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**AGEING AND INFLAMMATION WITH FOCUS ON END-STAGE RENAL DISEASE – GENETIC
AND EPIGENETIC FACTORS**

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AGEING AND INFLAMMATION WITH FOCUS ON END-STAGE RENAL DISEASE – GENETIC AND EPIGENETIC FACTORS

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ABSTRACT

The presence of ageing-associated disorders at a relatively young age in patients suffering from chronic kidney disease (CKD) has led to the hypothesis that CKD is characterized by accelerated ageing, resulting in a marked discrepancy between chronological and biological age. Factors that accelerate biological ageing, such as inflammation, oxidative stress, and toxins, impact the processes of cellular senescence and/or apoptosis, thereby shortening the life span of cells, and consequently, of the organism as a whole.

Numerous studies have linked increased cellular senescence and apoptosis to disorders commonly associated with ageing, such as cardiovascular disease (CVD), osteoporosis, and cognitive dysfunction – all of which are common in the uremic phenotype.

In **Study I**, we demonstrate that increased arterial gene expression of cyclin-dependent kinase inhibitor 2A (*CDKN2A*), a known inducer of cellular senescence, is associated with the presence of CVD and vascular calcification (VC) in CKD patients. Furthermore, there is a positive correlation between *CDKN2A* expression and the expression of matrix Gla protein (*MGP*) and runt-related transcription factor 2 (*RUNX2*), both of which are involved in osteogenesis. We also show a tentative relationship between a higher degree of VC and increasing p16^{INK4a} expression, a cognate protein of *CDKN2A*.

In **Study II**, we use telomere length as a biomarker of biological age, showing that CKD patients have shorter telomeres than non-CKD controls. In addition, our results indicate a possible association between longitudinal telomere length, folate, and immunosuppressive treatment in patients undergoing renal transplantation (RTx). This suggests that anti-metabolite therapy may have an impact on biological ageing in RTx patients.

In **Study III**, we show that the global methylation status in dialysis and RTx patients at baseline and after 12 months of renal replacement therapy (RRT) differs at several sites in the genome from that of age- and gender-matched healthy controls. Furthermore, differences in methylation between patients and controls can be found at CpG sites located in genes with known functional relevance to CKD, cellular ageing, CVD and/or metabolic disease.

Continuing our investigations of factors affecting epigenetic status, **Study IV** investigates the association between the degree of self-reported physical activity and global DNA methylation in Swedish seniors. In this study, we demonstrate that individuals who reported higher physical activity had less global DNA methylation than those who were less physically active.

Study V describes the application of a multifactorial mathematical model for predicting the presence of inflammation in a dataset generated from 225 incident dialysis patients. Eight of

the ten features with the highest predictive factor were single nucleotide polymorphisms (SNPs), suggesting a large genetic influence on inflammation in CKD patients.

In **Study VI**, the interplay between inflammatory status, genotype, and mortality is demonstrated in two cohorts of incident dialysis patients. The mortality was reduced in inflamed individuals carrying a 32 base-pair deletion in the C-C motif chemokine receptor 5 (*CCR5*) gene compared to individuals who were inflamed but lacked the deletion.

LIST OF SCIENTIFIC PAPERS

I. Arterial expression of Biological Ageing Factor *CDKN2A/p16^{INK4a}* and its Cognate Protein p16^{INK4a} in End-Stage Renal Disease; “A Man is Only as Old as His Arteries”

Karin Luttrupp, Dagmara McGuinness, Anna Witasp, Abdul Rashid Qureshi, Hannes Olauson, Annika Wernerson, Louise Nordfors, Martin Schalling, Jonaz Ripsweden, Lars Wennberg, Peter Bárány, Peter Stenvinkel, Paul G Shiels

Submitted manuscript

II. Accelerated Telomere Attrition Following Renal Transplantation – Impact of Anti-Metabolite Therapy

Karin Luttrupp, Louise Nordfors, Dagmara McGuinness, Lars Wennberg, Hannah Curley, Tara Quasim, Helena Genberg, John Sandberg, Isabella Sönnberg, Martin Schalling, Abdul Rashid Qureshi, Peter Bárány, Paul G Shiels, Peter Stenvinkel

Submitted manuscript

III. Global DNA Methylation Changes in Response to Renal Replacement Therapy – a Longitudinal Study in Chronic Kidney Disease Patients

Karin Luttrupp, Peter Bárány, Olof Heimbürger, Lars Wennberg, Peter Stenvinkel, Louise Nordfors

Submitted manuscript

IV. Physical Activity is Associated with Decreased Global DNA Methylation in Swedish Older Individuals

Karin Luttrupp, Louise Nordfors, Tomas J Ekström, Lars Lind

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V. Genotypic and Phenotypic Predictors of Inflammation in Patients with Chronic Kidney Disease

Karin Luttrupp, Malgorzata Debowska, Tomasz Łukaszuk, Leon Bobrowski, Juan Jesús Carrero, Abdul Rashid Qureshi, Peter Stenvinkel, Bengt Lindholm, Jacek Waniewski, Louise Nordfors

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VI. CCR5 Deletion Protects Against Inflammation-Associated Mortality in Dialysis Patients

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LIST OF ABBREVIATIONS

AE	Apparent error
ARF	Alternate open reading frame
ASCF	Abdominal subcutaneous fat
BMD	Bone mineral density
BMI	Body mass index
CAC	Coronary artery calcification
CCR5	C-C motif chemokine receptor 5
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CGI(s)	CpG island(s)
CI	Confidence interval
CKD	Chronic kidney disease
CPL	Convex and Piecewise-Linear
(hs)CRP	(high-sensitivity) C-reactive protein
CT	Computed tomography
CVD	Cardiovascular disease
CVE	Cross-validation error
DAB	Diaminobenzidine
DDR	DNA damage response
(T2)DM	(Type 2) Diabetes mellitus
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
E2F1	E2F transcription factor 1
EMPs	Endothelial microparticles
ESRD	End-stage renal disease
FDA	Food and Drug Administration
(e)GFR	(estimated) Glomerular filtration rate
GLM	General linear model
HD	Haemodialysis
HDL	High-density lipoprotein
HIV-1	Human immunodeficiency virus-1
HR	Hazard ratio
IHC	Immunohistochemistry
IL-6	Interleukin-6
IPA®	Ingenuity Pathway Analysis®
LD	Living donor
LDL	Low-density lipoprotein
LUMA	Luminometric methylation assay
MGP	Matrix Gla protein

NADPH	Nicotinamide adenine dinucleotide phosphate
NLRP3	NLR family pyrin domain containing 3
PCA	Principal component analysis
PEW	Protein energy wasting
RLS	Relaxed linear separability
RRT	Renal replacement therapy
RTx	Renal transplantation
RUNX2	Runt-related transcription factor 2
SASP	Senescence-associated secretory phenotype
SCB	Statistics Sweden
SNP(s)	Single nucleotide polymorphism(s)
SODs	Superoxide dismutases
TLR	Toll-like receptor
VC	Vascular calcification
VSMC(s)	Vascular smooth muscle cell(s)

1 INTRODUCTION

1.1 CHRONIC KIDNEY DISEASE

Chronic kidney disease (CKD) encompasses a variety of aetiologies with the common denominator of a progressive degradation of kidney function. It is divided into different stages according to glomerular filtration rate (GFR), where lower GFR reflects a more severe stage of the disease. The different stages are presented below in **Table 1**. The most severe stage of CKD, stage 5, is termed end-stage renal disease (ESRD), which occurs when the GFR is below 15 mL/min/1.73 m². At this stage, the patient requires renal replacement therapy (RRT) in the form of dialysis or renal transplantation (RTx) to survive.

CKD stage	GFR (mL/min/1.73 m ²)	Description
1	≥90	Normal renal function with abnormal urine findings or genetic predisposition to CKD
2	60-89	Mild reduction in renal function with abnormal urine findings or genetic predisposition to CKD
3a	45-59	Moderate reduction in renal function
3b	30-44	Moderate reduction in renal function
4	15-29	Severe reduction in renal function
5	<15	Very severe reduction in renal function

Table 1. Stages of chronic kidney disease².

1.1.1 Incidence and prevalence

The prevalence of CKD is growing worldwide, and has become a global health problem^{3,4}. About 10-12% of the population is currently estimated to suffer from CKD in Western countries^{4,5}, and in 2010, 2.62 million people worldwide received RRT⁶. Consequently, the societal cost for CKD treatment – in terms of medical costs and patient disability – is increasing rapidly^{3,5}.

Chronic kidney disease is a complex disease, with a number of contributing factors, some of which are known while others are as yet unknown. These factors may be genetic^{7,8} or environmental⁹, and lead not only to the development of CKD but also to its devastating comorbidities and complications.

1.1.2 Treatments

The two main kinds of RRT available are dialysis and RTx. Dialysis treatment can be divided into haemodialysis (HD) and peritoneal dialysis (PD). In the case of RTx, patients commonly receive additional medication, such as immunosuppressants.

1.1.2.1 Immunosuppressive (antimetabolite) therapy

Patients receiving a renal transplant are routinely on immunosuppressive medication with the purpose of preventing organ rejection. **Study II** focuses on the particular immunosuppressive medications azathioprine (Imurel®) and mycophenolic acid mofetil (CellCept®), both of which are antimetabolite drugs. These drugs inhibit two different enzymes in the purine synthesis pathway – amidophosphoribosyltransferase (azathioprine) and inosine monophosphate dehydrogenase (mycophenolic acid)¹⁰⁻¹² – which reduces cell proliferation, particularly in lymphocytes¹².

1.1.3 Comorbidities and their risk factors

The healthcare costs and the strain on CKD patients are not solely due to RRT. Patients suffering from CKD are prone to a long list of different comorbidities – cardiovascular disease (CVD)¹³⁻¹⁵, osteoporosis¹⁶, cognitive dysfunction¹⁶, frailty, protein energy wasting (PEW)¹⁷, and sarcopenia¹⁶, to name a few – all occurring at a much higher rate than in healthy subjects of the same age, indicating a substantial discrepancy between chronological and biological age^{16, 18, 19}.

The reason(s) why CKD patients exhibit such a markedly increased risk of age-associated diseases is largely unknown. Biologically relevant hypotheses include the presence of chronic low-grade inflammation²⁰, hypogonadism, increased oxidative stress²¹, elevated systemic levels of uremic toxins²², and, possibly, the effects of medications²³. These processes have all been associated with increased cellular senescence, giving rise to an accelerated biological ageing process.

1.1.3.1 Inflammation

An often observed phenomenon in CKD, and indeed in many other chronic diseases, is the presence of inflammation. Inflammation is often defined as an elevated (> 5 mg/L) systemic level of C-reactive protein (CRP), which is readily measured in blood²⁴. There are numerous other pro-inflammatory markers with functional roles in propagating inflammatory processes. Chief among these is interleukin-6 (IL-6), a universally expressed pro-inflammatory cytokine which has been suggested to be a more specific indicator of inflammation, PEW, and CVD risk than CRP^{25, 26}. The release of CRP and IL-6 can be induced by IL-1 β , a pro-inflammatory cytokine which requires an inflammasome to become active²⁷. The best studied inflammasome, NLR family pyrin domain containing 3 (NLRP3), can be activated by uric acid, among other factors²⁷. Interleukin-6 is also a major component of the senescence-associated secretory phenotype (SASP), which is defined by the secretion of a set of molecules by senescent cells (see below).

