TREATING VASCULAR DYSFUNCTION IN CHRONIC KIDNEY DISEASE: INTERVENTION WITH VITAMIN D

Kristina Lundwall
Treating vascular dysfunction in chronic kidney disease: intervention with vitamin D

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Kristina Lundwall

Principal Supervisor:
Jonas Spaak, Associate Professor
Karolinska Institutet
Department of Clinical Sciences
Danderyd Hospital
Division of Cardiovascular Medicine

Opponent:
Gunnar Sterner, Associate Professor
Lund University
Department of Clinical Sciences
Skåne University Hospital

Co-supervisors:
Gun Jörneskog, Associate Professor
Karolinska Institutet
Department of Clinical Sciences
Danderyd Hospital
Division of Internal Medicine

Torbjörn Linde, Associate Professor
Uppsala University,
Department of Medical Sciences
Uppsala University Hospital

Examination Board:
Anders Fernström, Associate Professor
Linköping University
Department of Medical and Health Sciences
Linköping University Hospital

Stefan Jacobson, Professor
Karolinska Institutet
Department of Clinical Sciences
Danderyd Hospital
Division of Nephrology

Angela Silveira, Associate Professor
Karolinska Institutet
Department of Medicine Solna,
Cardiovascular Medicine
To my mother, my shadow supervisor

Without you there would not have been any PhD degree. You have dedicated ours after ours helping me, sitting beside me, silently ignoring my sometimes very bad temper. You have the ability to irritate me the most by telling me the truth. Thank you.
“Believe in something, even if it means sacrificing everything”
Colin Kaepernick

“Just do it”
Nike
ABSTRACT

Background: Chronic kidney disease (CKD) is common, affecting 10-15% of the population worldwide. It is currently recognised by both cardiologists and nephrologists as a strong risk factor for cardiovascular events and death. During the last decades it has been shown that CKD leads to a state of activated renin angiotensin-aldosterone system and sympathetic nervous system, to endothelial dysfunction, chronic vascular inflammation, mineral bone disorder, and in late stages also to an acidic and uremic cell milieu. Together these disturbances create an advanced and rapidly progressing vascular disease, leading to vascular stiffening and calcification. CKD patients have chronically low levels of activated vitamin D, a vitamin now regarded as a hormone involved in a wide range of processes in the body. It affects immune cells, leading to a shift towards anti-inflammatory responses, and inhibits the production of oxidants. Vitamin D upregulates the expression of eNOS, a crucial enzyme in endothelial function, and downregulates the expression of renin. Accordingly vitamin D deficiency might affect several of the processes involved in the progressive vascular disease seen in CKD. The aim of this PhD project was to investigate the effects of intervention with vitamin D on measures and markers of vascular function, inflammation, and upstream epigenetic regulation in patients with CKD.

Methods and results: We performed a randomised placebo-controlled double blind trial (RCT) including 36 participants, with non-diabetic CKD stage 3-4. Patients were randomised to intervention for 12 weeks with 1 or 2 µg of paricalcitol, an active vitamin D analogue, or placebo.

In paper I, we investigated physiological measures of macro- and microvascular function as well as muscle sympathetic nerve activity. We found that treatment with 2 µg of paricalcitol attenuated a decline in endothelial function measured by flow mediated vasodilation and iontophoresis by acetylcholine, and that both treated groups showed ameliorated measures of microcirculation, compared to placebo.

In paper II, a milliplex assay was performed to assess cytokine expression before and after intervention. We found that treatment with both 1 and 2 µg of paricalcitol suppressed levels of PDGF and VEGF, cytokines known to be implicated in vascular function and atherosclerosis. We also examined microRNAs, by PCR-techniques, and detected a downregulation of microRNAs 432, 495 and 576, shown to be involved in atherosclerosis, platelet function and inflammation.

In paper III, concentrations of microparticles (MPs), and their expression of the vascular activation and atherosclerotic markers ICAM-1 and VCAM-1 were determined by antibody labelling and flow cytometry. We showed that treatment with paricalcitol induced a decline in the expression of ICAM-1 on MPs compared to placebo. The results from the combined investigation of cell specific MP profiles showed that treatment with 2 µg of paricalcitol resulted in sustained levels of endothelial, platelet and leukocyte MPs, in contrast to the other two groups where levels declined.
Paper IV used meta-analysis techniques to assess the overall effect-size post treatment in flow mediated vasodilation (FMD) after intervention with any vitamin D compound. Inclusion criteria were any stage of chronic kidney disease, and with no restrictions regarding underlying diseases. Four articles fulfilled the criteria, comprising 305 participants. The overall effect size was in favour of treatment with vitamin D. The results were strongest for the study with the youngest population, for treatment with 2 µg of paricalcitol and treatment with cholecalciferol.

Conclusions: In our examined population, vitamin D has positive effects on endothelial macro- and microcirculatory functions, suppress levels of atherosclerotic and inflammatory markers and maintain the production of microparticles, potentially due to a more normally functioning endothelium. Important questions that remain are whether these findings may translate to effects on hard endpoints, in which patient groups, and the optimal timing of initiation of treatment.
LIST OF SCIENTIFIC PAPERS


* = shared first authorship
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LIST OF ABBREVIATIONS

CVD cardiovascular disease
CKD chronic kidney disease
MDRD modification of diet in renal disease
NSTEMI non ST elevation myocardial infarction
PCI percutaneous coronary intervention
CRS cardiorenal syndrome
RAAS renin angiotensin-aldosterone system
SNS sympathetic nervous system
ROS reactive oxygen species
PDGF platelet derived growth factor
TGF-β transforming growth factor-β
IL interleukin
TNF-α tumour necrosis factor-α
NO nitric oxide
RONS reactive oxygen and nitrogen species
eNOS endothelial nitric oxide synthase
PTH parathyroid hormone
FGF23 fibroblast growth factor23
MBD mineral bone disorder
VDRAs vitamin D receptor analogues
VDR vitamin D receptor
Th T helper cell
AT1-receptor angiotensin II receptor-1
RCT randomised controlled trial
FMD flow-mediated vasodilation
PWV pulse wave velocity
PWA pulse wave analysis
LVM left ventricular mass
ACh acetylcholine
SNP sodium nitroprusside
RHI  reactive hyperaemia index
SEVR  subendocardial viability ratio
LDF  Laser Doppler flowmetry
SCORE  Systematic COronary Risk Evaluation
ICAM-1  intercellular adhesion molecule-1
VCAM-1  vascular endothelial adhesion molecule-1
vWF  von Willebrand factor
E-selectin  endothelial leukocyte adhesion molecule
MP  microparticle
EMP  endothelial microparticle
PMP  platelet microparticle
miR/miRNA  microRNA
eGFR  estimated glomerular filtration rate
ACEi  angiotensin converting enzyme inhibitor
ARB  angiotensin receptor blocker
AIx  augmentation index
CBV  capillary blood cell velocity
MSNA  muscle sympathetic nerve activity
WoS  Web of Science
ANOVA  analysis of variance
MLM  multilevel modelling
STANDmean ES  standardized mean difference effect size
VEGF  vascular endothelial growth factor
IP10  interferon-gamma induced protein 10
1 INTRODUCTION

1.1 THE CARDIORENAL SYNDROME

Cardiovascular disease (CVD) remains the main cause of death worldwide (1, 2). Well established risk factors are hyperlipidaemia, smoking, hypertension and diabetes, although in the last two decades, chronic kidney disease (CKD) has come forth with similar importance (1, 3). CKD including microalbuminuria is also common, affecting 10-13% of the general population (1, 2). The comorbidity of CKD and CVD is nowadays recognised in leading clinical guidelines for both cardiologists (4, 5) and nephrologists (6) (Table 1).

Table 1: Risk stratification for future CV disease/death. Based on ESC European Guidelines on cardiovascular disease prevention in clinical practices, Piepoli et al-16 (4).

<table>
<thead>
<tr>
<th>Low risk</th>
<th>Moderate risk</th>
<th>High risk</th>
<th>Very high risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCORE&lt;1%</td>
<td>SCORE ≥1% and &lt;5%</td>
<td>Cholesterol &gt;8 mmol/L (e.g. in familial hypercholesterolaemia)</td>
<td>Documented CVD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BP &gt;180/110</td>
<td>DM with target organ damage (e.g. proteinuria) or major risk factor (e.g. smoking, marked hypercholesterolaemia, marked hypertension)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DM (young people with type 1 DM without major risk factors at low or moderate risk)</td>
<td>Severe CKD (GFR &lt;30 mL/min/1.73 m2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moderate CKD (GFR 30–59 mL/min/1.73 m2)</td>
<td>SCORE ≥10%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCORE ≥5% and &lt;10%</td>
<td></td>
</tr>
</tbody>
</table>

SCORE= a person’s 10 year risk of CV death; BP=blood pressure; CKD=chronic kidney disease; DM diabetes mellitus; CVD=cardiovascular disease.

An eGFR <60 ml/min is known to be associated with cardiovascular death in the general population (7), but a recent meta-analysis of almost 2 million subjects showed that the risk of myocardial infarction started to increase even at a mild renal dysfunction with an eGFR below 90 ml/min (modification of diet in renal disease formula (MDRD)) (8).

Not only do CKD patients carry a high cardiovascular risk, they also have a worse prognosis once they suffer a cardiovascular event (2, 9, 10). This may be due to direct effects caused by the declining renal function and associated vascular disease, but it may also in part be due to that CKD patients are treated differently. Szummer et al (10) showed a correlation between kidney function and the percentage of early revascularization performed in non-ST elevation myocardial infarctions (NSTEMI), with less percutaneous coronary interventions (PCI) with declining kidney function. Several factors may contribute to fewer interventions in CKD, such as difficulties to correctly diagnose NSTEMI in CKD, fear of contrast-induced nephrotoxicity, and challenges with correct dosing.

The connection between heart and kidney disease comprises a wide spectrum of disorders often classified as the cardiorenal syndrome (CRS). The CRS is subgrouped into 5 types.
based on primary aetiology (2); here the focus will be on the chronic nephrocardiac type (Table 2).

**Table 2: Subgroups of the cardiorenal syndrome, based on Ronco et al -08, -14 (2, 11).**

<table>
<thead>
<tr>
<th>Type</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acute cardiorenal. Heart failure leading to acute kidney disease</td>
</tr>
<tr>
<td>2</td>
<td>Chronic cardiorenal. Chronic heart failure leading to kidney failure</td>
</tr>
<tr>
<td>3</td>
<td>Acute nephrocardiac. AKD leading to acute heart failure (uremic cardiomyopathy, AKD-related)</td>
</tr>
<tr>
<td></td>
<td>Chronic nephrocardiac. Chronic kidney disease leading to heart disease</td>
</tr>
<tr>
<td>4</td>
<td>(left ventricular hypertrophy, left ventricular diastolic dysfunction, chronic ischaemic heart disease due to kidney disease)</td>
</tr>
<tr>
<td>5</td>
<td>Secondary: Systemic disease leading to kidney and heart failure (sepsis, vasculitis, DM).</td>
</tr>
</tbody>
</table>

AKD = acute kidney disease; DM = diabetes mellitus.

