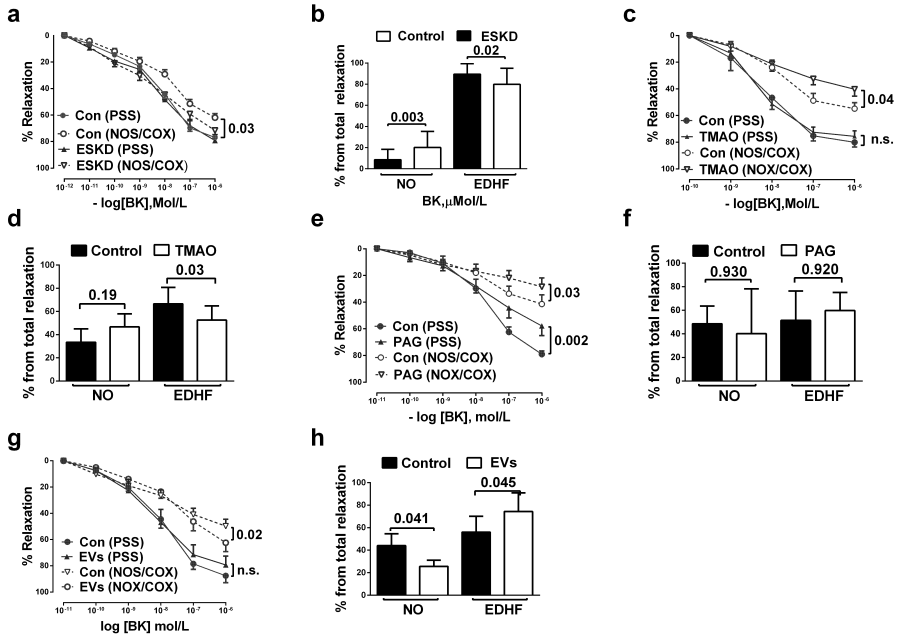

Figure 13: Concentration response curve to bradykinin (BK) induced relaxation in arteries from ESKD compared to controls in PSS, before and after NOS/COX inhibition in controls and ESKD (a); contribution of NO and EDHF in controls and ESKD (b); concentration response curve to BK induced relaxation in arteries from control compared to TMAO (c), PAG (e) and EVs (g) in PSS; before and after NOS/COX inhibition in control vs TMAO (c), PAG (e) and EVs (g); contribution of NO and EDHF in controls vs TMAO (d), PAG (f) and EVs (h). Comparisons were assessed by (a, c, e, g) two-way ANOVA and (b, d, f, h) Mann-Whitney U test. Statistical significance level was set to $p<0.05$; NO =Nitric oxide; EDHF=endothelium-derived hyperpolarizing factor; NOS=nitric oxide synthase; COX=cyclooxygenase; ESKD = end stage kidney disease; Con=control; TMAO= trimethylamine-N-oxide; PAG=Phenyl Acetyl Glutamine and EVs= extracellular vesicles; BK: Bradykinin; PSS: Physiological salt solution

We have observed a negative correlation between RHI and TMAO levels (**Paper I, fig. 10H**). This implies that an increase in plasma TMAO levels may lead to endothelial dysfunction and this finding led us to investigate the ex vivo effects of soluble TMAO on resistance arteries. The endothelium-mediated dilatation was not noticeably different between the TMAO exposed versus vesicle control groups (**Fig. 13 c**). However, when we

inhibited NOS/COX, we observed a shift in endothelium-derived factors after exposure to TMAO. NOS/COX inhibition indicated that the EDHF-mediated dilation was impaired by TMAO incubation (**Fig. 13d**). In support, cell culture and animal studies have demonstrated that TMAO has a negative impact on endothelial function by triggering inflammation and activating the ROS-TXNIP-NLRP3 inflammasome [83,87]. Conversely, the TMAO-mediated effect was reversed with treatment using the ROS inhibitor N-acetylcysteine.

On the other hand, the endothelium-mediated dilation in arteries that were incubated with PAG was notably lower than that of the control group (**Fig. 13e**). We propose that higher circulating PAG levels could lead to endothelial dysfunction, as evidenced by the reduced overall endothelial function observed after PAG incubation. Interestingly, the NO and EDHF ratio remained unaltered (**Fig. 13f**), suggesting that PAG targets both the NO and EDHF pathways. These NO and EDHF pathways normally work together and compensate for each other [188], so when both are impaired, overall endothelial function is reduced, which we observed after PAG incubation [178]. In contrast, incubation with TMAO only impaired the EDHF pathway, indicating that PAG is a more harmful uremic toxin for endothelial function compared to TMAO.

Additionally, we have observed a higher constriction induced by NE in patients with ESKD that had higher plasma levels of PAG (**Paper I, fig. 5 F**). This observation concurs with study by Nemet et al., which showed that PAG can upregulate adrenergic receptors, further supporting the observation of higher responses to NE in patients with higher PAG concentrations [77]. Moreover, a reduced endothelium-independent dilation observed in ESKD patients (**Paper I, fig. 5G**) with higher plasma PAG levels indicates that PAG not only causes endothelial malfunction, but also targets the downstream NO pathway, including VSMC in ESKD patients.

Finally, although beyond the scope of this thesis, a recent study of us has suggested that even IS has effects on endothelial function by downregulating contribution of NO [189]. IS has been suggested to be a leading toxin related to the increased CVD risk. Here we present additional toxins and their effects on vascular function and structure. Further studies should consider looking for additive effects of toxins, but the current finding that TMAO has direct effects on vascular function also implies a potential for interventions targeted to reduce TMAO production, e.g. via diet.

Previously, we confirmed that ESKD patients have a higher concentration of EVs, primarily derived from endothelial cells, leukocytes, and platelets and that ex vivo incubation of arteries with these EVs could contribute to EVA [190]. Our ex vivo experiments showed that the contribution of endothelial derived factors differed between EV treated vs. non treated vessels, further supporting the involvement of EVs on endothelial dysfunction by means of impairment of NO contribution (**Fig. 13g, h**). Based on these findings, we propose that circulating EVs in ESKD patients may act as uremic toxins in conjunction with other solutes.

