

Dissertation

MiRNAs as new putative Biomarkers for Monitoring of Mineral Bone Disorder and Vascular Calcification in Chronic Kidney Disease

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I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz“.

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Abbreviations:

1,25(OH) ₂ D...	1,25-dihydroxyvitamin D
25(OH)D...	25-hydroxyvitamin D
AGO...	Argonaut
anti-miR...	miRNA inhibitor
ATM...	ataxia telangiectasia mutata
bALP...	bone alkaline phosphatase
BMD...	bone mineral density
BMP...	bone morphogenic protein
BMP-2...	bone morphogenic protein-2
BMR2...	bone morphogenetic protein receptor type 2
BSP...	bone sialoprotein
Ca ²⁺ ...	calcium ion
CAD...	coronary artery disease
Cbfa1...	core-binding factor alpha 1
CBVD...	cerebrovascular disease
CDKN1a...	cyclin-dependent kinase inhibitor 1A
CHUK...	conserved helix-loop-helix ubiquitous kinase
CKD...	chronic kidney disease
CKD-MBD...	CKD-mineral bone disorder
CRP...	C-reactive protein
CT...	computer tomography
CTCL...	cutaneous T-cell lymphoma
CTNNBIP1...	beta-catenin-interacting protein 1
CTX...	beta-crosslaps
CVD...	cardiovascular disease
DUSP2...	dual specificity protein phosphatase 2
DXA...	dual-energy X-ray absorptiometry
E2F1...	E2F transcription factor 1
ERK...	extracellular signal-regulated kinase
ESRD...	end stage renal disease
FGF...	fibroblast growth factor
GFR...	glomerular filtration rate
GLA-protein...	gamma-carboxyglutamic acid protein
HDAC...	histone deacetylase

HF...	heart failure
IHH...	indian hedgehog protein
IKK α ...	Inhibitor of NF- κ B Kinase alpha
IL-10...	interleukin 10
IL-6...	interleukin 6
IL6ST...	interleukin 6 signal transducer
IL-8...	interleukin 8
IRAK4...	interleukin-1 receptor-associated kinase 4
KAT2B...	lysine acetyltransferase 2B
KDIGO...	Kidney Disease: Improving Global Outcomes
KT...	kidney transplantation
LATS2...	large-tumor-suppressor-kinase 2
MAPK...	mitogen-activated protein kinase
M-CSF...	macrophage colony-stimulating factor
M-CSFR...	macrophage colony-stimulating factor receptor
MEF2C...	myocyte enhancer factor 2C
MGP...	matrix GLA protein
MI...	myocardial infarction
miRNA...	micro ribonucleic acid
MPO...	myeloperoxidase
NFAT5...	nuclear factor of activated T-cells 5
NF-ATc1...	nuclear factor of activated T-cells, cytoplasmic 1
NFI-A...	nuclear factor 1 A-type
NF- κ B...	nuclear factor kappa-light-chain-enhancer of activated B cells
OC...	osteocalcin
OPG...	osteoprotegerin
OPN...	osteopontin
OSX...	osterix
PAH...	pulmonary arterial hypertension
PARP1...	poly(ADP-ribose) polymerase 1
PDCD4...	programmed cell death protein 4
PI3K...	phosphatidylinositol 3-kinase
PPARD...	peroxisome proliferator activated receptor delta
PTEN...	phosphate suppressor candidate 2
PTH...	parathyroid hormone

PTH-rp...	PTH related protein
RANKL...	receptor of NF- κ B ligand
RAS...	renin-angiotensin system
RISC...	RNA-inducing silencing complex
RNA-Pol...	RNA-polymerase
RPS6KB1...	ribosomal protein S6 kinase B1
RRT...	renal replacement therapy
RunX2...	runt-related transcription factor 2
SIBLING...	Small Integrin-Binding Ligand, N-linked Glycoprotein
STAT3...	Signal transducer and activator of transcription 3
STMN1...	stathmin 1
T2DM...	type 2 diabetes mellitus
TGF- β ...	transforming growth factor beta
TLR4...	toll-like receptor 4
TNFSF11...	tumor necrosis factor superfamily member 11
TNF α ...	tumor necrosis factor alpha
TOX...	thymocyte selection associated high mobility group box
TRAIL...	TNF-related apoptosis inducing ligand
TRAP 5b...	tartrate resistant alkaline phosphatase 5b
UTR...	untranslated region
VEGF...	vascular endothelial growth factor
VEGF-A...	vascular endothelial growth factor A
VSMC...	vascular smooth muscle cell

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Abstract:

Chronic kidney disease (CKD) is a widely spread disease, which is characterized by progressive deterioration of the functionality of the kidney. CKD is not only limited to negative effects on the kidney but is associated with a multifactorial dysregulation of bone metabolism and vascular calcification and is closely linked to increased cardiovascular disease (CVD) and concomitant bone disease, generally known as CKD-MBD (mineral bone disorder). These comorbidities are responsible for an increased mortality in CKD patients. Therefore, an early detection of bone related and cardiovascular problems in this patient group would help to improve the therapeutic approach.

In this study we aimed to investigate specific microRNA (miRNA) signatures in CKD patients as new biomarkers differentially regulated during the disease development, which would serve as indicators for vascular calcification and/or bone mineralization changes during CKD. An impact of kidney transplantation (KT) on these miRNA signatures was also monitored.

We evaluated a cohort comprising of 73 patients in CKD stages 3 to 5, 67 CKD patients after KT, and 36 healthy controls. Preliminary, a miRNA array investigating changes of miRNA expression in CKD patients was used to select 4 miRNAs which were quantified in the entire cohort. To characterize the different groups, a spectrum of biochemical parameters including markers for kidney function, inflammation, glucose, and mineral metabolism were measured and associated to the analyzed miRNAs using a univariate approach. Additionally a prospective study was started which includes CKD stage 5 patients listed for KT, with serum samples being collected on different time points, before, during and after the KT. Expression of miRNAs is compared between the time points.

The primary array results and bioinformatic evaluation lead to the selection of miR-223-3p, miR-93-5p, miR-142-3p and miR-146a-5p for further experiments. The relative expression of miR-223-3p and miR-93-5p from CKD patients in stage 4 and 5 was down-regulated compared to healthy controls, whereas miR-142-3p and miR-146a-5p did only show non-significant changes. After KT no down-regulation could be detected anymore for any of the 4 miRNAs, even when lower glomerular filtration rates (GFR) persisted. The levels were comparable to those in healthy controls. Associations for miR-223-3p and miR-93-5p were found for interleukin-6 (IL-6), eGFR levels, and by trend with interleukin-8 (IL-8), C-peptide, hematocrit, and parathyroid hormone (PTH).

The analysis of follow-up patients from the prospective study also indicated lower expression levels for miR-223-3p and miR-93-5p before KT.

Serum miRNA expression is altered in CKD patients. The miRNAs targeted, miR-223-3p and miR-93-5p, are putatively reflecting pathophysiological changes during CKD which are associated with inflammation, vascular calcification and changes in mineral and glucose metabolism. Identification of targets predicted for miR-223-3p and miR-93-5p strengthen these associations and indicate their possible effects during CKD-MBD. Further description and evaluation of the miRNAs and detection of additional miRNA signatures could contribute to future risk markers or future therapeutic targets in bone-, cardiovascular- and kidney-disease.

Zusammenfassung:

Chronische Nierenerkrankung (CKD) ist eine weit verbreitete Krankheit, die durch eine voranschreitende Verschlechterung der Nierenfunktion charakterisiert ist. CKD ist aber nicht auf die negativen Effekte auf die Nieren beschränkt, sondern ist mit einer multifaktoriellen Dysregulierung von Knochenmetabolismus und Gefäßverkalkung assoziiert. Es besteht daher eine enge Verbindung mit gesteigerten kardiovaskulären- und gleichzeitigen Knochenerkrankungen, welche als CKD-MBD (Mineral Bone Disorder) bezeichnet werden. Diese Begleiterkrankung sind hauptsächlich für die erhöhte Mortalität von CKD Patienten verantwortlich. Daher würde eine frühzeitige Erkennung dieser osteologischen und kardiovaskulären Probleme in dieser Patientengruppe helfen, die Therapieansätze zu verbessern.

Diese Studie zielt darauf ab spezifische microRNA (miRNA) Signaturen in CKD Patienten als neue Biomarker zu untersuchen, die während der Krankheitsentwicklung verändert sind und als Indikatoren für Gefäßverkalkung und/oder Probleme der Knochenmineralisierung während der Nierenerkrankung dienen. Einen Einfluss von Nierentransplantationen (KT) auf diese miRNA Signaturen wurde ebenfalls überprüft.

Die untersuchte Kohorte umfasste 73 CKD Patienten in den Stadien 3-5, 67 Patienten nach KT und 36 gesunden Kontrollen. Zu Beginn wurden mittels eines miRNA-Arrays Unterschiede im Serum miRNA Profil von CKD Patienten im Vergleich zu gesunden Kontrollen untersucht. Basierend auf diesen Resultaten wurden 4 miRNAs ausgewählt und diese in der gesamten Kohorte analysiert. Um die unterschiedlichen Patientengruppen zu charakterisieren wurde ein Spektrum verschiedener biochemischen Parameter gemessen, darunter Marker für Nierenfunktion, Entzündung, Glukose- und Mineralmetabolismus. Diese Parameter wurden mit Hilfe von univariater Statistik mit den analysierten miRNAs assoziiert. In einer zweiten prospektiven Studie von CKD Patienten im Stadium 5, die für KT gelistet sind, wurden Serumproben zu Zeitpunkten vor, während und nach der KT gesammelt und die miRNA Expression verglichen.

Die Array Ergebnisse und bioinformatische Evaluierung führten zur Selektion von miR-223-3p, miR-93-5p, miR-142-3p und miR-146a-5p für die weiteren Experimente. Bei CKD Patienten in den Stadien 4 und 5 war die relative Expression von miR-223-3p und miR-93-5p im Vergleich zu gesunden Kontrollen herunterreguliert. Gleichzeitig zeigten miR-142-3p und miR-146a-5p keinen signifikanten Unterschied. Nach der KT normalisierte sich die

Expression aller vier miRNAs wieder auf das Level der gesunden Kontrollen, auch wenn die glomeruläre Filtrationsrate (GFR) niedrig blieb. Assoziationen für miR-223-3p und miR-93-5p wurden für Interleukin-6 (IL-6), GFR Level und zu geringerem Ausmaß mit Interleukin-8 (IL-8), C-Peptid, Hämatokrit und Parathormon (PTH) gefunden.

Auch die Analyse der Patienten der prospektiven Studie, zeigte eine geringere Expression von miR-223-3p und miR-93-5p vor der KT.

Die miRNA Expression ist im Serum von CKD Patienten verändert. Deregulierung von miR-223-3p und miR-93-5p sind vermutlich ein Anzeichen von pathophysiologische Änderungen während der Nierenerkrankung welche mit Inflammation, vaskulären Veränderungen und Problemen des Mineral- und Glucose-Stoffwechsels assoziiert sind. Die bioinformatische Vorhersage von direkt beeinflussten Zielgenen für miR-223-3p und miR-93-5p bestärken diese Ergebnisse und deuten ebenfalls diverse Effekte während CKD-MBD an. Eine genauere Beschreibung der miRNAs und Detektion von weiteren miRNA Signaturen könnten in Zukunft zu neuen Risikomarkern und möglichen Therapieansätzen bei Kochen-, Gefäß- und Nierenerkrankungen dienen.

1. Introduction:

Chronic kidney disease (CKD), in later stages leading to end stage renal disease (ESRD), is a progressive loss of the kidney function. Comorbidities such as increased blood pressure, decreased erythropoietin synthesis with renal anemia or metabolic acidosis are major clinical factors. Possibly the most important and most frequent comorbidity which is associated with CKD is a multifactorial dysregulation of bone and mineral metabolism. This may lead to a typical disease, called chronic kidney disease-mineral bone disorder (CKD-MBD) and is associated with increased cardio- and cerebrovascular calcification in this group of patients. Disturbances of mineral metabolism including hyperphosphatemia, hypocalcemia as well as parathyroid hormone (PTH) levels are increasing during CKD, all of which contributes to the development of cardio- and cerebrovascular diseases as well as abnormalities in bone metabolism^(1,2).

The association of CKD with increased cardiovascular disease due to progressive vascular calcification is leading to high cardio- (CVD) and cerebrovascular (CBVD) mortality compared to the healthy population ⁽³⁾. Even after kidney transplantation, cardiovascular diseases are the most common cause of death, with CVD and CBVD being responsible for 44% of deaths occurring ⁽⁴⁾.

CKD-MBD is defined as a systemic disorder of mineral and bone metabolism and is a consequence of kidney disease. It is described as either one or a combination of abnormalities of calcium, phosphorus, PTH or vitamin D metabolism, as well as abnormalities of bone turnover, bone mineralization, bone volume, and extra skeletal calcification which are relevant to the cardiovascular system ⁽⁵⁾.

Vascular calcification was previously thought to be a passive process, caused by precipitation of minerals, which circulate in the body. Extensive research has revealed mechanisms in vascular calcification which seem to be similar to bone formation. Using in-vivo and in-vitro models, several proteins have been identified which are involved in both bone mineralization and regulation of vascular calcification of blood vessels. Other proteins expressed in the vessel wall and circulating proteins produced at distant sites inhibit the process of calcification ⁽⁶⁾.

The ability to identify and quantify vascular calcification or bone mineralization in late stage CKD patients may help to predict the likelihood of developing cardiovascular (and cerebrovascular) or osteologic events and might have consequences for their therapy. In this

context, circulating biomarkers may be a useful and convenient measure of a patient's individual risk of CKD-MBD and vascular calcification. Identification of the promoters and inhibitors of complications of mineral metabolism will allow developing future therapeutics strategies to modulate and inhibit the progression or even induce regression of these CKD comorbidities.

Current biomarkers for a risk prediction of comorbidities in CKD have shortcomings such as reduced sensitivity or insufficient specificity, thus not allowing an accurate prediction. In addition, it is not likely to find biomarkers that are really specific for a single disease only. A strategy using several biomarkers at the same time as patterns or profiles may resolve these limitations by adding accuracy and predictive power.

In order to improve disease prediction, finding new biomarkers and associating them with existing parameters is of utter importance for risk prevention and to find potential new therapy targets in CKD patients.

One of those new and promising biomarkers investigated in the regulatory processes are microRNAs (miRNAs). They are a family of post-transcriptional regulators of gene expression, with an approximate length of ~ 22 nucleotides. MiRNAs control many developmental and cellular processes in the eukaryotic organism. In mammals, this has been predicted to be more than 60% of all protein coding genes.

The discovery of miRNAs and their involvement in various biological processes suggested them as interesting players and indicators of changes in biological systems. They are very stable in serum and plasma even under unfavorable conditions and are potential surrogate markers for pathophysiological conditions. Circulating miRNAs were shown to be protected from endogenous RNase activities. There are several hypotheses how this is achieved. Protection may be occurring either by packaging into exosomes, association of the miRNAs with other molecules like protein complexes, or via miRNA modifications leading to resistance to RNase activity⁽⁷⁾.

Some distinctive patterns of circulating miRNAs for cardiovascular diseases have already been reported. These diseases include coronary artery disease (CAD), myocardial infarction (MI), heart failure (HF), hypertension, cardiovascular effects in type 2 diabetes mellitus (T2DM) as well as atherosclerotic diseases⁽⁸⁾.

This PhD thesis aimed to evaluate miRNA signatures which could potentially serve as a new class of biomarkers in combination with protein biomarkers for future risk prediction of bone-associated complications and vascular calcification in CKD.

2. Background

2.1 CKD

Chronic kidney disease is a major public health problem. In 2013, it was estimated that 8-16% of the world's population have developed a form of CKD ⁽⁹⁾. In Austria, approximately 400.000 people got CKD ⁽¹⁰⁾.

CKD is defined according to the presence of kidney damage and the reduced level of kidney function, irrespective of the type of kidney disease. In individual patients, CKD is defined as one of 5 stages according to the level of kidney function. Of course, classifications can differ due to selection of different cutoffs. In all cases, the CKD stage is defined by the level of glomerular filtration rate (GFR), with higher stages representing lower GFR levels. The end-stage of CKD is kidney failure, resulting in an urgent replacement of the function by hemodialysis or peritoneal dialysis and potential transplantation later on. If no measures can be taken the end point would be mortality ⁽¹¹⁾.

The course of CKD disease starts with kidney damage, with potential complications occurring alongside decrease of kidney function. These complications can include CVD, bleeding, infections, or systemic failure of several other organs, all of which are comorbidities important in increasing mortality ⁽¹²⁾ (Figure 1).

Other disturbances of mineral metabolism occurring during CKD are hyperphosphatemia, metabolic acidosis, and inflammatory processes. These complications are involving changes in a number of mineral and inflammatory parameters including PTH⁽¹³⁾, C-reactive protein (CRP)⁽¹⁴⁾, interleukin 6 (IL-6)⁽¹⁵⁾, interleukin 8 (IL-8)⁽¹⁶⁾, and, among others, tumor necrosis factor alpha (TNF α)⁽¹⁵⁾, interleukin 10 (IL-10)⁽¹⁷⁾ and myeloperoxidase (MPO)⁽¹⁸⁾.

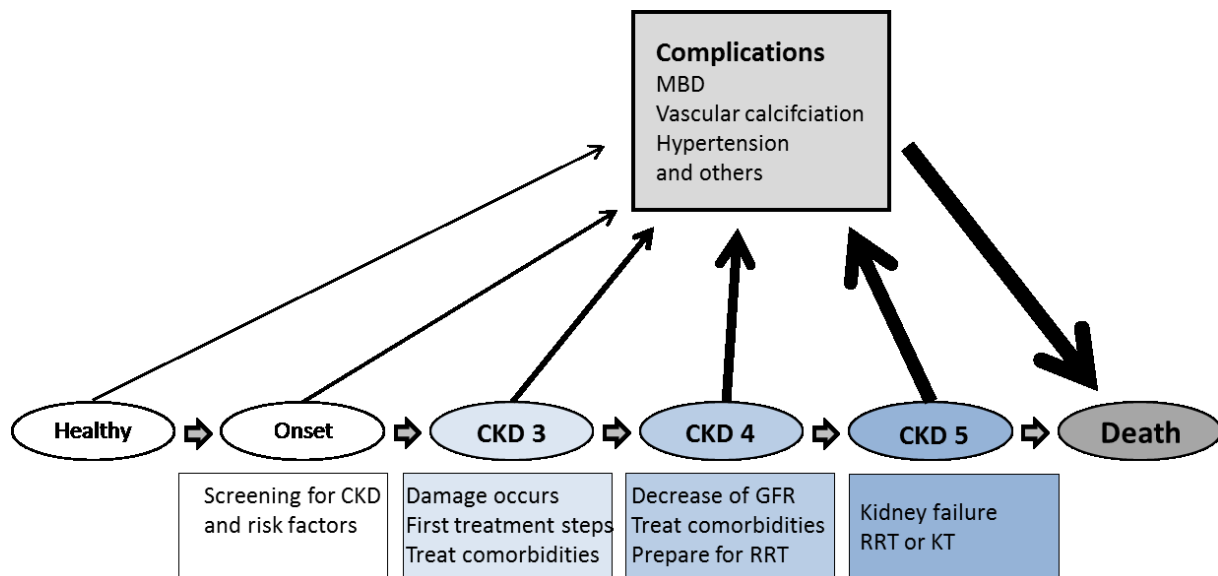


Figure 1: Potential course and development of CKD. Interventions that can be taken to prevent progression of the disease are indicated below the different stages. The thicknesses of the arrows indicate how prone the different stages of CKD are to complications that can occur during the disease, including bone disease, CVD, hypertension, and others.

The most common causes leading to CKD are diabetes mellitus and hypertension, being responsible for 70% of the CKD incidences. Generally, the first treatment option of CKD is to control these diseases and stop the progression. When ESRD is reached, renal replacement therapy has to be implemented. Patients almost always have to undergo hemodialysis or peritoneal dialysis, while subsequent renal transplantation depends on many medical and availability-associated factors.

The GÖG (Gesundheit Österreich GmbH) released information about the annual transplantation rate of kidneys. In 2015, 418 kidneys were transplanted in Austria; of these 63% were male recipients and 37% female. 62 kidneys were transplanted from living donors. 625 patients still have been on the waiting list for kidney transplantation (KT) in 2015 ⁽¹⁹⁾. In summary, in 2015, Austrian ESRD patients can be sub-grouped in 418 patients which underwent KT and another 1072 which started dialysis. Only 82 are registered for peritoneal dialysis, the majority of 990 are being hemodialysed ⁽²⁰⁾. The total number of dialysis patients in Austria at the end of 2015 was 4451 with 4073 undergoing hemodialysis and 378 undergoing peritoneal dialysis.

At the Division of Transplant Surgery of the Medical university of Graz 241 renal transplantations were performed from 2010 to 2014. At the moment, efforts are taken to further increase the KT number at the Medical University of Graz.

In the annual report of the Organ Procurement and Transplantation Network (OPTN), the five year graft survival for US patients receiving a deceased donor transplant was 73%, for living donor transplants it was 84%. Kidney transplantation was stated to be one of the most cost-effective surgical interventions with costs of 200.000\$ in the first year after KT. Yearly costs afterwards decrease to 30.000\$ and remain stable for the following years compared to approximately 83.000\$ costs of care for a dialysis patient per year ⁽²¹⁾.

2.2 Bone Metabolism

Bone is a complex connective tissue with high structural stability, underlying permanent reorganization by bone remodeling. It is an important protector for organs as well as hematopoietic stem cells and enables the activity of muscles. The skeletal elements are formed through two distinct developmental processes, intramembranous and endochondral ossification.

Intramembranous ossification is described as direct osteogenesis, where the mesenchymal precursor cells are converted directly to bone. This differs from endochondral ossification, which also involves mesenchymal cells, but incorporates an intermediate step in which a cartilaginous template indirectly regulates the growth and patterning of the developing skeletal element ^(22,23).