Inflammation has long been considered an aggravating, or potentially causative, factor in the development of CVD^{28, 29}. Not surprisingly, the presence of inflammation has also been

linked to an increased risk of overall and cardiovascular mortality³⁰⁻³². There are several studies showing that inflammatory processes are associated with both intimal and medial vascular calcification (VC), as reviewed by Amann³³. Proinflammatory cytokines facilitate the osteogenic transformation of vascular smooth muscle cells (VSMCs), either by direct action or indirectly through an increased level of oxidative stress³³.

While part of the immune response is dependent on environmental components, the inflammatory process is also dictated by genetic factors, which contribute to the inter-individual differences observed in response to challenges to the immune system. An example of a genetic factor that affects infection and inflammation is the C-C motif chemokine receptor 5 (*CCR5*) gene, where a deletion of 32 base pairs (*CCR5*Δ32) renders the resulting *CCR5* protein non-functional³⁴. The *CCR5* protein is expressed on the surface of T cells and macrophages, and is, among other things, involved in recruiting inflammatory cells to the endothelium following the binding of its ligands C-C motif chemokine ligand 3 (*CCL-3*), *CCL-4*, and *CCL-5*³⁵. It also functions as a co-receptor for the human immunodeficiency virus-1 (*HIV-1*)^{36, 37}. With a minor allele frequency of about 10% in populations of European descent^{34, 38}, the *CCR5*Δ32 variant is associated with restriction of *HIV-1* infection as well as slower progression to AIDS in *HIV-1*-infected individuals³⁴. In addition, *CCR5* and its ligands have been implicated in the atherosclerotic process³⁹⁻⁴², and the *CCR5*Δ32 variant is associated with an improved prognosis in patients with atherosclerosis⁴³⁻⁴⁷.

Exercise has been shown to reduce the level of high-sensitivity CRP (hsCRP), both in healthy individuals and in patients with heart disease⁴⁸. While the exact pathways are not yet defined, exercise appears to affect the expression of several inflammation-related genes in immune cells, such as toll-like receptor (TLR) genes, NF-κB, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, and superoxide dismutases (SODs)⁴⁹. In addition, exercise is considered to be a first line treatment for patients diagnosed with type 2 diabetes mellitus (T2DM), as it has been shown to improve markers of metabolic disease⁵⁰. There is also a link between exercise and CKD, as Viana *et al.* found that aerobic exercise might serve as anti-inflammatory therapy in CKD patients⁵¹. Overall, exercise and physical activity have been proposed to reduce ageing-related disorders, such as CVD, diabetes mellitus (DM), Alzheimer's disease, osteoporosis, and cancer⁵². Considering the pathophysiology of CKD, CVD, DM and inflammation, such interconnections are perhaps not surprising.

1.1.3.2 Vascular calcification and cardiovascular disease

The most prominent comorbidity seen in CKD patients is CVD. Concomitant with a decrease in renal function, there is an exponential increase in total and cardiovascular mortality^{14, 53}. The incidence and prevalence of CVD – and CVD mortality – is markedly increased in CKD patients compared to age-matched individuals in the general population^{13, 15}. There are

numerous forms of CVD, which is a complex disease in its own right. In the context of CKD, several CVD risk factors are present and have also been suggested to be involved in the pathogenesis of CVD in this patient group. For instance, the sustained presence of uremic toxins in CKD patients contributes to an increased CVD risk⁵⁴, partly through an elevated degree of oxidative stress^{55, 56}.

A major CVD risk factor is arterial stiffening and/or vascular calcification, a process which is termed arteriosclerosis. Atherosclerosis, a form of vascular disease, refers to the deposition of lipid particles in the intima of the arterial wall, resulting in the formation of fatty streaks and plaques that restrict the lumen and hence the blood flow. There are two different forms of VC, named after their site of occurrence – intimal or medial calcification. Vascular medial calcification is a major risk factor for developing CVD, and predicts cardiovascular morbidity and mortality in HD patients⁵⁷. The process of VC in CKD is characterised by the phenotypic transformation of VSMCs in the arterial wall, resulting in stiffening and an increased risk of CVD, such as cardiac failure and stroke^{22, 58}. Medial calcification, or Mönckeberg's sclerosis, is traditionally viewed as an age-related process, as it is commonly observed in elderly individuals⁵⁸. Although rare in the general population, medial calcification is prevalent in chronic diseases such as CKD⁵⁹, DM⁵⁹, osteoporosis⁶⁰, systemic lupus erythematosus³³, certain genetic disorders³³, and rare progeroid diseases²². In fact, medial and intimal calcification appear to be two distinct processes in CKD, and each has its own clinical symptoms and consequences^{33, 58}. Intimal calcification is related to atherosclerosis and the presence of a prominent inflammatory component in the form of macrophages⁶⁰, while medial calcification (arteriosclerosis) apparently lacks lipid deposits and is more closely associated with osteogenic transformation of VSMCs^{33, 60}.

Osteogenic transformation of VSMCs appears to be central to the VC process⁵⁴. When exposed to a stressor such as high phosphate, VSMCs have the potential to re-differentiate into an osteoblast-like phenotype, which stimulates and supports the calcification process in the vessel wall⁵⁴. This transition is aided by factors such as runt-related transcription factor 2 (RUNX2)^{61, 62}, while inhibitory factors such as matrix Gla protein (MGP), which attempt to reduce the amount of extracellular calcification, are typically down-regulated^{63, 64}. While overall expression of *MGP* is low in Mönckeberg's sclerosis, it is expressed at high levels in VSMCs from calcified regions⁶⁵.

1.2 AGEING

The concept of age traditionally refers to the number of days, months or years that have passed since an individual was born, also known as chronological age. Biological age is another form of age, which does not necessarily equal the chronological age of a person, but rather refers to the physical age of the entire organism – organs, tissues *etc.* – and is in essence a reflection of the cellular age.

Cells age more or less rapidly depending on their surroundings, a process which is termed senescence. If they age quickly, the biological age exceeds the chronological age, and *vice versa*. Increased senescence is hypothesised to result in organ and tissue dysfunction, which ultimately leads to disease conditions associated with premature ageing^{23, 66}. Whereas cellular senescence accumulates during normal ageing, there are diseases and environmental factors that accelerate the ageing process, such as smoking, chemotherapy, and kidney transplantation²³. Immunosuppressive treatment may further add to biological ageing, as it reduces the clearance of senescent cells²³.

1.2.1 Senescence

In order to protect the organism from unrestricted cellular replication, i.e. cancer, a number of intrinsic mechanisms are designed to terminate, or make latent, a particular cell after it has reached a critical number of cell divisions. These processes are vital not only to prohibit cancer, but also for the formation of tissues and organs, especially during foetal development and wound healing⁶⁷⁻⁶⁹. In addition, senescent cells accumulate in tissues during normal ageing, and are believed to directly contribute to the ageing phenotype²³.

The word *senescence* is derived from Latin and means “to grow old”. It is the process whereby old cells cease to replicate and become latent or dormant. A schematic overview of triggers of senescence is shown in **Figure 1**. While the cells no longer divide, they retain many of their functional characteristics and are fully capable of secreting various factors, thereby affecting nearby cells and tissues^{23, 66, 70}. In fact, senescent cells have been likened to carcinogenic cells, in that they both exhibit an increased resistance to apoptosis⁷¹⁻⁷³. Furthermore, senescent cells are potentially harmful as they may acquire a SASP, which involves the secretion of a panel of factors mainly consisting of growth factors, pro-inflammatory cytokines, metalloproteinases, and chemoattractants^{74, 75}. The exact composition of the SASP varies slightly depending on cell type and/or stressor – however, it is consistently pro-inflammatory in nature^{23, 74}. Due to the SASP, senescent cells have been proposed to be involved in causing, or aggravating, many chronic diseases characterised by low-grade inflammation, such as DM, Alzheimer’s disease, osteoarthritis, and cancer^{23, 70}. As reviewed by Burton *et al.*⁷⁶, IL-6 – a prominent SASP component – appears to promote osteoblast transformation, thereby further linking senescence, (local) inflammation, and VC. Because of the SASP, senescent cells are able to affect tissue and organ function disproportionately to their numbers.

Senescence of VSMCs has been repeatedly linked to VC^{65, 76, 77}. In a study by Buendía *et al.*⁷⁸, endothelial microparticles (EMPs) from CKD patient serum as well as EMPs from senescent human umbilical vein endothelial cells (HUVECs) initiated VSMC calcification and osteogenic transformation *in vitro*. Prelamin A, a protein associated with Hutchinson-Gilford progeria syndrome, is of particular interest in this context, as aged VSMCs express significantly

greater quantities of this protein than young VSMCs do⁷⁹. This accumulation of prelamin A is also associated with the presence of atherosclerosis in human arteries⁷⁹, and triggers osteogenic differentiation of VSMCs through activation of DNA damage signalling⁸⁰.

1.2.2 Apoptosis

Apoptosis, from ancient Greek “falling off”, describes the process of “programmed cell death”. It results in the removal of a particular cell, and can occur due to a number of reasons (summarized in **Figure 1**). The presence of apoptotic bodies is linked to VC, as they can serve as nidi for mineral nucleation, and have also been shown to initiate the process of calcification⁸¹⁻⁸³. In addition, dialysis treatment *per se* can induce apoptosis in arterial tissue, and this increase in cell death is associated with an elevated risk of CVD⁸⁴⁻⁸⁶.

Importantly, both senescence and apoptosis have been associated with atherosclerosis and endothelial dysfunction⁸⁷⁻⁹⁰.

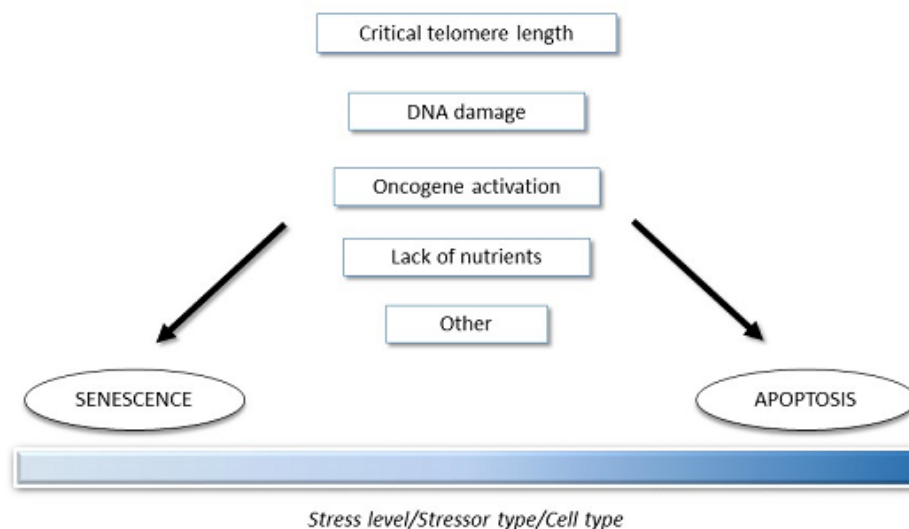


Figure 1. Triggers of senescence and apoptosis. Information adapted from Childs *et al.*⁹¹

1.2.3 Estimation of biological age – telomere length

There are several ways of assessing biological age. By far, the most common method is to measure telomere length and attrition rate.