Our results from **Paper I** have revealed a complex disease picture related to arterial senescence signature characterized by increased expression of senescence markers, calcification, and reduced anti-oxidative control. We have demonstrated that the contribution of endothelium-derived factors, particularly NO and EDHF, to vascular maintenance is selectively impaired by uremia. Our findings suggest that uremic toxins TMAO, PAG, and EVs may serve as a plausible link between the uremic environment and endothelial dysfunction. We have also observed impaired VSMC function, as evidenced by altered sensitivity to K^+ and reduced expression of contractile markers, indicating a switch towards a synthetic phenotype. These vascular abnormalities are closely associated with inflammation biomarkers such as TNF, IL-1β, PTX-3, and cathepsin B. Our sex-disaggregated statistics indicate that certain abnormalities are more prevalent in males than in females or vice versa.

4.4 Association of plasma amino acids and its metabolites with vascular function

Paper II investigates whether there are any differences in the plasma AA metabolomic profiles of patients with CKD and whether these differences are related to abnormal vascular maintenance. We have observed a significant decrease in the BH4/BH2 ratio in CKD-5 patients compared to non-CKD controls and CKD-3 participants. The plasma levels of BH2 and ADMA increased progressively with the advancement of CKD, whereas the plasma levels of BH4 remained similar between the three groups studied (**Fig. 14a, b, c**).

Under normal circumstances, BH4 is produced from GTP or through the reduction of oxidized BH2 when there is a demand for BH4 in the body [191]. However, under oxidative stress, which is commonly seen in CKD [192], BH4 acts as a scavenger for radicals and can be oxidized to BH2. The decreased BH4/BH2 ratio seen in our CKD-5 patients suggests that there may be a reduced conversion of BH2 to BH4 or reduced activity of dihydrofolate reductase [193]. We did not find differences in BH4 levels between CKD patients at different stages of the disease. Our findings align with and build upon the results of a study by Yokoyama et al., which showed no change in BH4 levels in CKD-5 patients compared to healthy individuals [194]. However, we have observed increasing BH2 levels as CKD progressed, which could have an impact on biological activity because BH2 competes with eNOS for binding [195]. Therefore, even though plasma BH4 levels did not change due to kidney dysfunction, the decreased BH4/BH2 ratio and elevated BH2 could contribute to the reduced production of NO in our CKD patients [195]. This is consistent with ex vivo results **(Paper I)** that showed impaired NO production in uremic arteries compared to controls. It is important to note that other mechanisms may also be involved for reduced NO, as studies have suggested that a pro-oxidative environment and its effects on NO bioavailability play a role in kidney failure models [196,197].

In patients with CKD, there was a notable increase in the levels of citrulline and proline compared to the control group as the disease progressed (**Fig 14d, e**). The liver synthesizes citrulline through the urea cycle, and subsequently metabolize it into arginine. In healthy

individuals, citrulline is filtered by the kidneys and excreted in the urine. The findings of our study indicated that citrulline concentrations were notably elevated in participants with eGFR levels below 45 mL/min. Moreover, the Arg/Cit ratio was progressively reduced with the advancement of CKD, indicating lower activity of arginosuccinate synthase and/or argininosuccinate lyase. Our findings support previous research showing higher plasma citrulline levels in CKD patients [198,199]. Additionally, citrulline has been found to be a predictor of adverse outcomes in CKD patients, such as CVD and mortality, suggesting it could be useful as a biomarker for predicting adverse outcomes in CKD patients.

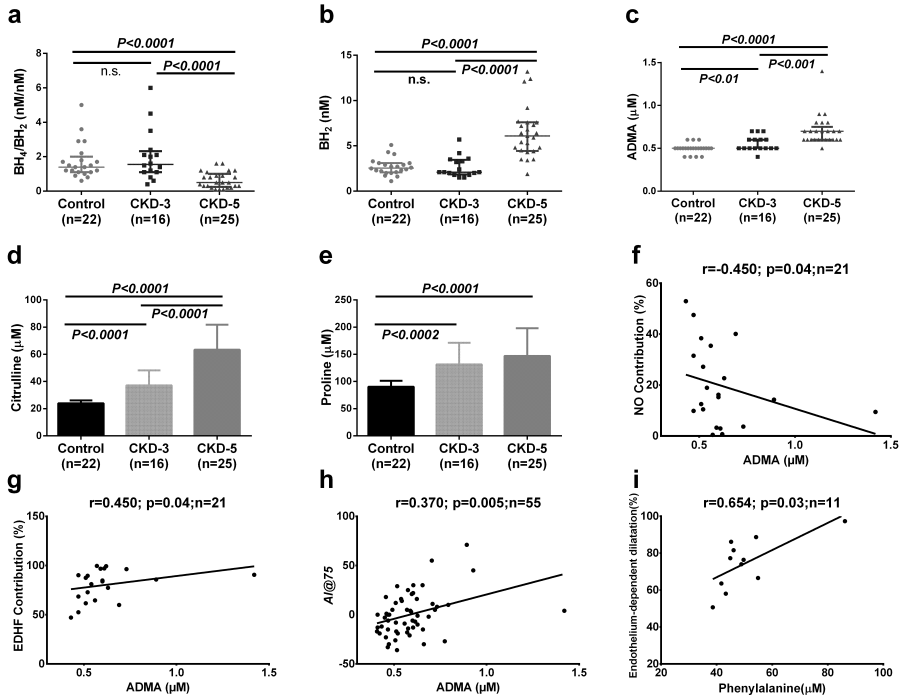

Figure 14: Plasma concentration of BH4/BH2 ratio (a), BH2 (b), ADMA (c), Citrulline (d) and Proline (e) between controls vs CKD-3 vs CKD-5 respectively; Correlations between NO contribution with ADMA ((f); ADMA with EDHF contribution (g); AI@75 with ADMA (h) and between endothelium-mediated dilatation and phenylalanine (i); significances were assessed by Mann–Whitney U-test (a, b, c, d, e) and Spearman's rank correlations- (f, g, h i); Statistical significance level was set to $p<0.05$; BH4 = tetrahydrobiopterin; BH2 = dihydrobiopterin; ADMA = asymmetric dimethylarginine; NO = nitric oxide; EDHF = endothelium-derived hyperpolarizing factor; CKD-3 = chronic kidney disease stage 3; CKD-5 = chronic kidney disease stage 5; n.s. non significance.