Bone modeling, as well as bone remodeling, are highly regulated processes. The Wnt signaling pathway affects both of these events. Wnt signaling was indicated to have important functions during embryogenesis, mitogenic stimulation, cell differentiation and, as before mentioned bone development and bone disease ^(24,25). In mice, the Wnt pathway was shown to execute crosstalk with other bone development pathways such as the PTH pathway and the TGF- β /BMP pathway. In vivo studies with defective Wnt ligands and antagonists resulted in osteoporosis and imperfect bone development ⁽²⁵⁾.

In adults, osteoblasts, osteoclasts and chondrocytes are the three major cell types involved in bone formation and resorption ⁽²⁶⁾.

The regulatory mechanisms of bone formation have been widely studied. One of the earliest molecules involved in the regulation process is Sox9. It is responsible for the formation of mesenchymal condensation ⁽²⁷⁾ and controls the expression of Core-binding factor alpha 1 (Cbfa1, or Runt-related transcription factor 2, RunX2), a transcription factor expressed in all

osteoblasts, but also in hypertrophic chondrocytes. *Cbfa1* is both, a transcriptional regulator of chondrocyte hypertrophy and the major regulator of osteoblast differentiation. It is required to produce bone extracellular matrix ⁽²⁸⁾. The shift of mesenchymal stem cells from chondrocyte to osteoblast differentiation involves, among others, fibroblast growth factor (FGF), bone morphogenic protein (BMP), PTH related protein (PTH-rp) and Indian hedgehog (IHH) protein ⁽²²⁾.

Osteoclasts are multinucleated cells. Their formation is controlled by parathyroid hormone (PTH) and other factors such as receptor activator of NF- κ B ligand (RANKL). These cells are specialized for acid secretion at the bone surface, dissolving mineral and enzymatically degrading the organic matrix after removal of the minerals. Secretion of PTH after the decline of calcium blood levels, leads to subsequent osteoclasts formation. The osteoclasts dissolve the bone material and restore the calcium levels in the systemic circulation ⁽²⁹⁾.

2.3 Vascular Calcification

There are different factors and diseases that are connected to vascular calcification. Some of those being aging, vascular injury but also clinical conditions like diabetes, atherosclerosis or Mönckeberg's media sclerosis ⁽³⁰⁾. However, a number of studies have shown that CKD patients generally have a high risk for developing vascular calcification because of factors that induce the phenotypic transformation of VSMCs to osteoblast-like cells. Just like osteoblasts during bone formation, these transformed VSMCs are capable of driving tissue mineralization outside the bone ⁽⁶⁾. Under normal conditions, inhibitors such as γ -carboxyglutamic acid protein (GLA-Protein) or circulating fetuin A are preventing soft tissue mineralization. Downregulation of these inhibitors is leading to dysregulation and the subsequent phenotypic transformation of VSMCs to osteoblast-like cells.

Two types of vascular calcification can be distinguished:

- media sclerosis (also known as Mönckeberg's sclerosis)

and

- atherosclerosis ⁽³¹⁾.

2.3.1 Media sclerosis

The deposition of mineral complexes exclusively in the media of the arterial vessel is characteristic for media sclerosis. It normally affects arteries which are less prone to develop atherosclerosis, for example abdominal visceral arteries, arteries of the thyroid and the breast and, arteries in the extremities. High frequency and severity of media sclerosis is detected in chronic renal failure, diabetes mellitus, osteoporosis and, in hypervitaminosis D ⁽³²⁾. Media calcification, frequently seen in CKD and/or diabetic patients causes arterial stiffness ⁽³³⁾, increased blood pressure and reduced compliance of vessels.

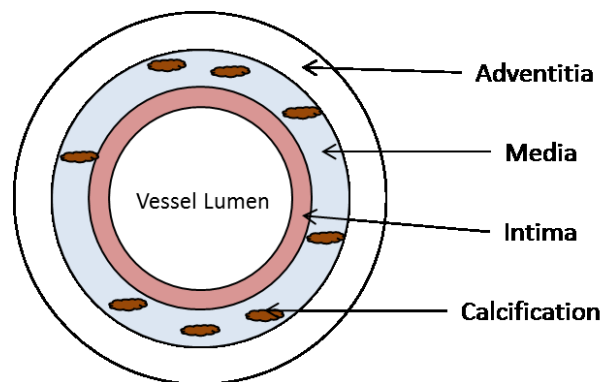


Figure2: During media calcification mineral complexes are solely seen in the media of the vessels. The intima is not affected. Results are stiffening and loss of elasticity of the blood vessels.

2.3.2 Atherosclerosis

Atherosclerotic calcification appears more frequently and is present mainly in the intima. In later stages of atherosclerosis, mineral complex deposition can occur in the media of the vessel ⁽³⁴⁾. While normal arteries have little or no intima, adaptive intima thickening by fibromuscular proliferation can be observed at sites where atherosclerotic plaque formation takes place ⁽³⁵⁾. The process of atherosclerosis is complicated and not fully understood. It involves lipid and cholesterol mechanisms, inflammation, blood clotting due to platelets and other important factors ⁽³⁶⁾. During the course of atherosclerosis, small areas show formation of increased mineral content in basal regions of the intima. They can enlarge and form organized patterns ⁽³⁷⁾. Deposition of mineral complexes near the vessel surface may lead to erosions which can be followed by plaque disruption and thrombosis ^(38,39).

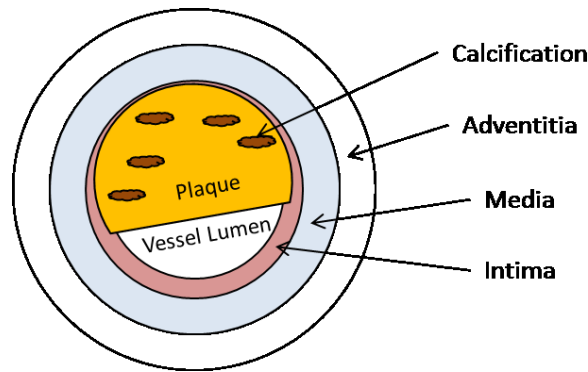


Figure 3: During atherosclerosis the intima and the vessel lumen are mostly affected. After a complicated process in which calcification of the intima occurs, a plaque forms which tightens the vessel lumen. Disruption of the plaque can lead to clotting of vessels and subsequently to a stroke.

2.3.3 Vascular smooth muscle cells

There are some differences between intima and media calcification, but a common feature of both forms is the transformation of vascular smooth muscle cells (VSMCs) to calcified cells⁽⁴⁰⁾. Intima calcification frequently involves macrophages and VSMCs in lipid-rich regions of the intima, but also along elastic lamellae, an area separating tunica intima and media, which is almost exclusively associated with VSMCs^(38,41).

VSMCs apparently have a major role in the mechanism of vascular calcification, especially in CKD patients. Interestingly, a study in young adults suffering from CKD stage 5 showed that a majority of the calcification observed was media calcification⁽⁴¹⁾.

In normal vessels, VSMCs are important for inhibiting calcification, for example through the uptake of inhibitors such as fetuin A⁽⁴²⁾. Subsequently VSMCs release vesicles containing inhibitors and thereby prevent soft tissue from calcifying⁽⁴³⁾. However, due to environmental triggers effecting their function during CKD, deposition of minerals as wells as changes in the cellular biology occur, leading to vascular calcification.

During vascular calcification, minerals are deposited in the vessel wall. These minerals have been shown to be basic calcium phosphate in apatite form, sometimes even hydroxyapatite, the same crystal found in bone. It was shown that in vivo, VSMCs bud matrix vesicles from their plasma membrane and that this particles are capable of concentrating calcium and phosphate in their environment. This behavior was previously shown in chondrocytes and osteoblasts during osteogenesis⁽⁴³⁾. In addition to this finding, other studies intensify the relation between bone formation and vascular calcification showing expression of osteogenic

factors in VSMCs. The first osteogenic factors observed in vascular calcification were matrix GLA protein (MGP), osteopontin (OPN) and bone morphogenic protein-2 (BMP-2) ^(44,45).

Arterial calcification is associated with a loss in smooth muscle markers in combination with the previously mentioned gain of osteogenic marker expression. This demonstrates an association of vascular calcification with smooth muscle cell phenotypic transition, in which several osteogenic proteins including osteopontin, osteocalcin, and the bone determining factor Cbfa1 are gained. All these findings suggest an important role for VSMCs in promoting vascular calcification ⁽⁴⁶⁾.

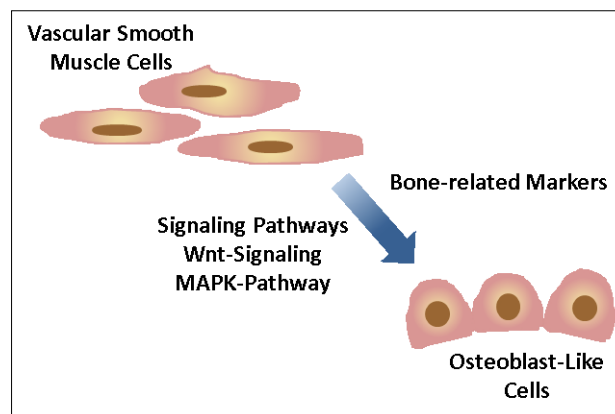


Figure 4: VSMCs undergo a differentiation to osteoblast-like cells. This differentiation is triggered via several pathways important for bone development and homeostasis and leads to calcification of media and/or intima.

Vascular calcification and bone formation share many similarities. But the main difference is that skeletal mineralization is a physiologic, complex regulated process whereas, vascular calcification, even though also highly regulated, is a pathological process leading to vascular diseases and further health complications.

2.4 CKD and bone

One of the main comorbidities of CKD is mineral bone disorder (MBD).

Changes during MBD include reduced bone mineral density (BMD), fractures, bone pain but also deformities and reduced growth velocity in children.

Patients with CKD-MBD show osteoporosis and renal osteodystrophy. Both are resulting in lower bone quality and subsequent fractures. In 10-40% of dialysis patients, bone fractures

will occur. At an age over 50, 50% of the patients may have fractures or other bone related incidences especially if they are in later stages of CKD ⁽⁴⁷⁾.

Bone disturbances during CKD have been reported to be associated with patient mortality. A long time study following patients over 10 years has shown that patients in CKD stage 5 have a higher mortality, when a hip fracture occurred. In fact, mortality was 2.7 times higher than in CKD patients without fractures and 2.4 times higher than in non CKD patients with hip fractures ⁽⁴⁸⁾. This has been confirmed by another study, when hip fractures in dialysis patients were compared to controls with matching cardiovascular risk scores, and mortality was found to be 2.15 times higher in the group with hip fractures recorded ⁽⁴⁹⁾.

Similar increases of mortality in hemodialysis and CKD patients have been shown for patients with vertebral, pelvic or long bone fractures ^(50,51), demonstrating the importance of bone tissue and mineral metabolism during renal disease.

In a chronic hemodialysis population, a reduced total hip bone mass has been suggested as an independent predictor of all-cause mortality ⁽⁵²⁾. In a study by West et.al male and female patients suffering from CKD stages 3 – 5 without renal replacement therapy showing lower BMD according to DXA and CT measurements had a higher risk to suffer from subsequent fractures ⁽⁵³⁾.

The “Kidney Disease: Improving Global Outcomes” (KDIGO) group suggests that during later stages of CKD, the measurement of PTH and bone alkaline phosphatase (bALP), which is directly related to bone turnover ⁽⁵⁴⁾, might be good predictors for CKD-MBD and fractures ^(47,54).

2.5 CKD and vascular calcification

It is well known that in parallel to the deterioration of kidney function an increase of vascular calcification can be detected. The degree of calcification is also higher than that compared to the normal healthy population. The progression and severity of vascular calcification has been shown to be the highest in CKD patients in general.

For CKD patients it has been indicated that media calcification of peripheral arteries can occur without intima changes and was shown to be more common in CKD patients compared to calcification diseases of other etiology ⁽⁵⁵⁾.

In the healthy population, body fluids are saturated with calcium and phosphate, but there are a number of inhibitors which regulate precipitation and actively inhibit systemic calcification in the circulation. These inhibitors, deregulated during CKD, are supporting the formation of calcification in soft tissue. They include fetuin A, matrix-Gla-protein (MGP) as well as osteoprotegerin (OPG). All of those have been hypothesized to be involved in vascular calcification during CKD in various extent and by different mechanisms ⁽⁵⁶⁾.

Fetuin A, a circulating inhibitor, was shown to be reduced in serum samples of patients during CKD. Contrary, MGP acts locally to prevent calcification. Under normal conditions, VSMCs in the vessels are containing significant amounts of fetuin A and MGP ⁽⁴²⁾. When these two inhibitors are decreasing, vessels will become more prone to calcification, especially if more calcium is available in the circulation ⁽⁵⁷⁾. The levels of OPG in the serum of CKD patients are increased. Higher OPG results in less bone turnover and thereby in less mineral taken up by the bone, resulting in a higher calcium load in the vessels ⁽⁵⁶⁾, making them prone to calcification.

2.6 Factors involved in mineral homeostasis during CKD

There are many factors which are important during mineral homeostasis, and are routinely measured in CKD patients to assess their mineral status.

2.6.1 Osteocalcin (OC, bone gla protein)

Osteocalcin is a bone-matrix protein which is produced by osteoblasts under the control of Runx2 and is highly regulated by PTH and Vitamin D, two hormones very important during mineral metabolism ⁽⁵⁸⁾. OC can act as a regulator of bone metabolism and mineralization but can also affect the activity of osteoblasts and osteoclasts and thereby bone remodeling. In mice, OC has even been shown to have a metabolic activity, with OC showing effects on blood glucose and insulin levels and as a result on obesity of the mice ⁽⁵⁹⁾. There are two forms of OC, the γ -carboxylated form and the uncarboxylated form. The first one is vitamin-K dependent and associated with its bone mineral functions, the uncarboxylated form is circulating in the blood, acting as a hormone increasing insulin secretion ⁽⁵⁸⁾.

During CKD, it was stated that the ratio of OC is shifted towards the uncarboxylated form. This was hypothesized to be in parts due to the abnormalities in vitamin-K metabolism ⁽⁶⁰⁾.

During CKD and the concurrent vitamin-K deficiency, uncarboxylated OC increases, leading to negative effects on insulin secretion and sensitivity which in turn stimulated cardiovascular calcification ⁽⁵⁸⁾.

2.6.2 Bone alkaline phosphatase (bALP)

bALP is a phenotypic marker of osteoblasts. It is thought to be highly important during vascular calcification and was shown to be expressed in atherosclerotic lesions as well as medial calcification. Functionally, bALP is suggested to release inorganic phosphate on the cell surface, and may remove calcification inhibitors like pyrophosphate and osteopontin ^(61,62). For CKD-MBD it was indicated that ALP might be an indicator for bone health ⁽⁴⁷⁾. However, high levels of the bone specific bALP have been associated with short-term mortality in dialysis patients. In the long term high bALP levels were also associated with increased cardiovascular mortality specifically ⁽⁶³⁾. Another study indicated that serum ALP was the only marker with a significant association with coronary artery calcification in hemodialysis patients ⁽⁶⁴⁾.

2.6.3 Tartrate resistant phosphatase 5b (TRAP 5b)

TRAP is available in two forms. The two isoform are distinguished by the presence of different carbohydrates. TRAP 5a contains sialic acid, TRAP 5b does not. When TRAP was purified from human bone, it has been shown that it represented the 5b form. Further it was indicated, that TRAP 5b was derived from osteoclasts ⁽⁶⁵⁾ and functions as a bone resorption marker ⁽⁶⁶⁾. A study in hemodialysis patients has indicated, that TRAP5b is significantly associate to PTH, OC or bALP and that an increase of TRAP5b serum levels is associated with higher cortical bone loss ⁽⁶⁷⁾. During CKD there are varying results for the diagnostic value of TRAP5b. In children suffering from CKD it has been described to be negatively correlated with OPG levels in CKD stage 5 patients ⁽⁶⁸⁾. In another study in peritoneal dialysis patients, an increase in TRAP5b levels was reported, but TRAP5b levels were not affected by severity or the use of calcitriol as counteracting measure. Therefore this study was not supportive for using TRAP5b as a marker of diagnostic value during kidney disease ⁽⁶⁹⁾.

2.6.4 Beta-crosslaps (CTX, C-terminal telopeptide of type I collagen)

CTX is widely accepted as a marker for bone resorption, being released in the blood stream during bone resorption ⁽⁷⁰⁾. In chronic hemodialysis patients, CTX has been associated with other bone markers like PTH or TRAP 5b which indicates its potential as a marker for bone metabolism changes also during renal bone disease ⁽⁷¹⁾. In a study in hemodialysis patients, CTX was shown to be a mineral metabolism marker that can be used alongside other markers like PTH, but cannot replace their essential roles monitoring CKD-MBD ⁽⁷²⁾.

2.6.5 PTH (Parathyroid hormone)

PTH is one of the principle regulators of calcium homeostasis. It stimulates release of calcium and phosphate from bone. In the kidney, it stimulates reabsorption of calcium and inhibits reabsorption of phosphate. PTH maintains blood calcium and phosphate concentration via multiple mechanisms ⁽³¹⁾.

Circulating PTH is increased during CKD due to the secondary hyperparathyroidism ⁽⁷³⁾. It has been shown that PTH as well as FGF23, are both increased before hyperphosphatemia first appears ⁽⁷⁴⁾. Rats receiving high concentrations of PTH develop massive media calcification, resembling calcification in CKD patients, even if they had normal renal function. High levels of PTH, which are often seen in hemodialysis patients, have an important role in vascular calcification, even if the levels for calcium or phosphorus might be normal or close to normal ⁽⁷⁵⁾.

2.6.6 Core binding factor 1 (Cbfa1, RunX2 – Runt-related transcription factor 2)

Core binding factor 1 (Cbfa1) is a transcription factor originally found in bone, belonging to the Runt-related transcription factor (Runx) family. It is a key regulator of osteoblast differentiation and function, playing a critical role in bone development and chondrocyte maturation ⁽⁷⁶⁾.

Cbfa1 is the only Cbfa family member which is also expressed in differentiated osteoblasts, and regulates gene transcription of a variety of osteoblast and chondrocyte specific genes (e. g. the smad6, osteocalcin, osteopontin, bone sialoprotein) ^(77,78).

Cbfa1 is expressed in smooth muscle cells, while analysis of the normal vessel wall demonstrated an absence of Cbfa1 ⁽⁷⁹⁾.

In a recent study, it has been shown that Cbfa1 directly binds to the promoter of RANKL and controls its expression, indicating an influence of Cbfa1 on the osteogenic differentiation of VSMCs by up-regulation of RANKL, thereby promoting macrophage migration and osteoblast-like cell differentiation during vascular calcification ⁽⁸⁰⁾.

It was demonstrated that Cbfa1 was expressed in both the media and intima in calcified arteries from renal transplant patients by different methods, but only minimal expression was observed in non-calcified vessels ⁽⁵⁵⁾. This supports the hypothesis that Cbfa1 is a key regulatory factor in the vascular calcification observed in CKD patients. Increased expression of Cbfa1 in calcified, but not in non-calcified arteries has been shown. Thereby there was no difference in samples from CKD patients or non-CKD controls ⁽⁸¹⁾.

2.6.7 Receptor activator of nuclear factor-kappa-B ligand protein (RANKL)

Receptor activator of nuclear factor-kappa-B ligand protein (RANKL) is a member of the tumor necrosis factor (TNF) superfamily. Binding of RANKL to its receptor RANK (Receptor activator of nuclear factor-kappa-B) regulates the formation of multinucleated osteoclasts as well as their activity and survival in bone remodeling and in a variety of pathologic conditions. Inhibition of RANKL binding to its receptor is achieved by its decoy receptor OPG ⁽⁸²⁾. Intriguingly, RANKL/RANK interactions also regulate T cell/dendritic cell communications, dendritic cell survival and maturation, and lymph node formation ⁽⁸³⁾.

In mice treated with warfarin to stimulate media calcification, the inhibition of osteoclast function by OPG, the RANKL decoy receptor, resulted in less arterial calcification ⁽⁸⁴⁾. RANKL and RANK were not detected in normal, non-calcified vessels, whereas they were detected in atherosclerotic vessels ⁽⁸⁵⁾.

Another in vivo model indicated the importance of RANKL on VSMC calcification via RANK binding and thereby increasing BMP4 via the NF- κ B pathway ⁽⁸⁶⁾.

There are contradicting reports about the effects of RANKL during CKD. There are discrepancies if using circulating RANKL levels to indicate calcification or mineralization problems during CKD is possible or productive ⁽⁶⁸⁾. However, preliminary results have indicated that RANKL might be responsible for the osteoblast-like activity of vascular smooth muscle cells ⁽⁸⁷⁾, which also occurs during CKD.

2.6.8 Osteoprotegerin (OPG)

OPG is a glycoprotein that is widely expressed by a variety of tissues, including bone and the vascular wall. It circulates in serum and serves as a decoy receptor for RANKL and TRAIL (TNF-related apoptosis inducing ligand), both members of the TNF ligand superfamily ⁽⁴⁰⁾.

OPG neutralizes the actions of RANKL and prevents bone resorption and bone loss ⁽⁸⁴⁾. OPG is thereby an indirect inhibitor of osteoclastogenesis, inhibiting the activation of RANK by RANKL which is essential for the maturation of osteoclast progenitors ⁽⁸⁸⁾. OPG and RANKL are found in normal arteries ⁽⁸⁹⁾ but histological examinations revealed a decrease in OPG expression in calcified regions compared to non-calcified regions ^(90,91). OPG knockout mice exhibit severe osteoporosis with a high incidence of fractures as well as media calcification of the aorta and renal arteries ⁽⁹²⁾.

A clinical study postulated that a high level of plasma OPG is predictive for a decreased survival of patients with CKD in later stages ⁽⁹³⁾. The fact that increasing OPG serum levels are associated with increased coronary artery as well as aortic calcification has not only been shown for CKD and subsequent hemodialysis patients, but is also seen in the general population ⁽⁵⁶⁾.

2.6.9 Vitamin D

Vitamin D is a steroid hormone. Two important forms are well established, the circulating storage form 25-hydroxyvitamin D (25(OH)D) and the active form 1,25-dihydroxyvitamin D (1,25(OH)₂D).