Several of the mechanisms underlying the chronic nephrocardiac syndrome have been investigated during the last decade, outlining a syndrome of advanced vascular disease and premature vascular ageing. CKD patients suffer from chronic inflammation, endothelial dysfunction, and mineral bone metabolism disorders, leading to vascular calcification and arterial stiffening (1, 2, 12, 13). Studies have also demonstrated an activated sympathetic nervous system (SNS) and activated renin-angiotensin-aldosterone system (RAAS), contributing to the vascular disease (2, 11, 14, 15). In later stages the acidotic and uremic milieu in the cells will accelerate the inflammatory process further. All these changes together create the perfect conditions for atherosclerosis, vascular stiffening and fibrosis (1, 2, 14, 16, 17).

### 1.2 VASCULAR DYSFUNCTION IN CKD

#### 1.2.1 RAAS and SNS activation

Both the RAAS and the SNS are highly activated in patients with CKD (2, 11, 14, 15). Renin release is stimulated by a decrease in renal perfusion pressure, and by sympathetic nerve stimulation (18). Furthermore, low levels of active vitamin D in CKD cause an upregulation of renin expression (19), and may contribute to the higher blood pressure seen in CKD, although the importance of this mechanism remains controversial (20). The sympathetic over-activation in CKD is likely due to a combination of renal injury, ischemia, chemoreflex dysregulation, and increases in sympahtoexcitatory substances (i.e. Angiotensin II) (21).
While these two systems are essential for homeostasis, a chronic activation is deleterious for the kidneys, vessels and the heart (14, 22). Sustained RAAS activation causes sodium and water retention, systemic vasoconstriction, fibrosis and oxidative stress (14, 22). Sustained SNS activation induces reactive oxygen species (ROS) production, reduces β-adrenoreceptor density and sensitivity, causes cardiomyocyte hypertrophy and subsequent heart failure, and vascular hypertrophy (23-25).

Angiotensin II, the potent end product of the RAAS system, is an important link between the RAAS and the activated immune system seen in CKD. Angiotensin II does not only stimulate central sympathetic outflow, but also activates and stimulates monocytes/macrophages, dendritic cells, NK-cell and neutrophils as well as proinflammatory T-cells, and induces reactive oxygen species (ROS) production chemotaxis and proinflammatory cytokine release (26-28). ROS also interplay with SNS, with increased levels by SNS signalling, and at the same time enhance SNS activity by promoting sympathetic outflow (23, 29) (Figure 1).

Figure 1: Angiotensin II, the immune system, and the connection to SNS, leading to vascular inflammation.

1.2.2 The immune system

The immune cells are currently not only regarded as major players in cancer and autoimmune diseases, but are also central in the development of atherosclerosis and hypertension (28-31). Current research has shown that both the innate and the adaptive immune system are implicated in the process (32, 33). Both these immune systems are further dysregulated in CKD patients, shown by dysfunctional immune cells, by increased levels of proinflammatory
cytokines and acute phase proteins (34-37). The starting point of this activation in CKD patients is not fully understood and probably multifactorial. Hypertensive stimuli and shear stress are thought to lead to the formation of neoantigens presented to T helper cells by dendritic cells (DCs) thereby activating them (28). An activated RAAS and SNS as described above also contribute, and in later stages uremic toxins play an important role as antigens in the body and lead to additional activation of the immune system (2, 16). Many cytokines produced by immune cells are shown to play an active role in the development of kidney injury and disease by enhancing inflammation, fibrosis and proteinuria. Among those are platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), interleukin-1 (IL)-1, IL-6 and tumour necrosis factor-α (TNF-α) (37-41). PDGF and VEGF are also thought to play an active role in the development of atherosclerosis and atherosclerotic plaques (42, 43). ROS, produced by the activated immune cells, are important in the development of endothelial dysfunction, and in keeping the feedback-loop mechanisms of inflammation active.

1.2.3 Endothelial dysfunction

Micro- and macrovascular endothelial dysfunction is evident from the early stages of atherosclerosis, and is a potent independent predictor of cardiovascular risk (17, 31, 44-46). Several studies have described endothelial dysfunction in patients with varying degree of CKD, and it plays an important role in the chronic nephrocardiac syndrome (17, 47-49). The endothelium controls vascular tone, permeability of immune cells and molecules, and haemostasis in the blood vessel. Endothelial cells biosynthesize several vasoactive substances, and in normal homeostasis keep the balance between vasodilatory, anti-inflammatory properties, and vasoconstrictive, proinflammatory properties. One of the most important vasoactive substances produced by the endothelial cells is nitric oxide (NO). NO is essential for a normal endothelial function and inhibits platelet aggregation, transcription of cell adhesion molecules, leukocyte adhesion and growth of vascular smooth muscle cells (50, 51). A central mechanism in the development of endothelial dysfunction is the uncoupling of endothelial NO synthase (eNOS). Reactive oxygen and nitrogen species (ROS/RONS) and NADPH oxidases are believed to play a major role in uncoupling endothelial nitric oxide synthase (eNOS), which turns the enzyme from a protective NO-producing enzyme, to a harmful one, leading to production of very potent oxidants (28, 51).

This loop mechanism created will lead to less NO bioavailability and high levels of ROS/RONS. The endothelial cell balance will shift towards a chronic vasoconstrictive, pro-inflammatory state, with not only activation of endothelial cells, but also activation of immune cells. The activated immune cells will produce more pro-inflammatory cytokines and ROS/RONS, infiltrate the vessel wall, leading to vascular inflammation and dysfunction, and in the end, atherosclerosis (28, 49, 52).
1.2.4 Arterial stiffness and calcification

Arterial calcification and stiffness are important in the development of vascular disease in CKD, where impaired vitamin D metabolism, calcium-phosphate imbalance, and hyperparathyroidism play important roles (2). Vascular stiffening is mainly the result of endothelial dysfunction, vascular inflammation, and arterial calcification.

All these processes lead to accelerated vascular disease in CKD, with diminished coronary blood flow and risk of myocardial infarction, risk of arrhythmias, or left ventricular hypertrophy and sudden cardiac death (1, 2, 49). The causation and interrelationship of these different aspects of vascular disease in CKD is however debated (12, 13). Vascular inflammation is an early hallmark of vascular disease, and studies have shown that it is followed by, but not coexisting with, calcification and fibrosis (13, 53, 54). Calcification and fibrosis seem to be due to low-grade long standing inflammation with pronounced structural changes (13, 55) and can be seen as the body attempting a healing process, in turn leading to arterial stiffening (13). The preceding endothelial dysfunction on the other hand has a strong inflammatory connection as described above. It is activated by inflammation, but also works as a promoter of the continuous inflammatory process, and as such is an early sign of the vascular disease process (17, 45, 46).

**Figure 2: An illustration of some of the complex mechanisms in CKD leading to CVD. The many feedback-loops in the network further enhance negative effects (also see Figure 1 and text for further details).**
1.3 VITAMIN D AS A TREATMENT OPTION

1.3.1 Vitamin D deficiency and CKD-MBD

Active vitamin D deficiency is common already from early stages of CKD, mainly due to the loss of renal function and availability of 1,25-α-hydroxylase, the vitamin D activating enzyme. Low levels of active vitamin D cause insufficient re- and absorption of calcium from the kidney and gut, which in turn stimulates release of parathyroid hormone (PTH). High levels of PTH stimulate calcium release from skeletal bones, augment absorption of calcium from the kidneys, and increase active vitamin D levels (56). Loss of kidney function also leads to retention of phosphate, which stimulates both PTH- and fibroblast growth factor 23 (FGF23) release, the latter a calcium-phosphate regulating hormone, with anti-vitamin D properties (57, 58). The changes in this axis in CKD are together known as CKD mineral bone disorder (MBD) (59) (Figure 3). There is an ongoing discussion on the importance of these different aspects of CKD MBD, but the regulation of FGF23-, phosphate- and vitamin D levels are all affected early in CKD patients (59).

Figure 3: The CKD-MBD axis. Ca=calcium; Pi=phosphate; PTH=parathyroid hormone; FGF23=fibroblast growth factor 23.
1.3.2 Vitamin D and CVD

Several observational studies, including all types of populations, have shown a strong association between vitamin D deficiency and an increased risk of cardiovascular events (60-68). The causality of this association is however still debated.

Table 3: Examples of epidemiological studies on vitamin D and cardiovascular disease.

<table>
<thead>
<tr>
<th>Author</th>
<th>Population</th>
<th>Participants</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Martins - 07</td>
<td>NHANES</td>
<td>15,088</td>
<td>25(OH)D levels correlate to CV risk factors</td>
</tr>
<tr>
<td>Wang - 08</td>
<td>Framingham Offspring</td>
<td>1,739</td>
<td>25(OH)D deficiency associated with incident CVD</td>
</tr>
<tr>
<td>Giovannucci - 08</td>
<td>Health Professionals Follow-up Study</td>
<td>18,225</td>
<td>Low levels of 25(OH)D associated with higher risk of MI</td>
</tr>
<tr>
<td>Kendrick - 09</td>
<td>Third National Health and Nutrition Examination Survey</td>
<td>16,603</td>
<td>Strong and independent relationship of 25(OH)D deficiency with prevalent CVD</td>
</tr>
<tr>
<td>Semba -10</td>
<td>Prospective cohort study of aging in Tuscany, Italy</td>
<td>1,002</td>
<td>Low serum 25(OH)D levels associated with higher risk of all-cause and CVD mortality.</td>
</tr>
<tr>
<td>Brondum-</td>
<td>Prospective cohort of general Danish population</td>
<td>10,170</td>
<td>Decreasing 25(OH)D levels associated with risk of IHD, MI, and early death</td>
</tr>
<tr>
<td>Jacobsen -12</td>
<td>Meta-analysis, association of 25(OH) vitamin D with CVD risk</td>
<td>65,994</td>
<td>Linear inverse association between 25(OH)D and risk of CVD</td>
</tr>
<tr>
<td>Wang -12</td>
<td>Meta-analysis, association of 25(OH)D, CV risk, MI and early death</td>
<td>82,982</td>
<td>Decreasing 25(OH)D levels associated with risk of IHD, MI, and early death</td>
</tr>
<tr>
<td>Brondum-</td>
<td>German population-based cohort aged 50–74</td>
<td>9,758</td>
<td>25(OH)D inversely associated with all-cause, CV, cancer and respiratory mortality</td>
</tr>
<tr>
<td>Jacobsen -12</td>
<td>Community dwelling type 2 DM patients followed to first CHD</td>
<td>2,607</td>
<td>25(OH)D deficiency an independent predictor of future CHD events</td>
</tr>
</tbody>
</table>

CVD=cardiovascular disease; MI=myocardial infarction; DM=diabetes mellitus; CHD=coronary heart disease; IHD=ischaemic heart disease.

Vitamin D treatment is an established treatment of hypocalcaemia and secondary hyperparathyroidism in patients with more advanced kidney failure. However, due to the findings in observational studies, the interest for vitamin D as a possible treatment option to affect cardiovascular risk in earlier stages of CKD has increased.

