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Graz, 23.08.2018

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List of Abbreviations

ADAR	Adenosine deaminase acting on RNA
AKT2	Protein kinase B 2; also known as AKT
BMI	Body-Mass-Index
cDNA	Complementary desoxyribonucleic acid
Cq	Quantification cycle
DXA	Dual Energy X-ray Absorptiometry
GLUT-4	Glucose transporter type 4
GTP	Guanosine triphosphate
hsa	human
HMGA2	High Mobility Group AT-Hook 2
IL-6	Interleukin-6
LNCaP cell line	Lymph Node Carcinoma of the Porstate
miR	microRNA
MMP-11	Matrix Metalloproteinase-11
mRNA	messenger RNA
NF- κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
25OHD	25(OH)vitamin D
1,25OH2D	1,25(OH)$_2$vitamin D
PCR	Polymerase chain reaction
PTH	Parathyroid hormone
Ran	Ras-related nuclear protein
RANK	Receptor Activator of NF-κB
RANKL	Receptor Activator of NF-κB Ligand
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RT-qPCR	Real-time quantitative polymerase chain reaction
STAT-3	Signal transducer and activator of transcription 3
T1DM	Diabetes mellitus type 1
T2DM	Diabetes mellitus type 2
TGF-β	Transforming growth factor beta
TH-17	T-helper cell 17
UniSp	Spike-in
USP1	Ubiquitin specific protease 1
VD	Vitamin D
Wnt	Wingless / Int-1

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Zusammenfassung

Einleitung: Die vielseitigen Effekte von microRNAs auf Zelldifferenzierung, Zellfunktion und Zellstoffwechsel sind Gegenstand von großem wissenschaftlichem Interesse. Die einzelsträngigen, nicht-codierenden microRNA-Moleküle regulieren wichtige Schritte im Glukose- und Knochenstoffwechsel. Das Ziel der vorliegenden Arbeit ist, Expressionsunterschiede von im Blut zirkulierenden microRNAs bei gesunden Männern zu finden, die 12 Wochen lang entweder mit Vitamin D oder Placebo behandelt wurden.

Methoden: Die Patienten wurden aus einer randomisiert kontrollierten Studie ausgewählt, in der gesunde Männer mit Vitamin D-Mangel (25(OH)Vitamin D < 30ng/ml) entweder mit 20.000 IU Vitamin D oder Placebo behandelt wurden. Von 4 Patienten mit deutlichem Anstieg von Serum-25(OH)Vitamin D-Werten wurden Blutproben an 3 Zeitpunkten (Studienvisite 1,2,3) analysiert und von 3 Patienten unter Placebo zu 2 Zeitpunkten (Studienvisite 1 und 3). Die RNA-Isolation und microRNA-Quantifizierung wurde mittels Exiqon RNA Panel durchgeführt.

Ergebnisse: Von 372 untersuchten microRNAs pro Probe, zeigten 111 in allen 18 Proben ein Signal. In den Proben von Patienten, die mit Vitamin D behandelt wurden, zeigten sich im Vergleich zum Ausgangswert bei 7 microRNAs signifikante, größer als 2fache Änderungen des Expressionsniveaus. Einige dieser microRNAs (z.B. miR-194-5p, miR-326) wurden in der Literatur bereits mit Glukosestoffwechsel und Karzinogenese assoziiert.

Diskussion: In dieser Studie konnte der direkte Effekt einer Vitamin D-Behandlung im Vergleich zu Placebo auf das Expressionsniveau relevanter microRNAs nachgewiesen werden. Die komplexe Regulation dieser microRNAs, die von ihnen regulierten Zielmoleküle, sowie deren Rolle im komplexen pathophysiologischen Zusammenspiel spezifischer Krankheiten ist Gegenstand weiterer Forschung.

Abstract

Introduction: MicroRNAs (miRNAs) and their multiple influences on cell differentiation, cellular function and metabolism are subject of intense research. These single-stranded, non-coding RNA elements have been detected at important steps in bone and glucose physiology. Our aim is to describe differentially expressed microRNAs in healthy men treated with vitamin D or placebo (PBO) for 12 weeks.

Methods: Out of a randomized controlled trial including vitamin D deficient (serum 25(OH)vitamin D < 30 ng/ml) healthy men treated with 20,000 IU of vitamin D or PBO per week, we randomly chose 4 patients with an increase of 25OHD serum levels (3 time points including baseline) and 3 PBO samples at baseline and final visit. RNA was isolated from serum samples and quantified by an Exiqon RNA panel.

Results: Out of 372 microRNA assays, 111 showed a signal in all 18 samples. In the samples with 25OHD increase, 7 microRNAs showed significant changes above 2fold in their expression levels compared to baseline. Some of them (e.g. miR-194-5p, miR-326) have already been associated with β-cell-function and carcinogenesis in previous studies.

Discussion: In this study, we were able to show a direct impact of vitamin D treatment on the expression of biologically relevant microRNAs in serum as compared to PBO. Identification of downstream targets of these microRNAs as well as their complex role in the pathophysiology of specific diseases have to be elucidated in future.

1 Introduction

MicroRNAs are small (about 22-24 nucleotides) non-coding RNA-molecules such as small interfering RNAs and small nuclear RNAs. Since they have been described for the first time in 1993, a lot of attention has been drawn to these novel regulators of the genome. Today, microRNAs are known to affect nearly every physiologic and pathophysiologic process throughout the whole body. MicroRNAs are present in every tissue and circulate in every body fluid. They interfere with the entire protein metabolism and crucially regulate both pre- and post-transcriptional gene regulation. Forming a complex with other proteins called RNA-induced silencing complex [RISC], microRNAs are able to interact with messenger-RNAs [mRNAs] on a posttranscriptional level repressing their translation. This repression affects all major cellular functions like differentiation, proliferation, survival and metabolism. MicroRNAs are thought to regulate more than 60 % of the human genome. In 2008, microRNAs became known to circulate in blood either being present in protein complexes or in microvesicles and exosomes. (1,2)

Nomenclature: MicroRNAs are marked by a miR-prefix followed by the number of identification (e.g. miR-1, miR-2,..). These numbers are given by the sequence of discovery; however, attention is paid on giving the same name to microRNAs with equal sequences explored in different species. Every species is marked by its specific prefix. A human microRNA is called “hsa”, e.g. hsa-miR-326”, a murine microRNA is called “mus”. In addition, microRNAs with similar sequences are distinguished by additional letters, e.g. miR-13a. In a microRNA database called miRBase (www.mirbase.org) all microRNAs known so far are listed systematically. (3)

1.1 MicroRNA-biogenesis

The process of microRNA-biogenesis starts inside the nucleus. First, a pri-microRNA gets transcribed by either RNA polymerase 2 or RNA polymerase 3 enzymes. Clustering on genomic level has been proven for many microRNAs. However, every single microRNA can be regulated separately inside these clusters. The pri-microRNA consists of a few 1000 nucleotides forming a hairpin stem, a terminal loop and two single stranded flanking regions. Some pri-microRNAs are modified by a deamination process called microRNA editing. Therefore, an enzyme called adenosine deaminase acting on RNA [ADAR] replaces adenosine with inosine. This replacement affects both downstream processing and target specifity of the microRNA edited. Still remaining in the nucleus, the pri-microRNA

is cleaved by an enzyme complex consisting of the RNAse 3 enzyme Drosha and the DiGeorge critical region 8 protein also called Pasha. In a co-transcriptional process, Drosha cleaves off a part of the hairpin stem to produce a so-called pre-microRNA, which consists of about 70 nucleotides. In some cases, Drosha mediated processing can be replaced by a process where intron-derived microRNAs are set free by splicing. As can be seen in figure 1, the mature pre-microRNA then gets exported into the cytoplasm by Exportin-5 associated with Ran-GTP. In the cytoplasm, the formation of an extensive protein complex called RISC loading complex begins in which further maturation of the microRNA and various structural alterations are accomplished. Cleaving off the terminal loop of the pre-microRNA, the RNAse 3 enzyme Dicer creates a microRNA duplex consisting of a guide and a passenger strand. As the guide strand remains in the RISC complex, the passenger strand gets expelled and degraded. The RISC complex consisting of the mature microRNA, argonaute proteins (which are the real effector proteins mediating mRNA degradation) and many other proteins is now ready to interact with other RNA molecules. (1)