The main action of 1,25(OH)₂D is to maintain the serum calcium level within the normal range, by increasing the efficiency of intestinal calcium resorption and by inducing progenitor cells in the bone to become mature osteoclasts, mobilizing calcium from bone. 1,25(OH)₂D increases the expression of alkaline phosphatase, OPN, and osteocalcin, all of which play important roles in bone mineralization. Dependent on the serum calcium level, 1,25(OH)₂D can be a potent stimulator of either bone resorption or bone formation ⁽³¹⁾.

Even in the early stages of CKD, a significant decrease of serum vitamin D is observed. The final hydroxylation step from 25(OH)D to 1,25(OH)₂D is mediated by 1 α -hydroxylase which is produced in the kidney, therefore 1,25(OH)₂D deficiency occurs during progression of CKD. 1,25(OH)₂D deficiency promotes PTH synthesis resulting in atypical calcium and

phosphate concentrations ⁽⁹⁴⁾. Studies suggest that systemic activation of vitamin D receptors (VDRs) may have direct effects on the cardiovascular system to decrease mortality in CKD and that vitamin D therapy may prolong survival in CKD patients by mechanisms that are independent of calcium, phosphate and PTH ⁽⁹⁵⁾. Low dosages of vitamin D are protective against aortic calcification in a VDR dependent manner in CKD animal models ⁽⁹⁶⁾.

2.6.10 FGF23 (Fibroblast growth factor 23)

FGF23, a hormone, is mainly produced by osteocytes. There are other cells secreting FGF23 such as pericyte-like cells in the bone marrow or in the lymph nodes, but their contribution to circulating FGF23 is most likely small ⁽⁹⁷⁾. FGF23 is involved in the endocrine regulation of mineral metabolism, especially in the regulation of phosphate and vitamin D metabolism. It decreases phosphate reabsorption because it downregulates sodium-phosphate cotransporters. It reduces the circulating levels of 1,25-dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}$) because it inhibits the enzyme 1α -hydroxylase and stimulates 24-hydroxylase, which degrades vitamin D. Furthermore, FGF23 inhibits the secretion of PTH ^(98–100).

It has been observed that FGF23 deficient mice exhibit similar phenotypes as klotho deficient mice, showing hyperphosphatemia and hypercalcemia as well as premature aging disorders such as arteriosclerosis, osteoporosis or skin atrophy. FGF null mice have severe growth retardation, osteopenia, a short life span, renal phosphate wasting as well as excessive mineralization in soft tissue, especially vascular calcification in the kidneys ⁽¹⁰¹⁾. Klotho binds directly to multiple FGF receptors, and this complex binds FGF23 with much higher affinity. So, klotho functions as a cofactor which is essential for activation of FGF23 signaling ⁽¹⁰²⁾.

FGF23 concentrations increase progressively as the renal function declines during CKD, but it does not change in response to phosphate deprivation or phosphate loading in healthy individuals ⁽¹⁰³⁾. In dialysis patients, higher FGF23 levels in serum are associated with higher mortality ⁽¹⁰⁴⁾.

2.6.11 Osteopontin (OPN)

Osteopontin (OPN) is a multifunctional matrix protein involved in diverse biological processes. It is highly expressed in chronic inflammatory and autoimmune disease. Besides

various other functions it functions as an inhibitor of mineralization, it prevents calcium deposits and inhibits vascular calcification ^(105,106).

OPN was shown to directly inhibit calcification in aortic smooth muscle cells in vitro and aortic valve calcification in vivo ⁽¹⁰⁷⁾. It binds integrins on the osteoclastic cell surface, which enhances the osteoclast attachment and thus promotes osteoclast function and enhances cell mediated releases of calcium from vascular deposits ⁽¹⁰⁸⁾.

Mice deficient in OPN and MGP developed spontaneous media layer calcification, which was enhanced compared to MGP-null mice. This result suggests that OPN has an inhibitory effect on vascular calcification in vivo ⁽¹⁰⁹⁾. OPN levels were elevated in mineralizing arteries of MGP knockout mice ⁽¹¹⁰⁾. Absence of OPN in an animal model was associated with impaired osteoclast function and confers protection against bone loss, but worsened arterial calcification ⁽⁴⁰⁾.

In ESRD patients OPN was abundant in the media layer of calcified vessels. This production of OPN has even occurred before calcification took place ⁽¹¹¹⁾.

2.6.12 Matrix Gla protein (MGP)

Matrix Gla protein (MGP) is a low-molecular-weight protein found in bone, cartilage and is highly expressed in kidney, cardiac valves and the media of arteries. It is a member of a family of mineral-binding proteins that also includes osteocalcin ⁽⁴⁰⁾.

MGP is present in matrix vesicles and serum and limits mineral nucleation ⁽⁴³⁾. A variety of cells express MGP in their arterial wall, for example vascular smooth muscle cells and endothelial cells where it helps to prevent vascular calcification ⁽¹¹²⁾. In in-vivo models, especially the undercarboxylated form of MGP is present in calcified vessels ⁽¹¹³⁾. MGP influences the effect on vascular calcification in two ways, by inhibiting calcium crystal formation and by influencing transcription factors that inhibit VSMC differentiation to the osteoblast-like phenotype ⁽¹¹⁴⁾.

Mice deficient in MGP develop media layer calcification. As a direct consequence of vascular calcification, they typically develop lethal aortic ruptures ⁽¹¹⁵⁾. MGP is downregulated in calcified vessels and cannot inhibit crystal growth by binding of Ca^{2+} ⁽¹¹⁶⁾.

In contrast, the MGP protein seems to accumulate in atherosclerotic lesions as a consequence of the feedback mechanism of MPG which prevents or minimizes further deposition of minerals ⁽¹¹⁷⁾.

MGP is also highly expressed by VSMCs. It is thought that MGP is an important inhibitor of vascular calcification, also in CKD patients ⁽¹¹⁸⁾.

2.6.13 Bone sialoprotein (BSP)

Bone sialoprotein (BSP) is a highly glycosylated and sulphated phosphoprotein that is found almost exclusively in mineralized connective tissues ⁽¹¹⁹⁾. It belongs to the SIBLING family (Small Integrin-Binding Ligand, N-linked Glycoprotein) ⁽¹²⁰⁾. As a bone matrix protein it is known to be involved in the regulation of bone development and maturation. BSP is highly expressed in bone, but its functions are still not fully known ⁽¹²¹⁾.

In cell culture experiments, it has been shown that BSP expression has a direct effect on osteoblast differentiation whereas restriction of BSP shows reduced osteoblast differentiation. ⁽¹²²⁾ Increased mRNA levels of BSP, osteopontin, osteocalcin and Cbfa1 were detected in calcified vessels ⁽¹²³⁾.

In calcified media, staining for BSP was significantly more intense in CKD patients compared with controls ⁽⁸¹⁾.

2.6.14 Fetuin A (alpha 2-Heremans-Schmid glycoprotein, AHSG)

Fetuin A is a circulating plasma glycoprotein with a high affinity for mineral and belongs to the cystatin superfamily of cysteine protease inhibitors and occurs predominantly in the liver ⁽¹²⁴⁾. It is a very potent inhibitor of apatite crystal formation in solutions containing calcium and phosphate and keeps calcium soluble ⁽⁴²⁾. Fetuin A is taken up by VSMCs at sites of vascular damage and is incorporated into and released within matrix vesicles, to inhibit mineral nucleation ⁽⁴²⁾. Fetuin A has been shown to be an important circulating inhibitor of calcification in vivo and is linked to inflammation due to its downregulation during systemic inflammation. Especially in patients with hemodialysis, fetuin A deficiency is proposed to be contributing to the reduction of vascular wall elasticity due to vascular calcification ⁽¹²⁵⁾.

Fetuin A knock-out mice show a perturbation in bone mineralization and suffer from fatal calcification of soft tissue (e.g. kidney, testis, skin, heart and vasculature) ⁽⁴²⁾. Fetuin A deficient mice already develop mild ectopic calcification, but when mice are fed mineral- and vitamin D-rich, or a high fat diet, they develop extensive and fatal vascular and soft tissue calcification which goes along with renal damage ⁽¹²⁶⁾.

In patients with CKD stage 5 fetuin A serum levels are lower compared to individuals of corresponding age and sex without renal disease ⁽¹²⁷⁾. Also patients with atherosclerosis or media sclerosis show reduced serum levels of fetuin A. Immunostaining of arteries from ESRD patients indicated localization of fetuin A at areas of calcification ⁽⁵⁶⁾.

2.6.15 Klotho

The enzyme Klotho is related to β -glucuronidases and exists in two forms, the full-length membrane bound form and the secreted form, produced by cleavage and release of the extracellular domain. The membrane bound klotho binds FGF receptors and activates FGF signaling by enhancing FGF23 activity ⁽¹⁰²⁾. The secreted klotho is a circulating peptide hormone and seems to be a key regulator of ion transport and insulin signaling ⁽¹²⁸⁾. The klotho gene is highly expressed in the kidney and was shown to be involved in aging. Klotho deficient mice show a short lifespan, infertility, skin atrophy, osteoporosis as well as media vascular calcification and atherosclerosis ⁽¹²⁹⁾. Further, klotho deficiency results in reduced osteoblast and osteoclast differentiation ⁽¹³⁰⁾. Overexpression of klotho in mice extended their life span ⁽¹³¹⁾ and prevented the vascular and skeletal abnormalities seen in klotho deficient mice ⁽¹²⁹⁾.

It has been shown that kidney impairment during CKD corresponds to klotho deficiency in the kidney, plasma and urine. The downregulation of klotho is not only a biomarker for kidney damage, but plays an important pathogenetic role in the progression of CKD as well as in its main complications, such as vascular calcification. This consequence has been shown in mice and humans ⁽¹³²⁾.

2.6.16 SMADs

SMADs are intracellular regulators, belonging to the TGF-beta superfamily, which mediate the spatial and temporal fine-tuning of local BMP effects ⁽¹³³⁾. BMPs are a group of multifunctional cytokines and control osteoblast differentiation and promote osteogenesis ⁽⁸²⁾.

SMAD6 knock-out mice show cartilaginous metaplasia of the media layer with ossification and the presence of bone marrow in the aorta ⁽⁴⁰⁾. SMAD6 has an essential role in the development and integrity of the cardiovascular system ⁽¹³³⁾.

SMADs have already been linked to CKD when the TGF-β/SMAD regulatory network was proposed as a potential therapeutic target to treat CKD, especially renal fibrosis and inflammation occurring in CKD. The authors focus on Smad2, Smad3 and Smad7 and miRNAs-21, -29 and -192 which might mediate their function ⁽¹³⁴⁾.

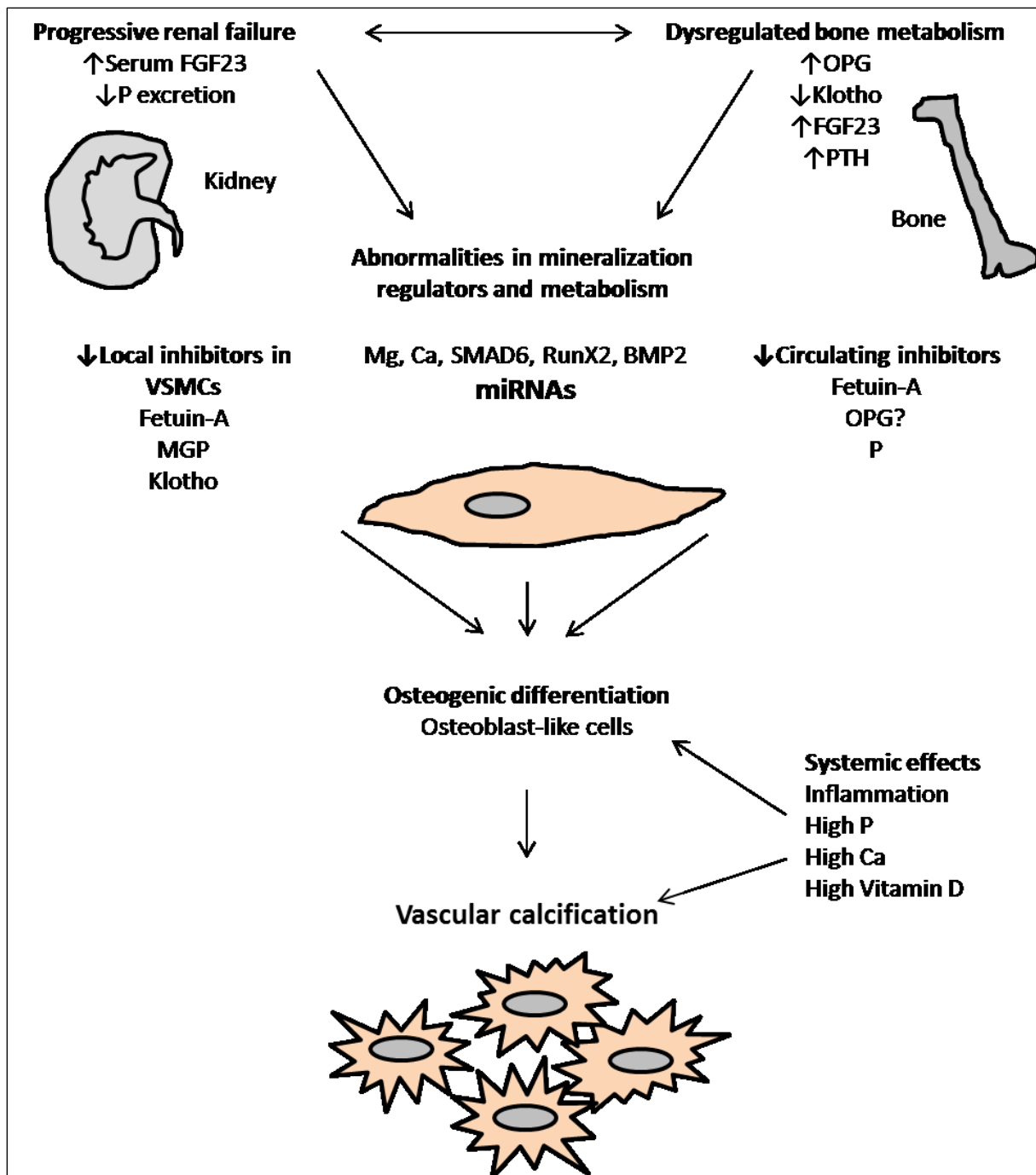


Figure 5: Simplified overview of mechanisms and factors involved in soft tissue calcification and bone resorption due to kidney failure during CKD. Mg...magnesium; Ca...calcium; RunX2...Runt-related transcription factor 2; FGF23...fibroblast growth factor-23; MGP...matrix Gla protein; OPG...osteoprotegerin; P...phosphate; PTH...parathyroid hormone; VSMC...vascular smooth muscle cell.

2.7 Micro RNAs (miRNAs)

MicroRNAs (miRNAs) are a large family of post-transcriptional regulators of gene expression. They are only about 22 nucleotides long and influence the regulation of gene expression on the mRNA level. As per December of 2016 in the miRNA database (miRBase, www.mirbase.org) there are 2588 mature human miRNAs annotated as well as 1881 human precursor miRNAs. It is hypothesized that mature miRNAs regulate transcription of more than 60% of all protein coding genes ⁽¹³⁵⁾, and thereby regulate many developmental and cellular processes in the eukaryotic organism. It has to be taken into consideration that every miRNA can regulate hundreds of mRNAs, meaning that one mRNA is influenced by different miRNAs, but one miRNA can also influence multiple mRNAs ⁽¹³⁶⁾. Functionally, miRNAs are quite similar to transcription factors or RNA-binding-proteins. They are expressed in a tissue and developmental specific manner, which indicates their importance for the development and the cell type and tissue specific expression of protein profiles ⁽¹³⁷⁾.

Only in 2008 it was discovered that miRNA are circulating in blood. Subsequently they have been detected in plasma, serum and almost all body fluids in animal and human samples. It was detected that during disease, miRNAs are expressed in different patterns. This leads to the hypothesis that miRNAs could function as fingerprints during disorders, enabling them as potential biomarkers indicating development of disease. Further, they could be targets for diagnosis and putative therapeutic intervention ⁽¹³⁸⁾⁽¹³⁹⁾. The fundamental role of miRNAs in development, cellular differentiation and metabolism also suggests an important role during diseases ⁽¹³⁷⁾.

2.8 MiRNA biogenesis

MiRNAs can either be transcribed from specific genes or they can be expressed from introns of protein-coding genes. MiRNA biogenesis starts with transcription of the so called precursor miRNA (pri-miRNA) by RNA-polymerase II (RNA-Pol II) in the nucleus. This pri-miRNA is approximately 1000 bases long and contains one or several local hairpin elements. The pri-miRNA undergoes different maturation steps. Drosha, a member of the RNase III family cuts a hairpin element which is also called stem-loop from the original nucleotide chain. The resulting product is an approximately 70 nt long pre-miRNA, still aligned in a hairpin structure ⁽¹⁴⁰⁾. Subsequent the protein Exportin 5 forms a complex with the pre-miRNA and in combination with the protein Ran-GTP releases the pre-miRNA from the nucleus into the

cytosol of the cell. The energy for this step is provided by GTP-hydrolysis⁽¹⁴¹⁾. In the cytosol another enzyme from the RNase III-family called Dicer binds and cleaves the pre-miRNA, resulting in an approximately 22 nt long miRNA duplex⁽¹⁴²⁾. AGO-proteins (Argonaut) bind to the RNA double strand. One of the RNA double strands, the so called passenger strand is degraded. This leaves the single stranded RNA bound to the AGO-proteins. The whole complex is labelled as the mature RISC-complex (RNA-induced silencing complex). In the RISC complex the mature miRNA is the recognition site guiding the whole complex to complementary sites in mRNAs⁽¹⁴³⁾ (Figure 6).

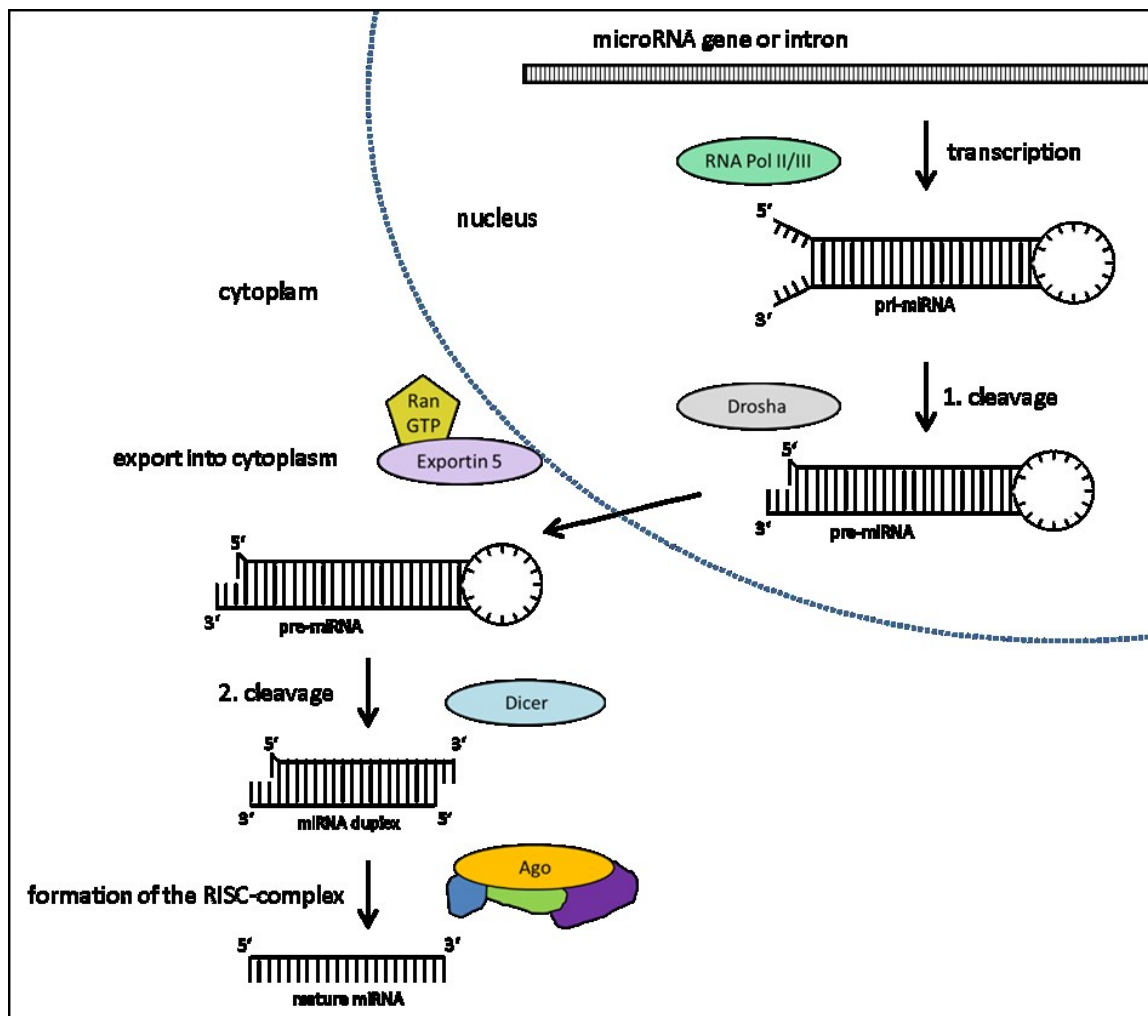


Figure 6: miRNA biogenesis starting with the transcription of a DNA template and ending with the formation of the mature miRNA bound to the RISC complex.

RNA POL...RNA-polymerase; RISC... RNA-induced silencing complex; Ago... Argonaut protein

MiRNA activity is executed by guiding the RISC-complex to miRNA-binding-sites on mRNAs. Binding sites for miRNAs are normally present in the 3'UTR (untranslated region)

in multiple copies. A suitable binding site is recognized by a small, 2-8 nt long region of the miRNA, the so called seed-region. A Watson-Crick base pairing between miRNA and mRNA results in the activation of the RISC complex. The initial pairing doesn't even have to be perfect and can contain mismatches and bulges ^(144–146).

Binding of the RISC complex to the mRNA facilitates its function. Its major function is inhibition of protein synthesis which can be executed in different ways (Figure 7):

1. miRNAs can mark mRNAs for degradation
2. miRNAs can repress translation
3. miRNAs can increase mRNA deadenylation ⁽¹³⁷⁾.