Our investigation found no significant changes in ornithine levels between groups. Ornithine acts as a transitional molecule in the urea cycle and is converted into citrulline. However, as CKD progresses to stage 5, the Cit/Orn ratio increased significantly, reflecting a rise in plasma citrulline levels. This suggests an increase in ornithine breakdown, leading to citrulline and possibly proline synthesis. The Pro/Orn ratio also increases with the progression of kidney disease, supporting the notion of increased proline production. These results suggest that decreased eGFR and impaired urinary excretion contributes to ornithine accumulation, thereby triggering the production of citrulline and proline.

In summary, **Paper II** highlights the importance of understanding the relationship between AA metabolism and endothelial and VSMC function in CKD patients. The results of this study indicate that uremia is linked to changes in AA metabolism, which may have implications for endothelium-dependent dilatation and vascular stiffness in the microcirculation. These findings highlight the importance of further exploring the effects of uremia on AA metabolism and the potential downstream effects on vascular function. These findings provide valuable insights into potential interventional strategies that could be employed to normalize AA metabolism and improve treatment outcomes for CKD patients. For instance, exogenous administration of AAs such as arginine [200], citrulline [201], and L-phenylalanine [202] has been proposed as a possible therapeutic approach for addressing vascular disease. However, the existing evidence is inconsistent and may be influenced by variations in dosage and route of administration.

4.5 Angiotensin-converting enzyme 2 in microcirculation of patients with chronic kidney disease

Patients with CKD are at a higher risk of severe COVID-19 due to their vulnerability to infections and higher prevalence of CVD resulting from EVA. In **Paper III** we focused on ACE2, which serves as a receptor for SARS-CoV-2 virus. The aim of this study was to investigate potential differences in circulating soluble ACE2 levels as well as expression of ACE2 and TMPRSS2 receptors in the microcirculation and subcutaneous adipose tissue between individuals with ESKD and non-CKD controls. Specifically, we aimed to assess whether variations in soluble ACE2 levels and ACE2 receptor expression in the vasculature may help to explain why CKD patients are at greater risk of SARS-CoV-2 virus infection. Measuring soluble ACE2 levels may provide insight into natural shedding processes, while analysis of ACE2 and TMPRSS2 receptor expression levels in the vasculature could offer important information regarding potential risk factors for viral infection.

The results of our study showed a significant increase in ACE2 levels in both the circulation and resistance arteries of ESKD patients in comparison to the control group (**Fig. 15a, b**). These findings are consistent with a previous study that reported increased levels of soluble ACE2 in patients with advanced stages of CKD [203]. The alterations in soluble ACE2 levels have been linked to the development of CVD and have been associated with conditions such as heart failure, stroke, myocardial infarction, and diabetes in a multinational population study [204]. The elevated levels of soluble ACE2 in ESKD patients suggest a possible role in the pathophysiology of CVD in this population. Several studies have demonstrated an association between higher soluble ACE2 enzymatic activity and a higher risk of atherosclerosis in CKD stage 3/5 patients [205]. In our study, we found a positive correlation between soluble ACE2 levels and IL-6, as well as an inverse correlation with cholesterol levels (**Paper III, table 2**). Using multivariable linear regression models, we determined that there was a significant independent interplay between IL-6, TNF, and cholesterol with soluble ACE2, after adjusting for age, sex, BMI, CVD, DM, and ACEi/ARB treatment.

Our study findings have important implications in the context of COVID-19. The positive correlation between soluble ACE2 and inflammation in ESKD patients indicates that they may have an increased risk of an exaggerated inflammatory response, leading to a cytokine storm and potentially worse outcomes in COVID-19. IL-6 is a key regulator of the SASP, and higher levels of IL-6 and soluble ACE2 in ESKD patients could indicate a state of senescence and EVA, as ESKD is appreciated as a clinical model of senescence [206]. These findings suggest that ESKD patients may have an increased susceptibility to COVID-19 due to their "cardiovascular system senescence status" as discussed earlier in **Paper I** and may potentially require special consideration in terms of prevention and management of the disease.

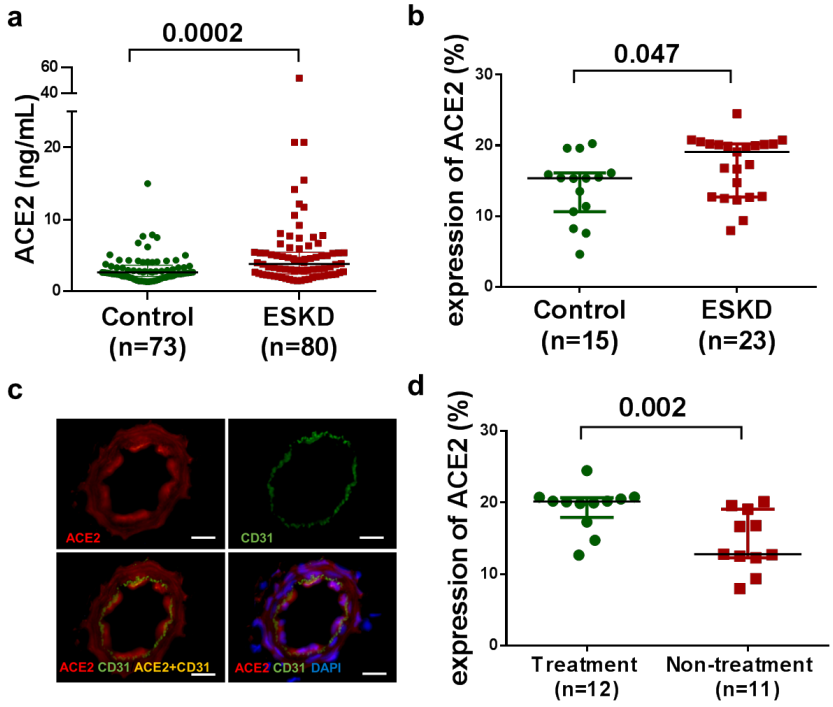

Figure 15: Serum concentration and tissue expression in resistance artery of angiotensin-converting enzyme (ACE2) in controls versus patients with end-stage kidney disease (ESKD) (a, b); Representative immunofluorescence staining of ACE2 expression and CD31 endothelial cell marker expression in resistance artery from patients with ESKD (c); ACE2 expression in resistance artery sections from patients with ESKD with or without ACE-inhibitor/angiotensin receptor blocker (ARB) treatment (d); Bar = 100μm; Comparisons were assessed by Mann-Whitney U test; Statistical significance level was set to $p<0.05$.