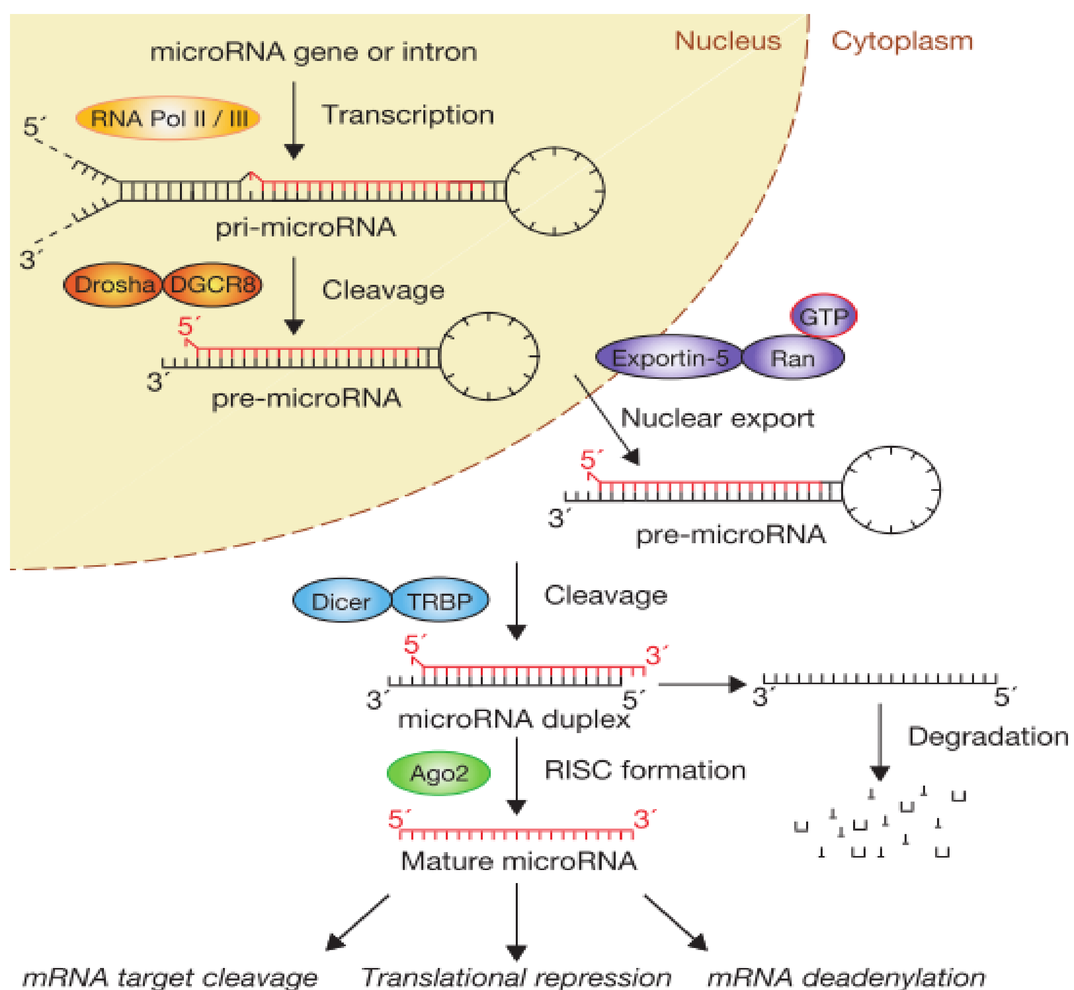

Figure 1: MicroRNA biogenesis. The different steps of microRNA biogenesis from transcription inside the nucleus to RISC-formation in the cytoplasm are shown. Reproduced with permission from (1).

1.2 MicroRNA function

Being part of the RISC ribonucleoprotein complex, the microRNA's task is to target other RNAs by the principle of complementary base paring. Thus, the microRNA anchors the functional RISC complex at its place of work, where the specific interaction of the target RNA and all the components of the RISC complex including the microRNA take place. In the majority of cases, the RISC complex gets guided to the 3′ untranslated region of a mRNA which is ready for translation at the ribosome. But mRNAs are not the exclusive targets of microRNAs. Having the ability to target every kind of RNA, microRNAs target all other species of non-coding RNA and even microRNAs themselves. The targeting of one single microRNA is not restricted to a RNA sequence of perfect complementarity. It seems to be more like a fluent targeting process where very short sequences of complementarity can attach the microRNA to its target in a delicate binding which is ready to melt in the next moment. The higher the grade of complementarity, the sooner the target gets degraded, whereas low complementarity only leads to a slowdown in translation. Amongst other things, this way of targeting is also responsible for the great amount of different sequences which can be bound by a single microRNA. Every single microRNA can target a great amount of mRNAs; vice versa one mRNA can be targeted by many different microRNAs. Being integrated in a fluent network of posttranscriptional regulation, the direct physiological impact of one specific microRNA is hard to establish. Furthermore, the function of microRNAs cannot be split up in a simple on/off. In terms of translation, microRNAs can be seen as inhibitory players in the translation equilibrium.

Under particular conditions, microRNAs are even able to increase the level of certain proteins. This can happen via stimulating promoter interactions of competitive effects on RNA-repressive proteins.

In summary, microRNAs are highly involved in the crosstalk of posttranscriptional gene regulation, influencing cell metabolism to a great extent. (4)

1.3 Circulating microRNAs

In 2008, fifteen years after the discovery of microRNAs, another significant finding about these novel regulators of the genome has been made: their circulation in nearly every body fluid, which opened up completely new perspectives on these small RNA-molecules. From then on, the impact of microRNAs was no longer restricted to translational repression inside the cell and microRNA were considered to act as signaling molecules throughout the whole body. Since then, a lot of research has been carried out and evidence has come up

that microRNAs are subject of targeted long-range transport between cells. MicroRNAs are able to circulate in exosomes, microvesicles, and high density lipoproteins. Furthermore they get attached to AGO-proteins. Microvesicles are formed by a process in which a part of the cell membrane is "cut off" and released into the extracellular space. (5)

1.3.1 Exosome formation

The attention will now be drawn to the more complex process of exosome formation which is run in a few steps. In a first step, the cell membrane invaginates forming an endosome. Then, the endosome itself is subject of multiple invaginations forming a multivesicular body which contains a great amount of membrane vesicles. The fusion of the endosome membrane and the cell membrane hereupon causes release of all the vesicles inside the endosome into the extracellular space. These vesicles are then called exosomes. (5)

1.3.2 Exosomal interaction with target cells

Carrying their specific cargo, exosomes are found in nearly every body fluid after exocytosis. The mechanisms of exosome targeting are still not well understood. In fact, exosomes can interact with their target cells in four different ways. First, transmembrane proteins located in the exosomal membrane and the cell membrane of the target cell can bind to each other in terms of signaling. Second, a fusion of exosomal and cellular membrane can cause direct release of the exosomal cargo into the target cell. Third, internalized exosomes get shuttled through the cell pushing towards adjacent cells in a process called transcytosis. The last way of interaction is the maturation of internalized exosomes to lysosomes. (5)

Although the exosomal cargo covers a wide variety of ingredients like lipids and proteins, microRNAs are supposed to be the main functional components. Evidence for specific alteration in microRNA profiles between exosomes and their releasing cells has been found. This finding suggests the existence of specific microRNA sorting mechanisms in the process of exosomal maturation. The exact process of sorting is still unknown, but there is evidence that particular sequences within the microRNA initiate its translocation into the immature exosome. This energy consuming process gets catalysed by specific enzymes. Enriching specific subsets of microRNAs in exosomes, cells are able to influence other cells in a new way of posttranscriptional communication. By targeted accumulation of microRNAs in cells and tissues all over the body, exosomes might play significant roles in nearly every physiological process. (5)

A study by Lee et al. (2015) gives an example for the interaction of cells and exosomes. In a cell culture model, they investigated microRNA levels and exosomes in papillary thyroid cancer. In these cancer cell lines, miR-146 and miR-222 were found to be enriched. Furthermore, the degenerated cells produced exosomes, in which high levels of those microRNAs were found. After isolation of the exosomal cargo, it was transferred back to its cells of origin. Interestingly, this autocrine treatment caused reduced rates of cellular proliferation within the cancer cells. (6) These findings suggest the hypothesis that the tumor cells try to create an antiproliferative environment in order to prevent further degeneration and proliferation.

1.4 Biological significance of microRNAs in diseases

In terms of microRNA and disease most research has been done in the fields of cancer, cardiovascular disease and metabolic diseases whereas associations can be found in nearly every subject area. An exemplary overview describes the following topics.

1.4.1 Diabetes mellitus

MicroRNAs have been reported to have a wide influence on the pathogenesis of diabetes mellitus. Both β-cell function and insulin resistance are affected by a substantial number of microRNAs. Suppressing growth-inhibiting genes, miR-375 seems to be an efficient regulator in β-cell development. Mice showed reduced β-cell mass after miR-375 knock out. Also, the differentiation from pluripotent stem-cells into mature β-cells is conducted by microRNAs. Some microRNAs (such as miR-375 and miR-7) showed specific in vitro expression patterns at every step in the process of differentiation. As mentioned above, microRNAs play a major role in insulin resistance. Apart from other intracellular signaling steps, the fusion of membranous vesicles containing GLUT-4 with the cell membrane of adipose and muscle cells is an important step in insulin response. Inhibiting GLUT-4 directly, miR-199 and miR-93/223 can lead to a decrease of insulin sensitivity. (7)

In biomarker research, a field of aspiring clinical importance, microRNAs become an issue of growing prominence. Being present in nearly every body fluid, microRNAs can potentially be used for early diagnosis of metabolic diseases. For example, miR-126 shows a reduced level of circulation in patients susceptible to diabetes mellitus type two (T2DM). (7)

In a study of 2014, evidence has come up that circulating levels of miR-326 were increased in patients with T2DM. (8) Another study showed reduced levels of miR-194 in muscle biopsies of both pre-diabetic and diabetic patients. (9)