Additionally, miRNAs have also been suggested to be able to silence DNA. DNA silencing was hypothesized to be achieved by some mammalian miRNAs when they directly affect gene expression on the transcriptional level via histone methylation ⁽¹⁴⁷⁾.

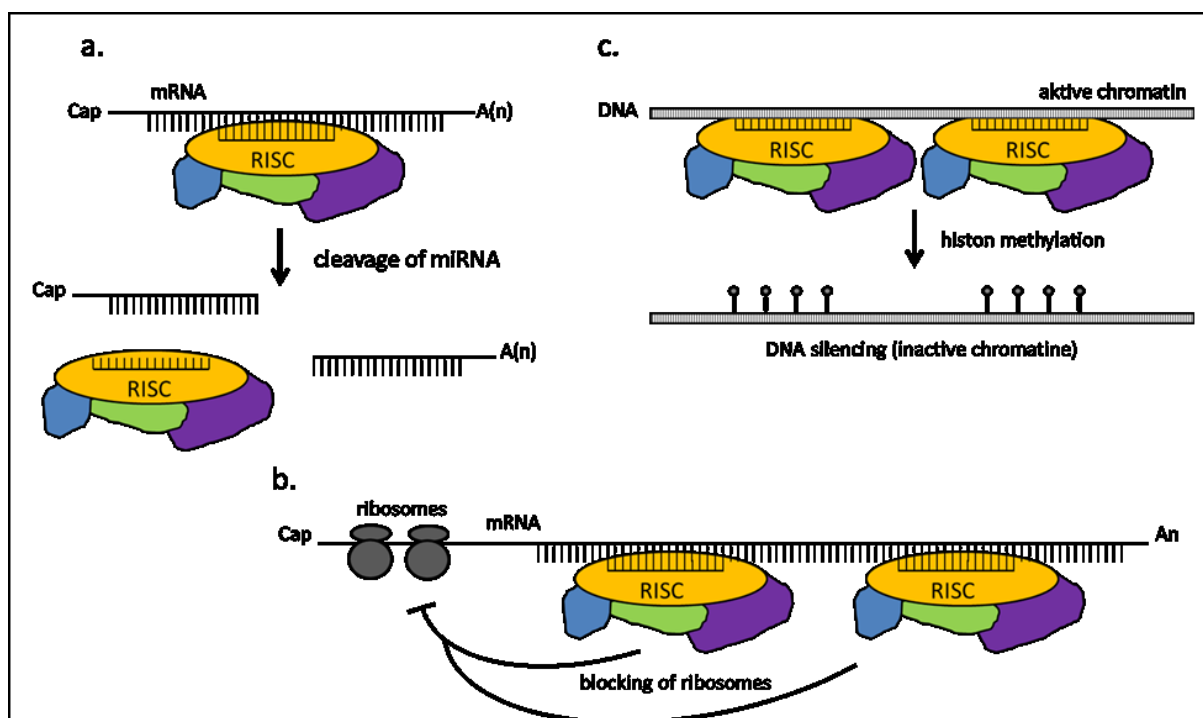


Figure 7: Effects of miRNAs, as part of the RISC-complex, on mRNA and DNA. a) the RISCcomplex binds via miRNA to the mRNA and induces cleavage of the mRNA. b) the RISC complex binds to mRNA via miRNA and blocks ribosomes inhibiting translation. c) the RISCcomplex binds to DNA via miRNA and induces histone methylation and thereby DNA silencing.

mRNA...messenger RNA; RISC... RNA-induced silencing complex; Cap... 5'-Cap structure; A(n)... poly(A)-tail;

2.9 MiRNAs as regulators of mineral metabolism

Physiological bone metabolism is based on a balance between the functions of osteoblasts and osteoclasts, resulting in deposition of new or resorption of old bone matrix.

The importance of miRNAs for development and function of both cell types was observed via a knockdown of the enzyme dicer. Knock-out mice missing dicer showed embryonic lethality due to imperfect development of the normal vertebral body during the gastrulation phase ⁽¹⁴⁸⁾. In a cell specific knock out of dicer in osteoblast precursor cells of mice, a complete lethality of the embryos with deformation of the cartilaginous skeleton and a missing bone formation was reported ⁽¹⁴⁹⁾. A similar effect was seen in human cells when dicer or drosha deactivation in human mesenchymal stem cells lead to inhibition of osteogenic differentiation ⁽¹⁵⁰⁾.

Specific miRNAs have been associated to bone metabolism. It was shown that a group of miRNAs (miR-23a, miR-30c, miR-34c, miR-133a, miR-135a, miR-205, und miR-217) lowers the expression of the osteogenic regulator Runx2 and thereby lower osteoblast differentiation ⁽¹⁵¹⁾.

Negative effects on bone formation have been indicated for miR-206. Expression of miR-206 has been declined in human osteoblasts during progression of osteoblast differentiation. Overexpression of miR-206 has been inhibiting further differentiation, whereas inhibition of miR-206 has induced differentiation. This effect is presumably mediated via connexin 43 (Cx43), a gap-junction protein in osteoblasts. Also in mice, overexpression of miR-206 in osteoblasts has been leading to lower bone mass and impaired osteoblast differentiation ⁽¹⁵²⁾.

MiR-29b has an inhibitory effect on TGF β 3 (transforming growth factor beta), HDAC4 (histone deacetylase 4), CTNNBIP1 (beta-catenin-interacting protein 1) and DUSP2 (dual specificity protein phosphatase 2), all of which are inhibitors of the cellular signaling cascades or transcription programs in osteoblasts. Thereby, through its inhibitory effect, miR-29b indirectly supports specific signaling pathways, such as the SMAD-, ERK- (extracellular signal-regulated kinase), MAPK- (mitogen-activated protein kinase), or Wnt-signaling-pathway leading to a positive effect on osteoblast differentiation and osteogenesis ⁽¹⁵³⁾.

There are few studies on the regulation of osteoclasts by miRNAs at the moment. When dicer has been deactivated in mature osteoclasts of mice, an increase of bone mass in trabecular bone has been observed due to a reduction of the amount of osteoclasts ⁽¹⁵⁴⁾. Another study has confirmed the increased trabecular bone volume in a dicer knock down indicating that the

mice used had diminished bone resorption with a reduction of osteoclasts, but unchanged osteoblast number ⁽¹⁵⁵⁾.

A feedback-loop positively influencing osteoclast development has been shown for miR-21. C-fos, a transcription factor, is inducing expression of miR-21, which itself is inhibiting PDCD4 (Programmed cell death protein 4). PDCD4 boosts expression of transcription factors important for osteoclastogenesis, among them c-fos. This feedback loop results in the start of differentiation of bone marrow macrophages (BMMs) to osteoclasts ⁽¹⁵⁶⁾.

In osteoclast precursor cells it has been shown that the transcription factor PU.1 stimulates the production of miR-223 and RANK (Receptor Activator of NF- κ B), an effect induced by M-CSF (macrophage colony-stimulating factor). MiR-223 in turn is repressing NFI-A (Nuclear factor 1 A-type), an inhibitor of M-CSFR (macrophage colony-stimulating factor receptor). Expression of M-CSFR, amongst other functions, is important for physiological development and function of osteoclasts ⁽¹⁵⁵⁾.

Mir-146a might have a negative effect on osteoclastogenesis. It has been shown to act as an inhibitor for the expression of different important factors of osteoclastogenesis, such as c-Jun, NF-ATc1 (nuclear factor of activated T-cells, cytoplasmic 1), PU.1 and TRAP (tartrate resistant acid phosphatase) ⁽¹⁵⁷⁾.

2.10 MiRNAs as biomarkers

The presence of miRNAs in circulating blood has indicated their potential as putative biomarkers for various diseases. They have been detected in serum and plasma of humans as well as different animal models. Detection of specific expression patterns of miRNAs in different cancers as well as other diseases lead to the conclusion that serum miRNAs could provide fingerprints for various diseases and thereby serve as potential biomarkers for early disease detection ⁽¹³⁸⁾.

One of the major challenges in diseases is the identification of suitable biomarkers that reliably indicate disease. Importantly, miRNAs are very stable in plasma also under unfavorable conditions, such as boiling, low pH, long-term storage at room temperature or multiple freezing-thawing cycles. Unexpectedly, the circulating miRNAs were even shown to be protected from endogenous RNase activity ⁽⁷⁾. This protection was hypothesized to be caused by several effects, because miRNAs are released from the cells into plasma, packed in

microparticles (exosomes, microvesicles, apoptotic bodies), bound to RNA binding proteins (Argonaut 2) or associated in lipoprotein complexes (high density lipoprotein, HDL), which prevents them from degradation ⁽¹⁵⁸⁾.

In a review by Hackl et al. ⁽¹⁵⁹⁾ they discuss miRNAs as biomarkers for bone disease and indicate roles as indicators for disease of the bone including osteoporosis, for example a correlation of miR-21-5p, miR-133a and miR-146a-5p with spinal and hip BMD in osteoporotic patients. According to the studies which are discussed, it is stated that circulating miRNAs are correlating to different skeletal conditions, but interpretation of systemic miRNAs is demanding, also because circulating miRNAs normally cannot be linked to a specific tissue or organ.

Some distinctive patterns of circulating miRNAs for cardiovascular diseases have already been found so far. The diseases include coronary artery disease (CAD), myocardial infarction (MI), heart failure (HF), hypertension, T2DM as well as atherosclerotic diseases ⁽⁸⁾. The importance of miRNAs on osteoblast differentiation, bone formation and bone homeostasis has been described in a review, showing that every important step and every pathway involved has been brought into connection with miRNAs ⁽¹⁶⁰⁾.

Involvements of miRNAs in the biology of VSMCs have been implicated in different publications, among them papers from Wu et al. ⁽¹⁶¹⁾, Cui et al. ⁽¹⁶²⁾ or Rangrez et al. ⁽¹⁶³⁾. In view of VSMCs undergoing a differentiation to osteoblast-like cells in the vessel wall of CKD patients, thus changing to bone forming cells ⁽¹⁶⁴⁾ it is interesting to look at miRNAs regulatory effects on VSMCs.

All cells involved in media and intima sclerosis, including VSMCs, potentially release miRNAs into the circulation. Differences in this secretion patterns due to developing calcification might be detected. Therefore an increase or decrease of specific miRNAs might indicate the disease in an early phase of CKD and measures could be taken to counteract in time.

Current biomarkers have shortcomings such as reduced sensitivity and insufficient specificity, which is a reason that they do not allow accurate diagnosis. It is quite hard to find biomarkers that are really specific for one disease. A strategy using different biomarkers at the same time may resolve these limitations by adding accuracy and predictive power. So, finding new biomarkers and associating them with existing parameters is of utter importance.

2.11 MiRNAs in disease

Different studies already indicated the importance of miRNAs as regulators during disease. For examples, in 2009, miR-2861 has been observed during the development of osteoporosis. Overexpression of miR-2861 has increased BMP2 (bone morphogenic protein 2) induced osteoblastogenesis. It has been shown that miR-2861 directly represses HDAC5 (Histone deacetylase 5), which itself induces degradation of Runx2. This suggests a positive influence of miR-2861 on osteoblast differentiation. In mice it has been observed that a diminished miR-2861 production leads to a decreased bone formation due to the same effect. Additionally, a lower level of miR-2861 has been discovered in bone tissue of osteoporotic patients ⁽¹⁶⁵⁾.

Increased miR-214 expression has been identified in tissue samples of patients with reduced bone formation. In mice, miR-214 has been shown to influence osteoblast differentiation and bone formation during bone diseases. This influence is probably transmitted through an effect of miR-214 on ATF4 (Activating transcription factor 4), one of the most important transcription factors for functional osteoblast development. Inhibition of miR-214 in mice has led to an improvement of bone mineral density ⁽¹⁶⁶⁾.

Considering the extensive functions of miRNAs during bone development and the onset and the course of bone diseases, it is obvious to investigate miRNAs potentially serving as targets for putative treatments. MiRNAs were suggested to be putative diagnostic biomarkers as well as potential targets for treatment of osteoporosis or healing of fractures in osteoporotic patients ⁽¹⁶⁷⁾.

As a treatment option, anti-miRs (miRNA inhibitors) and miRNA-mimics (synthetic miRNA replicas) are interesting with respect to their pharmacological or therapeutic value. However, due to the ubiquitous occurrence of miRNAs and the numerous different functions of single miRNAs, the choice of a suitable miRNA target might be difficult.

As an example, overexpression of miRNA-29a has been shown to be important for osteoblast differentiation. This is achieved via a positive feedback through the Wnt-signaling pathway ⁽¹⁶⁸⁾. In rats miRNA-29a has been described to protect bone formation from influences of glucocorticoids and a treatment with miR-29a could attenuate the negative effect of glucocorticoids on bone volume ⁽¹⁶⁹⁾. However, another study has indicated that miR-29a has a substantial role as an oncogene and can contribute to tumor development. For example,

miR-29a has been indicated to strengthen the formation of metastasis during colorectal cancer (170).

Hence, a therapeutic usage of miRNAs in cancer is dependent on extensive characterization and evaluation of the respective miRNA in the whole system. To date, there are no investigative trials for miRNA use in the therapy of non-cancer diseases, such as the pathology of calcification in vascular, bone and other tissues.

During this thesis we tried to find and evaluate differentially regulated miRNAs, circulating in serum of CKD patients, which are involved in the systemic changes leading to CKD-MBD and vascular calcification during the kidney disease. Finding such miRNA signatures could potentially lead to new biomarkers, which in combination with protein biomarkers have a higher potential to predict the risk of bone-associated complications and vascular calcification in CKD.

3. Materials and methods

3.1 Study populations

For this project, two separate study populations have been analyzed. The main focus was set on the first study in cooperation with the research group of Vanessa Stadlbauer, where patient recruitment was already finished and samples were collected. The second study had a prospective design with ongoing patient recruitment.

Study 1, was approved by the Research Ethics Committee of the Medical University of Graz, Austria, (23-056 ex 10/11). The study name is “Endotoxin, Neutrophil Function and Albumin in Renal Insufficiency” (ENARI) and is registered at www.clinicaltrials.gov under the registration number NCT01362569. The study was outlined and conducted by Vanessa Stadlbauer. Frozen samples and patient data were analyzed in cooperation with her group.

Study 2, called “Renal replacement and Calcification Study” (RCS) study, was also approved by the Ethics committee (EK Number 25-207 ex 12/13) of the Medical University of.

For both studies written informed consent was obtained from all patients and controls and the study was performed according to the principles laid out in the Declaration of Helsinki.

In the ENARI study samples, we determined if CKD and the concomitant osteogenic and vascular side effects affect the expression of circulating miRNAs and different surrogate markers. We analyzed the expression and protein levels of parameters in stored serum and plasma from CKD patients in stage 3–5, samples from post KT patients as well as healthy controls.

Inclusion criteria for the ENARI study were stage 3-5 CKD (defined as estimated glomerular filtration rate [eGFR]) and age above 18 years.

Patient samples have been frozen at -80°C in aliquots with no previous freezing-thawing.

In the RCS study, patients were included which are in CKD stage 5 and are undergoing dialysis, mostly peritoneal dialysis. Blood was drawn at every study visit. Study visits were planned at the inclusion date before KT, during KT and 6 months after KT. We wanted to determine if miRNA patterns change over the course of the disease before and after transplantation when looking at a single patient.

3.2 Patient cohorts

In the ENARI study, seventy-one patients with CKD stages 3 to 5 (without renal replacement therapy (RRT)), sixty-six CKD patients after KT (mean time since transplantation: 7 years, range 1 - 28), and 36 healthy controls were included in the cross sectional study. All CKD patients were assigned into subgroups according to their estimated glomerular filtration rate (eGFR) as calculated via CKD-EPI creatinine equation ⁽¹⁷¹⁾. A total of five groups was compared, including CKD stages 3-5 (eGFR: 30-44, 15-29 and below 15 [ml/min/1,73m³]) , patients after KT (eGFR: >15 [ml/min/1,73m³]) and healthy controls. Blood samples were drawn in the morning after a 12-hour overnight fast and serum as well as plasma samples were centrifuged according to a standardized protocol, aliquoted, and stored at -80 °C until analysis.

Until the 1st of December of 2016, thirty-one late stage CKD patients, all undergoing RRT have been included in the RCS study. Ten of these patients had already undergone transplantation surgery and two of them have attended the first post-KT study visit. Blood samples were drawn in the morning after overnight fasting at the scheduled study visits and at the day of transplantation.

3.3 RNA Isolation

Serum samples were used to isolate miRNAs via the miRNeasy Mini Kit (Qiagen, Hilden, Germany). 1200 µl Qiazol lysis reagent were added to 200 µl of serum or in case of the Nanostring isolations to 250µl of serum, respectively and incubated at room temperature for 5 minutes. 2 fmol of non-human mi-RNA (ath-miR-159a) were spiked in as an internal control. After mixing, 200 µl of chloroform were added, followed by 15 seconds of vortexing. After incubation for 3 minutes at room temperature, the samples were centrifuged for 15 minutes at 12000 g and 4 °C. The upper phase (approximately 800 µl) was transferred to a new tube and 1200 µl 100 % ethanol and 20 µl glycerol (Ambion, ThermoFischer, Waltham, USA) were added. The subsequent isolation was done using miRNeasy Kit column tubes and solutions following the manufacturer's instructions. RNA was eluted from the columns in 40 µl RNase-free water and stored at -80 °C. Quantification was not possible by using the local NanoDrop 2000 equipment due to the length of the isolated small RNAs. Thereby, standardization of the workflow was used to achieve comparable results.

3.4 miRNA screening

The digital multiplexed nanoString nCounter human miRNA expression assay® (nanoString Technologies) was performed using 10–30 ng total RNA isolated from a net volume of 250 µl serum as input material. Small RNA samples were prepared by ligating a specific DNA tag (miR-tag) onto the end of each mature miRNA according to the manufacturer's instruction. These tags have several purposes: they normalize the wide range of melting temperatures (Tms) of the miRNAs, provide a template to facilitate the use of the nanoString dual probe system, enable single base pair discrimination and specificity of highly homologous miRNA family members, and identify each miRNA species separately.

Hybridizations were carried out by combining each miRNA-miRTag sample with the nCounter Reporter probes in hybridization buffer and nCounter Capture probes overnight at 65°C for 16–20 hrs. Excess probes were removed using two-step magnetic bead based purification on the nCounter Prep Station (nanoString Technologies). Abundances of specific target molecules were quantified on the nCounter Digital Analyzer by counting the individual fluorescent barcodes and assessing the target molecules. Results were collected using the nCounter Digital Analyzer and processed according to the instructions by nanoString using the nSolver software. In a first pilot analysis, changes in miRNAs in 14 samples of CKD stage 5 patients compared to 10 samples of healthy controls were assessed. The nCounter multiplex platform is capable of detecting approximately 800 human miRNAs, 5 housekeeping genes (ACTB, B2M, GAPDH, RPL19, RPL10) and 5 non-mammalian miRNAs (cel-miR-248, ath-miR159a, osa-miR414, osa-miR442, cel-miR-254) of which we used two, namely cel-miR-248 and ath-miR159a.

3.5 qPCR

Quantitative PCR (qPCR) to determine miRNA expression was performed using TaqMan miRNA assays (Applied Biosystems, Foster City, CA). 3µl isolated RNA was used for reverse transcription to synthesize complementary DNA using TaqMan miRNA-specific primers and the TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA).

After reverse transcription, an amplification step using the TaqMan PreAmp Master Mix was conducted. Real-time PCR amplifications were performed using TaqMan miRNA assays (TaqMan MGP probes, FAM dye-labeled) on the LightCycler® 480 (Roche, Basel, Switzerland).

For all qPCRs, a maximum of 40 cycles was completed and the cycle number, at which the amplification plot crossed the threshold, was calculated (CT)⁽¹⁷²⁾. For normalization, the non-human miRNA ath-miR-159a (Microsynth AG, Balgach, Switzerland), was spiked in prior to isolation and was measured to calculate fold changes using the $\Delta\Delta$ CT method⁽¹⁷²⁾.

12 miRNAs were selected because of the results from the Nanostring screening and their putative involvement in pathways relevant to CKD, vascular calcification, bone mineralization, and VSMC biology according to relevant literature and bioinformatics tools (mirPath, TargetScan, miRDB) (miR-146a, miR-223-3p, miR-142-3p, miR-23a-3p, miR-93-5p, miR-1976, miR-4454, miR-631, miR-451a, miR-143-3p, miR-1283 and miR-1253). In addition to these selected miRNAs we also looked at two miRNAs that didn't pop-up in the array experiment but are already mentioned in the literature in similar settings (miR-145 and miR-125). The qPCR experiments were first carried out in a discovery group of random samples from all 5 available groups (CKD 3 – 5, KT, controls). Based on the constant reproducibility of experiments for 4 miRNAs (miR-223, miR-93, miR-142-3p, and miR146), a subsequent qPCR analysis of these miRNAs was performed in all samples.

In order to exclude the confounding element which is T2DM in our patients group (around 25% in all patients groups except the controls suffer from T2DM) we found it necessary to investigate the effect of T2DM on the expression differences as far as possible. Therefore we analyze the expression of all patients with and without diabetes separately and looked at the outcome for the fold changes between the groups.

3.6 Laboratory measurements

Serum and plasma parameters of bone biology, inflammation and glucose metabolism as well as kidney and liver function were measured in serum and/or plasma according to the different manufacturers' instructions.