1.3.3 Treatment or supplementation?

There is an ongoing discussion about how to treat CKD patients with vitamin D deficiency. Some argue that supplementation with the precursors cholecalciferol or ergocalciferol might be preferable over the active hormone calcitriol, or vitamin D receptor analogues (VDRAs, i.e paricalcitol). Many cells of the body are now known not only to have the receptor for
active vitamin D (VDR), but also the 1-α-hydroxylase, and are capable of producing active vitamin D locally. Studies have indicated potential drawbacks with active treatment, such as hypercalcaemia and hyperphosphataemia affecting vascular calcification, induction of adynamic bone disorder and also negative loop back mechanisms with downregulation of 1-α-hydroxylase and upregulation of 24-hydroxylase, an enzyme that degrades active vitamin D (69). Supplementation with the inactive form might give the positive pleiotropic effects of VDR-activation through local production of the active form and thereby activation of the VDR (69). Precursors seem to safely lower PTH and ameliorate 25OHD and 1,25OH2D3-levels, in all CKD stages, though at some less degree than active treatment, and without a rise in calcium and phosphate levels (70, 71). Active treatment on the other hand is correlated to higher calcium and phosphate levels (72-75). In a meta-analysis with both compounds Xu et al (75) did not however see a difference in calcium levels between precursors and active treatment. Some advocate a combination of supplementation with precursors and active treatment (76).

1.3.4 Cellular effects of vitamin D

On the cellular level, it is now clear that vitamin D not only regulates the bone-mineral metabolism, but also displays a wide range of effects in the body. Both the receptor and the Vitamin D activating enzyme are found in many different cell types, among them immune cells, vascular smooth muscle cells and endothelial cells (77-79). In the immune system, vitamin D seems to play a role as a balancing factor between pro- and anti-inflammation (78, 80). It has both antibacterial and proinflammatory effects by inducing production of antimicrobial peptides and bacterial killing by monocytes and macrophages. Vitamin D also mediates anti-inflammatory and anti-fibrotic responses by the suppression of proinflammatory cytokines, induction of anti-inflammatory cytokines, downregulation of the TGF-β pathway and differentiation of T helper (Th) cells towards the less inflammatory Th2 and anti-inflammatory regulatory T cells (74, 81).

In the endothelium, Vitamin D has been shown to decrease the expression of adhesion molecules, decrease levels of oxidative stress, upregulate the enzyme eNOS and thereby induce the production of NO (82-85). Vitamin D is also a negative regulator of the RAAS by a downregulation of renin expressing genes (86, 87), and by antagonistic synergy with the angiotensin II receptor-1 (AT1-receptor) (88-91).
1.4 INTERVENTIONS WITH VITAMIN D IN CKD

1.4.1 Studies on markers of inflammation, renal function and glucose metabolism

Three studies between year 2005 and 2010 showed that supplementation with vitamin D and treatment with VDRA in patients with CKD was associated with improved survival and reduced proteinuria (92-94). Meta-analyses on the topic confirm these results, and show that vitamin D affects residual proteinuria in CKD patients, on top of RAAS blockade (72-75). The effects are probably due to the anti-inflammatory actions (74) and the effects on the RAAS described above (86, 87, 91). Glucose metabolism is another interesting area, where one meta-analysis on dialysis patients shows positive effects on glucose control by treatment (95). When it comes to inflammatory markers, a couple of small randomised controlled trials (RCTs) have shown effects on proinflammatory cytokines and CRP (96-99), while Thethi et al (100) failed to show effects on similar parameters.
1.4.2 Studies on hard endpoints are lacking

Large and sufficiently long studies to evaluate hard endpoints are still lacking in this area. In a retrospective trial, Lishmanov et al (101) showed reduced cardiovascular events with vitamin D supplementation in patients with CKD. Meta-analyses on cardiovascular risk and mortality have been performed and show effect of treatment with vitamin D in CKD patients in observational studies (102, 103). One meta-analysis (104) investigated the effect on cardiovascular endpoints in controlled trials, but could not show any benefit of treatment. However, these results may be questioned since none of the included studies had cardiovascular endpoints defined à priori, and the study durations varied from only 3 weeks to 2 years.

1.4.3 Surrogate markers of CV risk

In the absence of hard endpoints, many studies have instead used surrogate markers of cardiovascular risk, such as flow-mediated vasodilation (FMD) and measures of arterial stiffness (pulse wave velocity or pulse wave analysis; PWV/PWA). Whereas PWV/PWA are complex measures of arterial structural changes, and are mainly measures of arterial stiffness (12, 105), FMD is primarily a measure of the capacity of the endothelial cells to produce and respond to NO (106). Still, PWV and FMD are interrelated (107) and both are predictors of cardiovascular risk (17, 45, 46, 108, 109).

1.4.4 Studies on FMD

During the last years a couple of studies have been performed investigating the effects of different vitamin D compounds on FMD (44, 100, 110-113). Most of them showed effects of supplementation or treatment on measures of FMD in CKD patients in stage 3-4 (44, 111-113). Kendrick et al (110) compared the effect of supplementation and active treatment, and did not detect any difference from baseline for the two groups. Neither did Theti et al (100), investigating the effects of paricalcitol in patients with diabetic nephropathy. These studies are systematically reviewed in the present thesis in study IV.

1.4.5 Studies on arterial stiffness and diastolic measures

Left ventricular hypertrophy and diastolic dysfunction is often a result of hypertension and arterial stiffness (114-116). Interventional studies with vitamin D on left ventricular mass
(LVM), diastolic measures and arterial stiffness have, compared to FMD, primarily shown negative results. The PRIMO trial failed to show effects on left ventricular hypertrophy and diastolic function in CKD patients. However, secondary and explorative analyses showed less hospitalizations due to cardiovascular events, and a tendency to positive effects on levels of NT-proBNP, and atrial volume indexes. Several studies investigating the effects of vitamin D compounds on measures of PWV and/or PWA in kidney disease patients have been performed during the last decade. The study durations varied from 8 to 44 weeks, patients were in kidney stage 3-5 and both active treatment and supplementation in different doses were used. Levin et al and Kumar et al showed positive effects of treatment, whereas the rest of the studies failed to show any difference in PWV and/or PWA post treatment.

1.4.6 Other non-invasive methods to assess vascular function

There are other non-invasive methods to assess vascular and endothelial function, such as investigations of skin microvascular reactivity by LDF during iontophoresis with acetylcholine (ACh) and sodium nitroprusside (SNP), laser Doppler fluxmetry (LDF), reactive hyperaemia index (RHI) and subendocardial viability ratio (SEVR). These are less validated as risk markers, but iontophoresis with ACh and SNP have been shown to correlate to CV risk factors, and might reflect microvascular dysfunction in the rest of the body. However, Jekell et al showed that iontophoresis by ACh (endothelium dependent), LDF and SEVR were not correlated to cardiovascular risk in terms of the Systematic COronary Risk Evaluation (SCORE) estimates in a population of hypertensive patients. This was in contrast to PWV and FMD that both significantly correlated to SCORE. FMD and PWV also correlated significantly to each other, whereas the other measures did not, raising important questions about what these measures exactly stand for in the vasculature. There are not many studies performed on the effects of vitamin D treatment with these measures in CKD patients. Dreyer et al showed positive effects of treatment with ergocalciferol on measures of iontophoresis. Pihlström et al, using active treatment, could not show any effects of intervention on measures of reactive hyperaemia index (RHI).

In conclusion, there are still conflicting results in the field of Vitamin D treatment as a way of affecting measures of cardiovascular risk in CKD. Factors such as study size and duration, study population, and the choice of outcome-measures are of importance to interpret these results.
Table 4: Interventions with Vitamin D in chronic kidney disease, on surrogate markers of cardiovascular risk. For study populations nr in () are patients analysed.

<table>
<thead>
<tr>
<th>Author</th>
<th>Duration</th>
<th>Sample size (nr)</th>
<th>CKD stage</th>
<th>Treatment</th>
<th>Dose</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zoccali (-14)</td>
<td>12w</td>
<td>89 (88)</td>
<td>3-4</td>
<td>paricalcitol</td>
<td>2μg daily</td>
<td>FMD</td>
</tr>
<tr>
<td>Chitalia (-14)</td>
<td>16 weeks</td>
<td>26</td>
<td>3-4</td>
<td>cholecalciferol</td>
<td>300000IU at baseline, after 8w</td>
<td>FMD, PWV/PWA, ICAM, VCAM, E-selectin, vWF</td>
</tr>
<tr>
<td>Theti (-15)</td>
<td>12w</td>
<td>60 (46)</td>
<td>3-4</td>
<td>paricalcitol</td>
<td>1μg daily</td>
<td>FMD</td>
</tr>
<tr>
<td>Kumar (-17)</td>
<td>16w</td>
<td>120 (117)</td>
<td>3-4</td>
<td>cholecalciferol</td>
<td>300000IU at baseline, after 8w</td>
<td>FMD, PWV</td>
</tr>
<tr>
<td>Kumar (-18)</td>
<td>16w</td>
<td>31</td>
<td>3-4</td>
<td>cholecalciferol</td>
<td>300000IU at baseline, after 8w</td>
<td>FMD, PWV, vWF</td>
</tr>
<tr>
<td>Kendrick (-17)</td>
<td>6 months</td>
<td>128 (115)</td>
<td>15-44 eGFR</td>
<td>cholecalciferol, calcitriol</td>
<td>2000IU, or 0,5μg daily</td>
<td>FMD</td>
</tr>
<tr>
<td>Marckmann (-12)</td>
<td>8 weeks</td>
<td>52</td>
<td>HD comp non HD</td>
<td>cholecalciferol</td>
<td>50000IU/w</td>
<td>PWV/PWA, vWF, IL-6, hsCRP</td>
</tr>
<tr>
<td>Hewitt (-13)</td>
<td>6 months</td>
<td>60</td>
<td>HD</td>
<td>cholecalciferol</td>
<td>50000IU/w: 8 w, monthly 4m</td>
<td>PWV</td>
</tr>
<tr>
<td>Mose (-14)</td>
<td>6 months</td>
<td>64</td>
<td>ESRD</td>
<td>cholecalciferol</td>
<td>3000IU daily</td>
<td>PWV, PW/Aix, Echo, cBP</td>
</tr>
<tr>
<td>Dreyer (-14)</td>
<td>6 months</td>
<td>38</td>
<td>3-4</td>
<td>ergocalciferol</td>
<td>50000IU/w: 1 month, monthly: 6m</td>
<td>PWV, iontophoresis, LDF, eNOS</td>
</tr>
<tr>
<td>Pihström (-17)</td>
<td>44w</td>
<td>77</td>
<td>renal transplant recipients</td>
<td>paricalcitol</td>
<td>2μg daily</td>
<td>PWV, RHI</td>
</tr>
<tr>
<td>Levin (-17)</td>
<td>6 months</td>
<td>119 (87)</td>
<td>15-45 eGFR</td>
<td>Calcifediol, calcitriol</td>
<td>5000IU or 0,5μg 3 times/w</td>
<td>PWV</td>
</tr>
<tr>
<td>Thadhani (-12)</td>
<td>48w</td>
<td>227</td>
<td>15-60 eGFR</td>
<td>paricalcitol</td>
<td>2μg daily</td>
<td>LVMI, echo</td>
</tr>
<tr>
<td>Naeini (-17)</td>
<td>6 months</td>
<td>64</td>
<td>ESRD</td>
<td>vitamin D pearls</td>
<td>50000IU/w:3m, every 3rd w: 3m</td>
<td>VCAM, ICAM</td>
</tr>
</tbody>
</table>

ITT=intention to treat; eGFR=estimated glomerular filtration rate; HD=hemodialysis; ESRD=end stage renal disease; FMD=flow mediated vasodilation; PWV/PWA=pulse wave velocity/analysis; vWF=von willebrand factor; echo=echocardiography; hsCRP=high sensitive CRP; cBP=central blood pressure; LDF=laser Doppler flowmetry; eNOS=endothelial nitric oxide synthase; RHI=reactive hyperemia index; LVMI=left ventricular mass index.