1.4.2 Cardiovascular Disease

MicroRNAs play a substantial role in the pathophysiology of heart failure, cardiac fibrosis, lipid metabolism and atherosclerosis. For miR-133, evidence has come up that its inhibition might lead to hypertrophic cardiomyopathy in patients and animal models by modulation of the β_1-adrenic receptor pathway. Besides its role in hypertrophy, miR-133 also affects fibrosis-regulating proteins like the connective tissue growth factor. Targeting these proteins, miR-133 is said to be antifibrotic. In terms of fibrosis, an opposite action is performed by miR-21. In mice, the deposition of extracellular matrix is increased, in this way the inhibition of miR-21 leads to enhanced cardiac function. Further, vascular function is affected by miR-21. The pro-atherosclerotic effects of miR-21 are conducted through enhanced proliferation of vascular smooth muscle cells. Many more effects on the cardiovascular system are subject of intense research. (10)

1.4.3 Bone

In the past few years, the understanding of regulatory processes in bone conducted by microRNAs has increased. To fulfil both its endocrine and its mechanic function in a proper way, bone and its cells need profound regulatory mechanisms. Due to extensive and constant turnover within this tissue, an intense crosstalk of bone forming osteoblasts and bone resorbing osteoclasts is needed to guarantee the functional balance between these cells. Mesenchymal osteoblasts with a single nucleus and multinuclear osteoclasts forming out of hematopoietic precursor cells are regulated in different layers including both hormonal regulation in service of calcium homeostasis and paracrine/autocrine mechanisms including cytokines/tissue hormones as well as microRNAs. Getting transported from one tissue to another in microvesicles/exosomes, microRNAs are suspected to have a wider influence on intercellular crosstalk as previously thought. A direct exchange of microRNAs with a profound impact on the translational balance in involved cells seems to be possible. Packing microRNAs into microvesicles via exocytosis, the cell is able to connect with other cells on a pre-translational level. Thus, micro RNAs affect both the function of single bone cells and the homeostasis of the whole tissue. Targeting mRNAs of important bone-regulating proteins and causing their degradation, microRNAs have a powerful role in bone homeostasis from embryonic development to proliferation, resorption and degradation of bone. (11)

An important mechanism in regulating osteoclastogenesis is the RANKL-RANK interaction. Secreted by osteoblasts RANKL binds the transmembranous RANK-receptor located on osteoclast progenitor cells and causes their fusion to mature osteoclasts via activation of NF-κB. This RANKL-RANK interaction can be inhibited by osteoprotegerin, which is produced by osteoblasts as well. Acting as a decoy receptor, osteoprotegerin binds RANKL and prevents its interaction with RANK. Depending on the relation of osteoprotegerin and RANKL osteoblasts are able to control osteoclastogenesis and bone resorption. (11)

A study on glucocorticoid-induced differentiation of osteoclasts uncovered a direct link between microRNAs and the RANKL-RANK-NF-κB pathway. Targeting RANKL-mRNA directly, miR-17 and miR-20a were able to repress the translation of RANKL-mRNA. Thus, miR-17 and miR-20a reduced the amount of RANKL protein and caused a decrease in dexamethason-induced osteoclastogenesis. (12)

However, the microRNA way of interference is not as straight as in the example just mentioned. In the regulation of Runx2, the complex and well-timed interplay of microRNAs can be shown. On the one hand Runx2 acts as a key transcription factor in osteoblast proliferation. Interacting with many other proteins it determines the differentiation of mesenchymal stem cells in either osteoblasts or chondroblasts and conducts the further proliferation through deep intervention into the cell cycle. On the other hand, Runx2 itself is subject of crucial regulation. Different subsets of microRNAs orchestrate the chronologic sequence in Runx2 expression levels needed for each special type of differentiation. Figure 2 shows the microRNA subsets needed do induce either osteocyte or chondrocyte differentiation. Interestingly, the subset inducing osteocytes is downregulated in early osteogenesis resulting in high protein levels of Runx2 and upregulated towards the end of differentiation resulting in low levels of Runx2. On the contrary, the subset for chondrogenesis is high at the beginning and low at the end, which causes a constant increase of Runx2 protein levels. (11)

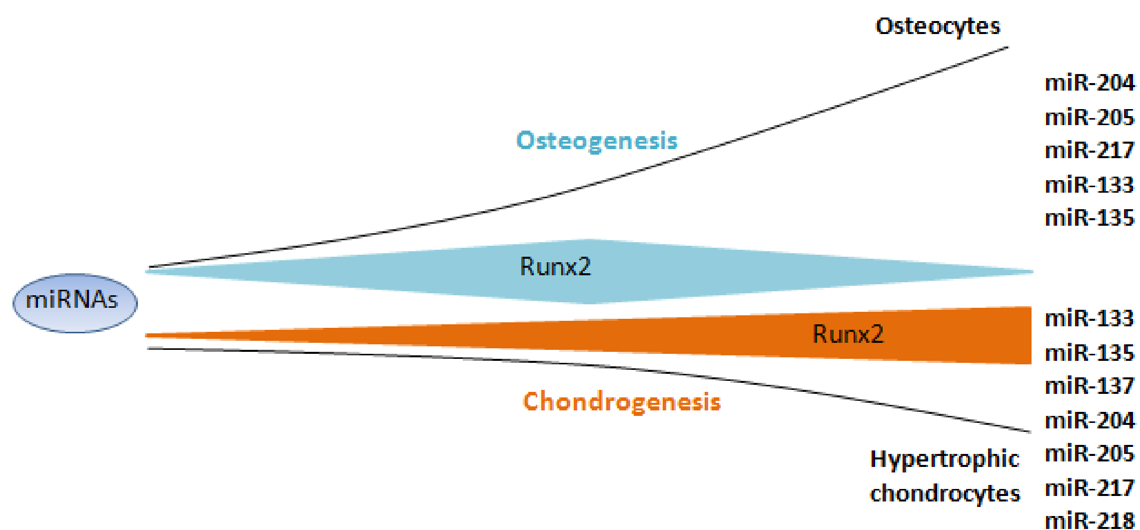

Figure 2: Runx2 regulation by microRNAs. The levels of Runx2 needed for either osteogenesis or chondrogenesis are demonstrated in progress of time. On the far right, the subsets of microRNAs regulating the diverse types of differentiation are listed. Modified from (11).

These findings suggest an induction of osteogenesis by an early rise of Runx2 protein levels, orchestrated by microRNAs. The omnipresent and profound regulation of key proteins in bone metabolism by micro RNAs offers a wealth of opportunities for artificial intervention. A future goal might be to find specific combinations of microRNAs and microRNA inhibitors for the treatment of bone disorders. (11)

1.4.4 Cancer

In the past few years, many new findings about the role of microRNAs in cancer have come up. Referring to this topic, the type of cancer which was investigated most is colorectal cancer, which is one of the leading causes of cancer death worldwide. Tumor progression in colorectal cancer takes a long time and is accompanied by step by step mutations of both tumor suppressor genes and oncogenes. Accumulating mutations can cause extensive deregulation of signaling pathways, enhanced proliferation and loss of epithelial differentiation. A great number of signaling pathways like Wnt, TGF-β, IL-6/STAT-3, KRAS, BRAF, inflammatory-pathways (NF-κB etc.) are involved into the carcinogenesis of colorectal cancer. MicroRNAs regulate every pathway mentioned. (13)

Dysregulation of Wnt signaling is frequently found in colorectal cancer. In Wnt signaling many proteins interfere in a complex sequence. B-catenin, an important transcription factor, which is also known to act in cellular adhesion, gets degraded by a protein complex called axin destruction complex, under normal conditions. This complex consists of the adenomatous-polyposis-coli-protein [APC-protein], axin and other proteins. Wnt binding to its receptor causes an inhibition of the axin destruction complex and an accumulation of

β-catenin in the cell leading to enhancement of cellular proliferation and changes in the cell phenotype. Besides its role in inherited cancer through mutations in the APC-gene, the APC-protein can be targeted by miR-135 a/b causing an increase in β-catenin levels. Vice versa, β-catenin upregulates miR-135. Further gain in Wnt signaling by microRNA 135 is caused by reducing the levels of secreted frizzled related protein 4, which degrades Wnt-proteins outside the cell. As Wnt signaling seems to be an important and highly dysregulated pathway in the progression of colorectal cancer, microRNAs regulating crucial parts of this pathway are promising for therapeutic use. (13)

A well-known player in carcinogenesis is the protein p53. Its accumulation in the cell gets triggered by DNA-damage. The main effects of p53 are the activation of DNA repair mechanisms and the induction of apoptosis. Thus, p53 is an important player in keeping up cellular integrity on genomic level. Besides many other effects the tumor-suppressor p53 drives the transcription of miR-34. Forming a feedback loop, miR-34 is enhancing the levels of p53 through targeting a p53 binding RING-finger protein. Furthermore miR-34 is enhancing the transcription of the p53 gene by interaction with its promoter. Another interesting effect of miR-34 is the inhibition of IL-6/STAT-3 signaling, which is essential for epithelial to mesenchymal transition and metastasis. Thus, miR-34 has multiple tumor-suppressive effects. (13)

1.5 Vitamin D and microRNAs

Active vitamin D, $1\alpha,25(OH)_2$vitamin D [1,25OH2D], also known as calcitriol is a liposoluble steroid hormone. Binding the nuclear vitamin D receptor, calcitriol is able to activate a large number of target genes. In clinical medicine as well as in our study, 25(OH)vitamin D [25OHD] is used for treatment. 25OHD is a pre-metabolite of the active 1,25OH2D-hormone (the detailed biogenesis will be demonstrated in the following section) which is the vitamin D metabolite of greatest efficacy. The activation of 25OHD to the active 1,25OH2D mainly happens in the kidney, but takes place cells throughout the entire body as well (14). So, the level of clinically used and measured 25OHD gives no information about the 1,25OH2D status in a specific cell. In cell cultures, 1,25OH2D is used for treatment. Thus, the results of in vitro and in vivo studies in context of vitamin D should be considered differentially, as the rate of conversion to effective 1,25OH2D in a specific cell is an unknown but important fact. (15)