Blood levels of IL-6, TNF α , IL-8, and IL-10 were measured using FlowCytomix™ Multiplex Technology (eBioscience, San Diego, CA, USA). IL-6R (human IL-6R Platinum ELISA, eBioscience, San Diego, CA, USA) and MPO (Myeloperoxidase ELISA K6631B, Immundiagnostik, Bensheim, Germany), OPG (Biomedica Medizinprodukte GmbH & Co KG, Vienna, Austria), TRAP5b (TRAcP 5b Elisa, Immunodiagnostic Systems, Boldon NE35 9PD, UK), FGF-23 (human FGF-23 ELISA Kit, Immntopics, Inc., San Clement, CA, USA)

were measured with standard ELISA technology. Vasopressin was measured with a radioimmunoassay (Vasopressin Direct Ria, Bülmann Laboratories AG, Schönenbuch, Switzerland). bALP (bone alkaline phosphatase), CRP (c-reactive protein), iron, ferritin, gamma-glutamyl transferase (GGT), glucose, calcium, magnesium, sodium, phosphate, potassium, urea, and uric acid were measured on an automated system via the Cobas® 8000/6000 modular analyzer series, PTH (parathyroid hormone), OC (osteocalcin), and CTX (beta-crosslaps) on the Cobas® 4000 analyzer series, PINP (procollagen type 1 amino-terminal propeptide) via electrochemiluminescence immunoassays on the cCobas e 411 (Roche Diagnostics, Rotkreuz, Switzerland). C-peptide was determined by the ADVIA Centaur XP Immunoassay System (Siemens Healthcare, Erlangen, Germany). Hematocrit was measured by ABL800 FLEX blood gas analyzer (Radiometer GmbH, Willich, Germany). 25(OH)vitamin D (25(OH)D), aldosterone and IGF-1 levels were assessed via an automated chemiluminescence immunoassay on the iSYS automated system (Immunodiagnostic Systems, Boldon NE35 9PD, UK). C-peptide was measured via a two-site sandwich immunoassay using the ADVIA centaur C-peptide assay (Siemens Healthcare GmbH, Erlangen, Germany).

3.7 Target predictions

To predict possible miR-223 and miR-93 targets which might play a role during CKD-MBD and vascular calcification, the online resources miRWalk 2.0 (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/path-self.html>) and TargetScan 7.0 (<http://www.targetscan.org>) were used. Predicted target genes and their conserved miRNA binding sites were annotated. Additionally, miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/>) was used to check for already validated targets. Using different webpages including Genecards (<http://www.genecards.org/>), UniProt (<http://www.uniprot.org/>) and NCBI (<https://www.ncbi.nlm.nih.gov/gene>) we investigated the functions and putative disease relations of predicted target genes.

3.8 Statistical analysis

Patient characteristics and biomarker levels are reported as mean $\pm$ standard error of the mean (SEM) unless otherwise stated.

Quantitative qPCR data are presented as mean $\pm$ standard deviation (SD). The expression levels of miRNAs were analyzed using the $2^{-\Delta\Delta CT}$ method ⁽¹⁷²⁾. To normalize the expression levels, the non-human miRNA ath-miR-159a was spiked in and used in the calculations. Changes of miRNA levels in CKD groups are documented as the relative fold change of patients compared to miRNA levels of healthy controls as reference. Quantitative data were compared using the Kruskal-Wallis test for non-parametric samples with post-hoc Bonferroni correction for multiple comparisons and adjustments for multivariate analysis.

To evaluate the associations between specific miRNA concentrations and biological markers, univariate linear regression analyses were used. Standardized coefficients B and their standard errors (SEs) were obtained.

In all cases, a p-value of <0.05 was considered statistically significant.

The following results are to a large part adapted from our own publication in the journal Bone (2017 Feb;95:115-123. doi: 10.1016/j.bone.2016.11.016. Epub 2016 Nov 17.) titled “MicroRNAs 223-3p and 93-5p in patients with chronic kidney disease before and after renal transplantation” ⁽¹⁷³⁾.

4. Results

4.1 Patients characteristics of CKD, KT and control groups in the ENARI study

In the first study, which was the basis for our publication in Bone ⁽¹⁷³⁾, 171 patients were recruited and subdivided into 5 groups, consisting of CKD stages 3 – 5, KT patients, and controls (Table 1). As expected, eGFR levels were lower in later CKD stages. The percentages of patients with concomitant T2DM are shown (Table 1).

Table 1: General characteristics of CKD patients, KT recipients and healthy controls.

Characteristic [Units]	Chronic Kidney Disease			KT (n=66)	Controls (n=34)
	CKD 3 (n=26)	CKD 4 (n=34)	CKD 5 (n=11)		
Age [years]	51±14	64±15 ^{c,d}	64±12 ^b	54±14 ^a	45±15
Sex [male]	69%	60%	73%	64%	47%
BMI [kg/m ²]	27.2±5.2	30.5±7.3 ^{a,d}	29±6.1	26±4.4	24.7±2.7
eGFR [ml/min/1.73m ³]	56.9±23.8 ^a	21.5±4.2 ^f	11.9±2.3 ^{c,f}	45.3±17.7 ^c	85±19.1
Diabetes [Yes]	31%	44%*	27%	26%	0%**
Time after Transplantation [months]	n.a.	n.a.	n.a.	86±86	n.a.

CKD patients were divided into 3 groups according to their eGFR (CKD 3 – 5). None of the CKD patients received hemodialysis (renal replacement therapy, RRT). Data are depicted as mean ± SD.

^a... p<0.05; ^b... p<0.01; ^c... p<0.001, compared to controls; ^d... p<0.05; ^e... p<0.01; ^f... p<0.001, compared to KT. Kruskal-Wallis tests for independent samples and Bonferroni post hoc testing for multiple comparison correction were used.

Diabetes incidence was compared using Chi-square test with post-hoc Bonferroni correction. *p=0.04; **p=0.0016;

n.a. not applicable.

4.2 Plasma and serum parameters in all subgroups

In all subgroups, levels of several surrogate parameters including inflammatory processes (IL-6, IL-8, IL-10, TNFα), glucose metabolism (glucose, C-peptide), electrolyte homeostasis (calcium, magnesium, sodium, potassium, phosphate), hematocrit, PTH, 25(OH)D and further parameters were measured, and are shown in Table 2. OC, CTX, bALP and TRAP levels were only determined in a subgroup of CKD patients.

Table 2: Serum parameters in CKD patients, KT recipients and healthy controls. (Adapted from our own publication ⁽¹⁷³⁾).

Parameter [Unit]	Chronic Kidney Disease			KT (n=66)	Controls (n=34)
	CKD 3 (n=26)	CKD 4 (n=34)	CKD 5 (n=11)		
Creatinine [$\mu\text{mol/l}$]	131 $\pm$ 42 ^{a,c,d}	248 $\pm$ 57 ^{a,b,e}	415 $\pm$ 90 ^{a,b,e}	157 $\pm$ 68 ^{a,c,d}	80 $\pm$ 11 ^{b,c,d,e}
Urea [mmol/l]	8.6 $\pm$ 3.6 ^{c,d}	18.2 $\pm$ 6.1 ^{a,b,e}	22.8 $\pm$ 3.6 ^{a,b,e}	11.6 $\pm$ 6.3 ^{a,c,d}	5.2 $\pm$ 1 ^{b,c,d,e}
IL-6 [pg/ml]	1.9 $\pm$ 3 ^a	2.4 $\pm$ 1.9 ^a	5.5 $\pm$ 5.3 ^a	3.3 $\pm$ 5.1 ^a	0.1 $\pm$ 0.4 ^{b,c,d,e}
IL-6R [pg/ml]	259 $\pm$ 67 ^a	260 $\pm$ 64 ^a	234 $\pm$ 55	224 $\pm$ 63 ^a	180 $\pm$ 47 ^{b,d,e}
TNF α [pg/ml]	3.1 $\pm$ 5.2 ^a	3.5 $\pm$ 4.6 ^a	2.6 $\pm$ 4.7	3.2 $\pm$ 5.2 ^a	0.7 $\pm$ 2.5 ^{b,d,e}
IL-8 [pg/ml]	20 $\pm$ 45 ^{a,c}	12.8 $\pm$ 9.2 ^a	73 $\pm$ 125 ^{a,e}	24.1 $\pm$ 4.7 ^a	1.5 $\pm$ 1.2 ^{b,c,d,e}
IL-10 [pg/ml]	2.1 $\pm$ 3.1 ^a	3 $\pm$ 2.3 ^a	4.5 $\pm$ 5.6 ^a	4.3 $\pm$ 7.8 ^a	0.1 $\pm$ 0.4 ^{b,c,d,e}
MPO [ng/ml]	402 $\pm$ 153	390 $\pm$ 181 ^a	469 $\pm$ 255	431 $\pm$ 179	522 $\pm$ 185 ^d
CRP [mg/l]	4 $\pm$ 5.9	4.1 $\pm$ 3.7	7.3 $\pm$ 6.3	3.6 $\pm$ 4	2.6 $\pm$ 3.7
Iron [$\mu\text{g/dl}$]	87 $\pm$ 27	74 $\pm$ 25	71 $\pm$ 22	87 $\pm$ 31	102 $\pm$ 39
Ferritin [ng/ml]	168 $\pm$ 127	139 $\pm$ 125	178 $\pm$ 114	170 $\pm$ 141	151 $\pm$ 127
Gamma GT [U/l]	34 $\pm$ 24	38 $\pm$ 28	52 $\pm$ 41	46 $\pm$ 41	28 $\pm$ 23
Glucose [mg/dl]	112 $\pm$ 37	125 $\pm$ 57	100 $\pm$ 10	108 $\pm$ 28	97 $\pm$ 26
C-peptide [pmol/mg]	209 $\pm$ 41 ^a	203 $\pm$ 34 ^a	220 $\pm$ 39	207 $\pm$ 26 ^a	256 $\pm$ 26 ^{b,d,e}
Calcium [mmol/l]	2.36 $\pm$ 0.11 ^b	2.36 $\pm$ 0.15 ^b	2.33 $\pm$ 0.17	2.45 $\pm$ 0.18 ^{d,e}	2.43 $\pm$ 0.09
Magnesium [mmol/l]	0.79 $\pm$ 0.07 ^{c,d}	0.9 $\pm$ 0.12 ^{b,e}	0.97 $\pm$ 0.13 ^{b,e}	0.75 $\pm$ 0.1 ^{a,c,d}	0.84 $\pm$ 0.04 ^{c,d}
Sodium [mmol/l]	140.7 $\pm$ 3.2	141 $\pm$ 2.5	143 $\pm$ 2.8 ^a	141.5 $\pm$ 2.4	140.2 $\pm$ 2.2 ^c
Phosphate [mmol/l]	1.01 $\pm$ 0.03 ^c	1.15 $\pm$ 0.04 ^b	1.52 $\pm$ 0.11 ^{a,b,e}	0.99 $\pm$ 0.04 ^{c,d}	1.03 $\pm$ 0.04 ^c
Potassium [mmol/l]	4.5 $\pm$ 0.5 ^a	4.5 $\pm$ 0.6 ^a	4.6 $\pm$ 0.7 ^a	4.5 $\pm$ 0.5 ^a	4 $\pm$ 0.3 ^{b,c,d,e}
OC [ng/ml] ¹	36.2 $\pm$ 29.7 ^c	57.7 $\pm$ 42.5	127.7 $\pm$ 84.4 ^{b,e}	55.4 $\pm$ 38.7 ^c	n.d.
PTH [pg/ml]	67 $\pm$ 40 ^{b,c,d}	133 $\pm$ 77 ^{c,e}	344 $\pm$ 235 ^{b,d,e}	123 $\pm$ 73 ^{c,e}	n.d.
CTX [pg/ml] ¹	400 $\pm$ 170 ^{c,b}	650 $\pm$ 360	1140 $\pm$ 580 ^e	710 $\pm$ 450 ^e	n.d.
bALP [$\mu\text{g/l}$] ¹	25.3 $\pm$ 9.5	28.8 $\pm$ 11	37.1 $\pm$ 12.9	29.8 $\pm$ 13.9	n.d.
TRAP [U/l] ¹	2.77 $\pm$ 0.58	3.05 $\pm$ 0.91	3.66 $\pm$ 1.09	3.98 $\pm$ 1.98	n.d.
25(OH)D [ng/ml]	29.9 $\pm$ 9.7	33.6 $\pm$ 15.9	30.4 $\pm$ 8	31.1 $\pm$ 12.8	n.d.
Hematocrit [%]	39.4 $\pm$ 4.8	36.8 $\pm$ 3.8 ^a	35.1 $\pm$ 4.6 ^a	37.6 $\pm$ 3.8 ^a	41.5 $\pm$ 3.1 ^{b,c,d}

CKD patients were divided into 3 groups according to their eGFR (CKD 3 – 5) and none of the CKD patients received hemodialysis (renal replacement therapy, RRT). Data are depicted as mean $\pm$ SD.

Significant differences between the groups: ^a...p<0.05; compared to control; ^b...p<0.05 compared to KT; ^c...p<0.05 compared to CKD 5; ^d...p<0.05 compared to CKD 4; ^e...p<0.05 compared to CKD 3 using Kruskal-Wallis tests for independent samples with Bonferroni post hoc testing for multiple comparison correction.

¹ Parameters were measured in a subgroup of 79% (OC), 80% (CTX), 50% (bALP) and 47% (TRAP) of samples from CKD 3-5 and KT patients.

n.d. not done

4.3 Overall low miRNA count in CKD stage 5 and healthy controls

The digital multiplexed nanoString nCounter human miRNA expression assay showed a quite low overall count of miRNAs. When comparing the abundance of miRNAs between the control and the CKD group (Table 3), it is evident that abundance of miRNAs is quite similar in the both groups.

Table 3: A list of miRNAs detected in human serum, arranged in their order of abundance. Listed is the abundance of different miRNAs in the serum of healthy controls and CKD patients. Also indicated are the average single counts (signals) detected in one sample

Gene Name	Percent abundance Control	Counts	Gene Name	Percent abundance CKD	Counts
hsa-miR-4454	5,09	601,83	hsa-miR-1283	3,90	413,27
hsa-miR-451a	3,25	383,73	hsa-miR-378e	1,73	183,44
hsa-miR-223-3p	2,93	346,77	hsa-miR-4454	1,70	180,20
hsa-miR-720	1,88	222,33	hsa-miR-720	1,61	171,04
hsa-miR-378e	1,76	208,38	hsa-miR-143-3p	1,45	153,24
hsa-miR-1283	1,59	188,47	hsa-miR-302d-3p	1,34	141,99
hsa-miR-25-3p	1,41	167,27	hsa-miR-25-3p	1,24	132,01
hsa-miR-1976	1,16	137,66	hsa-miR-320e	0,91	96,85
hsa-miR-302d-3p	1,13	133,69	hsa-miR-1253	0,85	90,64
hsa-miR-23a-3p	0,81	95,86	hsa-miR-630	0,61	64,95
hsa-miR-320e	0,67	78,69	hsa-miR-612	0,60	63,17
hsa-miR-22-3p	0,60	70,76	hsa-miR-574-5p	0,56	59,28
hsa-miR-631	0,59	69,73	hsa-miR-1183	0,49	52,17
hsa-miR-143-3p	0,58	68,53	hsa-miR-579	0,46	49,09
hsa-miR-612	0,54	63,52	hsa-miR-30e-5p	0,44	47,08
hsa-miR-574-5p	0,52	61,24	hsa-miR-4443	0,44	46,79
hsa-miR-30e-5p	0,50	58,84	hsa-miR-570-3p	0,42	44,95
hsa-miR-570-3p	0,49	58,28	hsa-miR-4516	0,41	43,59
hsa-miR-579	0,43	50,58	hsa-miR-514b-5p	0,39	41,52
hsa-miR-188-5p	0,43	50,36	hsa-miR-1827	0,37	39,14
hsa-miR-514b-5p	0,42	49,84	hsa-miR-1286	0,36	38,00
hsa-miR-1183	0,40	47,15	hsa-miR-451a	0,30	32,02
hsa-miR-1253	0,36	43,05	hsa-miR-520h	0,29	30,69
hsa-let-7g-5p	0,35	40,86	hsa-miR-548ai	0,29	30,30
hsa-miR-1827	0,33	39,5	hsa-miR-192-5p	0,28	30,07

hsa-miR-4516	0,32	38,2	hsa-miR-631	0,27	29,16
hsa-miR-888-5p	0,32	37,3	hsa-miR-188-5p	0,27	29,14
hsa-miR-142-3p	0,31	36,86	hsa-miR-542-3p	0,27	28,85
hsa-let-7b-5p	0,30	35,81	hsa-miR-1246	0,27	28,30
hsa-miR-222-3p	0,30	34,87	hsa-miR-4455	0,26	28,01
hsa-miR-146a-5p	0,29	34,53	hsa-miR-598	0,26	27,73
hsa-miR-1286	0,29	34,33	hsa-miR-1208	0,26	27,37
hsa-miR-520h	0,29	33,75	hsa-miR-22-3p	0,24	25,85
hsa-miR-93-5p	0,27	32,49	hsa-miR-216b	0,24	25,39
hsa-miR-4286	0,27	32,12	hsa-miR-376a-3p	0,24	25,03

miRNA expression was evaluated and changes between miRNAs between CKD stage 5 patients and healthy controls were depicted in a heat map produced via the nanoString nCounter software (Figure 8).

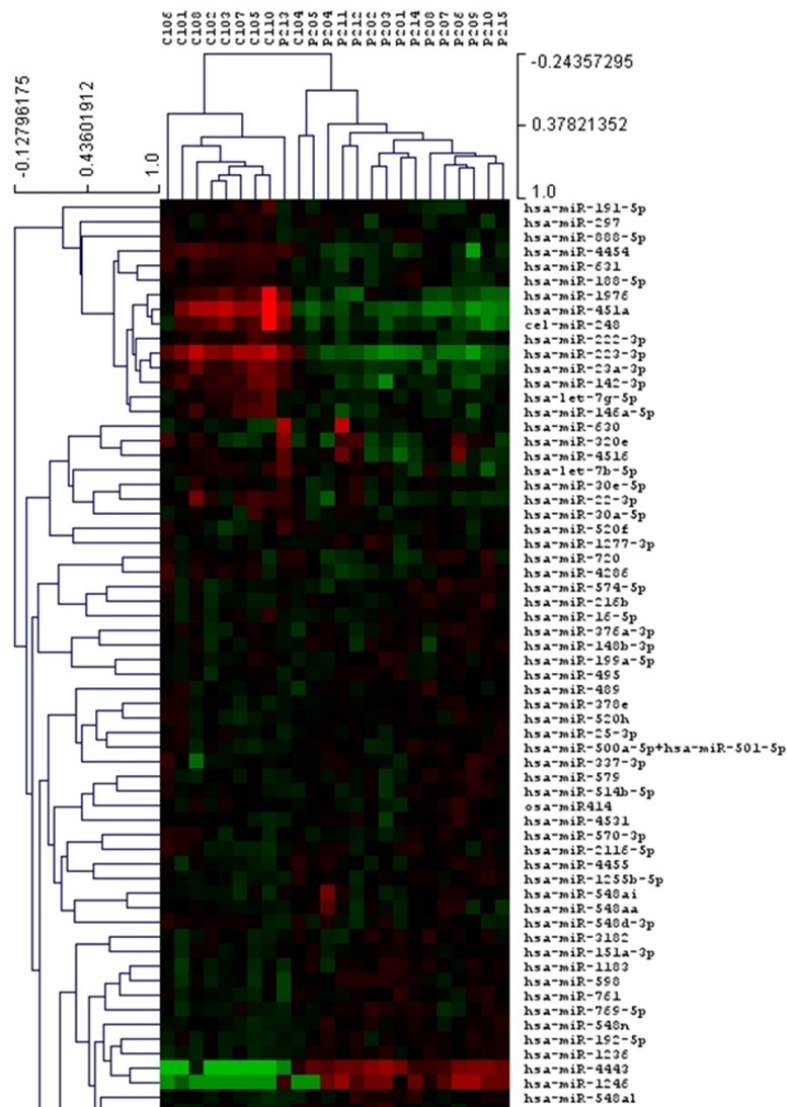


Figure 8: Identification of differentially expressed serum miRNAs if comparing healthy controls and CKD patients. Heat map was created with the Nanostring software. Controls (C101 – C110) are on the left side of the map, CKD patient samples (P201 – P215) are on the right side. (Green indicates low expression, red higher expression)

4.4 Selection of miRNAs

In a primary discovery step comparing serum of CKD stage 5 patients and healthy controls using the nanoString nCounter miRNA array analyzing 800 human miRNAs, expression difference for several miRNAs have been detected. 12 miRNAs (Table 4) were selected for further analysis based on expression differences seen in a miRNA array and their putative involvement in bone and calcification during CKD according to existing knowledge and databases.

Table 4: Fold changes of miRNA counts between CKD and healthy control serum samples using the nanoString nCounter miRNA array. Results are shown for miRNAs which showed a significant difference. MiRNAs which were subsequently tested via qPCR are highlighted. (also used as a supplement in our publication ⁽¹⁷³⁾).