1.4.7 Studies on endothelial markers in CKD

An activated and/or dysfunctional endothelium express activation markers such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), von Willebrand factor (vWF) and endothelial leukocyte adhesion molecule (E-selectin). They play an important role in the recruitment and migration of white blood cells and platelets to the site of inflammation. E-selectin mediates leukocyte rolling, the first step to slow down the flow of the leukocytes at the site of inflammation. ICAM-1 and VCAM-1 then induce leukocyte arrest whereas ICAM-1 initiates the crawling and intracellular transmigration of leukocytes into the vessel wall (127) (Figure 5).
Figure 5: The interaction of adhesion molecules and leukocytes (simplified). Modified from Ley et al (127). Published with permission from Springer Nature.

These events play an important role in the atherosclerotic process (128) and in line with this some studies have shown that levels of ICAM-1 correlate to cardiovascular events and death in CKD patients (129-131). VCAM-1 seems to correlate to intima media thickness in HD patients (132) and to measures of dyslipidaemia (130, 131). There are two studies performed showing lower levels of ICAM-1 and VCAM-1 after intervention with vitamin D, interpreted as a less inflammatory and less activated endothelium (44, 133).

1.5 MICROPARTICLES

Microparticles (MPs) are small cell membrane vesicles (100-1000nm) shedded from the parent cells, both during activation of the cell but also increasingly during stress and apoptosis. They are released in response to proinflammatory cytokines, acute phase proteins, ROS, and uremic toxins (134-136). They can be considered cellular biopsies from the tissues they are derived from, and are therefore promising biomarkers for various diseases. Microparticles contain mRNA, microRNA, receptors and proteins, and there are emerging evidence that they are biologically active, affecting cells around them, playing a role in intercellular communication (134-137). Different MPs have been shown to act in the production of proinflammatory cytokines, in promoting vascular inflammation and coagulation and regulating the production of ROS (135, 137).
1.5.1 The importance of subtypes of MPs

Not only their parent cell type but also the mechanism for their release; apoptosis, cell damage or activation, induce different patterns of surface markers, such as CD31, CD62E, CD62P, or CD144 (134, 138, 139). For endothelial microparticles (EMPs), it seems that the subtypes of EMPs reflect different aspects of endothelial function, and might be used to classify the type of endothelial injury occurring (134, 138). In acute coronary syndrome for example, there are mainly high levels of apoptotic (CD31⁺) EMPs (138, 140, 141) probably reflecting the acute endothelial cell injury due to ischemia. Jimenez et al (138), showed in an in vitro study that when apoptosis was induced in endothelial cells, activation markers on the cells and activation induced EMPs remained stable, while apoptotic markers on the cells and the production of apoptotic EMPs increased rapidly. Dr Lundström et al has also shown interesting results in patients with ischaemic stroke or TIA, where different subtypes of platelet microparticles (PMPs) reflect opposite correlations to prognosis in patients with ischemic stroke.¹ Depending on the cell origin and surface molecules, MPs also seem to exhibit opposite effects on apoptosis, inflammation and oxidative stress (134, 139, 142-145) (Figure 6).

Figure 6: Different actions of microparticles due to their release mechanisms.

¹ Published as part of the thesis “Platelet function and thrombin generation in ischemic stroke – clinical correlates and prognostic importance” 2018, by Annika Lundström, part III: Platelet microvesicles are elevated after ischemic stroke or TIA – specific subpopulations have different associations to prognosis
1.5.2 Endothelial microparticles and correlation to future CV risk

EMPs are novel biomarkers of endothelial dysfunction and injury (134, 135, 137, 146, 147). Levels of CD31-positive-CD41-negative (CD31⁺ CD41⁻) and CD144-positive (CD144⁺) EMPs correlate with physiological measurements of vascular function, such as FMD and PWV (148-152). The marked endothelial dysfunction in CKD patients is also reflected in higher levels of CD144⁺ and CD31⁻CD41⁻EMPs compared to healthy controls (147, 149, 153, 154). CD144⁺ or CD31⁻CD41⁻ EMPs, and also CD62E⁺ EMPs (155), may play a predictive role, and high levels correlate with cardiovascular morbidity and mortality in atherosclerosis, kidney failure, pulmonary hypertension and heart failure (146, 148, 155-157) (Table 5).

Table 5: Observational studies on microparticles in chronic kidney disease and interventional studies in all populations.

<table>
<thead>
<tr>
<th>Author</th>
<th>Population</th>
<th>MPs measured</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MPs in CKD and correlations to risk</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amabile 2005</td>
<td>44 ESRD, 32 healthy controls</td>
<td>CD31+CD41-, CD144+ EMPs</td>
<td>Correlation to FMD in ESRD</td>
</tr>
<tr>
<td>Boulanger 2007</td>
<td>34 HD patients</td>
<td>CD144+, CD31+41- EMPs</td>
<td>Correlation to measures of shear stress in ESRD.</td>
</tr>
<tr>
<td>Amabile 2012</td>
<td>81 HD patients</td>
<td>CD31+CD41- EMPs</td>
<td>Levels predicted all cause and CV mortality in ESRD after 50 months</td>
</tr>
<tr>
<td>Dursun 2009</td>
<td>33 pre-HD, 37 HD, 18 healthy controls</td>
<td>CD144+, CD146 + EMPs</td>
<td>Higher levels in CKD than healthy controls. PWV correlated to CD144</td>
</tr>
<tr>
<td>Faure 2006</td>
<td>45 CKD, 30 HD, 36 healthy controls</td>
<td>CD144+, CD146+EMP s</td>
<td>Higher in CRF and HD patients than in healthy controls</td>
</tr>
<tr>
<td>Chen YL 2015</td>
<td>68 CKD-CAD, 10 CAD, 10 healthy controls</td>
<td>CD31+42b- EMPs</td>
<td>Higher in patients with CAD than in patients with CAD and CKD.</td>
</tr>
<tr>
<td>Trappenburg 2012</td>
<td>27 CKD, 10 healthy controls</td>
<td>CD144+ EMPs, CD41+, CD62P+ PMPs</td>
<td>Higher levels in CKD4 and HD compared to controls</td>
</tr>
<tr>
<td><strong>Interventions (all populations)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Augustine 2014</td>
<td>119 patients referred for stress echo</td>
<td>CD31+41+PMPs, CD31+41-EMPs, erythrocyte MPs</td>
<td>Increasing levels after stress echo in patients with normal examination, but did not change in patients with CVD or pathologic stress echo</td>
</tr>
<tr>
<td>Tehrani 2013</td>
<td>20 type 1 DM patients</td>
<td>CD144+ EMPs</td>
<td>Levels tended to increase with Atorvastatin treatment</td>
</tr>
<tr>
<td>Mobarrez 2012</td>
<td>19 PAOD patients</td>
<td>CD144+ EMPs</td>
<td>Increasing levels with Atorvastatin treatment</td>
</tr>
<tr>
<td>Jia 2017</td>
<td>HUVEC cell line</td>
<td>AnnexinV+ MPs</td>
<td>MP release and superoxide generation significantly inhibited by 1,25(OH)2D3</td>
</tr>
</tbody>
</table>

ESRD=end stage renal disease; HD=hemodialysis; CAD=coronary artery disease; echo=echocardiography; PAOD=peripheral artery occlusive disease; HUVEC=human umbilical vein endothelial cell; CRF=chronic renal failure
1.5.3 Results from interventional studies

The production and thereby the levels of MPs also seem to be dependent on the capability of the cells to become activated. Augustine et al. (158) reported in a study of CD31\(^+\) EMP- and PMP-response to dobutamine-stress echocardiography, that patients with signs of coronary disease (wall motion abnormalities) on the examination did not react with elevated CD31\(^+\) EMPs or PMPs, whereas patients with a normal stress-test did. These results seem to indicate that a dysfunctional endothelium might be less reactive, not producing more MPs in response to stress or other events in the same way as in healthy controls or treated patients. Mobarrez et al (159) showed similar results in a study on atorvastatin treatment in patients with peripheral artery occlusive disease. Patients treated with atorvastatin produced more EMPs than the placebo group. This was interpreted as a sign of a more reactive and healthy endothelium since atorvastatin has known endothelium-cell protective effects (160). There are to our knowledge no interventional studies performed with vitamin D using MPs as outcome. Two in-vitro studies (83, 85) on endothelial cell lines show lower levels of CD31\(^+\) EMPs after vitamin D treatment indicating a protective effect against apoptosis (Table 5).

MPs are clearly affected by kidney disease, and some subtypes might be of use not only as predictors of risk, but also as markers to understand underlying mechanisms. Whether MPs may be used as markers of long-term outcome, is insufficiently studied.

1.6 EPIGENETIC REGULATION, A NEW AND EXPANDING FIELD IN CKD

1.6.1 Epigenetic regulation

CKD have a strong hereditary component, shown in epidemiological studies. Genetic wide association studies have however failed to find strong associations, implying the importance of other factors, such as epigenetic modifications (161, 162). Epigenetic regulation is the heritable interface between the rigid genome and the changing environment. It affects gene expression without changes in the nucleotide sequence, and therefore epigenetic changes are potentially reversible (162). For example, epigenetics are thought to be the reason for the interesting phenomenon “metabolic memory”, described in diabetes, where a period of impaired glucose control will induce cellular changes that will linger on even after periods of excellent treatment (40).

The most commonly discussed epigenetic regulators are microRNAs (miRs/miRNAs), post-translational modifications of nucleosomal histones and DNA methylation (Figure 7).
1.6.2 microRNAs

miRs are small RNAs (approximately 19-25 nucleotides) that regulate gene expression by binding to mRNAs, resulting in silencing of translation or degradation of that mRNA (40). These miRs can target hundreds of mRNAs, and their effects on gene expression can therefore be substantial (164). Because of this, miRs are not only believed to be future biomarkers for various diseases, but also highly interesting as targets for treatment (40). Several miRs have been associated with inflammation and kidney dysfunction (40, 162). miR-146 and miR-155 are recognized as key players in the immune cell response in chronic inflammation (161). miR-192 is well characterized, and promote fibrosis by upregulation of profibrotic genes, among them the TGF-β-pathway, known to be central in the development of renal fibrosis (162). There are several miRs thought to play a role in normal kidney function, among them the miR-30 family (162). As targets for treatment, there are trials performed on mice, where an anti-miR192 treatment induced changes in the phenotype of the mice with downregulation of mediators of renal fibrosis, and reduced proteinuria (40, 165).