1.5.1 Biogenesis and function of vitamin D

Unhydroxylated vitamin D - also known as cholecalciferol - is the preliminary stage of the active metabolite 1,25OH2D and can be received in two different ways. Either it is obtained from diet/ingestion of supplements or from activation in the epidermis. Therefore, 7-dehydrocholesterol, located in the stratum spinosum and stratum basale, is transformed by UVB radiation in a photochemical process. Thereafter, the gathered vitamin D is loaded on the vitamin D binding protein and translocated to the liver where hydroxylation to 25OHD is accomplished by the 25-hydroxylase enzyme. Getting bound to the vitamin D binding protein again, 25OHD can either remain in this state in terms of storage or undergo further activation. 25OHD reflects the body pool of vitamin D and is therefore a clinically relevant parameter, measured by several specific assays. As shown in figure 3, this activation mainly happens in the kidney but also in many other cells throughout the whole body (14). In the kidney, 25OHD is hydroxylated a second time by the 1α-hydroxylase enzyme in a parathormone (PTH) dependent process. Besides PTH, the 1α-hydroxylase gets activated by low calcium and phosphate levels. The product of this hydroxylation is 1,25OH2D, which is now ready to bind the vitamin D receptor. (15)

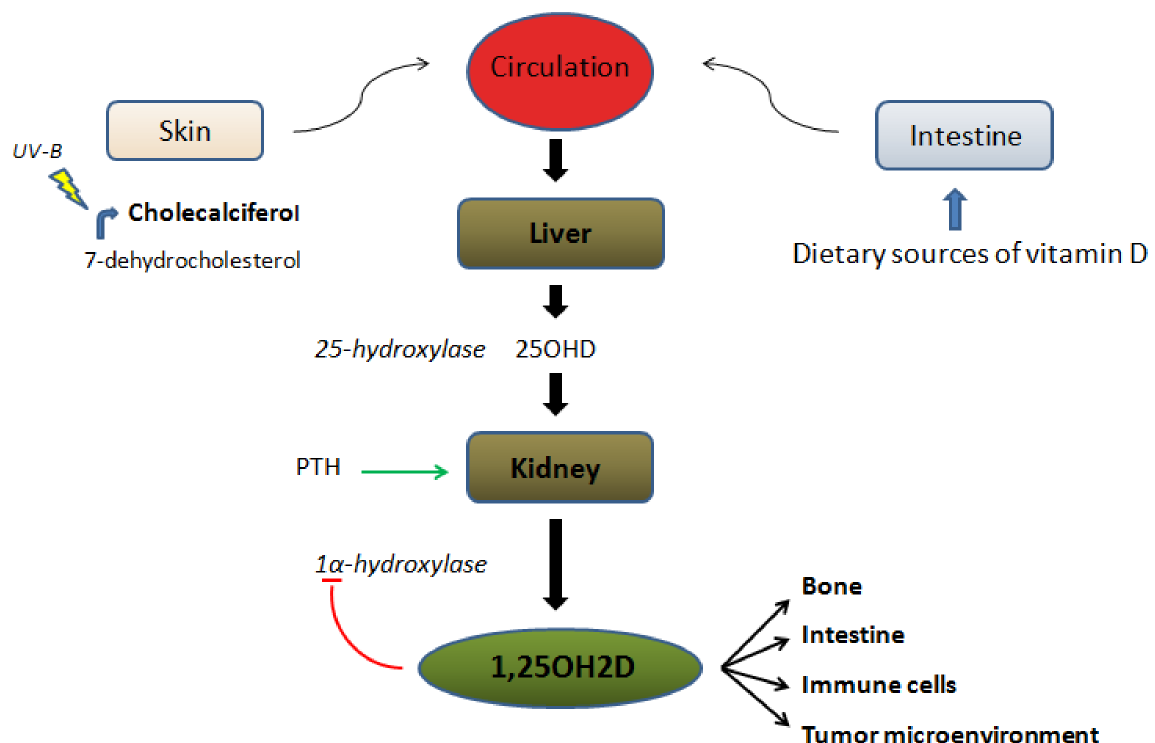

Figure 3: 1,25(OH)$_2$vitamin D-synthesis. The different steps of biogenesis including activation of 7 dehydrocholesterol by UVB-radiation in the skin, hydroxylation in the liver and the kidneys are shown. Modified from (16).

Forming a heterodimer, the vitamin D receptor is activated by 1,25OH2D which has the highest affinity to the receptor among the vitamin D metabolites. Acting as a ligand activated transcription factor, the vitamin D receptor directly interacts with the promoter regions of target genes after dimerisation. The vitamin D receptor itself is differentially expressed in various tissues, which is an important reason for the tissue dependent differences in the effecitve scope of 1,25OH2D effects. (15)
The most familiar effect of 1,25OH2D is the regulation of calcium homeostasis. Therefore, 1,25OH2D targets bone, intestine, kidneys and parathyroid glands. Changing the equilibrium of ion channels, 1,25OH2D increases calcium and phosphate uptake in intestinal cells. The 1,25OH2D-effects on bone including the RANK-RANKL system are complex and partially contradictory. In summary, 1,25OH2D is essential for adequate blood calcium levels and the lively crosstalk between osteoblasts and osteoclasts resulting in healthy bone formation. By repressing the transcription of the PTH-gene in the parathyroid gland, 1,25OH2D contributes to this bone anabolic effect. Vice versa, a 1,25OH2D deficiency results in increased PTH levels and high bone resorption. In the kidney, 1,25OH2D represses its own activation by inhibiting the 1α-hydroxylase enzyme and enhances calcium and phosphate absorption in the tubulus system. (17)
Further effects of vitamin D can be found in many fields including immunology, cardiovascular function, glucose homeostasis, and cancer. Some of these effects will be described in more detail in the following sections.

1.5.2 Vitamin D metabolites in cancer

Many studies published antiproliferative effects of vitamin D in cancer (18). Unfortunately, studies about microRNA dependent effects of vitamin D on cancer are mostly based on cell cultures. They all used 1,25OH2D for the investigation of vitamin D effects. The in vivo effects of vitamin D on carcinogenesis were investigated by supplementation of 25OHD. In vivo data about 25OHD and microRNAs in cancer is lacking at the moment. To differentiate between the in vitro studies, which used 1,25OH2D, and the in vivo studies using 25OHD their results get illustrated in two different sections.

1.5.2.1 Vitamin D and its microRNA-dependent influence on cancer cells in vitro

In prostate cancer, 1,25OH2D is said to have tumor suppressive effects. These findings are mainly based on cell culture experiments and supraphysiological doses of 1,25OH2D are needed to establish the tumor-suppressive effects in treatment. Nevertheless, early stage treatment of prostate cancer seems to be possible. (19)

In vitro studies showed miR-100 and miR-125 to be enriched by 1,25OH2D in prostatic cancer cells. As reduced levels were reported in multiple types of cancer, these two microRNAs are thought to be tumor suppressive. (20)

Another microRNA linking prostate cancer and vitamin D is miR-98, which is known to be tumor suppressive as well by interfering with the HMGA2 gene. This gene is essential for proliferation and tumor development. Enhancing the amount of microRNA-98 in both direct and indirect ways, 1,25OH2D was able to significantly slow down proliferation in a LNCaP cell line. (21)

Leptin, which is proportional to the body fat mass, is a risk factor for ovarian cancer in obese women. Enhancing the expression of human telomerase reverse transcriptase, leptin pushes the proliferation of ovarian cancer cells. MiR-498 targets the telomerase mRNA, decreasing proliferation of ovarian cancer in mice. (22)

1.5.2.2 Vitamin D and its impact on cancer cells in vivo

Many clinical studies investigated the relation of 25OHD levels in blood and the progression/prognosis of cancer. In 2014, Toriola et al. reviewed 26 studies in order to identify possible effects of 25OHD levels on the prognosis of various types of cancer. Significantly higher rates of survival in context of high levels of 25OHD were presented for breast cancer and colorectal cancer. In these tumor entities the majority of studies indicated anti tumor effects of 25OHD. For other types of cancer results were contradictory. (18) A review of 2010 suggests lower rates of colorectal cancer progression in patients with high levels of vitamin D, too. (23) In a study about acute myeloid leukemia, 97 patients were screened for 25OHD levels after diagnosis. Patients with low levels of 25OHD showed higher rates of recurrence. (24)

1.5.3 Vitamin D metabolites in immunology

In fields of immunology, a lot of in vitro and in vivo studies demonstrated an impact of vitamin D metabolites on immunologic cells and functions. In the following, the in vitro (based on 1,25OH2D) and in vivo (based on 25OHD) findings get described in two different chapters.