Gene Name	Counts CKD	Counts Control	Fold Change	p-value
hsa-miR-223-3p	21.84 ± 49.59	312,48 ± 205.78	-14.31	<0.001
hsa-miR-451a	28.57 ± 74.66	241.4 ± 163.4	-8.45	<0.001
hsa-miR-23a-3p	20.59 ± 15.36	86,17 ± 54.3	-4.18	<0.001
miR-106b-5p	9.87 ± 4.49	36.93 ± 19.38	-3.74	0.1
hsa-miR-142-3p	9.55 ± 6.81	33,93 ± 20.77	-3.55	<0.001
hsa-miR-93-5p	9.31 ± 3.06	29,93 ± 14.86	-3.21	<0.001
hsa-miR-1976	16.25 ± 16.93	50.78 ± 26.19	-3.12	<0.001
miR4454	179.73 ± 127.77	548.88 ± 119.3	-3.05	<0.001
hsa-miR-146a-5p	10.80 ± 3.35	31,34 ± 18.25	-2.9	<0.001
miR-512-5p	8.55 ± 6.93	22.83 ± 3.55	-2.67	0.057
miR-20a-5p + miR-20b-5p	10.44 ± 3.64	25.41 ± 13.14	-2.43	0.004
let-7g-5p	18.32 ± 3.43	43.96 ± 23.82	-2.41	0.001
miR-150-5p	10.13 ± 4.64	23.53 ± 11.26	-2.32	0.14
hsa-miR-631	30.16 ± 8.93	63.31 ± 20.08	-2.10	<0.001
miR-22-3p	30.12 ± 15.70	62.71 ± 42.10	-2.08	0.016
miR-1976	25.85 ± 21.50	50.78 ± 26.19	-1.96	0.04
miR-185-5p	11.39 ± 1.82	22.01 ± 11.21	-1.93	0.008
miR-191-5p	16.56 ± 2.67	29.82 ± 16.55	-1.80	0.082
miR-92a-3p	13.67 ± 6.37	21.92 ± 5.86	-1.60	0.006
miR-106a-5p + miR-17-5p	10.81 ± 3.94	17.25 ± 6.98	-1.60	0.066
miR-148a-3p	5.82 ± 3.31	8.65 ± 2.67	-1.49	0.062
miR-548d-3p	15.06 ± 4.58	21.88 ± 3.08	-1.45	0.002
miR-26a-5p	12.47 ± 4.25	17.94 ± 3.17	-1.44	0.007
miR-361-5p	12.36 ± 3.23	16.79 ± 3.95	-1.36	0.02
let-7b-5p	24.47 ± 18.26	33.15 ± 10.02	-1.35	0.02
miR-888-5p	26.40 ± 7.29	34.13 ± 8.61	-1.29	0.058
let-7b-5p	26.05 ± 20.26	33.15 ± 10.02	-1.27	0.08
miR-598	30.50 ± 8.79	23.81 ± 6.50	1.28	0.04
miR-216b	26.81 ± 8.18	20.66 ± 4.16	1.30	0.08
miR-3614-5p	27.31 ± 5.90	21.09 ± 5.94	1.30	0.03
miR-1286	41.41 ± 6.30	31.74 ± 5.20	1.30	0.001
miR-3934	25.69 ± 5.04	19.49 ± 4.52	1.32	0.007
miR-1183	57.18 ± 12.98	43.37 ± 10.30	1.32	0.01
miR-1236	24.95 ± 4.76	17.99 ± 4.25	1.39	0.002
miR-192-5p	33.66 ± 4.38	24.10 ± 5.64	1.40	0.0013
miR-578	22.99 ± 6.78	15.43 ± 3.51	1.49	0.0042

miR-520a-5p	9.05 ± 4.99	6.00 ± 1.56	1.51	0.082
miR-1185-5p	15.95 ± 1.68	10.46 ± 1.81	1.52	0.016
miR-384	8.68 ± 3.90	5.28 ± 2.23	1.64	0.0357
miR-573	8.17 ± 3.60	4.9 ± 2.01	1.67	0.084
hsa-miR-1253	94.07 ± 26.47	40,23 ± 28.79	2.34	<0.001
hsa-miR-1283	428.28 ± 167.33	197.4 ± 105.2	2.44	<0.001
hsa-miR-143-3p	159.09 ± 42.59	71.69 ± 32.95	2.49	<0.001

Fold changes were calculated using the nSolver software from nanoString Technologies. Significant results were analyzed using Mann-Whitney U test for 2 independent samples.

The microRNAs further analyzed were miR-146a, miR-223-3p, miR-142-3p, miR-23a-3p, miR-93-5p, miR-1976, miR-4454, miR-631, miR-451a, miR-143-3p, miR-1283 and miR-1253 from our screening data and miR-145 and miR-125 which we selected because its possible connection stated in the literature.

5 miRNAs, miR-4443, miR-631, miR-451a, miR-1976 and miR-1283 did not show enough signal during the qPCR to be evaluated.

In the preliminary qPCR experiment examining a ten selected samples, all other miRNAs show a fold change of at least 1.5 or higher according to our measurements (Figure 9).

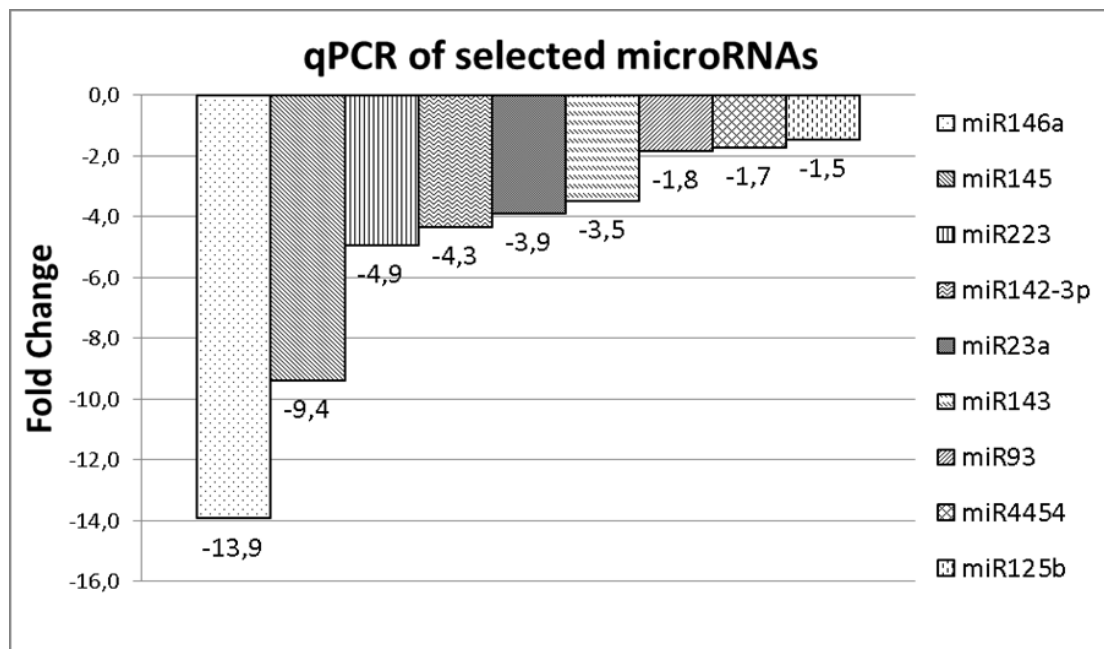


Figure 9: Fold changes of selected microRNAs if comparing samples from CKD patients to healthy controls. RNA was extracted from serum and measured with TaqMan small RNA assays with qPCR. Differences were measured via the $\Delta\Delta CT$ method. Fold changes were calculated via the formula in the literature.

When we started to analyze miRNA expression in all samples, only results for 4 miRNAs were convincing and produced sufficient fold changes and CT-levels. Those 4 miRNAs were miR-223-3p, miR-93-5p, miR-142-3p and mi-146a-5p.

4.5 MiR-223 and miR-93 are down-regulated over the course of CKD and normalize after KT

Systemic expression levels of miR-223-3p and miR-93-5p were significantly reduced in CKD patients in stages 4 and 5 when comparing them to CKD stage 3, healthy controls, and KT recipients (Figure 10A, B).

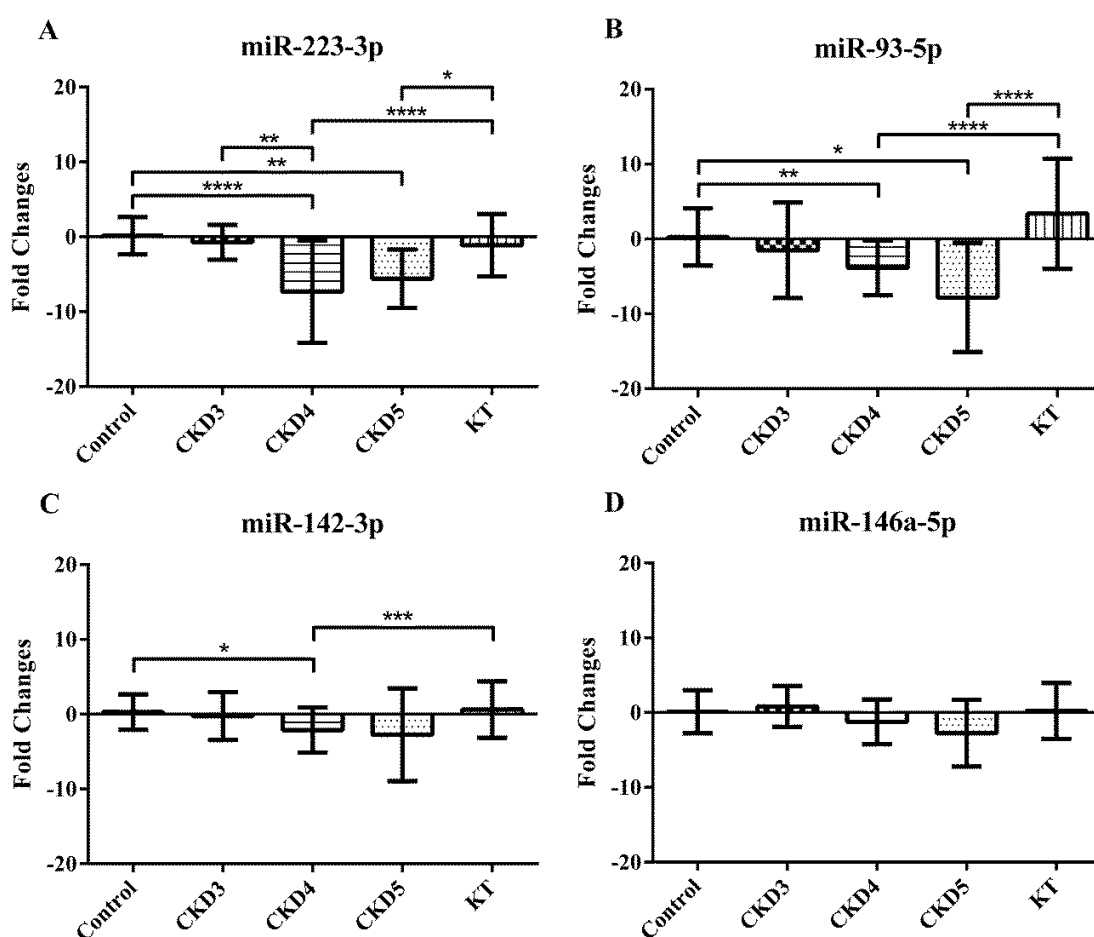


Figure 10: Fold changes of circulating miRNAs (miR-223-3p, miR-93-5p, miR-142-3p and miR-146a-5p) in CKD patients at different CKD stages and in patients after renal transplantation (KT) compared to healthy controls. CKD stages 3-5 (CKD 3...eGFR 30-44; CKD 4...eGFR 15-29; CKD 5...eGFR <15).

Data are depicted as mean $\pm$ SD. (* p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001).

MiR-223-3p was down-regulated in CKD stage 4 ($p<0.0001$ compared to controls; $p<0.01$ compared to CKD3; $p<0.0001$ compared to KT) and in CKD stage 5 ($p=0.003$ compared to controls; $p=0.06$ compared to CKD 3; $p=0.025$ compared to KT). After KT, expression levels reached levels of healthy controls again (Figure 10A).

MiR-93-5p also showed a stable down-regulation in all three CKD stages with a significant decrease in CKD 4 ($p=0.0093$) and CKD 5 ($p=0.048$) compared to healthy controls. After KT, expression was again normalized, with no significant difference compared to the control samples, but an increase compared to both CKD 4 ($p<0.0001$) and CKD 5 ($p<0.0001$) (Figure 10B).

Expression differences for miR-142-3p and miR-146a-5p which were detected in the miRNA array and primary qPCR experiments were attenuated in qPCR experiments in all CKD and KT groups. Nevertheless, miR-142-3p showed a small, but significant down-regulation in CKD 4, compared to healthy controls ($p=0.025$) and KT recipients ($p<0.001$) (Figure 10C). For miR-146a-5p, there was no statistically significant difference between any of the groups (Figure 10D).

Due to a considerable amount of T2DM cases of around 25% in all patients groups, we found it necessary to investigate the confounding effect of T2DM on miRNA expression as far as possible. Therefore we calculated the differences in expression for all 4 miRNAs according to manifestation of T2DM (Figure 11). However, there was no overall change of the results except for decrease in statistical power due to decrease in sample size.

Results seen in both Figures, 10 and 11, have been taken from our publication “MicroRNAs 223-3p and 93-5p in patients with chronic kidney disease before and after renal transplantation”⁽¹⁷³⁾, with Figure 11 being in the supplements of this paper.

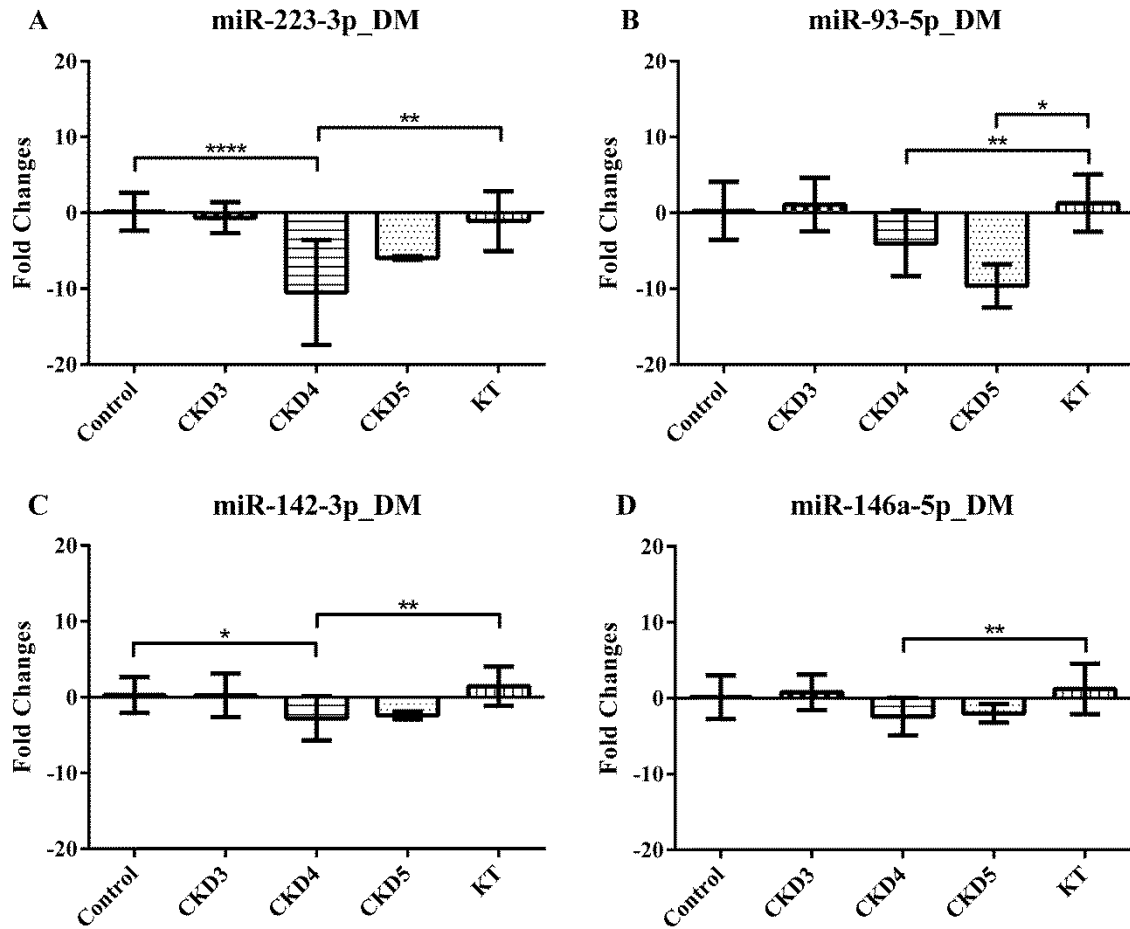


Figure 11: Fold changes of circulating miRNAs (miR-223-3p, miR-93-5p, miR-142-3p and miR-146a-5p) in CKD patients at different CKD stages and patients after renal transplantation (KT), all with T2DM, compared to healthy controls. CKD stages 3-5 (CKD 3...eGFR 30-44; CKD 4...eGFR 15-29; CKD 5...eGFR <15).

Data are depicted as mean $\pm$ SD. (* p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001).

4.6 Associations of miR-223 and miR-93 with relevant clinical parameters

Using univariate linear regression analyses for miR-223-3p and miR-93-5p we showed significant associations of the miRNAs with kidney function, hematocrit, inflammation, PTH, 25(OH)D, and glucose metabolism (Table 5, as shown in our own publication ⁽¹⁷³⁾).

Table 5: Regression analysis of miR-223-3p and miR-93-5p expression with parameters of kidney function, inflammation, mineral- and glucose-metabolism.

	Dependent variables	Statistical Associations			
		miR-223-3p		miR-93-5p	
		adjusted r^2	p-value	adjusted r^2	p-value
CKD 4	IL-6	0.155	0.018	0.136	0.043
	IL-8	0.092	0.061	-0.48	0.988
	eGFR	0.09	0.059	-0.01	0.388
	Hematocrit	0.09	0.272	0.127	0.049
	C-peptide	-0.024	0.575	-0.044	0.845
	OC	0.046	0.778	0.2	0.036
	PTH	-0.029	0.642	0.091	0.088
	CTX	0.226	0.015	-0.062	0.932
	Inorganic phosphate	0.026	0.391	-0.023	0.49
	25(OH)D	0.092	0.057	-0.045	0.977
CKD 5	IL-6	-0.091	0.582	-0.123	0.915
	IL-8	-0.67	0.503	0.16	0.138
	eGFR	0.584	0.01	0.41	0.027
	Hematocrit	0.137	0.175	0.59	0.006
	C-peptide	-0.136	0.847	0.492	0.014
	OC	0.517	0.041	0.384	0.06
	PTH	-0.058	0.461	0.339	0.058
	CTX	0.272	0.166	0.062	0.291
	Inorganic phosphate	0.052	0.301	-0.154	0.806
	25(OH)D	0.034	0.306	-0.143	0.969

A significant association of miR-223-3p with eGFR in CKD stage 5 with a trend towards an association in CKD stage 4 was indicated. In patients with CKD stage 4, miR-223-3p was significantly associated with levels of IL-6 and CTX. Furthermore, stage 4, miR-223-3p showed a trend towards an association with IL-8 and 25(OH)D (Table 5). In CKD stage 5, miR-223-3p showed a significant association with OC levels.

MiR-93-5p was significantly associated with eGFR in CKD stage 5 and with levels of IL-6 and hematocrit, both in CKD stages 4 and 5. In CKD stage 5, miR-93-5p was associated with C-peptide and a trend towards an association with PTH was detected. A trend towards an association of mi-93-5p with PTH was seen in CKD stage 4. OC was significantly associated with miR-93-5p in CKD stage 4 and showed a trend towards an association in stage 5 (Table 5).

4.7 MiRNA target prediction indicates binding of miR-223-3p and miR-93-5p to genes regulating bone metabolism during CKD

Based on predictions using miRWalk 2.0, miR-223-3p and miR-93-5p were found to interact with different signaling, differentiation or regulatory pathways including IL-6-signaling, TGF β -signaling, Wnt-signaling, and osteoblast differentiation. Genes showing a connection to these two miRNAs were further investigated using TargetScan 7.0.

For miR-223-3p prediction suggested a regulation of the following genes: CHUK (conserved helix-loop-helix ubiquitous kinase; IKK α), RPS6KB1 (ribosomal protein S6 kinase B1) and IL6ST (interleukin 6 signal transducer) (Table 6).

Table 6: Predicted targets for miR-223-3p with relevance for CKD-MBD and vascular calcification

Target Gene	miRWalk Pathway	3'UTR Position	Predicted consequential pairing of target region (top) and miR-223 (bottom)	Context++ score	P _{CT}
CHUK			5'...UUGCAAUCUAAUCUGAACUGACC...		
(IKK α)		917-923		-0.2	0.31
			3' ACCCAUAAACUGUUUGACUGU		
RPS6KB1	IL-6 Signaling		5' ...AUGUUGAUGACUCGAAACUGACA...		
		285-292		-0.22	0.29
			3' ACCCAUAAACUGUUUGACUGU		
IL6ST	IL-6 Signaling		5' ...UUUAAGCACUACAUAAACUGACU..		
		1882-1888		-0.23	0.41
			3' ACCCAUAAACUGUUUGACUGU		

Relevant genes were identified using the Pathway information retrieval system from miRWalk 2.0. Targets for miR-223-3p were confirmed using TargetScan 7.0. Predictions with a P_{CT} (probability of conserved targeting) score higher than 0.2 were considered ⁽¹⁷⁴⁾.

MiR-93-5p was predicted to regulate STAT3 (Signal transducer and activator of transcription 3), TNFSF11 (tumor necrosis factor superfamily member 11, RANKL), SMAD5 (SMAD family member 5), SMAD6 (SMAD family member 6), BMR2 (bone morphogenetic protein receptor type 2), PPARD (peroxisome proliferator activated receptor delta), and NFAT5 (nuclear factor of activated T-cells 5, tonicity-responsive) (Table 7).

Table 7: Predicted targets for miR-93-5p with relevance for CKD-MBD and vascular calcification

Target Gene	miRWalk Pathway	3'UTR Position	Predicted consequential pairing of target region (top) and miR-93 (bottom)	Context++ score	P _{CT}
STAT3	IL-6 Signaling		5' ...CUUUGAGCAAUCUGGGCACUUUU...		
		252-258		-0.23	0.74
			3' GAUGGACGUGCUUGUCGUGAAAC		
STAT3	IL-6 Signaling		5' ...CAUACUCCUGGCAUUGCACUUUU...		
		542-548		-0.19	0.73
			3' GAUGGACGUGCUUGUCGUGAAAC		
TNFSF11 (RANKL)	Osteoblasts		5' ...ACAUAUUUAACUGGUGCACUUUG...		
		617-623		-0.24	0.73
			3' GAUGGACGUGCUUGUCGUGAAAC		
SMAD6	TGF β -signaling		5' ...CAAACAAGAAAGACGCACUUUG...		
		249-255		-0.27	0.74
			3' GAUGGACGUGCUUGUCGUGAAAC		
BMPR2	TGF β -signaling		5' ...UGUUUUUUAAGUUUUGCACUUUU...		
		450-456		-0.22	0.74
			3' GAUGGACGUGCUUGUCGUGAAAC		
BMPR2	TGF β -signaling		5' ...UUUUUUUUAUUUAAGCACUUUC...		
		1767-1773		-0.17	0.72
			3' GAUGGACGUGCUUGUCGUGAAAC		
SMAD5	TGF β -signaling		5' ...UUAUCUUUUUUUUUGCACUUUU...		
		848-854		-0.22	0.74
			3' GAUGGACGUGCUUGUCGUGAAAC		
PPAR δ	Wnt-signaling		5' ...CAUAAGCACUGAAAUCACUUUAC...		
		1184-1190		-0.03	0.26
			3' GAUGGACGUGCUUGUCGUGAAAC		
NFAT5	Wnt-signaling		5' ...CCCUAUUGAUUUUUGCACUUUA...		
		5813-5820		-0.05	0.94
			3' GAUGGACGUGCUUGUCGUGAAAC		

Relevant genes were identified using the Pathway information retrieval system from miRWalk 2.0. Targets for miR-93-5p were confirmed using TargetScan 7.0. Predictions with a P_{CT} (probability of conserved targeting) score higher than 0.2 were considered⁽¹⁷⁴⁾.