Telomeres are the structures at the ends of the DNA chromosomes (**Figure 2**). They consist of a number of TTAGGG nucleotide repeats, which form a protective cap at the end of the DNA molecule to prevent DNA degradation and end-point fusion of chromosomes⁹². Due to a phenomenon known as the end-replication problem, the telomeres gradually shorten with each cell division until the so-called Hayflick limit is reached⁹³⁻⁹⁵. When this occurs, the

telomeres signal to the cell to cease cell division through the DNA damage response (DDR)⁹⁶⁻⁹⁸, thereby triggering cellular senescence.

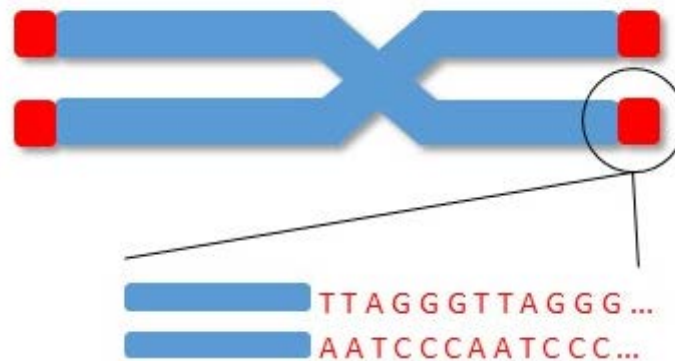


Figure 2. Schematic overview of the telomere.

Telomere attrition rate is determined not only by cellular proliferation, but also by factors such as persistent inflammation⁹⁹, oxidative stress¹⁰⁰, and hyperhomocysteinemia¹⁰¹, which are all part of the uremic phenotype. However, little is known about how drugs given during prolonged periods, such as immunosuppressants, affect telomere attrition.

1.2.3.1 Folic acid, methylation and telomere length

Folic acid, or folate, is the primary methyl donor in DNA methylation and nucleotide biosynthesis¹⁰²⁻¹⁰⁴. When folic acid levels are low, there is insufficient conversion of the nucleotide deoxyuridine monophosphate (dUMP) into the nucleotide deoxythymidine triphosphate (dTTP), resulting in increased misincorporation of dUMP into the growing DNA molecule during replication¹⁰⁵. This leads to decreased DNA stability and an elevated risk of DNA double strand breaks¹⁰⁵. A well-known clinical consequence of folate deficiency during pregnancy is spina bifida. Furthermore, low folic acid results in reduced DNA methylation, which in turn may affect telomere length, although findings are equivocal regarding the relationship between DNA methylation status and telomere length. A number of studies have focused on the association between telomere length and homocysteine levels, which are inversely correlated with folic acid¹⁰⁶. Whereas hyperhomocysteinemia and low folic acid are associated with an increased risk of CVD¹⁰⁷, as well as with decreased telomere length^{101, 108-110}. DNA hypomethylation has been associated with increased telomere length¹¹¹. Since higher folic acid has also been associated with decreased telomere length¹¹², the relationships between folic acid, telomere length, and DNA methylation status are as yet unclear.

1.2.3.2 Telomere length and disease

As many of the common chronic diseases are characterised by persistent low-grade inflammation, and inflammation is associated with telomere length¹¹³, a number of studies have investigated telomere length in patient populations with various forms of chronic disease. Truncated telomere length is associated with inflammatory diseases such as rheumatoid arthritis¹¹⁴, ulcerative colitis¹¹⁵, depression¹¹⁶, chronic obstructive pulmonary disease¹¹⁷, and CVD, both in the general population and in moderate CKD^{118, 119}. In addition, shorter telomeres are associated with premature mortality¹²⁰.

The link between inflammation and telomere shortening suggests that interventions which reduce the inflammatory burden could result in reduced telomere attrition. An example of such an intervention is physical exercise, which has been shown to reduce inflammation^{121, 122}.

1.2.4 Estimation of biological age – Cyclin-dependent kinase inhibitor 2A

The cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene is located in chromosomal region 9p21. It is a known tumour suppressor gene, and is often mutated in various forms of cancer^{123, 124}. The gene encodes at least three different proteins: p16^{INK4a}, p15^{INK4b} and a protein resulting from an alternate open reading frame (ARF)¹²⁵, all of which impair cell division and promote senescence. p16^{INK4a} inhibits the cell cycle by binding to the cyclin-dependent kinase 4 or 6 (CDK4/6) protein in the cytoplasm, thereby blocking its interaction with cyclin D¹²⁶. Thus, CDK4/6 is unable to phosphorylate the retinoblastoma (Rb) protein, which leads to the detainment of E2F transcription factor 1 (E2F1) in the cytoplasm and the transition from G1 to S phase of the cell cycle is therefore unable to take place¹²⁷. This eventually leads to irreversible cell cycle arrest, which contributes to cellular ageing. In **Study I**, we have focused primarily on the p16^{INK4a} isoform of the *CDKN2A* gene.

Through its actions as an inhibitor of the cell cycle, *CDKN2A* and its cognate proteins have been linked to increased senescence¹²⁸⁻¹³¹. In addition, *CDKN2A* expression has been proposed to be superior to telomere length as a marker of biological age^{128, 129}. Typically, p16 levels increase with age in most mammalian tissue types, including stem cells and β cells¹²⁷.

There are several stimuli that increase the *CDKN2A*, and/or the p16^{INK4a}, expression, such as oncogene (RAS)-mediated senescence and the DDR through p53¹²⁷. When the DNA is damaged, a response is activated to prevent the cell with defective DNA from dividing. As oxidative stress is a prominent feature in the uremic milieu, it is likely that this is a major inducer of p16^{INK4a} activity in this patient group.

1.2.4.1 *CDKN2A and disease*

The 9p21 region contains several single nucleotide polymorphisms (SNPs) that consistently show robust associations with CVD¹³²⁻¹³⁴. These associations, in combination with the findings regarding gene expression and protein levels, indicate that there is a functional relationship between this locus and CVD.

Through its effects on senescence, p16^{INK4a} has been proposed as a causative factor in the detrimental effects of ageing cells on organismal health²³. Using a transgenic mouse model to eliminate p16^{INK4a} –positive cells accumulating with age, Baker *et al.*¹³⁵ recently found that ageing-associated dysfunction in adipose and cardiac tissue was decreased. In addition, lifespan was extended up to 25% in mice that had undergone removal of p16^{INK4a} –positive cells, with no visible effect on tumorigenesis. Interestingly, clearance of p16^{INK4a} –positive cells in kidney was associated with a reduction in glomerulosclerosis¹³⁵, indicating that senescence might not just be a consequence of CKD, but possibly also a causative agent of it.

1.3 DNA METHYLATION

The DNA molecule can undergo various chemical modifications, which regulate the usage of the genetic material and are vital for appropriate gene expression according to external stimuli and cell phenotype. Such chemical modifications include the attachment of acetyl and methyl groups to different parts of the molecules that make up the DNA sequence and the nucleosome backbone¹³⁶. While acetyl groups are commonly attached to the histones of the nucleosome and are associated with a more open chromatin structure, a methyl group attached to the 5'-position of a cytosine nucleotide attached to a guanosine nucleotide is associated with a closed chromatin structure and reduced gene expression. Such methylation is termed 5'-CpG methylation, and is common throughout the genome¹³⁷. Methylation in promoter regions is typically associated with a repressive effect on gene activity. Regions of 200 base-pairs containing at least 50% guanine and/or cytosine residues, and where the observed/statistically expected ratio of CpG dinucleotides is at least 0.6, are termed CpG islands (CGIs)¹³⁸. CpG islands in particular are associated with gene silencing, and about 60% of all gene promoters in human cells contain CGIs¹³⁸. Another reason for the focus on 5'-CpG methylation is technical feasibility; today, there are genome-wide arrays that allow high-throughput, rapid analysis of methylation status of as many as 850,000 separate CpG sites, such as the Illumina Infinium HumanMethylation450 BeadChip¹³⁹ that was used in **Study III**. While this array places much emphasis on CGIs, it is important to keep in mind that an increasing number of studies have shown that it is the methylation status of regions with low numbers of CpG dinucleotides that appears to be important for cell- and tissue-specific differentiation^{140, 141}.

It has been proposed that inflammatory status may be affected by the degree of DNA methylation. Altered methylation of a number of genes encoding pro-inflammatory molecules, such as *IL-6*, *STAT3*, and *TLR* genes, has been linked to the presence of chronic, low-grade inflammation in obesity, DM, CVD, and CKD²⁷. Exercise, with its anti-inflammatory effects²⁷, has been shown to affect the methylation status of a number of genes associated with inflammation and/or metabolic disease, such as *TNF*¹⁴², *IL-10*¹⁴², *PGC-1 α* ¹⁴³, *PPAR- δ* ¹⁴³, and *RUNX1*¹⁴⁴.

1.3.1 Chronic kidney disease and DNA methylation

Uremic toxins appear to have the potential to alter the methylation status of the genome¹⁴⁵, possibly through their pro-inflammatory and oxidative stress effects. The fact that hyperhomocysteinemia is a prominent feature of CKD further adds to the concept of altered DNA methylation status in the uremic milieu. As mentioned previously, folic acid is the main methyl group donor in the cell, and reduced levels result in hypomethylation of the genome¹⁴⁵. Despite these biologically plausible theories, only a few studies have focused on 5'-CpG methylation in CKD.

1.3.1.1 Global DNA methylation studies

In a cohort of stage 3-5 CKD patients, Stenvinkel *et al.*¹⁴⁶ found that inflammation was significantly associated with a relatively higher degree of DNA methylation as assessed by the luminometric methylation assay (LUMA). Patients with hypermethylated DNA had a worsened survival compared to those whose degree of methylation was below the median.

Ingrosso *et al.*¹⁴⁷ found that the DNA from HD patients with hyperhomocysteinemia was hypomethylated compared to healthy controls. However, since their finding was not replicated in a study comprising stage 2-4 CKD patients¹⁴⁸, conclusions in this area are difficult and likely dependent on context and degree of renal dysfunction.