1.5.3.1 Vitamin D and its microRNA-dependent influence on the immune system in vitro

In the understanding of the immune system, microRNAs play an arising role in modulating immunologic processes. Investigating innate immune response, 1,25OH2D has been found to affect the equilibrium of cytokine production by inhibiting miR-155. This microRNA is able to repress the suppressor of cytokine signaling 1 gene (SOCS1), which is part of a negative feedback loop in macrophages preventing excessive cytokine production. Being down-regulated in a NF-κB dependent process by 1,25OH2D, miR-155 cannot perform its repressing function on SOCS1 anymore. Thus, 1,25OH2D plays an important role in suppressing excessive cytokine function preventing exuberant immune response. (25)

In terms of hepatitis C virus infections, an antiviral effect of 1,25OH2D through microRNA-130a has been found. (26)

Furthermore, a lack of 1,25OH2D is connected to severe alterations in microRNA levels in patients with systemic lupus erythematodes. (27)

1.5.3.2 Vitamin D and its influences on the immune system in vivo

Many clinical studies investigated the impact of 25OHD on the immune system (15). In two studies about cellular immunity, an association between vitamin D treatment and the function of regulatory T cells, which are important for immunomodulation, was found. In a blinded, placebo-controlled trial, 60 individuals were treated with high doses of cholecalciferol for 3 months. This led to significant higher rates of regulatory T cells whereas other cell types were not affected. These findings were supported by cell culture models treated with cholecalciferol. (28) In the second study, 15 subjects with freshly diagnosed T1DM were treated with cholecalciferol. Again, the rate and the functional level of the regulatory T cells were increased. (29) Another study found an inverse correlation between 25OHD levels and the rate of respiratory infections. In 800 soldiers, 25OHD levels were measured and associated with the days of illness in context of respiratory infections. Individuals with insufficient levels of 25OHD were absent significantly higher.

(30) All these findings indicate a beneficial role of vitamin D metabolites on immune functions.

To sum up, a great number of in vitro evidence about the functional conjunction of 1,25OH2D and microRNAs has been found in the last years. Wide fields of the intracellular 1,25OH2D effects seem to be executed or at least fine tuned by microRNAs. These discoveries open up many new possibilities for further investigation of therapeutic and diagnostic strategies based on microRNAs and vitamin D.

1.6 Objectives

This thesis intends to bring further light into potential systemic profiles of microRNAs during a vitamin D intervention. Considering microRNAs as potential biomarkers, we describe circulating microRNAs levels in blood. As data about circulating microRNAs in the context of vitamin D supplementation are lacking, we wanted to find out whether there are significant changes in expression profiles of specific microRNAs, trying to link our results with actual data about common diseases.

2 Materials and Methods

2.1 Sample collection based on clinical trial

The blood samples for miRNA-analysis were available from a clinical trial, investigating the effects of vitamin D supplementation on testosterone in vitamin D insufficient men. The trial was performed at the Department of Internal Medicine in cooperation with the Department of Urology and the ivF institute of the Department of Gynecology and Obstetrics at the Medical University of Graz, Austria. The microRNA analysis was then performed by Exiqon A/S, Denmark.

2.1.1 Study design

This single center study examined the effects of a 12-week vitamin D supplementation in two groups. Measuring the vitamin D effects on testosterone, the first group consisted of hypogonadal men with low levels of total testosterone and vitamin D. The control group consisted of vitamin D insufficient, but healthy men with normal levels of total testosterone. We used blood samples of healthy patients from the control group, to investigate the general effects of vitamin D supplementation on microRNAs in healthy subjects. Thus, we wanted to eliminate the endocrine interference of hypogonadism on circulating microRNAs. In each group, participants were randomly assigned either to be treated with vitamin D or placebo. To eliminate personal attachment in-between staff and patients, the study was carried out in a double-blind setting. Patients were recruited at the outpatient clinics of the departments mentioned above within 18 months. The study schedule contained a screening visit and three study visits. Blood samples were taken at every visit from 7:30 am to 9:00 am after fasting the previous night. In the screening visit, patients were assessed whether they passed the inclusion and exclusion criteria. In study visit 1, various checks were performed including oral glucose tolerance tests and DXA measurements. Blood samples for base line analysis (point 0) were taken at this visit. Four weeks after study visit 1, the second visit was held including a blood sample for assessment of short term effects of vitamin D supplementation. The last study visit was carried out 12 weeks after study visit 1, marking the end of treatment. Besides the blood sample for end-point analysis, this visit included further tests for glucose tolerance and bone density. (31)

2.1.2 Recruitment of healthy vitamin D deficient men

The inclusion and exclusion criteria for healthy control subjects are described below.

Inclusion criteria:

- Age older than 18 years and younger than 70 years.
- Calcidiol (25(OH)D) levels in blood lower than 30 ng/ml.
- Total testosterone levels higher than 3.0 ng/ml.

Exclusion criteria:

- Low blood calcium levels (< 2,7 mmol/L).
- Testosterone or calcitriol supplementation.
- Orchidectomy.
- Endocrine diseases.
- Hematocrit higher than 50%.
- Diseases of the lower urinary system.
- Prostatic tumors.
- Sleep apnea syndrome.
- Cardiac insufficiency.
- Levels of prostate specific antigen higher than >4 ng/ml.
- Chromosomal aberrations.
- Metabolic or endocrine medication.

2.1.3 Treatment

Patients were treated with either 20.000 IU of cholecalciferol (Oleovit D3® drops, Fresenius Kabi Inc., Graz, Austria) per week or with placebo including the same base material. All in all, the treatment lasted for 12 weeks, beginning with study visit 1 and ending with study visit 3. Due to the double-blind setting, neither patients nor the staff were informed about the randomization of the participants into either the treatment or the placebo group. Therefore, the ratio was 1:1. (31)

2.1.4 Selection of samples

Patients for microRNA analysis were selected out of the eugonadal control group to examine healthy individuals. Investigating the impact of vitamin D supplementation on microRNAs, we chose 4 patients with an increase in vitamin D levels after both 4 and 12 weeks of treatment. Of these patients, serum samples of all three visits were sent to Exiqon (0, 4 and 12 weeks). Vice versa, we chose patients under PBO at baseline and the final visit to exclude vitamin D dependant alterations in the placebo group. Out of these three patients, we only sent serum from study visits 1 and 3. All in all, we sent 18 samples (12 from patients treated with vitamin D and 6 from patients treated with placebo). Detailed characteristics of the selected individuals will be presented in the results section.

2.2 MicroRNA quantification

Serum samples were sent to Exiqon A/S, Skelstedet 16, 2950 Vedbaek, Denmark for microRNA quantification. In the following section, the miRNA quantification steps are described.

2.2.1 RNA isolation

The first step in the preparation of serum samples for the qPCR analysis was RNA isolation. Before centrifugation, the serum was defrosted and put on ice. Then tubes containing the serum samples were centrifuged for five minutes at 4°C with 3000 x g. For precipitation of proteins and lysis of exosomes and other membranous material 200 µL of centrifuged serum, 60 µl lysis solution, RNA spike ins and 20 µl protein precipitation solution were mixed together in a "FluidX tube". The final separation of the RNA was implemented by the "miRCURY™ RNA isolation Kit – Biofluids" from Exiqon. After isolation, the RNA was present in volumes of 50 µl.

2.2.2 Reverse transcription and real-time qPCR

After RNA isolation, the purified RNA was reverse transcribed into cDNA and transferred into well plates together with "ExiLENT SYBR® Green master mix". For this step, the "miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation, and cDNA synthesis kit" was used. In each well, a primer of the Human panel I was bound, guaranteeing a primer-specific amplification of one microRNA per well. Using the "Roche LightCycler® 480 Real-Time PCR System" in each of the 384 wells cDNA was amplified and Cq-values were determined by the "Roche Light cycler software". The following volumes were used: 10 µl RNA were transferred in 50 µl reactions of reverse transcription. PCR was performed with reaction-volumes of 10 µl with cDNA diluted 50 times.

2.2.3 Quality control

A melting curve was created by the "Light Cycler software" for each sample. In this step of quality control, called melting curve analysis, the primer efficacy is evaluated. Heating up the sample, the Light Cycler recognizes a fluorescent signal forming a peak during the highest dissociation of cDNA. If there is more than one peak in the melting curve, more than one cDNA template has been amplified and primers are suspected of unspecific binding. Only samples with one melting peak were further analysed.

To assess the purity of the RNA we wanted to examine, a negative control was performed. Therefore, a sample containing only the reagents, but no template was reverse transcribed

to uncover possible contaminations. Furthermore, only samples detected 5 cycles before the negative control were used for the final analysis.

Spike-ins are synthetic RNAs or DNAs which are added in a predefined amount at particular steps of the quantification process to assess the efficacy of these steps. In our experiment spike-ins were added to control reverse transcription, RNA extraction and qPCR. Cq-values for the added spike-ins will be presented in the results section.

Investigating the levels of extracellular microRNAs in blood, it is important to avoid hemolysis. The aim was to introduce only extracellular microRNAs into the process of quantification. Therefore, the ratio of intracellular miR-451, derived from erythrocytes, and extracellular miR-23a was analysed. Samples with a ratio of seven or higher are suspicious for hemolysis. The ratio for each sample is listed in the results section.

To balance deviations within different reactions, Cq values were normalized. Due to the high number of assays, the global mean normalization was considered to be most appropriate. Therefore, the Cq value of the assay was subtracted from the average Cq of all 18 samples.

$$\text{Normalized Cq} = \Delta Cq = Cq_{\text{(average of 111 microRNAs expressed in all 18 samples)}} - Cq_{\text{(microRNA assay of sample)}}$$

After quality control and normalization, differences in expression were calculated using the ΔΔCq-method following the style of the publication "Analyzing real-time PCR data by the comparative C_T method" (32).