Using miRTarBase we evaluated targets for miR-223-3p and miR-93-5p outside of the CKD-MBD spectrum, which have already been confirmed via molecular methods in other studies. Methods reported included reporter assays, western blot or qPCR. To limit potential results, we included targets that have been documented by more than one of these experiments, therefore increasing the reliability of the target miRNA interaction. Gene functions and associated diseases have been evaluated for the predicted targets via three well known databases; Genecards, Uniprot and the NCBI database (Table 8 and 9).

Table 8: Targets for miR-223-3p which have been validated with strong evidence in studies using reporter assays, western blot or qPCR. Targets prediction has been done using miRTarBase. Function and associated diseases have been checked with the Genecards, Uniprot and NCBI databases.

Target	Gene Name	Function	Associated Diseases	
MEF2C	myocyte enhancer factor 2C	chromatin binding, transcriptional activation	mental retardation	
STMN1	stathmin 1	regulation of the microtubule	acute leukemia	cancer
LMO2	LIM domain only 2	hematopoietic development	lymphoblast leukemia	t cell leukemia
E2F1	E2F Transcription factor 1	transcription factor	retinoblastoma	
RHOB	ras homolog family member B	cell survival	cancer	
NFIX	nuclear factor I/X	transcription factor		
NFIA	nuclear factor I A	transcription factor	leukemia	
Arid4b	AT rich interactive domain 4B	transcriptional repressor	breast cancer	
Il6	interleukin 6	cytokine	inflammatory processes	
Lpin2	lipin 2	lipid metabolism, phosphatase	inflammatory processes	
CHUK	conserved helix-loop-helix ubiquitous kinase	regulating NF- κ B		
FBXW7	F-box and WD repeat domain containing 7	ubiquitin protein ligase	lymphoblast leukemia	breast cancer
IGF1R	insulin-like growth factor 1 receptor	anti-apoptotic	cancer	resistance to IGF-1
LIF	leukemia inhibitory factor	cell differentiation	leukemia	
SP3	Sp3 Transcription factor	Transcription factor		
EPB41L3	erythrocyte membrane protein band 4.1 like 3	tumor suppressor	adenocarcinoma	lung cancer
SLC2A4	solute carrier family 2 member 4	glucose transporter	T2DM	
ARTN	artemin	neurotrophic factor	Parkinson's disease	tumor growth
FOXO1	forkhead box O1	transcription factor	cancer	breast cancer
HSP90B1	heat shock protein 90kDa beta (Grp94), member 1	molecular chaperone	tumor formation	lymphocytic leukemia
IRS1	insulin receptor substrate 1	mediates signals by insulin	cancer	T2DM
SCARB1	scavenger receptor class B, member 1	plasma membrane receptor for HDL	hepatitis C	coronary heart disease
PARP1	poly(ADP-ribose) polymerase 1	poly(ADP-ribosyl)transferase	T1DM	leukemia
SMARCD1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1	chromatin remodeling		

CDK2	cyclin-dependent kinase 2	cell cycle regulation	cancer	
ECT2	epithelial cell transforming sequence 2 oncogene	cell cycle regulation	cancer	
PTBP2	polypyrimidine tract binding protein 2	splicing	cancer	
TAL1	TAL bHLH Transcription factor 1	chromatin binding, transcription regulating	lymphoblast leukemia	
ATM	ataxia telangiectasia mutated	DNA damage response	lymphocytic leukemia	cancer
CYB5A	cytochrome b5 type A	membrane bound electron carrier	methemoglobinemia	
TOX	thymocyte selection associated high mobility group box	t-cell development		
PRDM1	PR/SET domain 1	transcriptional repressor	lymphoma	b-cell lymphoma

Table 9: Targets for miR-93-5p which have been validated with strong evidence in studies using reporter assays, western blot or qPCR. Targets prediction has been done using miRTarBase. Function and associated diseases have been checked with the Genecards, Uniprot and NCBI databases.

Target	Gene Name	Function	Associated Diseases	
TP53INP1	tumor protein p53 inducible nuclear protein 1	antiproliferative and proapoptotic	cancer	Alzheimer's disease
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	cell cycle progression regulator	cancer	leukemia
TCEAL1	transcription elongation factor A like 1	transcriptional regulation		
E2F1	E2F Transcription factor 1	DNA binding transcription activator	retinoblastoma	cancer
MAPK9	mitogen-activated protein kinase 9	serine/threonine-protein kinase	cancer	
VEGFA	vascular endothelial growth factor A	angiogenesis, growth factor	cancer	
ITGB8	integrin, beta 8	receptor activity, cell-cell interactions		
KAT2B	K(lysine) acetyltransferase 2B	histone acetyltransferase, transcriptional activation	HIV	
TUSC2	tumor suppressor candidate 2	tumor suppressor	cancer	
BCL2L11	BCL2 like 11	apoptosis induction	lymphoma	
PTEN	phosphatase and tensin homolog	tumor suppressor	cancer	
PURA	purine-rich element binding protein A	transcriptional activator	mental retardation	learning disability
LATS2	large tumor suppressor kinase 2	tumor suppressor	cancer	

All target predictions, from Table 6, 7, 8 and 9 were part of the supplementary information of our publication in Bone ⁽¹⁷³⁾.

4.8 MiRNA expression differences between CKD and KT patients with comparable eGFR

Comparing CKD 4 patients with an eGFR of 15 – 29 to KT patients with a similar eGFR level (eGFR 15 – 29), expression differed for miR-223-3p ($p < 0.01$), miR-93-5p ($p < 0.01$), and miR-142-3p ($p < 0.01$) and was significantly lower in CKD compared to KT patients with matched eGFR (Figure 12) for all three miRNAs. MiR-146a-5p did not show any difference between the two subgroups.

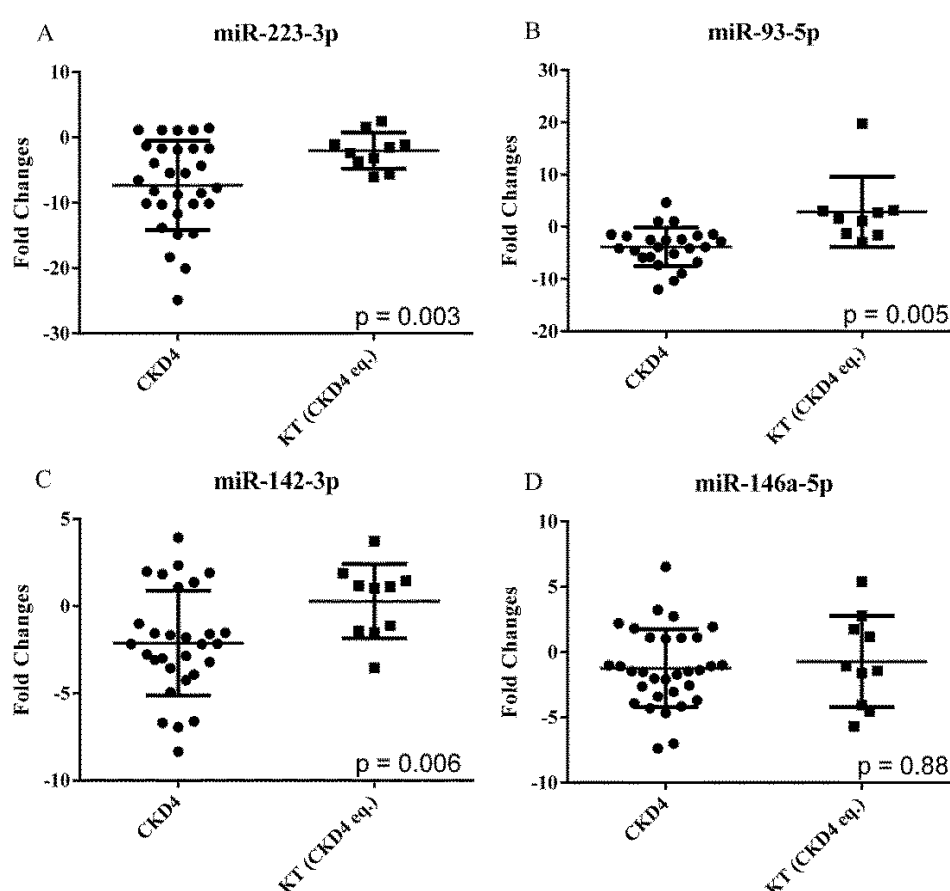


Figure 12: Comparison of miRNA expression between the CKD4 and KT patients with a similar eGFR (“KT (CKD4 equivalent)”, eGFR between 15 and 29 [ml/min/1.73m³]).

Data are depicted as mean $\pm$ SD. (Results were also presented in our publication ⁽¹⁷³⁾).

4.9 Patient characteristics of the RCS study

In our second study, the RCS study, 30 CKD patients were recruited. 10 underwent KT and 2 already had a first follow up examination at the end of this PhD thesis. General patient characteristics can be seen in Table 10.

Table 10: General patient characteristics of the RCS patients

Characteristic [Units]	CKD 5 (n=30)	KT (n=10)	Controls (n=2)
Age [years]	50±13	48±13	43±13
Sex [male]	69%	70%	100%
BMI [kg/m ²]	29±6.1	26±4.4	24.7±2.7
eGFR [ml/min/1.73m ³]	7.79±3.97	6.80±3.28	46.80±33.08
Time after Transplantation [months]			6

In the follow up study when comparing serum levels of different time points, levels of several parameters change in the KT group whereas levels between the CKD time point and the KT time point are similar. Differences for all measured parameters are shown in Table 11.

Table 11: Serum parameters of patients included in the RCS study. Measurements were undertaken at 3 different time points for the same individuals. Time points are CKD, KT and follow up (6 month after KT)

Characteristic [Units]	CKD 5 (n=30)	KT (n=10)	Follow up (n=2)
Creatinine [mg/dl]	8.24±3.78	8.67±2.92	2.14±0.91
Urea [mg/dl]	114.9±29.5	116.2±27.6	90.3±75.3
Uric Acid [mg/dl]	6.63±1.37	6.27±1.47	7.17±1.57
CRP [mg/l]	5.84±11.01	2.49±3.38	11.77±18.14
PTH [pg/ml]	322.7±195.89	257.5±117.2	206.9±157.4
bALP [μg/l]	21.13±11.27	19.48±12.56	30.17±5.55
TRAP [U/l]	4.14±1.91	3.58±1.90	4.29±2.45
25(OH)D [ng/ml]	21.46±9.45	19.74±10.73	33.40±9.92
CRP [mg/l]	4±5.9	4.1±3.7	7.3±6.3
C-peptide [ng/ml]	6.05±3.91	7.35±4.71	4.57±3.69
CTX [ng/ml]	1.81±1.33	0.97±0.47	1.13±0.78
Ery [count/μl]	20.97±41.47	19.19±23.32	64.60±99.07
OC [ng/ml]	186.1±96.9	112.8±98.3	59.2±11.3
FGF 23 [RU/ml]	1113±384	1170±266	534±381
ft3 [pg/ml]	4.22±0.79	4.39±0.94	6.07±3.29
ft4 [μg/dl]	14.77±2.63	15.12±1.74	22.23±8.37
Insulin [mU/l]	26.16±44.63	34.44±54.29	12.30±7.28

IGF1 [ng/ml]	205.25±68.36	172.62±73.66	218.57±48.01
Aldosterone [ng/dl]	12.87±12.94	26.63±41.14	19.33±8.02
OPG [pmol/l]	6.94±4.76	9.73±2.12	6.11±1.95
P1NP [ng/ml]	254.9±227.2	147.4±84.1	115.4±44.3
Renin [μ U/ml]	80.8±115.3	104.0±183.7	199.7±303.4
SHBG [nmol/l]	59.88±44.79	64.61±58.69	32.9±20.92
TSH [μ U/ml]	2.42±1.13	3.00±1.21	0.96±0.88
Vasopressin [pg/ml]	6.11±3.69	6.13±4.59	3.40±2.26
1,25(OH) ₂ D [pmol/l]	28.84±19.41	17.22±5.07	59.70±32.09
Rankl [pmol/l]	0.17±0.15	0.17±0.09	0.1±0.0
free Testosterone [pg/ml]	7.79±5.63	8.97±7.43	15.66±8.95
Testosterone [ng/ml]	1.76±1.36	1.64±1.29	3.36±1.65
VEGF [pg/ml]	133.8±175.4	111.3±92.5	27±18.4

4.10 miRNA expression changes during longitudinal follow-up

In study 2, the longitudinal study, samples from KT patients showed increased expression levels of both miR-223 and miR-93 six months after KT compared to the expression levels before transplantation. Of the two prospective patients receiving KT, miRNA-223 expression levels were 12 and 7 times higher than before surgery, respectively. For miR-93, changes after KT were similar, but less pronounced with three- and two-fold increases, respectively (Figure 13).

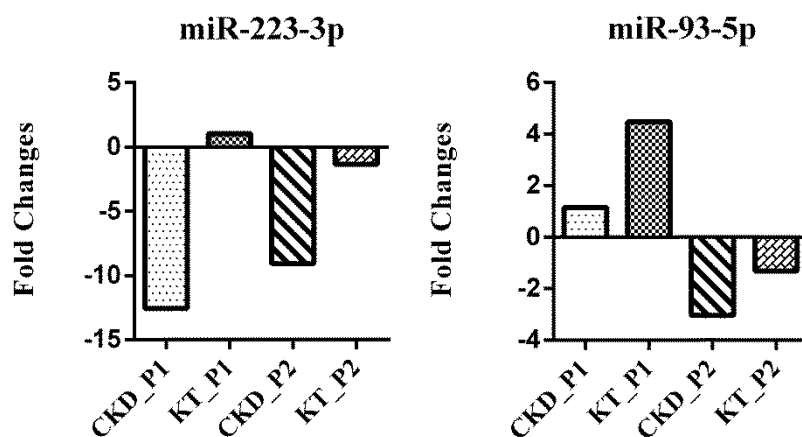


Figure 13: Fold changes of miRNA expression levels of miR-223-3p and miR-93-5p from RNA isolated from 2 CKD patients (P1 and P2) before (CKD) and 6 months after kidney transplantation (KT) shows increased expression after surgery. All fold changes are estimated compared to healthy controls.

5. Discussion

This PhD thesis aimed to analyze miRNA expression differences during CKD and after KT, and to find miRNAs indicative for CKD-MBD and vascular calcification as major comorbidities of CKD. We found significantly lower expression of miR-223-3p and miR-93-5p during later stages of CKD. These effects are reversed after kidney transplantation. The levels of expression for both miRNAs normalized, sometimes even exceeding levels of healthy controls. In addition we analyzed miR-142-3p and miR-146a-5p levels, indicating similar but only partly significant results. We report on associations of miR-223-3p and miR-93-5p with parameters of inflammation, mineral and glucose metabolism. Based on bioinformatic methods, target predictions suggested a regulatory role of these miRNAs in bone metabolism via different pathways. MiR-223-3p and miR-93-5p might be interesting new putative biomarkers for CKD-MBD, vascular calcification and bone diseases connected to CKD.

5.1 Associations of miR-223-3p, miR-93-5p, miR-142-3p and miR-146a-5p during bone metabolism

In addition to expression differences of miR-223-3p in our CKD study, this miRNA has recently been associated with several steps in bone metabolism including osteoclast differentiation, an osteogenic VSMC phenotype switch but also vascular calcification processes and inflammation, such as production of IL-1 β or the inflammasome complex, via various targets⁽¹⁷⁵⁾.

In a study in ApoE mice, a murine CKD model, miR-223 expression was up-regulated in the tissue of atherosclerotic aortas. By contrast, low levels of miR-223 in the circulation have been associated with calcification and advanced CKD-stages⁽¹⁷⁶⁾. This would go alongside our findings showing lower levels of serum miR-223-3p in a CKD background.

An in-vitro study supported this finding, showing that in VSMCs, expression of miR-223 was associated with inorganic phosphate metabolism. Phosphate overloads led to an increase in miR-223 expression as well as an increase in calcification of primary VSMCs⁽¹⁷⁷⁾.

Overall, in-vivo studies are pointing towards a decrease in systemic levels of miR-223 being indicative for negative metabolic effects, like calcification⁽¹⁷⁶⁾. This is in contrary to local effects of miR-223 where an increased expression seems to stimulate malignant processes.

Thus, local effects of miRNA-223 do contradict systemic findings. The systemic effects support our results and may suggest miRNA-223 to be an early systemic marker of calcification in CKD patients.

We have found a parallel systemic decrease of miR-93-5p during deterioration of CKD with a recovery after KT. Functional information on miR-93-3p is not as advanced as for miR-223-3p. Nonetheless, miR-93 has already been suggested as a potential regulator for the vascular endothelial growth factor (VEGF) as an important regulator during angiogenesis and vascular homeostasis. In type 1 diabetes mellitus (T1DM), downregulation of miR-93 was associated with increased systemic VEGF and IL-6 levels ⁽¹⁷⁸⁾. An interesting study from Long et al. conducted in isolated podocytes and in mice, associated this down-regulation of miR-93 and upregulation of VEGF with an increase in collagen and fibronectin in renal glomeruli which was associated with a deterioration of kidney function, thus diabetic nephropathy ⁽¹⁷⁹⁾.

There is an interesting link for miR-93 to Sp7, a transcription factor important during mineralization processes, which was shown to be inversely correlated with miR-93. Down-regulation of miR-93 was essential for high levels of Sp7 ⁽¹⁸⁰⁾. Notably, Sp7 has been shown to be an important factor during VSMC calcification, because it directs VSMC differentiation to osteoblast-like cells, a process known to be regulated by miR-125b ⁽¹⁸¹⁾. This link between Sp7 and miR-93 supports its role as an important factor during CKD-MBD and intensified calcification processes.

Alongside other miRNAs, namely miR-21, miR-23a, miR-24, miR-100, miR-122a, miR-124a, miR-125b and miR-148a, miR-93 expression has been shown to be upregulated in serum and tissue samples of osteoporotic patients with normal kidney function ⁽¹⁶⁷⁾. Interestingly, miR-93 was down-regulated in serum in osteoporotic patients with low traumatic fractures ⁽¹⁸²⁾, and was shown to inhibit osteoblast differentiation via reduction of transcription factor osterix (OSX) ⁽¹⁸⁰⁾. This results, even though contradictory show an association of miR-93 with bone mineral density and indicate that continuous research has to be conducted in this field.

MiR-142-3p and miR-146a-5p, which we also analyzed in detail, have been connected to mineralization and bone metabolism. For instance, in 2012 a study in human fetal osteoblasts, has described miR-142-3p to promote Wnt signaling, and thereby promoting osteoblastic differentiation. It was indicated that overexpression of miR-142-3p led to a reduction of important parameters like RunX2, OC and ALP, probably via direct targeting and inhibition

of the adenomatous polyposis coli (APC) gene ⁽¹⁸³⁾. Overexpression of miR-146a has been proposed to have an inhibitory effect on osteoclastogenesis, an effect which seems to be induced via RANKL and TNF α . However, a clear mechanism how osteoclastogenesis is affected by miR-146a has not been proposed yet ⁽¹⁵⁷⁾. In our study we could see a slight indication that miR-142-3p might be systemically downregulated during CKD and may thereby indicate bone and mineral deregulation. There seemed to be no systemic effect on miR-146a-5p in our study, but local effects cannot be excluded.

5.2 Bone metabolism and miRNAs in CKD

In a previous study, we investigated 25(OH)D, PTH, bALP, TRAP, OC, CTX, OPG and RANKL in a cohort of CKD patients in different stages with exclusion of patients showing traditional risk factors for cardiovascular events. Levels of the different parameters were measured from blood samples. The results on bALP and TRAP-5b, both markers of either bone formation or resorption were found to be independently predictive of CV events. There has been an increased prevalence of 25(OH)D and 1,25(OH)₂D deficiency with progression of CKD. Other markers, such as PTH and vitamin D as well as serum levels of RANKL, OPG, OC, and CTX have not been predictive for CV events ⁽¹⁸⁴⁾. A study which evaluates environmental and genetic risk factors for CVD is the so called LURIC study (Ludwigshafen Risk and Cardiovascular Health). They found an association of 25(OH)D and 1,25(OH)₂D to CVD, specifically to the renin-angiotensin system (RAS). Low levels of both forms of vitamin D were associated with an upregulated RAS, therefore to an upregulation of CVD risk such as atherosclerosis ⁽¹⁸⁵⁾.

When doing univariate association analysis, we saw associations for miR-93-5p and PTH in CKD stages 4 and 5. This might point to a direct or indirect interaction in later CKD stages between PTH and miR-93-5p, which might indicate a role of miR-93-5p during CKD-MBD via PTH. However, potential interactions between miR-93-5p and PTH are not known, but this certainly has to be further elucidated.

We found an association for miR-223-3p and OC in our study OC has been measured as bone formation parameter in CKD patients and controls. However, as OC may accumulate during renal failure ⁽¹⁸⁶⁾, results tend to be difficult to interpret.

Other bone associated enzymes which were measured in our study, namely TRAP5b and bALP, showed no associations to the candidate miRNAs, but this might also be due to the low

sample size in several groups. To our knowledge, until now, there are no publications that indicate a connection of TRAP or BALP to either of the two miRNAs miR-223-3p or miR-93-5p, indicating that there might not be an association between the markers and the two miRNAs.

5.3 Association of kidney function, and diabetes, with miRNA-223-3p, miR-93-5p, miR-142-3p and miR-146a-5p

Renal biopsies taken from CKD patients with progressive renal failure have been analyzed for its miRNA content. MiR-142-5p, miR-223 and miR-146b have been upregulated when compared to tissue biopsies of stable CKD patient, which supports a role of these miRNAs during deterioration of kidney function ⁽¹⁸⁷⁾.