1.3.1.2 Gene-specific DNA methylation studies

Differences in DNA methylation have been associated with changes in renal function as assessed by estimated GFR (eGFR) in a cohort of nearly 4 000 CKD patients¹⁴⁹. Using the Infinium HumanMethylation 450K BeadChip – similar to the method used in **Study III** in this thesis – the authors found several gene-specific differences in CpG methylation when comparing individuals with stable renal function to patients with decreasing eGFR. Genes displaying differences in methylation between the two patient groups include *NOS3*, *TGFB3*, *CLU*, *IQSEC1*, etc., and are known to be involved in pathways related to inflammation, renal fibrosis, oxidative stress, and epithelial to mesenchymal transition. In a study of genome-wide methylation profiling of HD patients and age- and gender-matched healthy controls, Zawada *et al.*¹⁵⁰ found a large number of atherosclerosis-related genes that were differentially methylated in the two groups. These genes had functions related to

inflammation, angiogenesis, cell cycle regulation, and lipid metabolism. Furthermore, methylation differences between diabetic patients with or without nephropathy were analysed by Sapienza *et al.*¹⁵¹, in an attempt to identify ESRD-specific genes while eliminating those with diabetes-induced methylation changes. Of the 187 genes displaying differences in methylation between the two groups, many had previously been associated with diabetic nephropathy and renal development, or had been found to be transcriptionally altered during dialysis treatment¹⁵¹. Judging by these studies, it appears that there are many potentially functionally relevant methylation changes in CKD patients, opening the door for new avenues of studies in this patient group. It is also interesting to note that genes involved in cellular proliferation and/or cell cycle regulation are repeatedly identified in these studies, further highlighting the importance of these processes in CKD.

2 AIMS

The overall aim of the thesis was to examine the involvement of genetic and epigenetic factors in inflammation, biological age and clinical outcome in ESRD.

Study-specific aims were as follows:

Study I: To determine the expression of the *CDKN2A* gene in arterial and muscle biopsies from ESRD patients and to investigate correlations with age, CVD, VC, and coronary artery calcification (CAC) score.

Study II: To determine longitudinal changes in telomere length in dialysis and RTx patients during their first 12 months of RRT, and to investigate possible associations with different types of immunosuppressive treatment.

Study III: To investigate potential differences in global 5'-CpG methylation between ESRD patients and age- and gender-matched healthy controls, and to examine longitudinal changes in global methylation in dialysis and RTx patients during their first 12 months of RRT.

Study IV: To investigate whether there is any association between the degree of physical activity and global DNA methylation in a group of Swedish senior citizens.

Study V: To identify factors associated with an inflamed phenotype in CKD through the use of a mathematical model enabling simultaneous analysis of a large number of genotypes and phenotypes.

Study VI: To investigate whether the *CCR5*Δ32 variant is associated with differences in mortality in ESRD patients with or without inflammation.

3 MATERIALS AND METHODS

More detailed information on Materials and Methods are found in the six studies included in the thesis.

3.1 COHORTS

3.1.1 KärI-Tx cohort

The KärI-Tx cohort is constituted by ESRD patients undergoing living donor (LD) RTx at the Karolinska University Hospital, Stockholm, Sweden. The cohort and its inclusion criteria have been described previously¹⁵². Briefly, whole blood, skeletal muscle, and abdominal subcutaneous fat (ASCF) were obtained from patients 1-2 days before LD RTx. Epigastric artery, ASCF, and muscle biopsies were obtained within 20 minutes after skin incision. Muscle samples were collected from the transverse abdominal muscle, or from the external or internal oblique muscle. All samples were placed in AllProtect Tissue Reagent (Qiagen) and stored at -70°C until RNA preparation. For immunohistochemistry (IHC), parts of the biopsies were fixed in 4% phosphate buffered formalin, embedded in paraffin, and cut into 1-2 µm thick tissue sections. Medial calcification category (0-3) for each patient was determined by an experienced pathologist using 1-2 sections of van Kossa-stained arterial tissue; the degree of VC in each category has been described previously¹⁵². In addition, degree of VC (%) was calculated as (calcified area)/(area of tunica media and intima) using a Nikon Eclipse E1000 light microscope and Fiji software. The degree of VC in the different categories is exemplified in **Figure 3**.

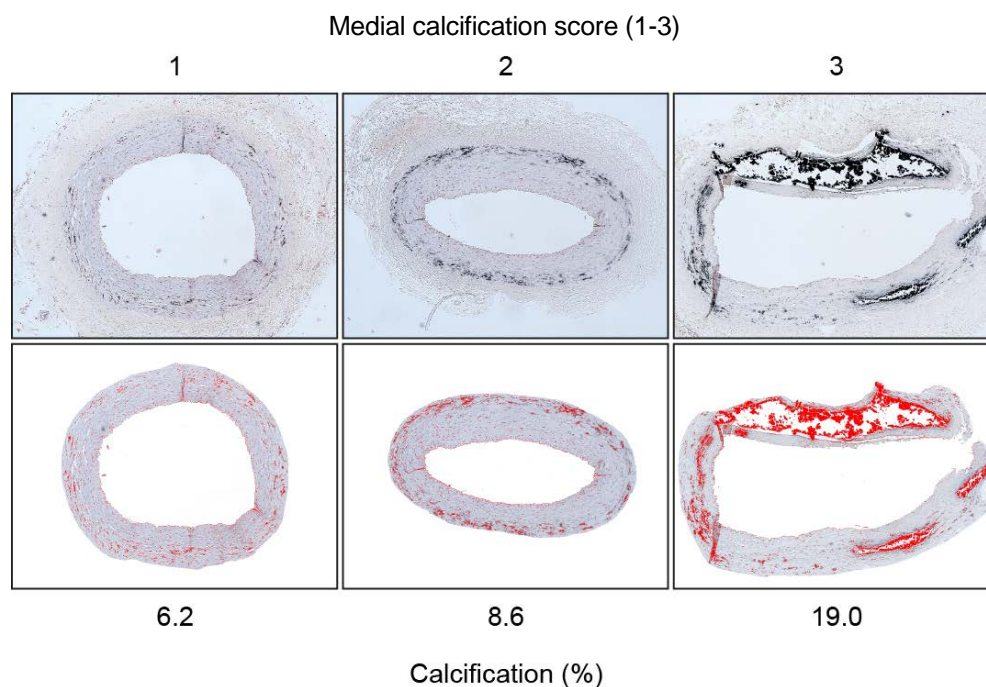


Figure 3. Degree of calcification in the KärI-Tx cohort.

Clinical CVD was defined as the presence of cerebrovascular, cardiovascular, and/or peripheral vascular disease. Total CAC score was obtained by adding CAC scores in the left main artery, the left anterior descending artery, the left circumflex artery, and the right coronary artery.

Patients from this cohort were included in **Study I** (n=81), **II** (n=47) and **III** (n=12).

3.1.2 MIA cohort

The Malnutrition, Inflammation and Atherosclerosis (MIA) cohort consists of incident dialysis patients at the Karolinska University Hospital, Stockholm, Sweden. More detailed information on inclusion criteria can be found in Stenvinkel et al¹⁷. Briefly, whole blood is obtained from all included subjects shortly before the initiation of dialysis treatment.

Patients from this cohort were included in **Study II** (n=49), **III** (n=12) and **V** (n=225).

3.1.3 PIVUS cohort

The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) cohort consists of individuals residing in Uppsala, Sweden, aged 70 years and above¹⁵³. Written invitations to participate were sent out within two months of the subjects' 70th birthdays. In total, 1,016 individuals agreed to be included in the study, corresponding to a participation rate of 50.1%. Subjects were carefully phenotyped for cardiovascular risk factors, and all individuals who provided a self-reported estimate of physical activity were included in the study (n=509).

This cohort was included in **Study IV** (n=509).

3.1.4 NECOSAD cohort

The prospective Netherlands Cooperative Study on the Adequacy of Dialysis (NECOSAD) cohort consists of ESRD patients enrolled at RRT initiation from 38 Dutch dialysis centres¹⁵⁴. All patients above the age of 18 years who had had no previous RRT were eligible for inclusion.

A part of this cohort was included in **Study VI** (n=413).

3.1.5 Control populations

In **Study II**, a control population established in Glasgow was investigated. These controls included individuals without overt renal disease residing in Glasgow, and have been described in part previously¹⁵⁵.

In **Study III**, a population-based, randomly selected cohort called PRIMA controls was used. Participants were selected using Statistics Sweden (SCB), and the only exclusion criteria was unwillingness to participate.

3.2 STUDY I

3.2.1 Protein and biomarker measurements

All measurements of protein and biomarker levels were done in whole blood, plasma, or serum samples, and were performed at the Clinical Laboratory at Karolinska University Hospital, Stockholm, Sweden. Levels of IL-6, IL-8, and TNF were measured using an Immulite 1000 Analyzer according to the manufacturer's instructions. Pentosidine levels were measured using reverse-phase HPLC with gradient separation under fluorescence detection (excitation-emission: 335/385 nm) and 8-OHdG was measured with a commercial competitive enzyme-linked immunosorbent assay kit. Validated routine methods were used for measuring hsCRP, parathyroid hormone, plasma cholesterol, triglycerides, high-density lipoprotein (HDL)-cholesterol, creatinine, albumin, calcium, phosphate, and D-vitamin.

3.2.2 RNA preparation and gene expression analysis

Total RNA was prepared from epigastric artery and muscle using TRIzol Reagent. RNA quality and concentration were determined using an Agilent 2100 Bioanalyzer and a NanoDrop spectrophotometer, respectively. From RNA, cDNA was prepared using random hexamers and gene expression was determined using individual TaqMan® gene expression assays for target genes *CDKN2A*, *MGP* and *RUNX2*. A minimum of one reference gene per gene assay was included.

3.2.3 Computed tomography

A 64-channel detector was used to obtain cardiac computed tomography (CT) data. The amount of calcium deposition in coronary arteries was determined by a radiologist, and CAC was calculated using Agatston score (based on Hounsfield units)¹⁵⁶. Total CAC score was calculated by adding CAC scores of the right coronary artery, the left circumflex artery, the left anterior descending artery, and the left main artery. Calcified plaques were considered to be present at > 130 Hounsfield units.

3.2.4 Immunohistochemistry

Immunohistochemical staining of p16^{INK4a} protein was performed using an anti-CDKN2A/p16^{INK4a} polyclonal antibody on paraffin-embedded sections of epigastric artery. Diaminobenzidine (DAB) and hematoxylin were used for staining and counterstaining, respectively. The negative control consisted of a section without the primary antibody.

3.2.5 Statistical analysis

Statistical significance was set at $p < 0.05$. All statistical analyses were performed using non-parametric methods, since *CDKN2A/p16^{INK4a}* distribution was non-normal. Hence, Wilcoxon rank sum test or χ^2 test was used for categorical comparisons; Spearman's rank correlation

was used for determining correlation between continuous variables; and multivariate analysis was done using general linear model (GLM).

3.3 STUDY II

3.3.1 Protein and biomarker measurements

All measurements were performed similarly to the description provided for study I (see section 3.2.1 for further details).