Expression levels of two different points of time were compared. Therefore, the ΔCq values of each point were correlated in the following way:

$$\Delta\Delta Cq = \Delta Cq_{\text{(point 2)}} - \Delta Cq_{\text{(point 1)}}$$

Hereinafter, the fold change was calculated using the formula:

$$\text{Fold change} = 2^{-\Delta\Delta Cq}$$

2.3 Statistical analysis

To determine if the data was adhering to the normal distribution, the Shapiro-Wilk test was used. ΔCq values of particular microRNAs among different study visits were compared by t-tests. Data not adhering to normal distribution was assessed by the Wilcoxon test. Fold changes higher/lower than ±2 were defined to be significant. *P*-values < 0.05 were considered to be statistically significant.

3 Results

3.1 Cohort characteristics

Four individuals treated with vitamin D and 3 individuals treated with placebo were selected for microRNA quantification in blood. Table 1 shows the detailed characteristics of the selected individuals. The average age of patients treated with placebo was 31, while subjects treated with vitamin D were on average 35 years of age ($p = 0.652$). We found no differences in baseline values between the placebo and vitamin D treated groups for body mass index [BMI] ($p = 0.670$), 25(OH)D ($p = 0.342$) and PTH ($p = 0.373$). 25(OH)D levels in the placebo group remained unchanged at 12.6 ng/ml at baseline and 11.1 ng/ml at the final visit (p 0.534). In vitamin D treated patients, levels started at 14.0 ng/ml and rose to 65.8 ng/ml after 12 weeks of treatment ($p = 0.003$). The BMI in the placebo group was 23.0 at baseline and 23.3 in the final visit ($p = 0.227$), whereas it started at 22.6 and ended at 22.9 in vitamin D treated individuals ($p = 0.394$). Levels of PTH in the placebo group were 51.6 pg/mL at baseline and 55.3 pg/mL at the final visit ($p = 0.785$). In vitamin D treated individuals, PTH was 48.4 pg/mL at the baseline visit and 47.0 pg/mL at the final visit ($p = 0.596$).

	Placebo (n = 3)			Vitamin D (n = 4)		
Study visit	First	Final	*p*-value	First	Final	*p*-value
Age (years)	31 ± 6		n.a.	35 ± 6		n.a.
BMI (kg/m^2)	23.0 ± 0.2	23.3 ± 0.5	$p = 0.227$	22.6 ± 1.7	22.9 ± 1.4	$p = 0.394$
25(OH)D (ng/mL)	12.6 ± 2.7	11.1 ± 1.1	$p = 0.534$	14.0 ± 0.6	65.8 ± 11.1	$p = 0.003$
PTH (pg/mL)	51.6 ± 6.3	55.3 ± 18.7	$p = 0.785$	48.4 ± 2.0	47.0 ± 3.6	$p = 0.596$

Table 1: Cohort characteristics. Age, BMI, vitamin D levels and PTH levels at baseline and final visit of both individuals treated with vitamin D and placebo are shown. *P*-values for age were not appropriate (n.a.).

3.2 Quality control results

Spike-ins were added to assess the efficacy of quantification steps.

UniSp2, UniSp4 and UniSp5 were added before RNA isolation. UniSp6 was added before reverse transcription. UniSp3 was added before qPCR. As can be seen in Figure 4, all spike-ins show approximately equal Cq-values after qPCR. This indicates consistent efficacy at every step in every sample.

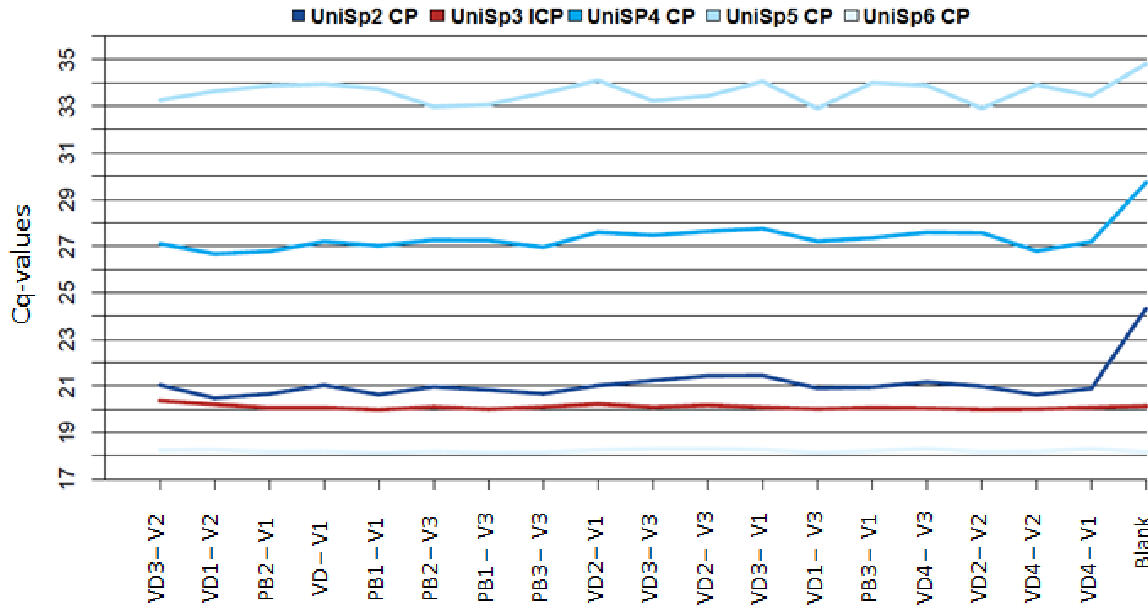

Figure 4: Spike-in Cq-values. Spike-ins were added at different points of analysis. Each color reflects one specific spike-in. On the x-axis, the samples were encoded in the following way: samples from subjects treated with vitamin D (n = 4) are titled VD;1,2,3,4. Samples from subjects treated with placebo (n = 3) are titled PB; 1,2,3. Study visits from 1 to 3 are titled V;1,2,3.

For assessment of hemolysis, the ratio of intracellular miR-451 derived from erythrocytes and extracellular miR-23a was analysed. Samples with a ratio of 7 or higher are suspected to be hemolytic. Figure 5 demonstrates the sample specific ratios. None of the samples shows a ratio higher than 7.

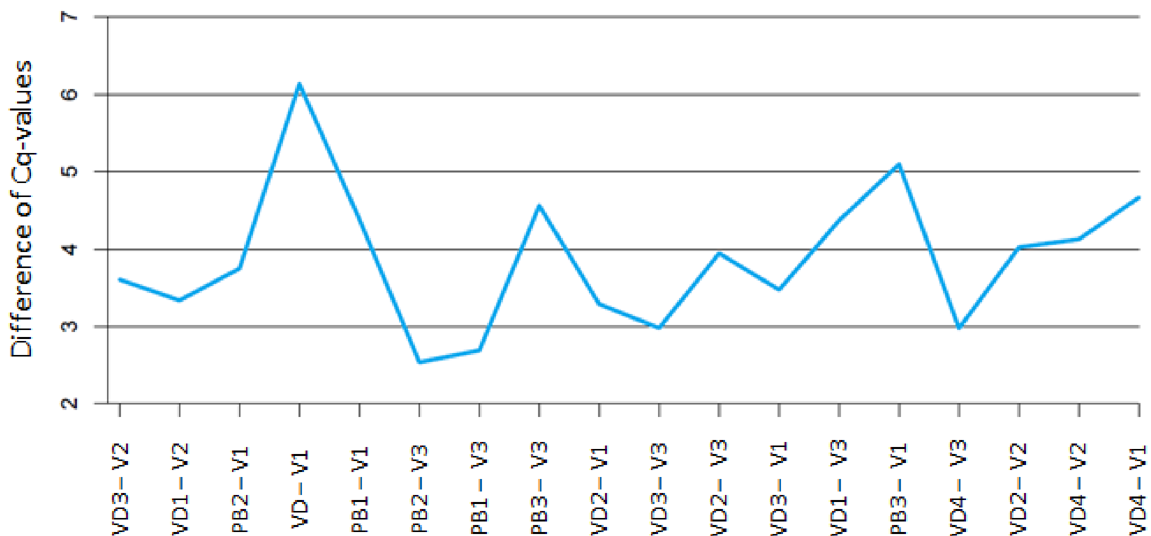

Figure 5: Ratio between miR-23a-3p and miR-451a. For each sample, the differences of extracellular miR-23a and intracellular miR-451 are shown. On the x-axis, the samples were encoded in the following way: samples from subjects treated with vitamin D (n = 4) are titled VD;1,2,3,4. Samples from subjects treated with placebo (n = 3) are titled PB; 1,2,3. Study visits from 1 to 3 are titled V;1,2,3.

3.3 Real time qPCR results

We quantified 372 real time qPCR assays per sample. 111 assays showed a signal in all samples and the average number of assays detected per sample was 175. In figure 6, the microRNA-content and average Cq-values of each sample are presented. The average Cq-values varied between 29 and 32.