In 2011, Neal et al. showed that the amount of circulating miRNAs was generally decreased in plasma samples of patients with ESRD receiving hemodialysis as well as in CKD stage 4 patients. Total circulating small RNAs as well as five specific miRNAs were measured. By measuring miRNA levels in urinary samples, clearance via the kidney was excluded, as miRNA levels did not change in urine ⁽¹⁸⁸⁾. Similar effects have been observed in our array results, when comparing counts and abundances for CKD stage 5 patients and healthy controls. However, there is a selectively decreased expression of systemic compared to locally expressed miRNAs in CKD, probably suitable as specific disease-associated patterns, thus ideal for biomarker research.

Diabetes is a major factor for impaired kidney function in a large group of CKD patients. In an earlier study, miR-223 levels have been shown to be decreased in T2DM compared to healthy controls, which might be due to a change in miR-223 regulation early in the disease manifestation ⁽¹⁸⁹⁾. Also miR-142-3p was shown to be differentially regulated during T2DM. In a cross sectional study in 93 men, 48 of them with T2DM, miR-142-3p was shown to be increased during T2DM ⁽¹⁹⁰⁾. For miR-146a a reduction in serum has been detected during T2DM, when comparing 56 T2DM patients to 40 healthy controls ⁽¹⁹¹⁾. This reduction of miR-146a in T2DM patients has been associated to circulating levels of IL-6, IL-8 and TNF α as well as insulin resistance⁽¹⁹²⁾. In our study, we investigated expression differences for miR-223-3p and miR-93-5p in CKD patients with and without T2DM. However, there were no major differences in miRNA expression levels. We concluded that inflammatory processes during CKD might be overwhelming to cause miRNA down-regulation and disturbances of

glucose metabolism might be less important in these circumstances. However, an increased subgroup sample size might be necessary to see if there are typical effects of T2DM on miR-223-3p and miR-93-5p regulation in CKD patients.

5.4 Inflammatory effects of miRNA-223-3p, miR-93-5p, miR-142-3p and miR-146a-5p

Systemic cytokine levels, namely IL-6 were associated with a decrease in miR-223-3p and miR-93-5p expression in our study. Some reports have been suggesting a regulatory effect of miR-223 on IL-6 via STAT3, but IL-6 was also found to inhibit miR-223 via a negative feedback loop⁽¹⁹³⁾. In addition, we found respective targets by bioinformatic pathway prediction, among them IL6ST (Interleukin 6 Signal Transducer) and RPS6KB1 (Ribosomal Protein S6 Kinase beta-1), which supports an involvement of miR-223 in the IL6 regulation.

In turn, miR-93 was indicated to interact with STAT3⁽¹⁹⁴⁾, which implies that also miR-93 might be regulatory for IL-6 via STAT3⁽¹⁹³⁾. The associations of miR-93-5p with IL-6 in CKD stages 4 and 5 seen in our results, support previous papers showing a putative anti-inflammatory effect of miR-93. MiR-93 directly targets IRAK4 (interleukin-1 receptor-associated kinase 4) mRNA, thereby inhibiting the pro-inflammatory effects of IRAK4 on NF- κ B signaling⁽¹⁹⁵⁾, indicating an anti-inflammatory effect of miR-93. Down-regulation of miR-93, as seen in our study, might strengthen the pro-inflammatory effect of IRAK4, also an important regulator for cytokines like IL-6 and a driving force for renal failure during CKD⁽¹⁹⁶⁾. Considering these results, miR-93 might be upregulated during conditions when the immune system is suppressed or downregulated. This would go along with our results, when miR-93-5p was significantly up-regulated in the KT group in comparison to CKD patients in stages 4 and 5. Thus, this might be an effect of the immunosuppressive medication in KT patients, where the resulting immunodeficiency can also lead to infections or cancers⁽¹⁹⁷⁾.

It has been reported that miR-223 might be a regulator of the NF- κ B and the toll-like receptor 4 (TLR4)-pathways, both of which have been described as important for the development of CKD^(198,199). An increase of miR-223 might lead to an increase in NF- κ B due to effects of miR-223 on the inhibitor of NF- κ B kinase α (IKK α) in macrophages. This resulted in a conditioning of macrophages towards an immune response, but without further harmful systemic autoimmune effects⁽²⁰⁰⁾.

We also looked into a potential association between miR-93-5p and IL8, because IL-8 was hypothesized to be a direct target of miR-93 due to potential target sites in the 3'-UTR of IL-8 mRNA. It has been associated with IL-8 up-regulation in IB3-1 cells which mimic an inflammatory state during fibrotic lung disease ⁽²⁰¹⁾. Nonetheless, in our study no associations between miR-93-5p and IL-8 have been detected, probably due to the small sample size in the subgroups or potential other confounding factors.

MiR-142-3p has recently been shown to have a pro-inflammatory effect in monocyte-derived dendritic cells. Several target genes of miR-142-3p have been analyzed with an effect on inflammatory pathways, including IL6ST, STAM, BCL2L ⁽²⁰²⁾. An effect on inflammation for miR-146a is well known and described in different settings. In human adipocytes miR-146a was shown to be upregulated during inflammatory conditions and lead to a reduction of IL-6 and IL-8 and thereby to a lower inflammatory response ⁽²⁰³⁾. Also during an inflammatory skin disease like psoriasis, miR-146a has been acting in an anti-inflammatory way via IL-17 suppression, shown in mice ⁽²⁰⁴⁾. A closer look into those two miRNAs during inflammatory effects alongside chronic kidney disease might also reveal additional, not yet confirmed connection.

5.5 Predicted targets for miRNA-223-3p and miR-93-5p and their role during CKD-MBD

To get a better understanding of the functions of miR-223-3p and miR-93-5p we analyzed putative targets of these miRNAs using TargetScan and have identified IKK α , RPS6KB1 and IL6ST mRNAs. The latter two are active in the IL-6 signaling pathway. RPS6KB1 is an effector of the PI3K (phosphatidylinositol 3-kinase) pathway which is important for ROS downstream signaling. It has been shown to be increased in a rat model of CKD on a high calcium-phosphate diet. Rats developed vascular calcification and VSMC differentiation to osteoblast-like cells affected by RPS6KB1 in combination with IL-6 and TNF ⁽²⁰⁵⁾. In mice, IL6ST was an important factor during osteoblast differentiation in response to PTH ⁽²⁰⁶⁾.

Predicted targets for miR-93-5p have been found in a number of regulators, such as STAT3, TNFSF11 (Tumor Necrosis Factor Superfamily Member 11, RANKL), SMAD5 (SMAD Family Member 5), SMAD6 (SMAD Family Member 6), BMR2 (Bone Morphogenetic Protein Receptor Type 2), PPARD (Peroxisome Proliferator Activated Receptor Delta), and

NFAT5 (Nuclear Factor Of Activated T-Cells 5), all of which are well-known regulators of mineral and vascular changes in CKD.

As mentioned in chapter 5.4, miR-93 has been connected to STAT3 in a setting of human cancer, as well as stem cell differentiation ^(194,207). STAT3 is a ubiquitous protein which plays an essential role in different pathways, also during osteoblast differentiation. It was shown to regulate osteoblast differentiation via a signaling pathway including LIF (Leukemia inhibitory factor), STAT3 and SOCS3 (Suppressor of Cytokine Signaling 3). In bone marrow stromal cells (BMSCs) the LIF/STAT3/SOCS3 cascade leads to a degradation of β -catenin, an important regulator of osteoblast differentiation ⁽²⁰⁸⁾.

RANKL represents an important link between bone and vasculature ^(209,210). RANKL is an essential molecule for osteoclastogenesis even though it is not only present in osteoclasts ⁽²¹¹⁾. The RANKL/RANK/OPG signal transduction involves different pathways and not only effects osteoclastogenesis. RANKL has been shown to be expressed by osteoblasts as well as T cells ⁽²¹²⁾. RANKL as well as RANK knock-out mice have been developing osteoporosis, but did also show effects on the development of lymph nodes ⁽²¹³⁾, which is indicative for their potential importance in the immune system.

SMAD5, SMAD6, and BMPR2 are all involved in BMP signaling. In the pluripotent cell line C2C12 as well as in rat aortic smooth muscle cells, SMAD5 has been identified as an upstream regulator of the transcription factor RUNX2, with activated SMAD5 inducing osteoblast differentiation via RUNX2 ^(214,215). SMAD6 plays a role as an inhibitor of BMP signaling ⁽²¹⁶⁾ and as an important factor for the normal development and homeostasis of the cardiovascular system ⁽¹³³⁾.

BMPR2, the receptor for BMP-2, is activating SMAD proteins and downstream signaling and thereby supports a potential switch of VSMCs to the osteogenic phenotype ⁽²¹⁷⁾. In mice, loss of BMPR2 resulted in increased bone mass due to osteoblasts activation ⁽²¹⁸⁾.

PPAR δ has been shown to be overexpressed in VSMCs during vascular lesion formation, suggesting a role during VSMC modulation in atherosclerosis ⁽²¹⁹⁾.

The transcription factor NFAT5 up-regulates the expression of RUNX2 via the transcription factor SOX9⁽²²⁰⁾, thus supporting the osteogenic transformation of VSMCs, which might also be enhanced by simultaneous inflammation, due to a stimulatory effect of NFAT5 on cytokine expression ^(221,222). An effect on calcification and bone development as well as inflammation

would go alongside our hypothesis for the regulatory effects of miR-93-5p but also miR-223-3p.

5.6 Predicted targets outside the CKD-MBD spectrum for miR-223-3p and miR-93-5p and their functions

For both miRNAs, potential targets outside of the CKD-MBD spectrum include various factors with a number of functions, localizations and disease associations, mainly in cancer topics due to the broad information available for oncological miRNA targets.

In case of miR-223-3p, examples for confirmed targets are

- MEF2C (myocyte enhancer factor 2C)
- STMN1 (stathmin 1)
- E2F1 (E2F transcription factor 1)
- PARP1 (poly(ADP-ribose) polymerase 1)
- ATM (ataxia telangiectasia mutate) and
- TOX (thymocyte selection associated high mobility group box).

MEF2C is a chromatin modifying protein that can target genes and thereby activate or repress gene activity. It has been shown to be directly targeted by miR-223 and that miR-223 and MEF2C were negatively correlated ⁽²²³⁾. MEF2C itself has been shown to increase during the course of chronic myeloid leukemia until the point of an acute leukemic phase ⁽²²⁴⁾.

STMN1 is a microtubule destabilizing protein which plays a role in mitosis and as an oncoprotein in several cancer types. In gastric cancer, an increase of STMN1 was correlated with poor disease-specific survival. MiR-223 has a direct and negative regulatory effect on STMN1, which is seen as a prognostic and therapeutic marker for gastric adenocarcinoma ⁽²²⁵⁾. Another interesting aspect of miR-223 and STMN1 interactions is its role during inhibition of the Dengue virus ⁽²²⁶⁾.

In granulopoiesis, miR-223 has been shown to be an inhibitor of E2F1, an important protein for cell cycle control and tumor suppression ⁽²²⁷⁾.

In a rat model for pulmonary arterial hypertension (PAH), a condition defined by vasoconstriction, inflammation and increased SMC proliferation in the arterial wall, the restoring of miR-223 expression was able to reverse the PAH effects. This was thought to be triggered by a direct interaction of miR-223 with PARP1 ⁽²²⁸⁾. Downregulation of PARP1 upon miR-223 overexpression has also been detected in esophageal adenocarcinoma, where cancer cells might be more prone to react to chemotherapy treatment ⁽²²⁹⁾, due to the downregulation of the DNA damage repair

The ATM protein is a protein kinase important during DNA damage repair caused by radiation therapy. In a study on radiation sensitivity of cancer cells, miR-223 overexpression lowered the expression of ATM making the cells more sensitive to radiation. MiR-223 was therefore hypothesized as helpful during cancer targeting ⁽²³⁰⁾.

TOX, which is important in the development of CD4⁺ T-lymphocytes, has been shown to be targeted by miR-223 in cutaneous T-cell lymphoma (CTCL). Reduction of miR-223 in CTCL cells had an upregulating effect on TOX which might contribute to the progression of CTCL ⁽²³¹⁾.

Predicted targets for miR-93-5p include

- CDKN1a (cyclin-dependent kinase inhibitor 1A)
- VEGF-A (vascular endothelial growth factor A)
- KAT2B (lysine acetyltransferase 2B)
- PTEN (phosphate suppressor candidate 2) and
- LATS2 (large-tumor-suppressor-kinase 2).

CDKN1A normally acts as a tumor suppressor. In a human granulosa-like tumor cell line, miR-93 has been shown to directly target and inhibit CDKN1A, thereby promoting cell proliferation ⁽²³²⁾.

VEGF-A is an essential factor during angiogenesis and vascular homeostasis, which is directly targeted and bound by miR-93 in the 3'UTR. In-vitro and in-vivo experiments indicate that miR-93 might play a role on microvascular complications during diabetes via VEGF-A ⁽¹⁷⁹⁾. In endometrial stroma cells, low miR-93 resulted in higher levels of VEGF-A, which in turns leads to deterioration of endometriosis ⁽²³³⁾.

MiR-93 has been shown to be overexpressed in an osteosarcoma cell line, inducing cell proliferation via induction of the cell cycle. This effect is carried out by directly targeting and downregulating PTEN, a tumor suppressor and negative regulator of proliferation in different cell types. ⁽²³⁴⁾.

The tumor suppressor LATS2 has been shown to be inhibited by miR-93 leading to promotion of angiogenesis and metastasis in brain tumor cells ⁽²³⁵⁾.

This overview indicates how important miRNAs are in every aspect of metabolism, development and disease, especially cell growth regulation in cancer. This indicates the importance of miRNAs as future therapeutic agents or therapeutic targets. However, the complexity of the regulatory interactions in the whole systems has to be considered, evaluated for its specificity and potential detrimental effects.

5.7 Study conditions and specific challenges

In order to minimize the potential effects of kidney impairment on the expression levels of our candidate miRNAs when comparing CKD patients, KT patients and healthy controls, we analyzed subgroups of these patient groups with a similar GFR. In fact, we found significant differences for both miR-223 and miR-93 expressions, with higher levels in the KT group of the same GFR. In our hands, this indicates a specific downregulation of both miRNAs during CKD disease, which appears to be independent of kidney function.

Expression of other miRNAs analyzed in our study, such as miR-142-3p, showed a similar trend of downregulation in higher CKD stages. However, expression differences did not reach statistical significance, probably due to pronounced scattering and the restricted sample size of the subgroups. Interestingly, miR-142-3p has already been indicated as an important factor in the Wnt-signaling pathway, influencing osteoblast differentiation ⁽¹⁸³⁾, which was shown to be regulated by several miRNAs during various signaling steps ⁽¹⁶⁰⁾.

Other miRNAs previously reported to be involved in calcification and mineralization processes during normal kidney function, among them also miR-146 ⁽²³⁶⁾, had a stable expression profile in CKD and KT patients and did not show a significant difference to levels in healthy controls.

Interestingly, there was a study on a specific downregulation of miR-146a plasma levels in CKD patients, but only when they underwent acute exercise. During normal activity, miR-146a was not changed ⁽²³⁷⁾. However, another study in 63 CKD patients varying from stages 1-5 has associated higher levels of miR-146a in plasma with impaired renal function and an increase in arterial stiffness ⁽²³⁸⁾. We did not see changes in miR-146a-5p in our patient collective, but due to these contradictory results, considering miR-146a-5p in subsequent studies with a similar setting should not be excluded.

Also possible interaction between miR-146a and Runx2 has been stated, when expression differences were analyzed in human dental follicle cells ⁽²³⁹⁾. The expression of miR-146a in cartilage makes it a potential candidate for expression also in bone or calcified tissue⁽²⁴⁰⁾. Considering that we were able to detect differences for 146a-5p in serum in the primary array experiments, it might be worth to look into more detail, even though we could not confirm these differences in our cohort based on the subsequent qPCR experiments.

Despite observing increased inorganic phosphate levels in CKD patients, we were not able to document an association of miR-223-3p or miR-93-5p with systemic phosphate levels in our study patients. This is probably due to the contribution of tissue-specific or intracellular phosphate or the heterogeneity of the investigated groups. However, the importance of high phosphate levels for bone mineralization in CKD has already been described previously ⁽²⁴¹⁾, even identifying an effect at only slightly higher levels of phosphate ⁽²⁴²⁾. Several studies have already indicated an interaction between miRNAs, including miR-223 or miR-125b, and abnormal phosphate levels leading to health complications during CKD ^(243,244). An influence of higher inorganic phosphate on an increase of VSMC migration and calcification which is modified by miR-223 has also been described ⁽¹⁷⁷⁾.

We focused our study on overall changes of miRNA patterns in serum. Whole serum determination of miRNAs was therefore our main focus to investigate the role of miRNAs as potential minimally invasive biomarkers that would be easy to analyze. Previous work has suggested that miRNAs can also be systemically transported in exosomes and bound to several proteins in serum, two reasons, why miRNAs might have a high stability ⁽²⁴⁵⁾. The main reasons why serum analyses are very common and often favored, is the easy handling which could potentially be done in a routine laboratory. Of course, further analyses of the miRNA origin, of specific tissues and cells, will be necessary to investigate underlying mechanisms.

Strengths of this work are an overall large sample size and the presence of all late CKD stages without the confounding influence of RRT. Furthermore, we used a large control group, and a variety of parameters was measured during the different stages of the disease, after KT and in healthy controls. Important serum parameters including interleukins and markers of glucose and bone metabolism have been investigated.

The primary miRNA array screening results have been confirmed by individual qPCR validation. However, the smaller group sizes in several CKD subgroups might have reduced the possibility to detect underlying regulatory associations and there was no possibility to perform multivariate analyses. Nonetheless, the subgrouping via eGFR was necessary due to the distinct characteristics of each CKD stage.

5.8 Conclusion

Based on our findings and previously published literature, we found two candidate miRNAs, miR-223-3p and miR-93-5p, which might be interesting systemic markers during CKD and should be further investigated for their potential direct effects on bone metabolism and vascular calcification as well as VSMC differentiation. Due to various influences on a large number of pathways, miR-223-3p and miR-93-5p are of great interest during CKD disease development and its comorbidities, both because of their association with inflammation and impact on bone metabolism. Predicted potential downstream targets of miR-223-3p and miR-93-5p do indicate an involvement in osteoblast and VSMC differentiation during CKD-MBD with possible diagnostic and/or therapeutic potential.

5.9 Future Aspects

We assume that miR-223-3p and miR-93-5p are playing a regulatory role during kidney disease. It is important to increase knowledge on pathways and functions in which these miRNAs are involved. In view of the use of serum samples, it would also be of great interest to know about the origin and processing of these miRNAs. However, miRNAs are ubiquitously expressed in tissues and organs and it is generally considered very difficult to match circulating miRNAs to a specific tissue where they have been secreted. For example, miRNA-223 has been shown to be derived from bone, adipose tissue, cardiac muscle tissue

and hematopoietic cells ⁽²⁴⁶⁾. The kidney might therefore be another source for this miRNA. To our knowledge, there is no publication on tissue specificity of miR-93 available yet.

It will be very important to look at predicted targets for miR-223-3p and miR-93-5p for putative effects in in-vitro experiments using VSMCs, bone cells or endothelial cell culture. Assessing the targets which are immediately affected by the miRNAs will help to decipher their function during normal conditions and disease.

For the assessment of miRNAs as diagnostic biomarkers, which was our major point in this study, it would be beneficial to find panels of differentially regulated miRNAs. These panels could be made available via an easy to handle laboratory technique and could be used as a novel lab measurement in clinical conditions.

MiRNAs have become a major field in medical science. Their ubiquitous presence and activity has raised vital new questions about the functions of the human body. Understanding how miRNAs regulate every aspect of development and homeostasis is of utmost importance, because it also offers great possibilities for disease detection and treatment. Trying to understand the different roles of miRNAs is one of the most exciting research fields in the future.

6. References

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Appendix:

Publications:

Ulbing, M; Kirsch, AH; Leber, B; Lemesch, S; Münzker, J; Schweighofer, N; Hofer, D; Trummer, O; Rosenkranz, AR; Müller, H; Eller, K; Stadlbauer, V; Obermayer-Pietsch, B

“MicroRNAs 223-3p and 93-5p in patients with chronic kidney disease before and after renal transplantation”

Bone. 2017; 95:115-123

Presentations:

Barbara Obermayer-Pietsch, Matthias Ulbing, Alexander Kirsch, Schweighofer Natascha, Bettina Leber, Sandra Lemesch, Alexander Rosenkranz, Helmut Müller, Kathrin Eller, Vanessa Stadlbauer

Specific microRNA signatures in CKD patients focusing on the risks of calcifications and ROD

ASBMR Abstract Book. 2015; -ASBMR 2015 Annual Meeting; OCT 9-12, 2015; Seattle, Washington, USA. [Poster]

Matthias Ulbing, Alexander Kirsch, Natascha Schweighofer, Bettina Leber, Sandra Lemesch, Alexander Rosenkranz, Helmut Müller, Kathrin Eller, Vanessa Stadlbauer, Barbara Obermayer-Pietsch

Specific MicroRNAs as Biomarkers in CKD Patients at High Risk for Calcifications and ROD?

IBMS BoneKEy 13, Article number: 673 (2015) Poster Presentations. 2015; -4th Joint Meeting of ECTS and IBMS; APR 25-28, 2015; Rotterdam, The Netherlands. [Poster]

Matthias Ulbing, Daniela Hofer, Alexander Kirsch, Natascha Schweighofer, Bettina Leber, Sandra Lemesch, Alexander Rosenkranz, Helmut Müller, Kathrin Eller, Vanessa Stadlbauer, Barbara Obermayer-Pietsch

MicroRNAs: Biomarker für Gefäßkalzifizierung bei chronischer Niereninsuffizienz?

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Ulbing, M; Kirsch, A; Schweighofer, N; Leber, B; Lemesch, S; Rosenkranz, A; Eller, K; Müller, H; Stadlbauer, V; Obermayer-Pietsch, B;

Micro-RNAs as new putative biomarkers for vascular calcification in CKD patients

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micro-RNA als neuer Biomarker zur Beobachtung von vaskulärer Verkalkung bei chronischen Nierenerkrankungen

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Ulbing, M; Schweighofer, N; Rosenkranz, A; Müller, H; Eller, K; Obermayer-Pietsch, B;

Micro-RNA als neuer Biomarker zur Beobachtung von vaskulärer Verkalkung nach Nierentransplantationen.

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