3.3.2 Telomere length measurements

Telomere length in whole blood was measured using the protocol developed by Cawthon, which is based on the quantitative PCR and Southern blot methods to determine the length of the telomeric region relative to a single-copy gene present in the genome¹⁵⁷. Briefly, all samples were analysed in triplicate using *36B4* as a single-copy reference gene. The sample signal derived from the run using the telomere primers was divided by the signal obtained using *36B4* primers, and all samples were compared to a reference DNA sample.

Telomere attrition ($\Delta T/S$) was calculated as (telomere length at 12 months) – (telomere length at baseline).

3.3.3 Statistical analysis

All analyses were performed using non-parametric tests. For categorical data, Wilcoxon rank sum test/ χ^2 test or Fisher's exact test was used. Spearman's rank correlation coefficient was used for analysis of continuous data and Wilcoxon matched-pairs signed rank test was used for paired analysis of baseline and 12 month data.

3.4 STUDY III

3.4.1 Global DNA methylation analysis

Global methylation status of bisulfite-treated DNA was assessed using the Illumina Infinium HumanMethylation450 BeadChip. Included in the study were 12 patients from the MIA cohort, 12 patients from the KärI-Tx cohort and 24 healthy controls from the PRIMA cohort. For each patient, there was a baseline sample and a second sample obtained 12 months after therapy initiation. The degree of methylation was defined as the signal intensity of methylated alleles divided by the added intensity signals of methylated + unmethylated alleles.

3.4.2 Statistical analysis

For normalisation of data from the Illumina Infinium HumanMethylation450 BeadChip, quantile colour adjustment was used. Bioconductor packages lumi and limma were used for further data analysis. First, limma was used to find CpG sites that displayed different

methylation status when comparing patients to controls. Group comparisons of these sites were then made as follows: (1) dialysis patients *versus* RTx patients at baseline; (2) dialysis patients *versus* RTx patients after 12 months; (3) dialysis patients at baseline *versus* dialysis patients after 12 months; (4) RTx patients at baseline *versus* RTx patients after 12 months. In addition, CpG sites that remained differently methylated compared to controls in both patient groups over time were noted. Results were considered significant if the false discovery rate (P_{FDR}) was less than 0.01. All probes overlapping DNA sequences containing SNPs were removed from analysis.

3.4.3 Pathway analysis

Using the output from the statistical analysis, CpG sites displaying large differences in methylation in any of the above-mentioned comparisons that had a gene annotation were entered into Ingenuity® Pathway Analysis (IPA®).

3.5 STUDY IV

3.5.1 Participant selection

Subjects in the PIVUS cohort were grouped according to the degree of self-reported physical activity:

Group 1 – Light physical activity < 2 times/week and no heavy physical activity (n=56);

Group 2 – Light physical activity > 1 time/week and no heavy physical activity (n=290);

Group 3 – Light physical activity > 1 time/week and heavy physical activity 1-2 times/week (n=129);

Group 4 – Light physical activity > 1 time/week and heavy physical activity > 2 times/week (n=34).

3.5.2 Measurement of global DNA methylation

Global DNA methylation was measured using LUMA, described in detail by Karimi *et al*¹⁵⁸. Briefly, 250-750 ng of DNA prepared from whole blood was subjected to enzymatic restriction treatment by either a combination of EcoRI (New England Biolabs), MspI (New England Biolabs), and Tango™ buffer (Fermentas), or EcoRI, HpaII (New England Biolabs), and Tango™ buffer. Samples were incubated for four hours at 37°C and subsequently analysed using Pyrosequencing®. For each sample, the MspI-specific and HpaII-specific peak heights were normalised using EcoRI as an internal control, and the normalised values were used to calculate the HpaII/MspI ratio. As HpaII is methylation-sensitive whereas MspI is not, a higher HpaII/MspI ratio indicates a lower level of global DNA methylation.

3.5.3 Statistical analysis

For testing the association between physical activity and DNA methylation, ANOVA analysis was used. For studying the involvement of potential confounders (gender, fasting glucose, systolic and diastolic blood pressure, serum triglycerides, body mass index (BMI), low-density lipoprotein (LDL)- and HDL-cholesterol, and smoking status), ANCOVA analysis was performed.

3.6 STUDY V

3.6.1 Patient selection

From the MIA cohort, a set of 225 individuals with less than 5% missing data was eligible for analysis using the relaxed linear separability (RLS) method. Features that had been measured in a minimum of 65% of the patients were included in the feature set. Nearest neighbour imputation was used to estimate missing data. In total, 57 anthropometric or biochemical measurements (phenotypes) and 228 genotype features were entered into the model, which was asked to differentiate between inflamed and non-inflamed patients. Presence/absence of inflammation was defined as hsCRP above/below median (5 mg/L), respectively.

3.6.2 Relaxed Linear Separability method

The application of the RLS method in this particular dataset has been described previously by Bobrowski et al¹⁵⁹. Briefly, the RLS method, combined with Convex and Piecewise – Linear (CPL) criterion functions was used to identify features that differ between inflamed and non-inflamed patients. The combination of features with the best predictive power was determined by the smallest apparent error (AE) and the lowest cross-validation error (CVE). A factor value, indicating predictive strength, was obtained for each feature included in the RLS model. For comparison purposes, univariate analysis (Spearman’s rank correlation coefficient) and principal component analysis (PCA) were performed on all features included in the resulting RLS model.

3.7 STUDY VI

3.7.1 Clinical data and definitions

In the NECOSAD cohort, the first blood and urine samples were collected three months after dialysis initiation. In the MIA cohort, blood and urine samples were obtained near dialysis initiation. In this study, presence of inflammation was defined as an hsCRP level > 10 mg/L.

Causes of death and primary kidney disease was categorised according to definitions from the European Renal Association-European Dialysis and Transplantation Association (ERA-EDTA)¹⁶⁰. In both cohorts, cardiovascular mortality was defined as myocardial ischemia and infarction, haemorrhage due to ruptured vascular aneurysm, hyperkalaemia, hypokalaemia,

cardiac failure/fluid overload/pulmonary oedema, cardiac arrest due to unknown cause(s), mesenteric infarction, cerebrovascular accident, or unknown cause of death.

3.7.2 Genotyping of CCR5Δ32 polymorphism

3.7.2.1 NECOSAD cohort

In the NECOSAD cohort, genotyping of the CCR5Δ32 variant was performed using a PCR-based allelic discrimination assay (5' nuclease assay), as described by Clark *et al*³⁸.

3.7.2.2 MIA cohort

In the MIA cohort, genotyping of the CCR5Δ32 variant was performed using PCR amplification followed by fragment size separation on 3% agarose gel with ethidium bromide staining.

3.7.3 Statistical analysis

Hardy-Weinberg equilibrium was calculated using the gene counting method. The main outcomes were set to all-cause and cardiovascular mortality within 5 years of follow-up. For categorical variables, χ^2 analysis was used, and one-way ANOVA was used for analysis of continuous data. Survival analysis was performed using the Kaplan-Meier method, with log-rank test to calculate differences in survival between groups. Hazard ratios (HR) of adjusted (gender, BMI, age at inclusion, DM, presence of CVD, and dialysis modality) as well as unadjusted mortality rates were calculated using Cox's proportional-hazard analysis. To examine the interaction between inflammatory status and CCR5Δ32 genotype, patients were divided into four groups: (1) *CCR5* ins/ins, hsCRP \leq 10 mg/L; (2) *CCR5* ins/ins, hsCRP > 10 mg/L; (3) *CCR5* ins/del or del/del, hsCRP \leq 10 mg/L; (4) *CCR5* ins/del or del/del, hsCRP > 10 mg/L.

4 RESULTS AND DISCUSSION

4.1 STUDY I

Patients suffering from CKD experience an increase in CVD morbidity and mortality disproportionate to their chronological age^{13, 15}, suggesting an accelerated biological ageing process. In this study, gene expression of biological age marker *CDKN2A/p16^{INK4a}* was analysed in epigastric artery and muscle obtained from ESRD patients, and possible differences in expression according to degree of VC, CAC score, and presence or absence of CVD, were investigated. Furthermore, arterial *CDKN2A/p16^{INK4a}* expression was associated with arterial expression of *MGP* and *RUNX2*, both of which are involved in osteogenic transformation of VSMC⁶¹⁻⁶⁴.

4.1.1 Age and *CDKN2A/p16^{INK4a}* expression

When analysing the correlation of *CDKN2A/p16^{INK4a}* expression with age, the association was significant in muscle ($p=0.0003$, $\rho=0.33$), but did not reach statistical significance in artery ($p=0.07$, $\rho=0.28$).

4.1.2 Cardiovascular disease, vascular calcification and *CDKN2A/p16^{INK4a}* expression

There was no significant difference in muscle *CDKN2A/p16^{INK4a}* expression according to CVD status. However, *CDKN2A/p16^{INK4a}* expression was significantly higher in arteries from patients with CVD compared to those with no CVD ($p=0.001$) (**Figure 4**).

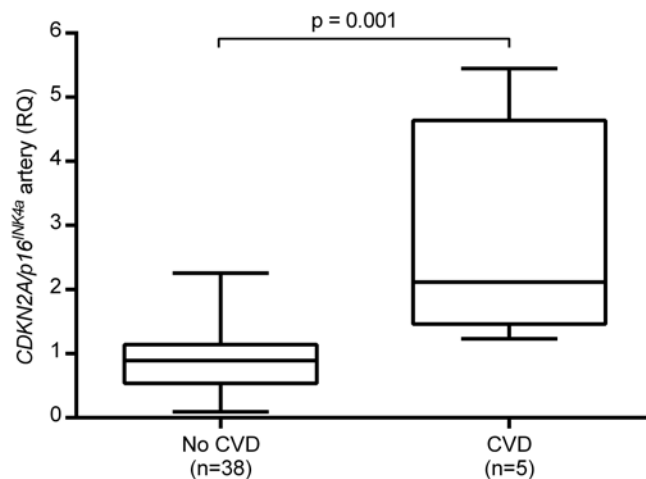


Figure 4. Arterial *CDKN2A/p16^{INK4a}* expression in ESRD patients with and without CVD.

Similarly, while there was no significant correlation between VC score and *CDKN2A/p16^{INK4a}* expression in muscle, arterial *CDKN2A/p16^{INK4a}* expression was significantly and positively correlated with VC score ($p=0.01$, $\rho=0.4$), and with degree of CAC by CT heart ($p=0.008$, $\rho=0.46$) (**Figure 5A-B**).