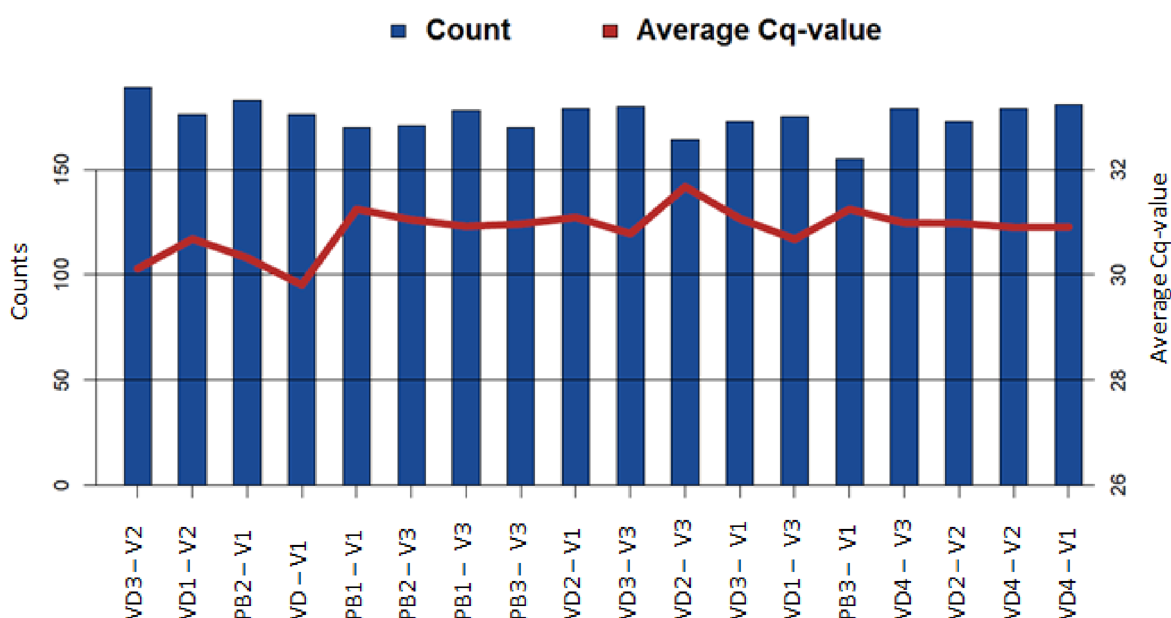

Figure 6: MicroRNA content. The blue bars of the chart representing the number of detected miRNAs per sample whereas the red line reflects the average Cq-value of each sample. On the x-axis, the samples were encoded in the following way: samples from subjects treated with vitamin D (n = 4) are titled VD;1,2,3,4. Samples from subjects treated with placebo (n = 3) are titled PB; 1,2,3. Study visits from 1 to 3 are titled V;1,2,3.

3.3.1 Differentially expressed microRNAs after 4 weeks of treatment with vitamin D

Comparing baseline Cq-values from study visit 1 (pre-treatment vitamin D) and Cq-values from study visit 2 (4 weeks of treatment with vitamin D), 7 microRNAs showed significant changes in their expression levels after 4 weeks of vitamin D treatment. In figure 7, the graphic distribution of the compared microRNAs is demonstrated in a volcano plot. In a coordinate system, fold changes are plotted on the x-axis and *p*-values plotted on the y-axis using logarithmic scales. Highly significant microRNAs with high fold changes can be seen at the upper right and the upper left of the volcano plot.

Hereinafter, the microRNAs passing the significance level of $p < 0.05$ when comparing the study visits by a t-test are listed in table 2.

MicroRNA name	Average ΔCq pre-treatment VD	Average ΔCq after 4 weeks VD	Fold change	*p*-value
miR-133b	-4.5	-2.6	-3.6 *	0.0076
miR-215-5p	-2.2	-3.0	1.7	0.011
miR-194-5p °	-1.2	-2.3	2.1 *	0.012
miR-192-5p °	-0.73	-1.5	1.7	0.021
miR-let-7e-5p °	-3.4	-2.3	-2.3 *	0.021
miR-let-7a-5p °	1.9	2.4	-1.4	0.027
miR-200a-3p	-5.0	-5.6	1.5	0.038

Table 2: Significant microRNAs after 4 weeks of treatment with vitamin D. The 7 most significant microRNAs are ranked by *p*-value. Average ΔCq values of the visits compared are shown in column 2 and 3 (VD means vitamin D). Significant fold changes with values higher than ±2 are marked by a *-symbol. The °-symbol marks miRNAs with consecutive alterations also after 12 weeks of treatment.

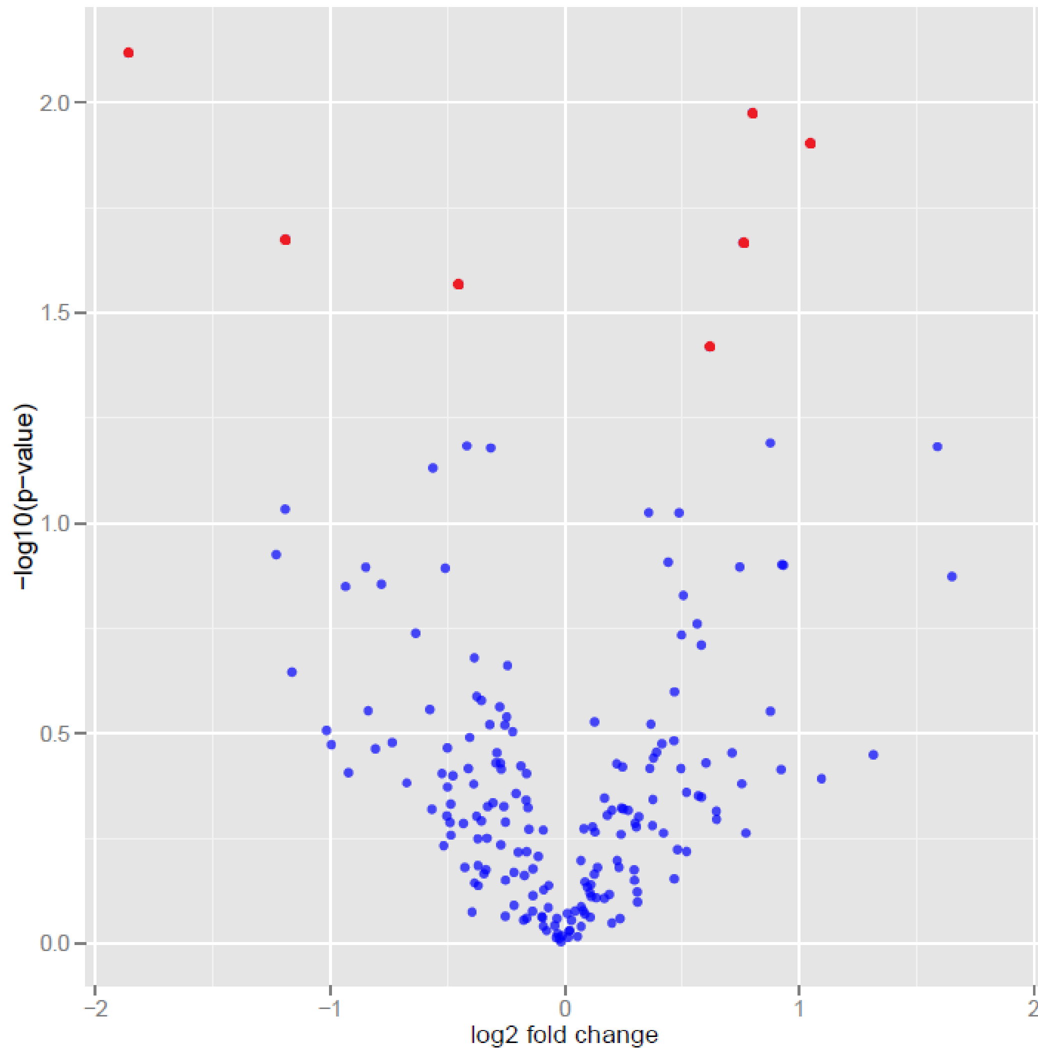

Figure 7: Differentially expressed microRNAs. On the x-axis, fold changes are plotted on a log2 scale and on the y-axis, *p*-values are plotted on a –log10 scale. The seven most significant microRNAs after 4 weeks of treatment are marked in red. These microRNAs passed the significance level with a cutoff of $p < 0.05$. MicroRNAs on the upper right (above 1 on the log2 fold change scale) and the upper left (below -1 on the log2 fold change scale) showed both significant fold changes and passed the significance level cutoff.

MiR-194-5p showed the highest positive fold change of 2.1 (p = 0.012) after 4 weeks of treatment. We also found increasing expression levels with miR-215-5p (fold change = 1.7, p = 0.011), miR-192-5p (fold change = 1.7, p = 0.021) and miR-200a-3p (fold change = 1.5, p = 0.038) after four weeks of treatment. We found 2 members of the let-7 family significantly decreased after 4 weeks of treatment. MiR-let-7e-5p was decreased with a fold change of -2.3 (p = 0.021) and miR-let-7a-5p with a fold change of -1.4 (p = 0.027). The strongest decrease after 4 weeks treatment with a fold change of -3.6, combined with the highest level of significance (p = 0.0076) was demonstrated by miR-133b.