Several miRs are also thought to be implicated in cardiovascular disease (164). For example, downregulation of the miR133 family seems to be implicated in hypertrophy and fibrosis of the heart (164, 166). MiR 495 probably acts in the process of platelet reactivity, and downregulation of miR495 was shown in a mouse model to ameliorate vascular recovery
after ischemia (167, 168). However, here there are conflicting results, where another mouse model has shown that miR495 was downregulated in ischaemic tissue, and showed anti-inflammatory properties (169). An upregulation of a cluster of miRs, among them miR432, seems to be implicated in the atherosclerotic process (170). Upregulation of miR 432, as well as miR 576, also seems to be implicated in the proinflammatory response, a possible pathway of enhancing vascular dysfunction (171, 172).

Epigenetic regulation plays an active role in kidney disease and cardiovascular disease, as in most other diseases, and might be of importance both as predictors of risk, and as possible targets for treatment. For the potential role of epigenetic regulation in interventions there is still very limited knowledge due to the lack of studies in the field.
2 AIMS OF THE THESIS

The overall aim of this thesis was to reach a higher understanding of cardiovascular disease mechanisms and treatment options in chronic kidney disease patients, with focus on the role of vitamin D on different aspects of vascular dysfunction.

The specific aims of the four studies are as follows:

Study I: To investigate if paricalcitol treatment affects measures of muscle sympathetic nerve activity, as well as measures of macro- and microvascular function in moderate CKD.

Study II: To analyse the effect of paricalcitol treatment on the proinflammatory profile and epigenetic modulators, measured by microRNAs, in moderate CKD.

Study III: To assess the effect of paricalcitol treatment on microparticle expression of ICAM-1, and VCAM-1, as well as the effect on the profile of endothelial, platelet and leukocyte microparticles in moderate CKD.

Study IV: To investigate, using overall effect size in a meta-analysis, if there is evidence in existing publications of an effect of vitamin D treatment or supplementation on endothelial function measured by flow-mediated vasodilation.
3 MATERIALS AND METHODS

3.1 STUDY POPULATIONS AND STUDY DESIGN

3.1.1 The SOLID trial; Study I, II and III

Patients (n=36) in study I, II and III were recruited from the Department of Nephrology at Danderyd University Hospital, Stockholm, Sweden, from 2010 to 2013. They were randomised in a double blind manner (12 in each group) to receive 1 µg paricalcitol, 2 µg paricalcitol or placebo. Due to the influence of sun, patients were not recruited during summer. Inclusion criteria were an estimated glomerular filtration rate (eGFR) of 15–59 ml/min/1.73 m² calculated from plasma creatinine using the MDRD formula, age >20 years, a plasma PTH level of 3.7–53 pmol/L, a Ca level <2.6 mmol/l, serum albumin >30 g/L and being on stable antihypertensive medication with no change in angiotensin converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB) medication during 2 months before enrolling in the trial. Exclusion criteria were nephrotic syndrome, diabetes mellitus or any treatment with vitamin D or its analogues that could not be discontinued, acute renal failure during the last 3 months, and if expected to need dialysis within 6 months. Patients were also excluded if they had known renal artery stenosis, severe kidney stones, uncontrolled hypertension (repeated measures of a brachial blood pressure >150/100 mm Hg) or other severe disease (active cancer, AIDS/HIV, severe congestive heart failure).

The study protocol was approved by the regional Ethics Committee in Stockholm, and registered on clinicaltrials.gov (SOLID study; NCT01204528). All patients provided written informed consent.

Figure 8: Flow chart of the SOLID trial. echo=echocardiography.

3.1.2 Study IV

Study IV was a meta-analysis investigating the effect of vitamin D on FMD. Inclusion criteria were chronic kidney disease in any stage and with any underlying cause, intervention with any vitamin D compound, a study design with a placebo or non-treatment control group, and outcome in form of flow mediated vasodilation. Exclusion criteria were combination
treatments with vitamin D and calcium, or comparison to other vitamin D compound or calcimimetics, without a non-treatment control group.

3.2 STUDY METHODS

Study I, II, and III started with two weeks of placebo run in, followed by 12 weeks of treatment with 1 or 2 µg paricalcitol, or placebo. Venous blood samples were drawn in the morning, after 12 h fasting and 20 min rest, at baseline and post treatment.

3.2.1 Study I

3.2.1.1 Arterial Stiffness

To assess arterial stiffness we used applanation- tonometry (SphygmoCor, AtCor Pty, NSW, Australia). The equipment was used to acquire a peripheral waveform from which pulse wave velocity (PWV) was measured and pulse wave augmentation index (Alx) was calculated. (105). To determine PWV, two waveforms were sequentially recorded (carotid- radial artery and carotid-femoral artery). The R-wave of a simultaneously recorded ECG was used as a reference frame, and the transit time of the pulse wave was then determined. The distance was measured and the PWV was determined. To determine Alx, a central waveform was assessed by the software, and the augmentation between the first and second systolic pressure peak of the waveform was calculated. The augmentation was then expressed as a percentage of the total pulse pressure.

3.2.1.2 Large Vessel Endothelial Function

Macrovascular endothelial function was assessed by FMD (106). The brachial artery diameter was measured by a vascular ultrasound device with a 9-MHz linear transducer (Vivid 7 Dimension, GE Medical system, Horten, Norway). Endothelial dependent vasodilation was investigated by inducing ischemic reactive hyperaemia with a pneumatic tourniquet inflated to 250 mm Hg for 5 min. When released, vasodilation was measured as the relative change from rest in brachial artery diameter at 30, 60 and 90s. After 10 min of rest, endothelial independent vasodilation was assessed by the administration of 0.4 mg of sublingual glycerol trinitrate. The relative change from rest in brachial artery diameter was calculated from measurements in diameter 4 min after administration.

3.2.1.3 Skin Microvascular Function – Perfusion Imaging

Skin microvascular function was investigated by laser Doppler perfusion imaging before, during and after iontophoresis with acetylcholine (ACh; Sigma-Aldrich AB, Stockholm, Sweden) and sodium nitroprusside (SNP; Hospira, Inc., Lake Forest, Ill., USA) (173). ACh and SNP, diluted in deionized water, were used to examine endothelium dependent and independent skin microvascular function, respectively. Iontophoresis is a non-invasive technique using a small electric current for drug administration across the skin. Electrode
chambers (LI611 Drug Delivery Electrode Imaging, Perimed, Järfälla, Sweden) were attached to the volar side of the left forearm and filled with either ACh (2%) or SNP (2%). A battery-powered iontophoresis controller (Perilont 382b, Perimed, Järfälla, Sweden) provided a direct current (0.1 mA for 60 s) for drug iontophoresis. ACh was delivered with an anodal and SNP with a cathodal charge. Skin microcirculation was measured by laser Doppler perfusion imaging (Periscan PIM II, Perimed, Järfälla, Sweden) and expressed in arbitrary units (AU). Skin microcirculation was recorded continuously for 10 and 14 minutes after iontophoresis of ACh and SNP, respectively. Peak microvascular flux was determined. At our laboratory, the mean coefficient of variation of peak microvascular flux after iontophoresis of ACh and SNP were 11 and 20%, respectively.

3.2.1.4 Capillary Blood Cell velocity

Blood cell velocity in single nailfold capillaries of the great toe was measured during videophotometric capillaroscopy (174). Capillary blood cell velocity (CBV) was determined by two videophotometric windows positioned along the arterial side of the capillary axis detecting variations in optical density when blood cells and plasma gaps are passing through the capillary. The variations in light intensity are then converted into an electronic signal. Given the distance between the windows and the time delay between similar events in the upstream and downstream windows, CBV can be continuously recorded. CBV was measured during resting condition and during post-occlusive reactive hyperaemia, performed by a small pressure cuff at the proximal phalanx of the great toe. The cuff was inflated to a pressure of 200 mm Hg for 1 min, and peak CBV (mm/s), time to peak CBV (s), as well as relative change from rest (%) were measured. The temperature was continuously recorded during the measurements of CBV We have a good reproducibility in our laboratory (175).

3.2.1.5 Sympathetic Activation

Muscle sympathetic nerve activity (MSNA) was recorded using microneurography by a tungsten microelectrode inserted percutaneously into a muscle fascicle of the peroneal nerve (176). The signal was digitized (Powerlab Neuroamp, ADInstruments, Bella Vista, Australia) and pulse-synchronised bursts were analysed by a blinded investigator. Heart rate was recorded by a standard electrocardiogram, and blood pressure was measured with an automatic cuff. MSNA was quantified as bursts per minute (SNA/min) and as bursts per 100 RR intervals (SNA/RRI).

3.2.2 Study II

3.2.2.1 Characterization of the expression of immune modulators in plasma

Using the Luminex technique, a Milliplex 26-plex (Millipore corp.) was performed to assess the expression of a wide spectrum of cytokines before and after 12 weeks of paricalcitol treatment or placebo. In the Luminex technique, the company prepares microspheres, or beads, numbered to allow differentiation, and then covered in different antigen-specific
antibodies. They are then put together in different patterns allowing a multiple of molecules to be identified in one well simultaneously. The test-sample was then added by us and the expression was quantified by labelled detection antibodies (Figure 9).

**Figure 9:** The Luminex® technique. Published with permission from Luminex® and ThermoFisher Scientific.

### 3.2.2.2 Reverse transcription polymerase chain reaction (RT-qPCR) to assess miRNA expression

We used miRCURY Ready-to-Use PCR Human panel I + II V1.M (EXIQON miRNA qPCR panel) (Figure 10) to assess the possible changes in miRNA expression in plasma. It was a ready to use kit involving a human miRNA panel, with a first step of reverse transcription (RT), of miRNA to DNA, followed by real-time PCR amplification with miRNA specific primers. For the first step, Universal cDNA synthesis kit II (miRCURY LNATM Universal RT microRNA PCR, EXIQON) was used. We then applied miRNA specific primers and ExiLENT SYBR Green master mix kit (miRCURY LNATM Universal RT microRNA PCR, EXIQON) for RT-qPCR. Quality control of the RNA isolation was performed by RNA spike-in (UniSp2, UniSp4, UniSp5), and cDNA synthesis control was assessed by UniSp6 in the RT-reaction. In addition DNA spike in (UniSp3) was added in all samples.