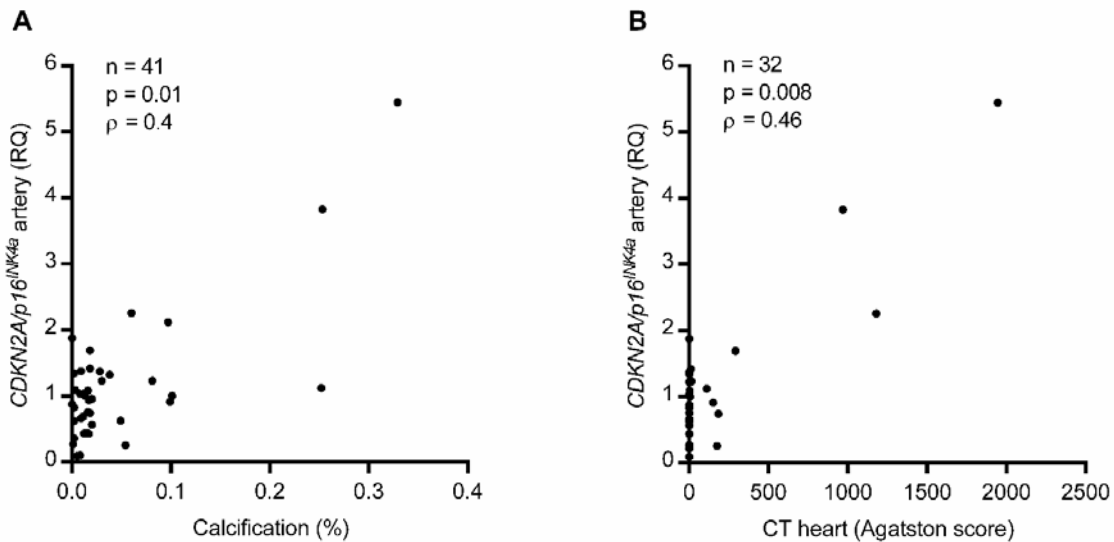


Figure 5A-B. Correlation between arterial $CDKN2A/p16^{INK4a}$ expression and VC (A), and between arterial $CDKN2A/p16^{INK4a}$ expression and CAC score as measured by CT heart (B).

4.1.3 Osteogenic markers and $CDKN2A/p16^{INK4a}$ expression

Arterial $CDKN2A/p16^{INK4a}$ expression was significantly and positively correlated with both *MGP* ($p=0.007$, $\rho=0.41$) and *RUNX2* ($p=0.046$, $\rho=0.33$) expression in artery.

4.1.4 Arterial expression of $p16^{INK4a}$

Immunohistochemical analysis of arterial $p16^{INK4a}$ was performed in a subset of patients with varying degrees of VC (n=4). The protein expression appeared to mimic the gene expression, in that higher degrees of VC were associated with increasing $p16^{INK4a}$ expression.

4.1.5 Discussion

Study I showed significant associations between higher arterial expression of biological age marker $CDKN2A/p16^{INK4a}$ and the presence of VC and clinical CVD. This indicates a relationship between elevated cellular senescence and CVD. As numerous SNPs in the vicinity of *CDKN2A* have been robustly associated with CVD, the findings strengthen the hypothesis that there is a functional relationship between $CDKN2A/p16^{INK4a}$ and CVD. The correlation between increasing senescence, VC and CVD is consistent with current literature, where endothelial dysfunction and atherosclerosis has been linked to the presence of senescent cells⁸³⁻⁸⁶.

Numerous factors present in the uremic milieu may contribute to an elevated level of cellular senescence – inflammation, oxidative stress and uremic toxins, to name a few. The persistent exposure to these factors is likely to accelerate the cellular ageing process, as shown in **Figure 1**.

The positive correlation between arterial expression of *CDKN2A/p16^{INK4a}* and osteogenic factors *MGP* and *RUNX2* suggests a link between cellular senescence and osteogenic potential in arterial tissue. While *RUNX2* stimulates the osteogenic transition of VSMCs, *MGP* is a calcification inhibitor. The positive correlation between *CDKN2A/p16^{INK4a}* and *RUNX2* indicates a link between senescence and osteogenic transformation, thereby confirming other studies^{65, 76, 77}. The positive correlation between *MGP* and *CDKN2A/p16^{INK4a}*, while seemingly counter-intuitive, may reflect an attempt to inhibit an ongoing calcification process, and accords with previous findings⁶⁵.

With the exception of an association with chronological age, all significant findings were observed in arteries only. As skeletal muscle is considered post-mitotic¹⁶¹, the lack of associations in muscle is biologically plausible. In addition, the link to VC is functionally relevant in artery, but not in muscle, and as VC is a major risk factor for CVD, this may explain the lack of significant associations in muscle. Similarly, CAC score was positively associated with *CDKN2A/p16^{INK4a}* expression in artery, further indicating that arterial *CDKN2A/p16^{INK4a}* expression may be a functional biomarker for biological vascular age in ESRD patients.

Some study limitations should be mentioned. The study population is a select group of patients with a relatively low CVD prevalence, and in some regards a healthier clinical profile than older, more severely ill, ESRD patients. The number of arterial RNA samples was reduced because of insufficient RNA quality, which was due to small biopsies. As all RNA samples were prepared using a homogenized tissue sample, it is impossible to conclude whether there were any cell-specific differences in *CDKN2A/p16^{INK4a}* expression. Furthermore, statistical analysis of the IHC data could not be performed due to the low number of samples. Hence, more samples should be analysed in order to confirm the findings in this pilot study. In addition, it is important to remember that since VC is a locally occurring phenomenon in the artery, the gene expression profile and the calcification and/or CAC score may not correlate even though the patient is calcified.

In summary, Study I indicates that cellular senescence is related to VC, and consequently, CVD in an ESRD population. In addition, the findings suggest a link between osteogenic potential and senescence, thus providing a partial explanation for the association between accelerated biological ageing and calcification.

4.2 STUDY II

Patients suffering from CKD have been found to have decreased telomere length compared to healthy individuals¹¹⁹, indicating accelerated biological ageing. However, the impact of RRT on the degree of telomere attrition has not been studied. In addition, it is not known what impact immunosuppressive treatment has on telomere length. To this end, Study II focused on measuring whole blood telomere length longitudinally in ESRD patients undergoing dialysis or RTx, to compare it to telomere length in a non-CKD population, and to assess possible differences in telomere length between different anti-metabolite immunosuppressive therapies in the RTx patient population.

4.2.1 Telomere length in patients and controls

At baseline, both dialysis patients (median age 55 years) and RTx patients (median age 45 years) had significantly shorter telomeres than the non-CKD controls (median age 58 years) ($p=0.0007$ and $p=0.03$, respectively), despite being significantly younger ($p<0.0001$).

Following 12 months of RRT, the degree of telomere attrition was significantly greater in RTx than in dialysis patients ($p=0.008$). Notably, there was no significant difference in telomere length between the two patient populations at baseline.

4.2.2 Telomere length and folic acid

In RTx patients at baseline, but not in dialysis patients, telomere length was significantly correlated with levels of folic acid ($p=0.04$, $\rho=-0.33$) and homocysteine ($p=0.04$, $\rho=0.29$).

4.2.3 Telomere length, folic acid and anti-metabolite therapy

Following 12 months of RRT, RTx patients receiving the anti-metabolite mycophenolic acid displayed a larger degree of telomere attrition than those treated with azathioprine ($p=0.007$), whose attrition resembled that seen in dialysis patients.

After 12 months, RTx patients receiving mycophenolic acid had higher folic acid levels than those treated with azathioprine ($p=0.008$), despite the absence of a significant difference between the groups at baseline ($p=0.25$).

4.2.4 Discussion

This study supports the notion that ESRD patients undergo premature ageing and are biologically older than non-CKD controls. Although the controls were chronologically older than the ESRD patients, they had longer telomeres, lending further credibility to the hypothesis regarding the progeric nature of ESRD.

Our finding that RTx patients showed a larger degree of telomere attrition than dialysis patients following 12 months of RRT was unanticipated. This could be due to a number of factors. The RTx patient group was younger than the dialysis patients, and attrition rate is

higher in younger subjects¹⁶². Likewise, BMI has been shown to have an effect on telomere attrition rate¹⁶³.

Another possible explanation lies in the immunosuppressive treatment. When RTx patients were analysed separately according to anti-metabolite treatment, the difference in telomere attrition between RTx and dialysis patients was entirely due to telomere attrition in the mycophenolic acid-treated group. Also, the reason for the observed differences in folic acid levels according to type of antimetabolite treatment is unknown. However, previous studies show that while both mycophenolic acid and azathioprine inhibit purine synthesis, thereby theoretically affecting the amount of the folic acid-derivative tetrahydrofolate, treatment with mycophenolic acid is associated with lower homocysteine levels than treatment with azathioprine¹⁶⁴. Consequently, as was observed in the current study, levels of folic acid should be higher in patients treated with mycophenolic acid. As folic acid and DNA methylation could impact telomere length, albeit in an unknown direction, it is likely that antimetabolite treatment caused the observed differences in folic acid, and telomere attrition.

A number of caveats should be considered. The current study would benefit from a more well-defined control population, preferably from the same general population as the patients. Also, as the immunosuppressive treatment of the RTx patients was non-randomized, it is possible that there is some unidentified clinical characteristic that separates the groups which has an impact on telomere length and/or folic acid. In addition, as it is not possible to infer any causal relationships between folic acid, telomere length, and/or immunosuppressive treatment, the findings in this study serve as a foundation for further research. Cell culture experiments would be of great interest to address the possible connections between medication, folic acid, and telomere attrition.

While previous studies investigated these topics, none examined folic acid/homocysteine, longitudinal changes in telomere length, and immunosuppressive treatment simultaneously. The potential impact of commonly used immunosuppressive treatment on biological ageing is of particular importance in this vulnerable patient group, which is constantly exposed to numerous factors that may predispose to premature ageing.

4.3 STUDY III

In CKD, there are several systemic factors that have the potential to impact the methylation status of the genome, such as inflammation, oxidative stress, and uremic toxins^{27, 142-145}.

While global DNA methylation has been investigated in CKD patient populations previously¹⁴⁶⁻¹⁵¹, the possible impact of RRT is unknown. Study III aimed to investigate the changes in global DNA methylation in patients receiving dialysis treatment or undergoing RTx, comparing baseline samples to samples obtained after 12 months of RRT. In addition, age- and gender-matched healthy controls were included to illustrate normal methylation status, and to aid in the identification of “CKD-specific” methylation alterations.

4.3.1 Methylation in patients versus controls

Both patient groups were compared to the control group, before and 12 months after RRT initiation. In addition, CpG sites that were differently methylated compared to controls in both patient groups, and at both time points, were identified and those located in a gene were further analysed with IPA®. Results are summarized below.

4.3.1.1 Distribution of significant CpG sites in different genomic regions

The majority of the significant CpG sites in RTx patients were located in regions unrelated to genes (64.3% at baseline and 64.1% after 12 months). In dialysis patients, a higher percentage of the significant CpG sites were located in promoter-associated regions (42.4% at baseline and 51.2% after 12 months).

In RTx patients, a majority of the significant CpG sites were not located in a CGI-related structure (46.3% at baseline and 47.1% after 12 months), whereas the significant CpG sites in dialysis patients were predominantly located in the island region (46.5% at baseline and 59.0% after 12 months).

Furthermore, in RTx patients, a larger proportion of the significant CpG sites were located in enhancers, than in dialysis patients (33% versus 18% at baseline, 33% versus 14% after 12 months).