The levels of soluble ACE2 were found to be similar in both men and women with ESKD and in control groups, which is different from previous studies that have found higher levels of soluble ACE2 in males compared to females, particularly in the general population with heart failure [207]. The hormonal changes associated with menopause may explain why there were no significant differences between males and females in the study as postmenopausal women tend to have higher levels of soluble ACE2 compared to premenopausal women [207].

Our results are consistent with earlier studies, demonstrating that ACE2 is expressed in both endothelial cells and VSMC in the resistance artery of both ESKD patients and non-CKD controls (**Fig. 15c**) [208,209]. The increased expression of ACE2 in the arteries of patients with ESKD may suggest a potential role in VC [210]. Zhang Q et al., has reported that elevated serum ACE2 is involved in VC in patients with CKD [210]. However, there are also contradicting findings suggesting that ACE2 may protect against VC by modulating the expression of calcification-related genes to attenuate atherosclerotic lesions shown in an animal study [211]. However, the exact mechanisms by which ACE2 exerts these effects are still not fully understood and further research is needed to fully elucidate the role of ACE2 in VC. Additionally, the presence of higher levels of ACE2 expression in the arteries of ESKD patients suggests that they are more susceptible to be infected by SARS-CoV-2 and therefore experiencing more severe symptoms of COVID-19. However, there were no significant differences in adipose ACE2 expression between ESKD patients and control groups. Nevertheless, as recent research shows a correlation between high visceral adiposity and the severity of COVID-19, it is possible that ACE2 expression in adipose tissue may contribute to the cytokine storm associated with the disease [212].

Our study found no significant difference in soluble ACE2 levels between males and females. However, we did observe that in control subjects, females had a significantly higher expression of ACE2 in the arterial wall than males. In contrast, male ESKD patients had significantly higher ACE2 expression in the arterial wall compared to male controls (**Paper III, Fig. 4b**). Surprisingly, there was no significant difference in ACE2 expression between female ESKD patients and controls. Although we appreciate that further studies are needed including more samples, we believe that a sex disaggregated approach could bring more light in the disease pathogenesis in respect to sex differences that were recently appreciated in relation to COVID 19 infection [213].

No differences in TMPRSS2 expression were observed in resistance arteries and adipose tissue between ESKD patients and control groups. Detectable levels of TMPRSS2 expression were found in the endothelium of resistance arteries in both ESKD patients and controls, despite previous difficulties in detecting TMPRSS2 expression in microvascular endothelial cells (except during active angiogenic or tubulogenic responses) [214]. However, the functionality of TMPRSS2 cannot be predicted by expression levels alone, as it is regulated by nitrosylation [215]. The activity of NO synthase and subsequent NO production may potentially affect viral infection of the endothelium by modulating the activity of TMPRSS2.

To summarize, this study offers new insights into how ESKD may impact the binding and priming process of SARS-CoV-2. Our findings indicate that ESKD patients exhibit elevated levels of soluble ACE2 in circulation, along with increased expression of ACE2 in the vasculature. Moreover, our results demonstrate that ACE2 receptor expression is higher in ESKD patients receiving ACE-inhibitor/angiotensin blocker treatment (**Fig. 15d**). These findings may have implications for clinical decision-making concerning the

management of COVID-19 in ESKD patients. Moreover, our data indicate that additional studies are needed to evaluate possible sex differences in specific treatment regimens for different comorbidities that are present in ESKD.

4.6 Effects of mPGES-1 inhibition on artery tone in patients with chronic kidney disease

The management of pain in individuals with CKD poses a significant challenge. This is due to CKD patients having an increased susceptibility to negative drug effects as a result of changes in their metabolism and excretion. The use of COX inhibitors in managing pain and inflammation has been recognized for many years. However, the use of traditional NSAIDs and COXIBs carries a risk of adverse side effects, such as gastrointestinal ulcers and CVD complications. To address this issue, there is a promising alternative treatment strategy that involves the pharmacological inhibition of mPGES-1, which is the terminal synthase responsible for producing PGE_2. This approach spares antithrombotic PGI_2, which helps to prevent adverse side effects [140]. Therefore, it is of great importance to conduct preclinical studies on the vascular effects of mPGES-1 inhibitors.

In **Paper IV,** we have used resistance arteries obtained from subcutaneous fat biopsies of ESKD patients and non-CKD participants to evaluate the effectiveness of mPGES-1 inhibitors under "inflammatory" and normal conditions. The outcomes of our investigation indicate that constriction induced by NE was notably reduced upon inhibiting mPGES-1 using compounds CIII and 118 in resistance arteries derived from ESKD patients (**Fig. 16a**). The decrease in contractile responses is consistent with our prior observations of decreased NE tone in control arteries [140]. Furthermore, an experiment was conducted to assess the effect of mPGES-1 inhibition using 118 on arterial relaxation induced by ACh. The findings revealed that the use of mPGES-1 inhibitor 118 significantly enhanced relaxation in arteries obtained from both ESKD patients and controls (**Fig. 16b**). This enhanced relaxation upon ACh stimulation might be attributed to the modulation of prostanoid generation favoring shunting to PGI_2. Studies has shown that mice lacking COX-2 had reduced relaxation in their aortic rings when exposed to ACh due to inhibition of eNOS by ADMA [216]. However, the deletion of mPGES-1 did not increase ADMA production, suggesting a preserved involvement of the NO pathway, which may explain why inhibiting mPGES-1 enhanced relaxation in resistance arteries [217]. Our result indicates that the inhibitory compounds used can affect resistance artery tone, leading to increased blood flow and decreased resistance. These findings are consistent with previous research showing that mPGES-1 inhibition can attenuate NE-induced vasoconstriction in larger human internal mammary arteries and the saphenous vein [218].

One question that remains is whether mPGES-1 inhibitors primarily affect resistance artery tone through shunting to PGI_2, as seen in larger arteries and veins [218], or whether additional pathways may be involved depending on the origin of the blood vessels or the disease of the subjects. To investigate this query, we conducted experiments to evaluate the impact of COX-2 inhibitors NS-398 and Etoricoxib, along with non-selective COX-1/COX-2

inhibitor indomethacin, on NE-induced constriction in resistance arteries from non-ESKD controls. The results showed that these inhibitors had no effect on constriction (**Fig. 16 d, e, f**). In addition, in ex vivo experiments using IL-1β-stimulated resistance arteries, the COX-2 inhibitor NS-398 completely blocked the production of PGE_2 and PGI_2, while the mPGES-1 inhibitor CIII and 118 reduced PGE_2 levels but not PGI_2 levels (**Fig. 16 g, h**). The observations indicate that COX-2 may undergo upregulation in resistance arteries when subjected to artery culturing following stimulation with IL-1β, and that PGI_2 derived from COX-2 might play a role in the diminished vasoconstriction exhibited by resistance vasculature upon administering mPGES-1 inhibitors.