PCR reaction was assessed by MicroAmp™ optical 384-well reaction plates with an ABI 7900 (Life Technology). As the amount of RNA in a sample is too small for exact determination of concentrations, the biofluid input amount in the PCR reaction was used as recommended by the manufacturer.
3.2.3 Study III

3.2.3.1 Assessment of microparticle concentrations

As previously described (178), plasma was centrifuged at 2,000 G for 20 min to attain platelet poor plasma, which was then frozen in aliquots at -80 °C until analysis. Platelet poor plasma was thawed and again centrifuged at 2,000 G for 20 min. The supernatant was subsequently centrifuged at 13,000 G for 2 min. 20 µL of the supernatant was incubated in the dark with lactadherin-FITC (BLAC-FITC, Coatech AB, Kläesholmen, Sweden) which binds to phosphatidylserine (PS), together with CD62E-APC (Thermo Fisher Scientific, Waltham, MA, USA), CD41-APC (Beckman Coulter, Brea, CA, USA), CD62P-PE (Thermo Fisher Scientific, Waltham, MA, USA), CD154-APC (Thermo Fisher Scientific, Waltham, MA, USA) CD45-APC (Beckman Coulter, Brea, CA, USA), CD106-PE (VCAM-1, Abcam, Cambridge, UK) and CD54-PE (ICAM-1, Abcam Cambridge, UK). MPs were defined as particles less than 0.9 µm in size, positive to lactadherin. All samples were analysed using a Beckman Coulter Gallios flow cytometer (Beckman Coulter, Brea, CA, USA), and the MP-gate was calibrated using Megamix beads (FSC; 0.5 µm, 0.9 µm and 3.0 µm, BioCytex, Marseille, FR).

![miRCURY-technique diagram](image)

**Figure 10:** The miRCURY-technique. First step is the synthesis of cDNA (reverse transcription (RT)) from existing miRNA in the sample. Next steps are amplifications of the cDNA produced in the first step by real-time PCR, using miRNA specific primers, and quantification of the process with help of SYBR-green. Modified from the miRCURY handbook (177). Published with permission from QIAGEN (formerly EXIQON).
3.2.4 Study IV

A systematic literature search of PubMed/Medline, Web of Science (WoS), Embase and Cochrane trials and reviews was performed. Search results were restricted to controlled trials and to the English language. We used the MeSH-terms for vitamin D as well as kidney disease, and all terms listed beneath. The selected articles were coded with a prespecified extraction form including study length, number of participants, vitamin D compound and dosage, age, CKD stage, other treatments and baseline laboratory measurements such as vitamin D status. Methodological study quality and risk of bias were assessed by the Jadad Score, which gives 1-5 points for blinding, randomization and risk of incomplete outcome data (the account of all screened and included patients) (179). Selective outcome reporting was also assessed during the screening and selection process. To assess publication bias, rank correlation and a funnel plot were both performed. We also searched ClinicalTrials.Gov for unpublished articles matching our inclusion criteria.

3.3 STATISTICAL ANALYSES

The SOLID study was a novel hypothesis-testing study, where the study-size was based on a treatment-induced change in MSNA. The intention was to study first non-diabetic CKD, and then diabetic CKD. Whether vitamin D affected MSNA was unknown, and an estimated moderate-large effect size (f=0.36) showed that we needed 36 patients in each group (non-diabetic and diabetic CKD). We included a pre-specified interim analysis after the first group of patients (non-diabetic CKD; n=36). At that interim analysis, we found no significant change in MSNA. Instead of pursuing the hypothesis that MSNA might be affected in diabetic CKD, due to limited resources, we instead decided to turn to secondary explorative outcome measures.

Descriptive statistics were presented as means and proportions. Between group differences in means at baseline were examined by one-way analysis of variance (ANOVA), by the Chi²-test, or the Fischer´s exact test when appropriate.

In paper I, the effects of intervention (paricalcitol) and time were investigated for vascular function and MSNA by using repeated measures two-way ANOVA as well as by multilevel modelling/mixed model (MLM) to improve the statistical power. We also examined the effects of intervention and time within groups, by paired t-test.

In paper II, non-parametric tests were used, as data was non normally distributed. Using the same patients, changes in cytokine expression was investigated by Kruskal Wallis (pre- and post-measurements separated), followed by Wilcoxon matched-pairs signed rank test for within group comparisons over time and Mann Whitney U-test for two independent group comparisons. For miRNA, the delta change (post - pre measurements) as well as fold change (post/pre measurements) were determined and the between-groups comparison was then
performed by non-parametric Kruskal Wallis and Mann Whitney U-test for two independent group comparisons.

In paper III, changes in microparticle-levels and their expressions in the SOLID patients, was tested using two-way repeated measures ANOVA (ICAM-1, VCAM-1) and, for the profile of subclasses of MPs, two-way repeated measures MANOVA. Post hoc analyses were performed with one-way repeated measures MANOVA (main effect of time) and paired t-test for within group changes.

Paper IV, a meta-analysis of treatment with vitamin D to CKD patients, was analysed as post treatment comparisons, calculating standardized mean difference effect size (STANDmean ES). Weighted standard deviation and then Hedges g were used to calculate effect sizes (STANDmean ES) for each study (180, 181). A positive value indicated an effect in favour of treatment. The overall effect size was then assessed by a fixed effects model (180). A random effects model was also performed as a secondary analysis. I² statistics were performed to assess heterogeneity.

Statistical analyses were performed using SPSS software, version 22 and 24 (SPSS Inc., Chicago, IL, USA) and SAS (version 9.3, SAS Institute Inc., 2002–2010).

3.4 ETHICAL CONSIDERATIONS

The SOLID trial was performed in accordance with the declarations of Helsinki. All patients provided and signed written informed consent. The study was registered at ClinicalTrial.Gov as SOLID study; NCT01204528.

The meta-analysis was performed in accordance with the PRIMA guidelines and MOOSE checklist. All included studies assured the use of the declarations of Helsinki and of written informed consent.
4 RESULTS

4.1 THE SOLID STUDY; PAPER I, II AND III

4.1.1 Patient characteristics

Thirty-six patients were included, and 35 completed the trial. The one person who did not complete the trial was in the placebo group, and experienced feelings of dizziness from the study drug (placebo) and did not perform the post-treatment measurements. No major adverse events were found during the study period, and there were no hospitalizations. Baseline characteristics showed that those in the placebo group were slightly older with more previous cardiovascular disease, and a tendency to higher number of patients with polycystic kidney disease as aetiology of CKD.

Table 6: Baseline characteristics in the SOLID trial as mean and (SD) or nr and (%).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Placebo (n=12)</th>
<th>Paricalcitol 1μg (n=12)</th>
<th>Paricalcitol 2 μg (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>70.8 (10.0)</td>
<td>66.1 (7.9)</td>
<td>59.1 (11.6)</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>9 (75%)</td>
<td>11 (92%)</td>
<td>8 (67%)</td>
</tr>
<tr>
<td>Smokers (current)</td>
<td>1 (8%)</td>
<td>1 (8%)</td>
<td>0</td>
</tr>
<tr>
<td>BMI</td>
<td>28.1 (2.4)</td>
<td>26.4 (3.5)</td>
<td>26.8 (2.8)</td>
</tr>
<tr>
<td>CKD duration (years)</td>
<td>10.3 (8.8)</td>
<td>5.8 (6.0)</td>
<td>9.7 (10.5)</td>
</tr>
<tr>
<td>Cause of CKD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>3 (25%)</td>
<td>4 (33%)</td>
<td>4 (33%)</td>
</tr>
<tr>
<td>Polycystic disease</td>
<td>4 (33%)</td>
<td>2 (17%)</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>4 (33%)</td>
<td>3 (25%)</td>
<td>4 (33%)</td>
</tr>
<tr>
<td>Other cause</td>
<td>0</td>
<td>2 (17%)</td>
<td>2 (17%)</td>
</tr>
<tr>
<td>CVD at inclusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>3 (25%)</td>
<td>2 (17%)</td>
<td>0</td>
</tr>
<tr>
<td>Atrial Fibrillation</td>
<td>1 (8%)</td>
<td>1 (8%)</td>
<td>0</td>
</tr>
<tr>
<td>Stroke</td>
<td>3 (25%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TIA</td>
<td>1 (8%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Heart failure</td>
<td>0</td>
<td>0</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>Aortic aneurysm</td>
<td>0</td>
<td>1 (8%)</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE-i/ARB</td>
<td>11 (92%)</td>
<td>9 (75%)</td>
<td>9 (75%)</td>
</tr>
<tr>
<td>β-blockers</td>
<td>6 (50%)</td>
<td>8 (67%)</td>
<td>4 (33%)</td>
</tr>
<tr>
<td>Ca flow-inh</td>
<td>10 (83%)</td>
<td>8 (67%)</td>
<td>4 (33%)</td>
</tr>
</tbody>
</table>

BMI=body mass index; CKD=chronic kidney disease; TIA=transient ischaemic attack; ACE-i=angiotensin converting enzyme inhibitor; ARB=angiotensin receptor blocker; Ca-flow-inh=calcium-flow inhibitors. Reprinted in courtesy of American Journal of Nephrology:2015, 42(4):265-73, by permission of S. Karger AG.
The three groups did not differ in eGFR, duration of CKD or serum creatinine levels. They were also well matched in routine biochemistry status, measures of vascular function (FMD and PWV), microcirculatory function except peak CBV (iontophoresis and videophotometric capillaroscopy), and measures of sympathetic nerve activity (MSNA). Measures of cytokine expression and concentrations of microparticles were also well matched between groups at baseline (Table 6).

4.1.2 Routine laboratory findings post treatment

There were no significant changes in albumin, UACR or CRP-levels post treatment. Neither were there any significant changes in phosphate, 25OH-vitamin D, or calcium. However, calcium did change slightly in absolute numbers with highest levels post treatment in the 2 µg treatment group. PTH was significantly suppressed by treatment (two-way ANOVA p=0.02 for treated groups combined, p=0.006 for 3 groups comparison) in a dose dependent manner.

4.2 VASCULAR MEASUREMENTS; PAPER I

There was a significant decrease in FMD across all groups after 3 months study (MLM main effect of time p=0.006). However, when within group changes were examined, the 2 µg treated group showed preserved endothelial function (paired t-test p=0.54) (Figure 11a).

For iontophoresis by acetylcholine the results were similar, with a borderline significant decrease across all groups, (repeated measures ANOVA, main effect of time p=0.06) but preserved function with no significant decrease for the 2 µg treated group (paired t-test, p=0.65) (Figure 11b).

We also performed videophotometric capillaroscopy. There were however several patients where this measure could not be performed. The treated groups were collapsed into one and MLM performed on 26 patients. The results showed borderline significant interaction, with ameliorated microcirculatory function in the treated groups, and declining function in the placebo group, measured as peak CBV (MLM p=0.06). Within group changes also showed ameliorated function for the treated group with borderline significance (paired t-test p=0.05) (Figure 11c).
We did not detect any change by intervention and time in PWV, PWAix or MSNA. For MSNA there was only complete data for 24 patients, but with MLM, 11 of those with incomplete data could contribute with values in the analysis.

4.3 PROINFLAMMATORY CYTOKINES AND miRNAs; PAPER II

When comparing pre- and post-measurements of a range of proinflammatory cytokines, VEGF and PDGF were significantly decreased in the two treated groups, but remained unchanged in the placebo group (Wilcoxon matched pairs for within group changes). Levels of interferon gamma induced protein 10 (IP10), a cytokine central in the enhancement of the inflammatory response, were also decreased for treated patients, but only significantly for the 2µg treated group (Wilcoxon matched pairs) (Table 7).
Table 7: Concentrations of cytokines (pg/ml) pre- and post intervention, including p-values. Reprinted in courtesy of BMC Nephrology: 2017, 18(161), in line with the open access agreement for Springer Nature.