4.3.1.2 CpG sites with largest methylation difference

In RTx patients at baseline, the 10 CpG sites with the largest methylation difference compared to controls were located in *TTC15*, *HLA-DRB5*, *PNKD*, *PRKAG2*, *FXYD2*, *BCL11B*, *NOSIP*, *IQSEC1*, *DGKA*, and *RARG*. After 12 months, CpG sites in *SSH3*, *HOXD13*, *EHD1*, *ACOT7*, *FAM24B*, *CDKL2*, and *RDH10* showed the largest fold change. In dialysis patients at baseline, the CpG sites with the largest methylation differences were located in the genes *D2HGDH*, *CDK6*, *AGAP3*, *PNKD*, *HIVEP3*, *RNASEH2C*, *ZNF562*, *CSGALNACT2*, and *COL6A3*. After 12 months of dialysis, the CpG sites with the largest fold change were located in *D2HGDH*, *PNKD*, *EID2*, *AGAP3*, *ETS1*, *MRFAP1*, *GBX1*, *RNASEH2C*, and *ZNF224*.

4.3.1.3 Genes with several significant CpG sites

In dialysis patients at baseline, the genes containing the largest number of significant CpG sites included *SKI*, *PRDM16*, *TRIM26*, *HDAC4*, *ZMIZ1*, *LOC146880*, *GFI1*, *HIVEP3*, and *BAHCC1*, whereas after 12 months, the largest number of significant sites were found in *MAD1L1*, *MYL9*, *LOC284837*, *SKI*, *PTPRCAP*, *PRDM16*, *MRFAP1*, *RPS11*, *FANCF*, *HDAC4*, and *C17orf64*. In RTx patients at baseline, the genes with the highest number of significant sites included *MAD1L1*, *HDAC4*, *RPTOR*, *TBCD*, *NCOR2*, *RASA3*, *KCNQ1*, *ANKRD11*, and *BCL11B*. After 12 months, *RASA3*, *MAD1L1*, *TBCD*, *RPTOR*, *DDR1*, *BANP*, *ARID3A*, *FOXK1*, *ITPKB*, and *HDAC4* had the highest number of significant CpG sites.

4.3.1.4 CpG sites with persistent differences in methylation

In total, 413 genes containing at least one significant CpG site remained differently methylated in both patient groups compared to controls over 12 months of RRT.

4.3.1.5 Ingenuity Pathway Analysis®

To investigate whether methylation differences between patients and controls were located in genes related to any particular biological function, all differently methylated CpG sites with a gene affiliation were entered into IPA®. This was done for both patient groups at both time points, as well as for those CpG sites that remained differently methylated compared to controls in both patient groups. Several of the canonical pathways were related to cell growth and/or cell proliferation, cell cycle regulation, cancer, and immune response.

4.3.2 Discussion

Until now, potential changes in global methylation during RRT have been unexplored. Our results showed that over 12 months of dialysis, or 12 months after RTx, significant differences in DNA methylation between ESRD patients and age- and gender-matched healthy controls were reduced. This could be a reflection of the observed normalisation of the toxic uremic milieu that occurs both in dialysis and RTx patients.

Many of the differently methylated CpG sites were located in genes with functions related to apoptosis, cell cycle regulation, and cell survival, such as *ANKRD11*¹⁶⁵, *ARID3A*^{166, 167}, *BANP*¹⁶⁸, *BCL11B*¹⁶⁹, *EID2*¹⁷⁰, *ETS1*¹⁷¹, *GFI1*¹⁷², *HIVEP3*¹⁷³, *MAD1L1*¹⁷⁴, *MRFAP1*¹⁷⁵, *NCOR2*^{176, 177}, *SKI*^{178, 179}, *ZMIZ1*¹⁸⁰, and *ZNF224*¹⁸¹. This is especially interesting given the association between cellular senescence and CKD-related comorbidities such as CVD. It also points to a potentially altered rate of biological ageing in CKD patients.

In addition to the link with cell cycle regulation and cellular senescence, several of the differently methylated CpG sites were located in genes associated with kidney disease, or with prominent CKD comorbidities such as CVD, DM and inflammation. Examples of these include *BCL11B*¹⁸², *COL6A3*^{183, 184}, *DDR1*^{185, 186}, *DGKA*¹⁸⁷, *HDAC4*¹⁸⁸, *KCNQ1*^{189, 190}, *NCOR2*¹⁹¹,

PRDM16^{192, 193}, *PRKAG2*^{194, 195}, and *RPTOR*¹⁹⁶. Inflammation may be the common denominator in this context, as it is associated with both CVD, DM¹⁹⁷, and CKD²⁰, and accelerates cellular ageing^{16, 19}.

While treatment effects on methylation of specific CpG sites are intriguing, the gene affiliation of the CpG sites whose methylation status remained unchanged when comparing patients and controls at either time point, is of equal interest. It is tempting to speculate that this set of genes is specific for CKD, as their methylation status was unaltered despite a normalisation of renal function. Interestingly, pathway analysis of this group of genes revealed a connection to biological ageing, and ageing-associated processes, such as oxidative stress and insulin/insulin growth factor signalling. IGF-1 signalling has been robustly associated with biological ageing¹⁹⁸, and it also induces STAT3 signalling¹⁹⁹. Meanwhile, Rac signalling is related to STAT3²⁰⁰, and is associated with various cardiac diseases and renal podocyte damage²⁰¹. Loss of the *PTEN* tumour suppressor gene induces cellular senescence²⁰². In addition, the *PTEN* promoter methylation status has been associated with the metabolic syndrome²⁰³, and PTEN protein levels have been associated with muscle wasting, a common feature of CKD²⁰⁴.

The differences in distribution of differently methylated CpG sites between the two patient populations were surprisingly consistent over 12 months of RRT. While the underlying reason(s) for this are unknown, it is possible that it reflects differences between the two groups. Indeed, ESRD patients who are not on the RTx waiting list and remain on dialysis have been found to have worse general health and long-term survival compared to patients who are on the waiting list²⁰⁵. In our dataset, patient characteristics between the groups were rather similar overall. However, dialysis patients had higher levels of IL-6, both at baseline and after 12 months, indicating an elevated degree of inflammation compared to patients selected for LD RTx. IL-6 is a prominent member of the SASP²⁰⁶, which has been hypothesized to aggravate, or even cause, age-related diseases²⁰⁷. Finally, the differences in methylation status could be due to differences in medication. RTx patients started immunosuppressive regimen 1-2 days before the baseline sample. Since the effects of steroid treatment on global methylation status are largely unknown²⁷, this possibility clearly motivates further study.

4.4 STUDY IV

Global methylation status has been associated with inflammation²⁰⁸, CVD²⁰⁹, CKD, and ESRD¹⁴⁶. Inflammation can be reduced with physical activity^{48, 52}, and there have been studies demonstrating a connection between methylation status of individual genes and amount of exercise²¹⁰⁻²¹². This study aimed to investigate the relationship between self-reported physical activity and global DNA methylation in a cohort of senior citizens (aged \geq 70 years) residing in Uppsala, Sweden.

4.4.1 Physical activity and DNA methylation

Individuals with a higher amount of self-reported physical activity had a significantly lower degree of global DNA methylation (ANOVA $p=0.01$).

After adjusting for cardiovascular risk factors (gender, BMI, systolic and diastolic blood pressure, serum triglycerides, LDL- and HDL-cholesterol, fasting glucose, and smoking status), the association remained significant (ANCOVA $p < 0.05$).

4.4.2 Discussion

The results of this study demonstrate that more physical activity is associated with a lower level of DNA methylation, a finding which remained significant after adjusting for numerous potential confounding factors that are risk factors for CVD. As physical activity reduces the presence of inflammation^{48, 52}, it could be hypothesised that lower circulating amounts of pro-inflammatory cytokines and other inflammatory factors contribute to alterations in the methylation status of the DNA molecule. This accords with previous findings in CKD patients, where a higher degree of inflammation was associated with increased global DNA methylation¹⁴⁶. However, as information regarding individual inflammatory status was lacking in this study, it is not possible to show whether DNA methylation and degree of inflammation were correlated in this particular cohort. The results could reflect a worsened overall health status in individuals with low *versus* high physical activity.

4.5 STUDY V

Being a complex disease, CKD is characterized by the presence of multiple underlying factors, which interact to form the uremic phenotype. Studying all of these factors separately is not only expensive and time-consuming, but also greatly increases the risk of false-positive associations as the number of tests performed rapidly accumulates. We explored the possibility to obtain a more comprehensive assessment by simultaneously entering a large number of genetic and/or anthropometric data points into an advanced mathematical model in order to identify features predictive of a specific outcome.

Study V was performed as a follow-up study of a previous study from our group, where the RLS method was used to identify features predictive of presence/absence of inflammation in a cohort of 225 incident dialysis patients¹⁵⁹. In the present study, we describe the results in more detail.

4.5.1 Factor summary

In the foregoing study¹⁵⁹ we showed that it was possible to separate inflamed from non-inflamed CKD patients (defined as a CRP level above or below 5 mg/L, respectively) using the RLS method. In the current study, a total of 57 anthropometric or biochemical data points and 228 genotype features were entered into the model. Of these, the smallest AE (0%) and CVE (1.8%) was obtained when 60 features were included in the analysis. Thirty-one of these 60 features were genetic, whereas the remaining 29 were anthropometric or biochemical.

All 60 features were also included in a PCA as well as univariate analysis using Spearman's rank correlation coefficient. The results showed that there was no single component that could explain a great degree of the variance in the dataset (maximum 7%), and a combination of the ten features with the highest factor values could explain only 40% of the variance. In addition, the univariate analysis identified 17 statistically significant features ($p < 0.05$) as opposed to the 60 features included in the RLS model, demonstrating that the RLS model provides added value to the conventional approach.

4.5.2 Strongest factors

The ten features with the highest predictive factor values included eight genetic features and two anthropometric or biochemical features. Specifically, these were polymorphisms in the *NAMPT*, *CIITA*, *CEP89*, *BMP2*, *NOS3*, and *PIK3CB* genes, and levels of fibrinogen and bone mineral density (BMD).

4.5.3 Discussion

We found many advantages using a mathematical method that enables simultaneous study of a large number of features. First, the number of statistical tests performed are reduced,

thereby minimising the risk of false-positive findings. Second, it represents a biologically relevant way of observing mechanisms and processes, as each feature entered in the model is considered in context with other features and not in isolation. Third, it is a less biased way of analysing the dataset, as the inclusion of all available information in the model precludes preconceptions of which feature(s) should be studied. That being said, it is important to remember that the original collection of data is obviously subject to bias.

The usefulness of this type of analysis becomes evident when subjecting the same dataset to PCA and univariate analysis. Not unexpectedly, PCA failed to identify any principal component explaining a substantial part of the variance; even when including 10 features, the variance explained only amounted to 40%. For complex diseases, such as CKD, PCA is not an entirely suitable tool. Likewise, univariate analysis is inappropriate in complex disease, albeit for different reasons. Statistically, it is unsound to perform a large number of separate tests as it greatly increases the risk of false-positive results, while correction for multiple testing can be too stringent and result in false-negative findings. The 17 features that reached statistical significance following univariate analysis were all consistent with the RLS factor values, i.e., a feature identified as having a positive effect on the presence of inflammation in the RLS model also had a positive p -value in the univariate analysis.