Upon conducting our investigation, we discovered that concurrent exposure to the IP receptor antagonist CAY10441 failed to reinstate NE-induced vasoconstriction in resistance arteries derived from both ESKD patients and controls. However, in control arteries, co-incubation with CAY10441 improved maximal constriction compared to other antagonists, but not to the extent seen with the mPGES-1 inhibitor 118 alone. The results of our study suggest that the vasoactive consequences of mPGES-1 inhibition could differ across distinct vascular beds, and that pathways beyond shunting to PGI_2 might also play a role in human microcirculation.

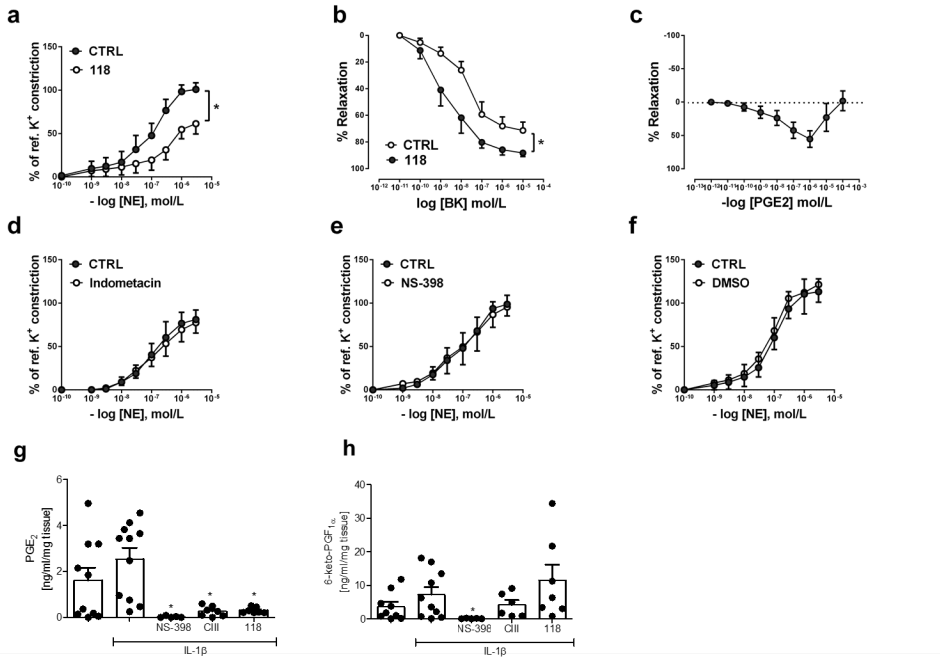

Figure 16: Effects of mPGES-1 inhibition on resistance artery tone in non-CKD controls. Panel (a) and (b): The effect of inhibitor 118 on NE-induced constriction and ACh-induced dilation, respectively; Panel (c): Biphasic effects of PGE_2 on vascular tone; Panel (d), (e), and (f): The effect of COX inhibitors on NE-induced constriction; Panel (g) and (h): The levels of PGE_2 and PGI_2 (6-keto-PGF1α) in cultured arteries incubated with IL-1β and COX-2 or mPGES-1 inhibitors; Statistical analysis: Two-way ANOVA was used to compare the concentration-response curves in panels (a), (b), (d), (e), and (f), while the student's t-test was used to compare prostaglandin levels in panels (g) and (h); The significance level was set to $p<0.05$.

It was previously demonstrated that both PGI_2 and PGE_2 have dual effects on resistance arterial constriction and relaxation, depending on receptor availability. Specifically, PGI_2 and PGE_2 can cause vascular relaxation via the IP and EP4 receptors at low nanomolar concentrations, but at higher micromolar concentrations, they can be constrictive. Our study focused on observing the effects of PGE_2 on human resistance arteries and found that at nanomolar concentrations, it caused vascular relaxation but increased tension development at micromolar concentrations (**Fig. 16c**). Although we could not verify that PGE_2-EP4 signaling was the reason behind the reduced vascular tone following mPGES-1 inhibition, local changes in PGE_2 levels due to mPGES-1 inhibition may still contribute to the reduction in vascular tone through EP2 signaling. Additionally, it is also plausible that the concurrent decrease in PGE_2 and preservation of PGI_2 may create an environment in the blood vessels with antioxidant characteristics, which may help retain NO availability and promote vasodilation.

Our examination of prostanoid receptors (EP1, EP2, EP3, EP4, IP, and TP) revealed their presence in both endothelial cells and VSMCs. We observed that the expression of mPGES-1 was predominantly found in VSMCs. Previous studies have identified the expression of mPGES-1 in VSMCs, endothelial cells, fibroblasts, and immune cells, which may play a role in the local formation of PGE_2 in vessels (**Paper IV, fig. 5**) [219,220].

Corroborating this idea, recent research has highlighted the link between mPGES-1 expression and oxidative stress in CVD. In a study using aortic segments from mPGES-1 knockout mice treated with angiotensin-II infusion, it was found that increased mPGES-1 expression was associated with elevated levels of ROS, reduced bioavailability of NO, and activation of PGE_2-EP1/EP3 signaling [221]. Thus, we propose that the reasons for the decreased NE-induced constriction and increased ACh-induced relaxation in resistance arteries upon mPGES-1 inhibition, are likely to be a result of a combined interplay between various factors, such as the presence of PGI_2, a reduction in the levels of local PGE_2, redirection towards other prostanoids, as well as the influence of other vascular factors.

Results from **Paper IV** provide new insights into the prostaglandin system and its effects on vascular function, particularly in patients with ESKD. Our findings suggest that inhibition of mPGES-1 in resistance arteries leads to a significant reduction in adrenergic vasoconstriction and an increase in ACh-induced dilation. Additionally, our results demonstrate that PGE_2 has a biphasic effect on vascular function, inducing dilation at nanomolar concentrations and constriction at micromolar concentrations. These findings have important implications for the development of novel therapies that target the prostaglandin system for the treatment of CVD. Additional investigations are necessary to evaluate the feasibility of targeting mPGES-1 as a therapeutic approach in patients with ESKD and other CVD.