The two treatment groups were also collapsed into one, to obtain better power, and we then detected a significant decrease in a wide range of cytokines (Wilcoxon matched pairs), in comparison with placebo where there were no significant changes during the study (Figure 12).

Figure 12: Changes in cytokine levels pre-post treatment in the collapsed treatment groups. P-values indicated in the figures. There were no significant changes in the placebo group.

To determine possible changes in miRNAs post treatment, we first performed a pilot study of five randomly selected patients on 2 µg paricalcitol treatment. The top five (p-value and fold change) changed miRNAs were miR 133a, miR432, miR 495, miR 576 and miR 874. These were then analysed in all 36 patients. We chose to include also the five patients in the pilot study in the final analysis to obtain better statistical power. MiR 432, miR 495 and miR 576 were all significantly downregulated by 2µg of paricalcitol treatment (Kruskal-Wallis and post hoc Mann-Whitney U-test comparisons of delta value and fold change) (Figure 13). The effect was similar when collapsing the treatment groups.
**Figure 13:** The five top ranked miRNAs validated in all patients, as fold change (post/pre measurements). Significant change by treatment indicated with p-value. Reprinted in courtesy of BMC Nephrology: 2017, 18(161), in line with the open access agreement for Springer Nature.

### 4.4 ENDOTHELIAL MICROPARTICLES AND VASCULAR BIOMARKERS; PAPER III

In this study, we investigated the change in expression of ICAM-1 and VCAM-1 on microparticles as well as the change in total MP profile during intervention.

There was a change by treatment in the expression of ICAM-1 on MPs, seen by a significant interaction (repeated measures two-way ANOVA p=0.04), with augmented levels in the placebo group, and decreased levels in the treated groups (Figure 14a). This was not seen for the expression of VCAM-1 on MPs, where the levels remained stable during intervention (repeated measures two-way ANOVA p=0.52).

To avoid multiple comparisons, a two-way repeated measures MANOVA was used, to investigate the changes in cell-specific MP subtypes (CD62E+ EMPs, CD41+ PMPs, CD41+CD62P+ PMPs, CD41+CD154+ PMPs and CD45+ LMPs). There was a significant decrease in all MP subtypes during the study (repeated measures MANOVA, main effect of time p=0.001), with a tendency to interaction between treatment and time (repeated measures two-way MANOVA p=0.08). Post hoc analyses with one way repeated measures MANOVA showed that the findings were due to sustained levels in the 2 µg (p=0.85), and significantly decreasing levels in the 1 µg (p=0.04), and placebo group (p=0.005) Figure 14b).
Focusing on EMPs, further analyses demonstrated that EMP levels were not significantly changed for either the 1 or 2 µg treatment groups during the study (1 µg, paired t-test, p=0.10, and 2 µg, p=0.3), while decreasing in the placebo group (paired t-test, p=0.002). In absolute numbers, a pattern of dose dependent change was observed (Figure 14c).

**Figure 14**: Pre and post measurements of microparticles. MP=microparticle; CD62E\(^+\)MPs=EMPs
4.5 OVERALL EFFECT SIZE BY PARICALCITOL TREATMENT ON FMD; PAPER IV

4.5.1 Study selection and population

In total, 1,744 articles were found searching the databases. After screening of title and abstract 304 articles remained, and 14 were selected for full review. Of these, four studies met the full inclusion criteria (Figure 15). However, one of the studies comprised two treatment groups (1 and 2 µg of paricalcitol; study one in the present thesis) and after discussion, this study was split in two, using the same placebo group as control, thus resulting in five studies in the final analysis.

![Flow chart of the selection process](image)

**Figure 15**: Flow chart of the selection process. WoS=Web of Science; Reprinted in courtesy of BMC Nephrology: 2018, 19(247), in line with the open access agreement for Springer Nature.

All included patients were in CKD stage 3-4. Mean age was 59.9 years, ranging from 44-65 years. Study size varied from 24-120 participants, and study duration from 12 to 16 weeks. One study investigated the effect of cholecalciferol in two oral doses of 300 000 IU at baseline and after 8 weeks, while the others used paricalcitol 1 or 2 µg daily (Table 8).
Table 8: Baseline characteristics of the included studies in the meta-analysis.

<table>
<thead>
<tr>
<th>Author</th>
<th>Zoccali (-14)</th>
<th>Lundwall 2μg (-15)</th>
<th>Lundwall 1μg (-15)</th>
<th>Theti (-15)</th>
<th>Kumar (-17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Country</td>
<td>Italy</td>
<td>Sweden</td>
<td>Sweden</td>
<td>USA</td>
<td>India</td>
</tr>
<tr>
<td>Duration</td>
<td>12w</td>
<td>12w</td>
<td>12w</td>
<td>12w</td>
<td>16w</td>
</tr>
<tr>
<td>Sample size (nr)</td>
<td>89, analysis on 88</td>
<td>24, ITT</td>
<td>24, ITT</td>
<td>60, 55 completed, analysis on 46</td>
<td>120, analysis on 117</td>
</tr>
<tr>
<td>CKD stage</td>
<td>3-4</td>
<td>3-4</td>
<td>3-4</td>
<td>3-4</td>
<td>3-4</td>
</tr>
<tr>
<td>Treatment</td>
<td>paricalcitol</td>
<td>paricalcitol</td>
<td>paricalcitol</td>
<td>paricalcitol</td>
<td>cholecalciferol</td>
</tr>
<tr>
<td>Dose</td>
<td>2μg daily</td>
<td>2μg daily</td>
<td>1μg daily</td>
<td>1μg daily</td>
<td>300000IU at baseline and after 8 week</td>
</tr>
<tr>
<td>Baseline 25(OH)D (nmol/l)</td>
<td>35.5</td>
<td>65.1</td>
<td>66.7</td>
<td>1.25-OHD: 34.5 (pg/ml)</td>
<td>33.2</td>
</tr>
<tr>
<td>Age (mean)</td>
<td>62.5</td>
<td>65.0</td>
<td>68.5</td>
<td>62.5 (median)</td>
<td>44.2</td>
</tr>
<tr>
<td>ACEi/ARB (%)</td>
<td>N/A</td>
<td>Non-diabetic patients</td>
<td>Non-diabetic patients</td>
<td>Diabetic nephropathy</td>
<td>Non-diabetic patients</td>
</tr>
<tr>
<td>Jadad score</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Underlying condition/DM</td>
<td>N/A</td>
<td>FMD, PWV, echo, ionicophoresis, microcirc</td>
<td>FMD, PWV, echo, ionicophoresis, microcirc</td>
<td>FMD</td>
<td>FMD, PWV</td>
</tr>
<tr>
<td>Outcome</td>
<td>FMD</td>
<td>FMD, PWV, echo, ionicophoresis, microcirc</td>
<td>FMD, PWV, echo, ionicophoresis, microcirc</td>
<td>FMD</td>
<td>FMD, PWV</td>
</tr>
</tbody>
</table>

ACEi=angiotensin converting enzyme inhibitor; ARB=angiotensin receptor blocker; DM=diabetes mellitus; ITT=intention to treat; FMD=flow mediated vasodilation; PWV=pulse wave velocity; echo=echocardiography. Reprinted in courtesy of BMC Nephrology: 2018, 19(247), in line with the open access agreement for Springer Nature.

4.5.2 Study quality and bias

Both the Jadad score and the Cochrane handbook 5.1 were used to evaluate study quality and risk of biased data. Included studies had Jadad scores from 3 to 5, indicating median to high quality. There were no signs of selective outcome reporting when performing the literature screening and selection. Overall, this indicated a low risk of biased data. Rank correlations and visual inspection of a funnel plot did not indicate publication bias. This conclusion was also strengthened by the search in ClinicalTrials.Gov, where there were no unpublished trials of interest to our inclusion criteria.

4.5.3 FMD outcome

The five included studies comprised 305 patients. Patients were well matched in terms of FMD at baseline in each study. The fixed effects model analysis showed a positive effect of vitamin D treatment on FMD (STANDmean ES 0.78, 95% CI 0.55-1.01) (Figure 16). A random effects model performed as a secondary analysis also showed significantly positive effects of treatment (STANDmean ES 0.67 95% CI 0.06-1.29). The heterogeneity was substantial for the fixed effects model ($I^2=84\%$), but minimal for the random effects model ($I^2=0\%$). There were too few studies included to perform a meta-regression to statistically investigate the heterogeneity in the fixed model.
Figure 16: Forest plot of effect sizes in the included studies. Overall effect size in a fixed effects model. Reprinted in courtesy of BMC Nephrology: 2018, 19(247), in line with the open access agreement for Springer Nature.
5 GENERAL DISCUSSION

In the SOLID study we show that 2 µg of paricalcitol to patients with moderate CKD preserves macro- and microvascular endothelial function measured by FMD and iontophoresis by acetylcholine, and seems to improve microcirculatory measures, in line with previous findings (44, 82, 111-113). We also show that this population, with few previous cardiovascular events, has a rapid decline in endothelial and microcirculatory functions.

Further investigations in our patients demonstrate that treatment with paricalcitol improves the inflammatory profile, measured by cytokine release, also in line with previous findings in small RCT:s (96-99). The effects were most pronounced for the vascular inflammatory cytokines VEGF and PDGF showing again the positive effects of vitamin D on vascular cells. The upstream regulation, measured by the change in microRNAs, was also changed by treatment, showing lower expression of microRNAs with a connection to atherosclerosis, platelet function and inflammation (167, 168, 170-172). These results, however in a new field, give an indication of the possible upstream epigenetic effects of vitamin D on our laboratory and physiological findings.

To further explore the vascular function in our patients, as well as investigate new possible biomarkers of cardiovascular disease, we assessed the microparticle profile pre- and post-treatment, as well as their expression of vascular activation markers. To our knowledge, no studies have previously investigated the effects of vitamin D on the MP profile in CKD patients. Our results on the microparticle expression on ICAM-1, showing lower expression with treatment, are in line with previous findings (44, 133) on soluble ICAM-1. The assessment of cell-specific MPs shows interesting and intriguing results, with decreasing production of MPs over time in all cells, however preserved by 2 µg of treatment, much like our physiological vascular results of FMD and iontophoresis. These findings are new and demand further research, but might indicate that a dysfunctional vasculature with surrounding cells does not have the ability to produce as much MPs, as healthy cells have. This interpretation is strengthened by a study of Augustine et al (158), showing lower MP levels after a dobutamine stress echocardiography in patients with signs of coronary disease, than in patients with a normal examination.