Many of the 60 features included in the RLS model are known to be associated with inflammation. These include, but are not limited to, the rs3091244 SNP in the *CRP* gene, as well as fibrinogen and IL-6 levels. This shows that the RLS model is able to identify validated inflammatory markers, thereby adding further credibility to its usefulness in complex datasets such as this.

Features that were not found to be predictive of inflammatory status are also of interest. Both DM and CVD, two conditions commonly associated with systemic inflammation, failed to add predictive value to the RLS model. This indicates that in CKD patients, features other than these two common comorbidities are more powerful determinants of the presence or absence of inflammation in the uremic milieu.

Some caveats of the study should be considered. It is important to note that no information regarding causality can be derived from the RLS analysis. In addition, the features included in the model cannot be studied separately, as the factor strengths and relative relationships between the features are only valid in this particular combination and setting. Finally, the results presented in this study should be replicated in a separate cohort, with similar genetic and anthropometric/biochemical data available.

4.6 STUDY VI

The presence of a persistent, low-grade inflammation that is commonly seen in CKD and ESRD patients has been hypothesised to exacerbate CVD and many of the comorbidities observed in the uremic milieu^{20, 28, 29}.

This study sought to investigate the relationship between presence of inflammation, CCR5Δ32 genotype, and outcome in two separate populations of incident dialysis patients.

4.6.1 Genotype frequency

In the NECOSAD cohort (n=413), the *CCR5* deletion allele was present at a frequency of 19.4% (ins/del heterozygote and del/del homozygote individuals), whereas the frequency in the MIA cohort (n=302) was 18.6%. Genotype distributions were in Hardy-Weinberg equilibrium in both populations (NECOSAD p=0.21, MIA p=0.22), and were not significantly different (p=0.96). In the NECOSAD cohort, patients with the deletion allele more frequently used anti-hypertensive medication (p=0.01).

4.6.2 Mortality rates according to inflammatory status and CCR5Δ32 genotype

In the NECOSAD cohort, there was no significant difference in CRP levels between patients with and without the *CCR5* deletion allele (p=0.22). Inflamed patients had a higher mortality rate than non-inflamed patients (log rank: p<0.01). This was true for cardiovascular, non-cardiovascular, as well as for all-cause death, and was confirmed by Cox regression analysis. When further dividing the patients according to presence/absence of the *CCR5* deletion allele, individuals with hsCRP > 10 mg/L carrying the ins/ins genotype had the highest mortality rate. Inflamed patients with the deletion allele had a reduced mortality hazard ratio, especially for cardiovascular mortality. CRP levels were associated with cardiovascular mortality in ins/ins homozygotes, but not in patients carrying the deletion allele

Results in the MIA cohort confirmed the findings in the NECOSAD cohort. However, while differences in cardiovascular mortality were significant, there was no significant difference in non-cardiovascular mortality between the four different groups when patients were divided according to inflammation and genotype status.

When combining the two cohorts, confidence intervals (CI) were improved, and the overall results remained the same as those initially seen in the NECOSAD cohort.

4.6.3 Discussion

This study highlights the interplay between a clinical biomarker (inflammation) and genotype (CCR5Δ32) on mortality, and is an example of interactions between genotype and environment. The fact that the results were similar in two independent patient populations strengthens the study.

Considering that the CCR5 protein is involved in recruiting inflammatory cells to the endothelium³⁵, and that the CCR5Δ32 variant has been associated with atherosclerosis⁴³⁻⁴⁷, it is not surprising that the differences in mortality were particularly pronounced for cardiovascular mortality in both patient cohorts.

Some shortcomings of the study are worth noting. First, mechanistic studies are needed before any conclusions can be made regarding a functional association between the CCR5Δ32 variant, inflammation, and mortality. Second, the CRP level used to define inflammation status was based on a single measurement. An attempt was made to partly counteract this by excluding samples with an hsCRP of 50 mg/L or more. The hsCRP cutoff of 10 mg/L can also be discussed. Still, this is a commonly used cutoff level, and replacing it with the median hsCRP level as a cutoff yielded the same results.

As presence of the CCR5 deletion allele appears to attenuate the effects of inflammation on mortality, especially with regards to cardiovascular mortality, it is tempting to speculate about the potential effects of a CCR5 antagonist. Since animal studies have indicated that such a drug could have beneficial effects on organ rejection²¹³, it could be of interest for clinical use in ESRD. The only such drug currently approved for use in humans is maraviroc, and it only has Food and Drug Administration (FDA) approval for HIV-1-infected individuals with R5-tropic virus²¹⁴. DeJesus *et al.*²¹⁵ found that HIV-1 patients who received maraviroc had better lipid profiles and a reduced need of lipid-lowering treatment compared to patients who received the standard drug regimen of combivir + efavirenz, supporting the idea of a potential benefit of a CCR5 antagonist in populations at high risk of CVD. As animal studies have also demonstrated benefits of blocking CCR5 with regards to atherosclerosis and CVD^{45, 47, 216, 217}, performing studies of CVD prevalence in HIV-1 patient populations with/without maraviroc treatment should be of great interest.

5 SUMMARY AND CONCLUDING REMARKS

The research field of cellular senescence, CKD, CVD, and inflammation is highly dynamic and rapidly evolving. The findings discussed in the manuscripts and introduction of this thesis may be applicable not only to CKD and/or ESRD patients, but also to other disorders characterized by inflammation, premature ageing, and CVD, such as rheumatoid arthritis, DM, and systemic lupus erythematosus.

Cardiovascular disease is a prominent feature of several public health priorities, ranging from metabolic diseases to autoimmune disorders and psychiatric illnesses. While these diseases may differ with regard to causative factors, they all share an inflammatory component, which in turn results in exaggerated oxidative stress, increased cellular proliferation and DNA stress. These factors induce cellular senescence, which has detrimental effects on vascular tissue in addition to the harmful consequences of the systemic inflammation *per se*. In vasculature, inflammation and senescence promote VC and arterial stiffness, two established risk factors for CVD. Hence, the common denominator between chronic debilitating diseases with an over-representation of CVD might be inflammatory-driven accelerated biological ageing (so-called “inflammaging”). **Study I** in this thesis demonstrates a link between the tumour suppressor gene (and known inducer of senescence) *CDKN2A/p16^{INK4a}* and VC/CAC score in arteries from ESRD patients undergoing LD RTx. This relationship is further related to osteogenic transformation, as the expression of *CDKN2A/p16^{INK4a}* was positively correlated with *MGP* as well as with *RUNX2*. The concept of advanced cellular ageing in ESRD is further discussed in **Study III**, where a genome-wide analysis of DNA methylation revealed significant differences in methylation between patients and controls in genes related to cellular proliferation and/or cell cycle regulation, as well as in genes previously associated with CVD, CKD, and/or DM. Biological age is also the focus of investigation in **Study II**, as longitudinal changes in telomere length were measured in ESRD patients during their first year of RRT (either dialysis or LD RTx). Unexpectedly, we found that higher telomere attrition was linked to RTx as opposed to dialysis treatment. Folate and immunosuppressive therapy are tentatively related to telomere length, highlighting the ageing potential of immunosuppressants. In addition, since ESRD patients, while significantly younger, had shorter telomeres than the non-CKD controls, this observation further adds to the concept of CKD as a progeric state.

Inflammation is a common feature of CKD and many disorders associated with premature ageing, and is linked to an accelerated cellular ageing. In addition, inflammation has been proposed to be associated with alterations in the epigenome, a hypothesis supported by **Study IV**. Here, elderly individuals engaging in regular physical activity – known to have anti-inflammatory effects – had relatively less methylated DNA than individuals who did not exercise. The complex nature of inflammation and mortality is exemplified in **Study VI**, where the effects of inflammatory status on outcome – particularly with regard to

cardiovascular mortality – is modified by the presence of a particular genetic variant in the *CCR5* gene. The importance of the genetic background for inflammation is highlighted in **Study V**, in which we report that 31 of 60 predictive features of inflammation were genetic.

Together with existing literature, the studies in this thesis support the importance of biological ageing and inflammation in CVD and CKD, and provide novel ideas for research avenues for the future.

6 FUTURE PERSPECTIVES

The studies performed in this thesis form a basis for several possible future investigations. Expanding the p16^{INK4a} IHC experiments from **Study I** is highly pertinent, as the sample size in the study was too small to be analysed statistically. In addition, it would be interesting to perform basic mechanistic studies on the interplay between CDKN2A/p16^{INK4a}, MGP, RUNX2 and calcification – preferably *in vitro*, and possibly while adding uremic serum to the cell culture. Although **Study II** suggests an intriguing association between immunosuppressive therapy and biological ageing in RTx patients, much remains to be explored until our observations can have clinical implications. *In vitro* studies and replication in an independent larger cohort are necessary, and the inclusion of other markers of biological age would also be beneficial. Further investigation of the epigenome in CKD is also warranted, with in-depth confirmation of methylation status and effects on gene and protein expression. As 5-CpG methylation is only one of many possible epigenetic alterations, much remains to be done – in CKD and other diseases – in the areas of histone modification and microRNA effects. Future research should take into consideration the interplay between environmental exposure and genetic background, a complex relationship which requires time and solid statistical expertise to address. Finally, all of the studies included in this thesis – and indeed much of the research being conducted – would benefit from a design where the hypothesis is followed all the way from DNA to protein effects. While time-consuming, this is the only way to ensure that observations made at a genetic level are functionally relevant – and to what extent protein effects are caused by genetic, as opposed to environmental, factors.

While it is difficult to make any prophecies regarding therapeutic potential in this field at such an early stage, there are several venues that deserve deeper investigation. The prospects of using a CCR5 antagonist have already been mentioned, and exploring the effects of the commercially available drug maraviroc on CVD should be an attainable goal. In addition, the field of senolytic drugs is a novel and fascinating area of study. Specific clearance of senescent cells can be achieved by different drugs already in use for other clinical indications. As senescent cells have been likened to cancer cells, in that both cell types successfully avoid entering apoptosis⁷³, drugs normally used for cancer treatment have been shown to successfully and specifically eradicate senescent cells⁷³. Animal models have shown promise in this field, as the elimination of senescent cells in *Ercc1*^{-Δ} mice improved cardiac function and exercise capacity, delayed symptoms and disease related to ageing, and resulted in an extended lifespan⁷³. Clearly, this is a promising avenue of research and would greatly benefit from more investigation. As each senolytic drug appears to be rather cell type-specific⁷³, screening tests need to be undertaken so as to identify suitable drug candidates for different tissues.

Overall, the field of cellular senescence in ageing-associated disorders would greatly benefit from a translational approach by researchers specialized in different fields coming together and exchanging ideas, as there are likely more things in common than there are separating factors. It is time to start viewing chronic, debilitating inflammatory diseases in a different light – not as separate organ-specific entities, but as varying clinical manifestations of a shared molecular pathogenesis provoked by persistent inflammation.

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