4.7 Senolytics as a novel therapeutic alternative

In **Paper I** we described a senescence signature contributing to the development of EVA phenotype in resistance arteries from microcirculation in patients with ESKD. These findings led us to test the effects of senolytics on secreted SASP from resistance arteries of ESKD patients and to characterize the nature of EVs after senolytics treatment. After culturing resistance arteries with a combination of senolytics (D+Q) for 24 hours, there was a notable decrease in the levels of SASP factors such as MCP-1, IL-1RA, IL-6, IL-8, IL-13, IL-16, CD40L, and PAI-1 compared to the DMSO control, as seen in **Paper I, fig. 11A**. Furthermore, administering senolytics to resistance arteries from patients with ESKD resulted in a significant reduction in the expression of class II HLA, endothelial marker CD31, TF-coagulation marker CD142, and EPCAM marker CD326 on EVs. Conversely, NK/T-cell and mesenchymal marker CD44 were found to have higher expression levels in the EVs isolated from supernatants compared to controls (**Paper I, fig. 11B**). While additional research is required to confirm these results under different experimental conditions, the proposed "hit-and-run" treatment regimen with senolytic agents, such as D+Q for a 24-hour incubation period, has the potential to modulate SASP from senescent cells in resistance arteries of ESKD patients. Our findings align with a recent phase one pilot study on patients with diabetic kidney disease, in which a similar combination of D+Q treatment lowered plasma SASP factors and senescent cell burden, in adipose tissue [147]. In addition, treatment with senolytic agents may have an impact on the origin of EVs released from isolated arteries, as previously suggested [222] and may serve as a component of the SASP. This may suggest that further studies should focus on the identification of various proteins and RNA subtypes carried by EVs with implications for novel biomarker and drug-target discoveries.

Overall, we suggest that the treatment with senolytic agents has the potential to decrease the senescent cell burden in resistance arteries of ESKD patients, as well as modulating the phenotype of EVs released from isolated arteries. The observed changes in protein expression on the surface of EVs may have implications for the use of EVs as biomarkers of vascular and immune function after senolytic treatment, although further research is ongoing to confirm these results under different experimental conditions.

5 SUMMARY AND PERSPECTIVES

The current thesis generated various novel findings related to vascular maintenance in uremia which are illustrated below in **figure 17**. The initial observation is that arteries from the microcirculation of CKD patients manifest the EVA phenotype, which is characterized by an upsurge in senescence and calcification markers and a decline in anti-oxidative control, proliferative, and contractile markers. Our study demonstrates that uremia compromises vascular maintenance by impairing contribution of endothelium-derived factors, predominantly NO, and we propose that there is a subtle balance in CKD most likely depending on several existing comorbidities, age, disease duration or vintage. When NO bioavailability is impaired, the EDHF pathway takes over to compensate for the impaired function of NO, thereby keeping the overall endothelium-mediated vasodilation preserved. However, with the persistence of CKD over a prolonged period, the bioavailability of both NO and EDHF reduces, resulting in the disruption of overall endothelium function, including mostly the predominant role of NO in vascular maintenance not only toward vascular tone, but its involvement in other important functions like cell signaling, proliferation, adhesion among others.

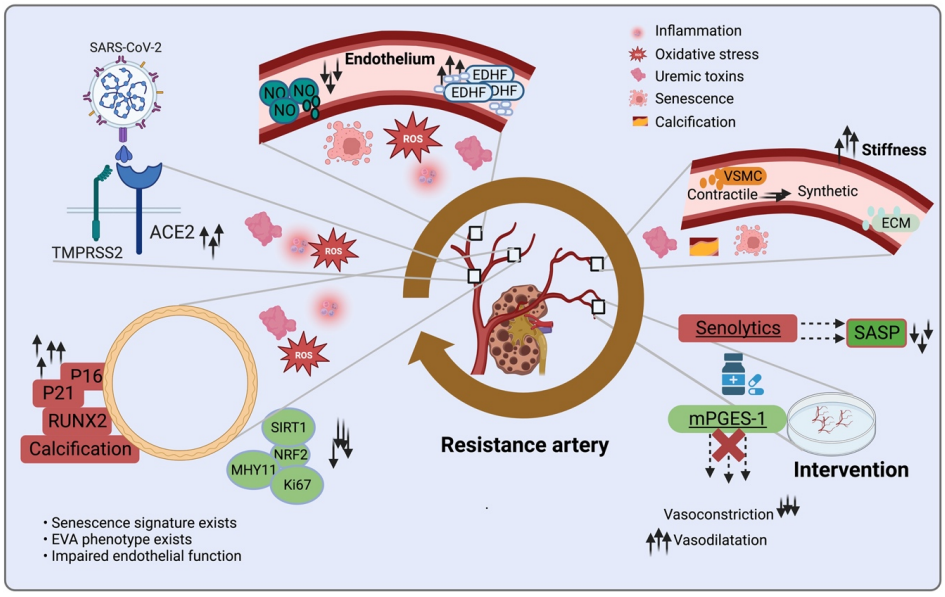

Figure 17: Schematic overview of the PhD thesis investigating small artery dysfunction and its contribution to early vascular ageing and cardiovascular complications in patients with chronic kidney disease. The illustration depicts the mechanisms underlying small artery dysfunction in ESKD patients, including changes in endothelium-derived factors and vascular stiffness modulated by inflammation, uremic toxins, oxidative stress, senescence and calcification. It also shows a higher ACE2 in ESKD patients. Moreover, analyses on potential therapeutic targets, such as senolytics and mPGES-1 inhibitors, for the prevention and treatment of cardiovascular complications are highlighted.

Reduced NO contribution is supported by impaired AA metabolism in CKD with decreased BH4/BH2 ratio and elevated ADMA levels. In addition, ADMA is associated with higher vascular stiffness and reduced NO contribution. Additional research is necessary to

investigate the potential implications of AAs and their metabolites on vascular function and structure, thus reinforcing the plausibility of utilizing AAs as a therapeutic strategy.

There is a distinct variation in the expression of ACE2 in the circulation and vasculature between the ESKD and general population. Enhancing a more comprehensive understanding of the sex predisposition of ACE2 could shed light on potential mechanisms of COVID-19 pathology in high-risk CKD patients, thus paving the way for the development of personalized treatment approaches.