Treatment with vitamin D in CKD patients seems to lower albuminuria on top of RAAS-blockade (72-75), but the effects on vascular measurements are inconclusive. Most studies on inflammatory markers, iontophoresis and FMD have shown effect of treatment (44, 82, 111-113, 133) but as outlined in the background, most studies using PWV/PWA have failed to show significant changes by treatment. The reasons for this discrepancy in findings might be many, but one important issue may be in the methods of measuring vascular function. PWV/PWA are complex measures of both arterial stiffening and calcification (PWV/PWAix) (12), and beta-2 induced vasodilation (PWA after administration of beta 2-adrenoceptor stimulation) (109), whereas FMD is primarily a measure of the capacity of the endothelial cells to produce and react to NO (106). As such, FMD is mainly a measure of function, not
structure, and is therefore probably an earlier sign of vascular disease, than PWV/PWAix. It is therefore likely easier to affect FMD in short duration studies, since it does not require structural changes to occur. Another important aspect in understanding these results are the cellular effects of vitamin D, with antioxidative, eNOS-upregulating actions, seen in in vitro-studies (44, 83, 85). These are direct pathways for an ameliorated NO-production by the endothelial cells. Even though PWV and FMD are clearly different measures of vascular structure and function, they are interrelated (107, 126) and are both independent predictors of cardiovascular risk (17, 45, 46, 109, 126).

In our last study, we aimed to further understand the diverging results of interventions with vitamin D on vascular function. As all studies performed have been quite small, they all have limited statistical power. We consequently performed a meta-analysis assessing the effect of vitamin D compounds in patients with CKD, and in accordance with the pathways for vitamin D actions, and the short duration of studies, we used FMD as single outcome. Our meta-analysis showed that vitamin D does affect endothelial function in terms of NO production in a positive direction.

The meta-analysis also gives interesting clues to which patients that might benefit the most from Vitamin D treatment. While all patients were in CKD stage 3 to 4, four studies had populations originating from western countries with a mean age of 63.9 years, while the study with the strongest effect size had a population originating from India, with a mean age of 44.2 years (111). It is plausible that the structural vascular changes were not as pronounced when entering the study for these latter patients. Kumar et al also used cholecalciferol, raising questions about the potentially negative effects on calcium- and phosphate metabolism by active vitamin D treatment. Of interest is the sub-study by Zoccali et al (182), on the PENNY trial (113), showing that the effect of paricalcitol on FMD was most pronounced in patients with the lowest rise in phosphate, and abolished in those with the highest rise.

Interventional studies on CKD patients have suffered from inconclusive and negative outcomes in many fields of research. Chronic kidney disease comprises a wide spectrum of underlying disorders, from hypertension, to autoimmune diseases, vasculitis, infections, diabetes type 1 and 2, and the inherited polycystic disease. The CKD diagnosis also includes patients with an almost normal kidney function, through the whole range of renal dysfunction, to dialysis patients with a very complex picture of disturbances due to both their kidney failure and the dialysis per se. When interpreting interventional studies on this group of patients, both the underlying disorder and the stage of kidney disease has to be taken into cautious consideration. In the field of vascular disease, questions have been raised if it is even possible to reverse or ameliorate the advanced vascular disease seen in later stages of chronic kidney disease (12, 13).

The studies performed with vitamin D treatment in CKD patients are too small and of too short duration to answer questions on hard endpoints. PWV/PWA and FMD are both predictors of cardiovascular risk, whereas other measures of vascular function, such as iontophoresis and SEVR are less validated (126). Our meta-analysis shows that there are still
very few patients included in controlled studies investigating these more validated measures of vascular risk. There were no found studies assessing hard endpoints as an à priori outcome in our search.

5.1 LIMITATIONS

The major limitations in our RCT are the small number of participants and short duration of intervention. The power calculation was made for a significant change in MSNA, and not for FMD, and therefore we may have a lack of power in the study and a risk of type-2 errors. From this aspect, it might have been wiser to use only a two-group scheme, comparing placebo to treatment, however at the time of the study design it was not known which dose to use. The use of a predictor instead of hard endpoints is another limitation, however closely linked to the short duration, which makes hard endpoints impossible to use.

The meta-analysis has similar limitations with each included study being small and of short duration, and in addition there are few studies performed. It makes the generalizability of our findings limited, but also highlights the need for properly sized studies in the field.

6 CONCLUSIONS

Treatment with vitamin D improves inflammation and preserves measures of endothelial function in patients with CKD stage 3-4. Early intervention, before manifest structural vascular changes have occurred, is probably needed for adequate effects. The question whether to use active treatment or supplementation remains, but some results are in favour of supplementation. Another remaining question is of other aspects of CKD-MBD, where studies indicate that combination therapies, controlling different aspects of the CKD-MBD axis, might be the right way forward. The study lengths are too short and the number of study participants is still too few to answer questions on hard endpoints in this field. However, the lack of evidence on hard endpoints due to these facts, is not to be confused with no evidence, and hopefully the future will give us conclusive answers.
7 FUTURE PERSPECTIVES

We show effects of vitamin D treatment in CKD patients on vascular function and inflammation, but the results in this area are not conclusive, like in many of the research areas of intervention in kidney diseases. In the field of vitamin D and CKD, many questions remain a decade after the pleiotropic effects of vitamin D were first acknowledged.

There are important issues of research methodology in this area. Underlying disease and CKD stage have to be taken into a more appropriate consideration when designing studies. Is it feasible to believe that we can change the late and advanced vascular disease seen in CKD stage 5 and dialysis patients? Would it not be better to lose some of the generalizability in the very broad spectrum of CKD, and instead perform studies on more specified underlying conditions?

Relevant research questions have to be asked and appropriate methods used pertaining to the examined population and the study length. If it is not possible to use hard endpoints, which predictors should be used, and in which populations? Are the outcomes used good enough to answer our questions?

How can we perform larger and longer studies in this area? Here, the usage of already existing registries, registry RCT:s is an interesting field, which gives the possibility to perform larger and longer studies that cost less money and without the need of industry involvement. Here, the Swedish Renal Registry and the SWEDEHEART registry might be of use.

There are also remaining questions regarding the different aspects of CKD-MBD. This axis is brilliantly designed by nature to control itself, and maybe our attempts to correct one side of the axis are too imprecise, tipping the other side over. We need more thorough analyses of our results, to understand why some patients respond to treatment, and some do not. This was elegantly performed by Zoccali et al (182), showing that the effect of paricalcitol on FMD was clearly correlated to the individual rise in phosphate levels in the examined patients. Can we predict which patients that might respond and who will not? Can we from early stages combine treatments affecting the axis to better balance the effects? Here, we have to widen our views and cooperate in the different research fields of CKD-MBD.
Nedsatt njurfunktion är en av våra vanligaste folksjukdomar och drabbar 10-15 % av världens befolkning. Hjärt-kärlsjukdomar är en annan stor folksjukdom, och är dessutom orsaken till flest dödsfall i världen. De senaste årtionderna har det alltmer uppmärksammats att patienter med njursvikt också i hög utsträckning drabbas av hjärt-kärlsjukdom. Det är faktiskt så att de flesta patienter med njursvikt inte dör av sin njursvikt, utan av hjärt-kärlsjukdomar som hjärtinfarkt eller stroke. Orsakerna har utforskats och vi har nu en god förståelse av hur njursvikt ger en avancerad och snabbt progredierande kärlsjukdom, med stela, kalkinlagrade blodkärl. Startpunkten är sannolikt en kombination av förändringar i kärlen på grund den minskande njurfunktionen med störningar i vitamin D, kalk och fosfatmetabolismen, aktivering av immunförsvar och påföljande kronisk inflammation, och dysfunktion i det innersta kärllagret, endotelet.

I detta doktorandprojekt har vårt mål varit att få en bättre förståelse för kopplingen mellan njursvikt och hjärt-kärlsjukdom, med fokus på vitamin D-brist, kärlfunktion och inflammation.

Vitamin D genomgår sitt slutliga aktiverande steg i njuren, vilket medför att njursvikt leder till låga nivåer av aktivt vitamin D. Vitamin D är klassiskt känt som en viktig del inom kalkiumomsättningen, då det behövs för upptaget av kalkium. Dock har studier de senaste årtionderna visat att vitamin D har betydligt fler funktioner än så. Dess receptor finns spridd i hela kroppen, och aktiveringen sker inte bara i njuren, utan även i andra celler, bland annat immunceller. Vitamin D har en viktig funktion i just immunförsvar, där det både förbättrar försvaret mot bakterier, men också verkar antiinflammatoriskt, och minskar nivåerna av oxidanter i kroppen.

Det inre kärllagret, endotelet, har en mycket viktig funktion i kärlen då det styr kärlens sammandragning vilket i sin tur påverkar blodtrycket. Endotelet aktiverar och styr också immunceller och blodplättar, och endoteldysfunktion är en av de tidiga hörnstenarna i åderförkalkning, ateroskleros. Endoteldysfunktion har därför också visat sig korrelera till risk för framtida hjärt-kärlhändelser. En viktig del i endoteldysfunktion är inflammation orsakat av aktiverade immunceller samt höga nivåer av oxidanter, bildade både från aktiverade immunceller och från endotelet självt. Vitamin D är därför intressant som en möjlig väg att påverka njursviktspatienters förhöjda risk för hjärt-kärlsjukdom, genom att verka antiinflammatorisk och antioxidativt på blodkärlens endotel.

Våra patienter var i genomsnitt 65 år gamla, och hade en måttligt nedsatt njurfunktion (medel estimerad njurfunktion GFR 40ml/min). De hade få tidigare hjärtkärlhändelser.

I studie I visade vi att behandling med 2 µg paricalcitol ledde till en bevarad endotelfunktion, medan denna försämrdes i placebogruppen, och gruppen som behandlades med 1 µg paricalcitol. Mikrocirkulationen förbättrades för båda behandlingsgrupperna, men försämrdes för placebogruppen.

I studie II undersökte vi inflammationsprofilen mätt i form av cytokiner, före och efter behandling. Här såg vi en minskad inflammation av både 1 och 2 µg behandling, dock tydligast visat med minskande nivåer av VEGF och PDGF, två cytokiner direkt kopplade till kärlfunktion och ateroskleros. Här undersöktes också epigenetisk påverkan i form av microRNA, som styr vilka mRNA som kommer att aktiveras och därmed bilda proteiner. Vi visar lägre nivåer av tre microRNA, kopplade till ateroskleros, blodplättss funktion, och inflammation.


Studie IV var en meta-analys av alla genomförda behandlingsstudier med vitamin D till njursviktspatienter, där utfallet var endotelfunktion mätt med flödesmedierad vasodilatation. Vi hittade fyra publicerade studier, med sammanlagt 305 deltagare. Den sammanlagda effekten av behandling med vitamin D visade på en förbättrad endotelfunktion vid behandling. Vår meta-analys ger också indicier på att det kan vara bättre att behandla från yngre ålder, och tidigare i förloppet av njursvikt.

Detta doktorandprojekt visar att vitamin D har positiva effekter på kärlfunktion och inflammation. Vi behöver dock mer forskning för att kunna välja rätt patienter att behandla och vi bör sannolikt starta behandlingen tidigt, innan kärlsjukdomen hunnit bli alltför uttalad. Vi behöver också förstå hela aterosklerosprocessen vid njursvikt bättre, då det sannolikt krävs behandlingar från flera håll och samarbete inom forskningsfältet för att bättre balansera systemet och minska dessa patienters aggressiva kärlsjukdom.
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