We also report that inhibition of mPGES-1 has vasoactive effects on resistance arteries in ESKD patients, leading to a reduction in vasoconstriction and an improvement in endothelium-dependent dilation. This pharmacological intervention was found to improve vascular tone in both CKD patients and the general population. Our results indicate that selective inhibition of mPGES-1 could represent a novel treatment option in CKD, but also in conditions characterized by endothelial dysfunction, such as severe Raynaud's phenomenon, pulmonary arterial hypertension, myocardial infarction, and heart failure. As senolytic treatment of uremic arteries in **paper I** shows a trend towards reduction in the SASP, future studies should test the effects of senolytics alone or in combination with Nrf2 agonists on EVA in CKD in an extended number of samples and more details related to the assessment of vascular function and structure.

An obvious strength of this study is the use of human materials in all **papers I-IV**. We have utilized a novel and comprehensive approach, combining in vivo and ex vivo techniques, including biochemical marker measurements, immune staining, isolated small artery bioassays, and wire-myography technique to investigate the underlying mechanisms and potential therapeutic targets. This interdisciplinary approach adds depth and rigor to the findings. While **Paper I** has comprehensively characterized the EVA phenotype and senescence signature, it is important to note that there is currently no universally accepted method or biomarker for defining EVA. This should be taken into consideration when interpreting the conclusions drawn from the study. Although the Kärl-tx cohort was utilized in all four **papers I-IV,** it is important to note that the results may not be generalizable to all ESKD patients, as the cohort consists of patients selected for living donor kidney transplantation who tend to be younger and healthier than the broader ESKD population. As the numbers of transplanted patients undergoing dialysis were low and there were no differences in any parameters assessed between dialysis vs non dialysis participants, we pooled both dialysis and non-dialysis group for the study in **paper I-**this deserves further investigation in larger groups. All four investigations in this thesis were conducted using a cross-sectional design and subsequent studies may expand on these findings by investigating the impact of transplantation on vascular function particularly in the same **Kärl-tx cohort,** for enabling longitudinal assessment of any changes that may occur.

The study for **paper II** was conducted on a relatively small sample size, which may limit the generalizability of the results to a larger population. While findings from **paper III** suggest that higher ACE2 levels may increase susceptibility to SARS virus infection,

further research is needed to experimentally test the interaction between the virus and ACE2 in individuals with varying levels of ACE2 expression. In **paper IV** we did not investigate the long-term effects of the identified therapeutic intervention with mPGES-1 inhibition and further research is needed to determine their effectiveness and safety over time. Taken together, the findings of this PhD thesis have important clinical implications and provide a basis for future research. One potential direction is to investigate the efficacy and safety of the identified therapeutic targets in larger, longitudinal studies.

In summary, the characteristics of EVA in microcirculation include functional and structural abnormalities towards reduced contribution of endothelium-derived factors and increased stiffness. It shows enhanced presence of senescence cell burden, including SASP, reduced features for antioxidant defense and a shift towards presence of synthetic VSMCs phenotype that together will drive an unfavorable development towards an increased risk of CVD. The modulation of vascular maintenance with senolytics, mPGES-1 inhibitors and AA supplementations provide novel treatment endeavors in ESKD to improve the uremia-associated cardiovascular complications. The sex specific occurrence of ACE 2 expression in the vasculature, as well as circulation, not only further strengthens its importance for EVA and cardiovascular health in CKD, but also supports the suggestion to apply sex as a biological variable in the preclinical and clinical research.

Conclusion: EVA phenotype is observed in the microcirculation in patients with ESKD, and pharmacological interventions targeting senescence, mPGES-1 inhibition and AA metabolism may improve vascular maintenance and reduced CVD complications in this population.

6 ACKNOWLEDGEMENTS

I feel immensely grateful for the privilege to pursue my PhD at Karolinska Institutet's Department of Clinical Science, Intervention and Technology where I had the opportunity to collaborate with exceptional scientists whose contributions were valuable in the success of this research.

I would like to express my sincere gratitude to my principal supervisor, **Karolina Kublickiene**, whose guidance, encouragement, and support were invaluable throughout my PhD journey. Without her, this achievement would not have been possible. Your support and guidance have been instrumental in my growth as a researcher, from my initial naive state as a foreign student to becoming an independent researcher. I am grateful for your patience in reviewing my papers, and for helping me prepare better presentations, even on weekends when it must have been a bit of a torture for you.

I am deeply grateful to my co-supervisor **Peter Stenvinkel,** for your unwavering support and guidance throughout this journey. Despite your busy schedule, along with the scientific input sometimes you even made time to assist me with logistics support such as ethics, MTA, or patient information. I would like to express my heartfelt appreciation to my co-supervisor, **Anna Witasp,** for your invaluable scientific input, guidance, and support throughout my PhD journey. Your suggestions and feedback were valuable in shaping my research. I am also grateful for your support with the Ersta hospital samples. I would like to express my deepest gratitude to **Per-Johan Jakobsson** for being my co-supervisor, bringing prostaglandin to our lab and of course for introducing me to a wonderful colleague Julia Steinmetz-Späh. The collaboration was fruitful and contributed greatly to my thesis journey. I would like to extend my heartfelt gratitude to **Mathias Loberg Haarhaus** for being my co-supervisor. Mathias provided me invaluable insights and knowledge, particularly in the area of calcification. Thank you, **Dara Mohammad** for your mentorship and offering invaluable guidance, not only in academic matters but also in various aspects of life beyond academia.

I am deeply grateful to all the participants who has given the consent to take part in the study and the research nurses at KBC specially **Ulrika Jensen, Anneli Lindberg, Yvonne Ekelow** and **Anna Johansson** for your invaluable assistance in collecting samples for my research. Thank you very much **Ulrika** for your continued support in providing patient demographic information whenever I visited you. I would like to express my gratitude to **Awahan Rehman,** our lab engineer, for the significant support she provided during the wire myography experiment and ordering reagents. Additionally, I enjoyed the conversations about your homeland, East Turkistan. I find myself missing the presence of our former colleagues, **Liam Ward, Monica, Ann-Christin Bragfors Helin** and **Neja Mudrovcic.** Thank you Annki for your encouragement in learning the Swedish language. I would like to thank all of my colleagues from our Renal lab; **Sam Hobson**, thanks mate for your support in everything. As the only male colleague in a group dominated by women,

Sam was a much-needed ally, and I am thankful for the opportunity to have had manly talks with him and we published seven papers together. Also thank you for looking on the English time to time on my scientific contents. And of course, my favorite **Angelina Schwarz,** I truly appreciated our conversations about topics such as the environment, diet, and fitness, which were always motivational and insightful. Additionally, I am grateful for your willingness to provide me with critical feedback on my work or illustrations from time to time. Thank you, Angelina, for your support, friendship, and valuable contributions to my work. I am grateful to **Leah Hernandez,** for assisting with the staining. Thank you, **Linn Berg,** for all of your administrative support. From the very beginning of this journey one person supported with paperwork from behind the screen, and she is **Agneta Wittlock,** you are such a sweetheart. I would like to express my heartfelt appreciation to our former administrator **Evianne Larsson** for the support she has provided me at the beginning of my PhD journey, particularly with regards to the registration process and visa processing.

During my PhD I had the opportunity to collaborate and learn from many enthusiastic scientists. I want to thank all present and former collaborators: **Abdul Rashid Qureshi, Bengt Lindholm, Annika Östman Wernerson, Hannes Olauson, Peter Barany, Magnus Söderberg, Paul G Shiels, Anna Levin, Moustapha Hassan, Marina Korotkova, Vincenzo Cantaluppi, Amaryllis Van Craenenbroeck, Carla Avesani, Gwladys Revechon, Tamar Tchkonia** and **James L Kirkland.** I would like to express my sincere gratitude to **Thomas Ebert** for generously sharing his expertise in statistics. Your knowledge and insights have been invaluable and greatly appreciated. Thank you for being a true gem. Thank you very much **Marius Kublickas** for inquiring about the well-being of my family and offering assistance. Thank you, **Lu Dai** for the wonderful conversations we shared, and the collaborative visit we made to the Mayo Clinic. Thank you **Rui He** for always being such a friendly and welcoming, keep smiling, you have a wonderful husband **Wenyi Zheng,** I should play again table tennis with you Wenyi. I would like to extend my deepest appreciation to my fellow colleagues at Neo for the warm and friendly conversations we have shared specially **Mona-Lisa Malarte, Soophie Olfat, Amit Kumar, Isabel Runneberger, Sumonto Mitra, Igor Fontana, Nina Boskvic** and **Rakesh Kumar,** Thank you **Urmimala Chatterjee** Boudi, for always spreading positivity around you, I miss the conversation with **Partha Bose** Dada.

Throughout my PhD journey, probably the best thing happened was collaborating with **Julia Steinmetz-Späh,** who proved to be an invaluable colleague. Julia was always there for me when I encountered methodological or technical difficulties, and I am deeply grateful for your unwavering support. In addition, I greatly appreciated your companionship during the tedious and lengthy experiment days. I am honored to have worked with such a talented and dedicated person. I am grateful to **Karin Larsson** for helping me with the cytokine array assay. I had the pleasure of collaborating with **Erdem Aybay**. I would like to thank **Federico Pietrocola** and **Yvonne Giannoula** for the collaboration and introducing new aspects of senescence in my project. I express my gratitude to M**agnus Bäck, Hildur Arnardottir, Sven-Christian Pawelzik, Zihan Qin,**

and **Simin Zhang** for their collaboration on a variety of matters in the lab. Thank you for the enjoyable conversations we've had, Sven. I am grateful to all the Masters students **Michaela, Giulia Tosti, Claudia Tucci, Shno Alsalhi, Leah Hernandez, Irene Peterlongo, Chiara Leotta, Eline Roeyen, Asrah AL Moussaui and Elisa Sartirana** came to our lab and who I had the opportunity to involve, actively or passively, in supervising or guiding with experiments. I have also gained a lot of knowledge and experience from working with you. Thank you. I would like to take a moment to thank **Elsa Jonsson Stenberg** for the delightful conversations we had regarding Swedish society. Her company was truly enjoyable, and the discussions were both insightful and enlightening.

I would like to express my gratitude to my colleagues **Victoria Arfuch Prezioso, Shaghayegh Bayati,** and **Fabio Baldivia Pohl,** whom I had the pleasure of meeting during a career course and have since become wonderful friends with. I particularly want to thank them for the brilliant idea of organizing the tour of restaurants showcasing different cultures, which was a truly memorable experience. Thank you, Victoria, Shaghayegh, and Fabio.Outside the network of my department, I am grateful to one person, and we call each other BOSS! from the first day, **Risul Amin.** Thank you boss for generously sharing your knowledge in staining and for discussing many creative business ideas. I would also like to express my gratitude for your invaluable support time to time when I faced reagent shortages in the midst of my research projects. I feel compelled to express my deepest gratitude to **Azmir Hossain** for his unwavering encouragement, which played a pivotal role in my decision to come to Sweden to pursue my PhD. Without his support, I might not have even considered embarking on this journey. I would like to express my heartfelt appreciation to **Shukarna Ahmed** for always reaching out to me and inquiring about my well-being which meant a great deal to me. I was truly inspired by the story of your PhD journey of Japan, **Sadia Monzur**, thank you for sharing it.

I would like to take this opportunity to express my appreciation to my friends from **SMS, No Vacancy Group,** and **Rajshahi University**. Your presence in my life has brought me immense joy and happiness. Moreover, I would like to extend my heartfelt gratitude to all the Bhabis here in Sweden for the delectable food that you have so kindly prepared. I would like to express my appreciation to all my family and friends particularly my Parents (**Abba, Amma),** my Brothers **Dr Abu Sayed, Dr Newaje Ahmmad** and **Amir Hamza** my Sister in laws **Dr Fardousi Begum** and **Jerin,** my sweetheart Nephew and Nieces **Tabassum, Bushra, Zarif** and **Mahveen Hasan** my **Uncles** and **Aunts** particularly **Dr Abul Hossain** (Khalu I still remember the day, how you were shorting your PhD thesis books after printing) and my **Cousins** for supporting me throughout this journey. **Finally**, I would like to express my sincere gratitude to my wife, **Nushrat Jenin,** for her unwavering support and encouragement throughout my academic journey. I am also deeply grateful to my daughter, **Lubaina,** and son, **Waarid,** for their love and understanding during the times when I had to focus on my studies. Without their constant love and support, this achievement would not have been possible.

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