3.3.2 Differentially expressed miRNAs after 12 weeks of treatment with vitamin D

After comparing the baseline Cq-values from study visit 1 with the end point Cq-values from study visit 3, we found 16 miRNAs to be differentially expressed in a significant (p < 0.05) way. In the volcano plot, the graphic distribution of the 16 significant miRNAs is shown. (Figure 8)

After comparing the baseline and endpoint Cq-values of patients treated with vitamin D, 16 microRNAs were found to be significant. They are shown in table 3.

miRNA name	Average ΔCq pre-treatment VD	Average ΔCq after 12 weeks VD	Fold change	*p*-value
let-7e-5p °	-3.4	-2.1	-2.6 *	0.0028
miR-329-3p	-5.8	-4.1	-3.2 *	0.0072
miR-421	-3.3	-4.2	1.9	0.0086
let-7a-5p °	1.9	2.5	-1.5	0.0095
miR-127-3p	-4.9	-3.4	-2.8 *	0.0097
let-7c-5p	-1.6	-0.89	-1.7	0.012
miR-194-5p °	-1.2	-2.3	2.2 *	0.019
miR-192-5p °	-0.73	-1.5	1.6	0.019
miR-347b-5p	-2.1	-1.6	-1.3	0.023
miR-423-3p	-0.49	0.19	-1.6	0.025
miR-326	-6.2	-4.4	-3.5 *	0.025
miR-185-5p	3.8	3.0	1.8	0.031
miR-30c-5p	0.69	1.1	-1.3	0.032
miR-339-5p	-1.7	-1.0	-1.7	0.041
miR-551b-3p	-4.3	-5.4	2.1 *	0.043
miR-23b-3p	2.4	2.7	-1.3	0.048

Table 3: Significant microRNAs after 12 weeks of treatment with vitamin D. The 16 most significant microRNAs are ranked by *p*-value. Average ΔCq values of the visits compared are shown in column 2 and 3 (VD means vitamin D). Significant fold changes with values higher than ±2 are marked by a *-symbol. The °-symbol marks miRNAs with significant alterations also after 12 weeks of treatment.

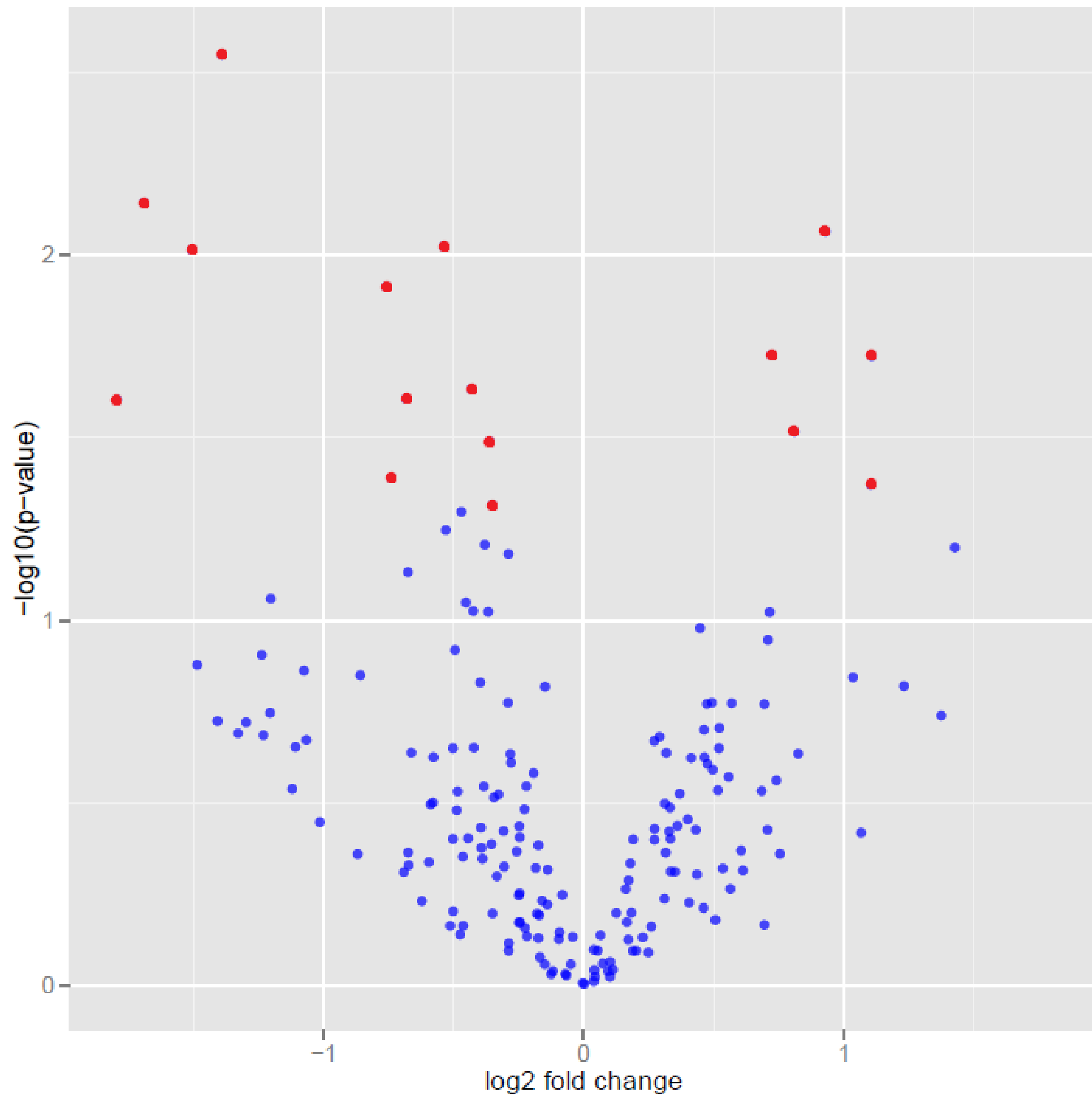

Figure 8: Differentially expressed microRNAs. On the x-axis, fold changes are plotted on a log2 scale and on the y-axis, *p*-values are plotted on –log10 scale. The 16 most significant microRNAs after 12 weeks of treatment are colored in red. These microRNAs passed the significance level with a cutoff of $p < 0.05$. MicroRNAs on the upper right (above 1 on the log2 fold change scale) and the upper left (below -1 on the log2 fold change scale) showed both significant fold changes and passed the significance level cutoff.

The miRNA with the largest positive fold change of 2.2 again was miR-194-5p (p = 0.019). Furthermore, miR-551b-3p (fold change = 2.1, p = 0.043), miR-421 (fold change = 1.9, p = 0.0086), miR-185-5p (fold change = 1.8, p = 0.031) and miR-192-5p (fold change = 1.6, p = 0.019) showed positive fold changes. We found 11 miRNAs to be expressed decreasingly. Those were miR-347b-5p (fold change = -1.3, p = 0.023), miR-30c-5p (fold change = -1.3, p = 0.032), miR-23b-3p (fold change = -1.3, p = 0.048), let-7a-5p (fold change = -1.5, p = 0.0095), miR-423-3p (fold change = -1.6, p = 0.025), let-7c-5p (fold change = -1.7, p = 0.012), miR-339-5p (fold change = -1.7, p = 0.041), let-7e-5p (fold

change = -2.6, p = 0.0028), miR-127-3p (fold change = -2.8, p = 0.0097), miR-329-3p (fold change = -3.2, p = 0.0072) and miR-326 (fold change = -3.5, p = 0.025).

Four microRNA showed significant changes of expression both after 4 and 12 weeks. In table 2 and 3 they are marked with a °-symbol. Two members of the let-7 family showed a decrease of expression at both points of time. Let-7a-5p was decreased -1.4fold after 4 weeks and -1.5fold after 12 weeks of treatment. Let-7e-5p was decreased -2.3fold after 4 weeks and -2.6fold after 12 weeks of treatment.

Two microRNAs were increasingly expressed at both points of time. Mir-192-5p was increased 1.7fold after 4 weeks and 1.6fold after 12 weeks of treatment. Mir-194-5p was increased 2.1fold after 4 weeks and 2.2fold after 12 weeks of treatment.

3.3.3 Differentially expressed microRNAs after 12 weeks of vitamin D compared to placebo

We also compared the end-point Cq values of patients after 12 weeks placebo-treatment with the end-point Cq values of patients after 12 weeks of vitamin D treatment. We found 5 miRNAs with significant ($p < 0.05$) changes in their expression level. In the volcano plot, the 5 most significant miRNAs can be seen at the top of the plot (Figure 9).

Five microRNAs passed the significance level of $p < 0.05$. They are listed in table 4.

miRNA name	Average ΔCq after 12 weeks VD	Average ΔCq After 12 weeks Pb	Fold change	*p*-value
miR-30e-3p	-4.4	-3.1	-2.5 *	0.021
miR-15b-5p	1.7	2.0	-1.3	0.022
miR-128-3p	-3.3	-4.2	1.8	0.041
miR-98-5p	-3.9	-4.7	1.8	0.041
miR-450a-5p	-4.0	-5.4	2.6 *	0.049

Table 4: Significant microRNAs after 12 weeks of treatment with vitamin D compared to placebo. The 5 most significant microRNAs are ranked by p-value. Average ΔCq values of the visits compared are shown in column 2 and 3 (VD means vitamin D). Significant fold changes with values higher than ±2 are marked by a *-symbol.

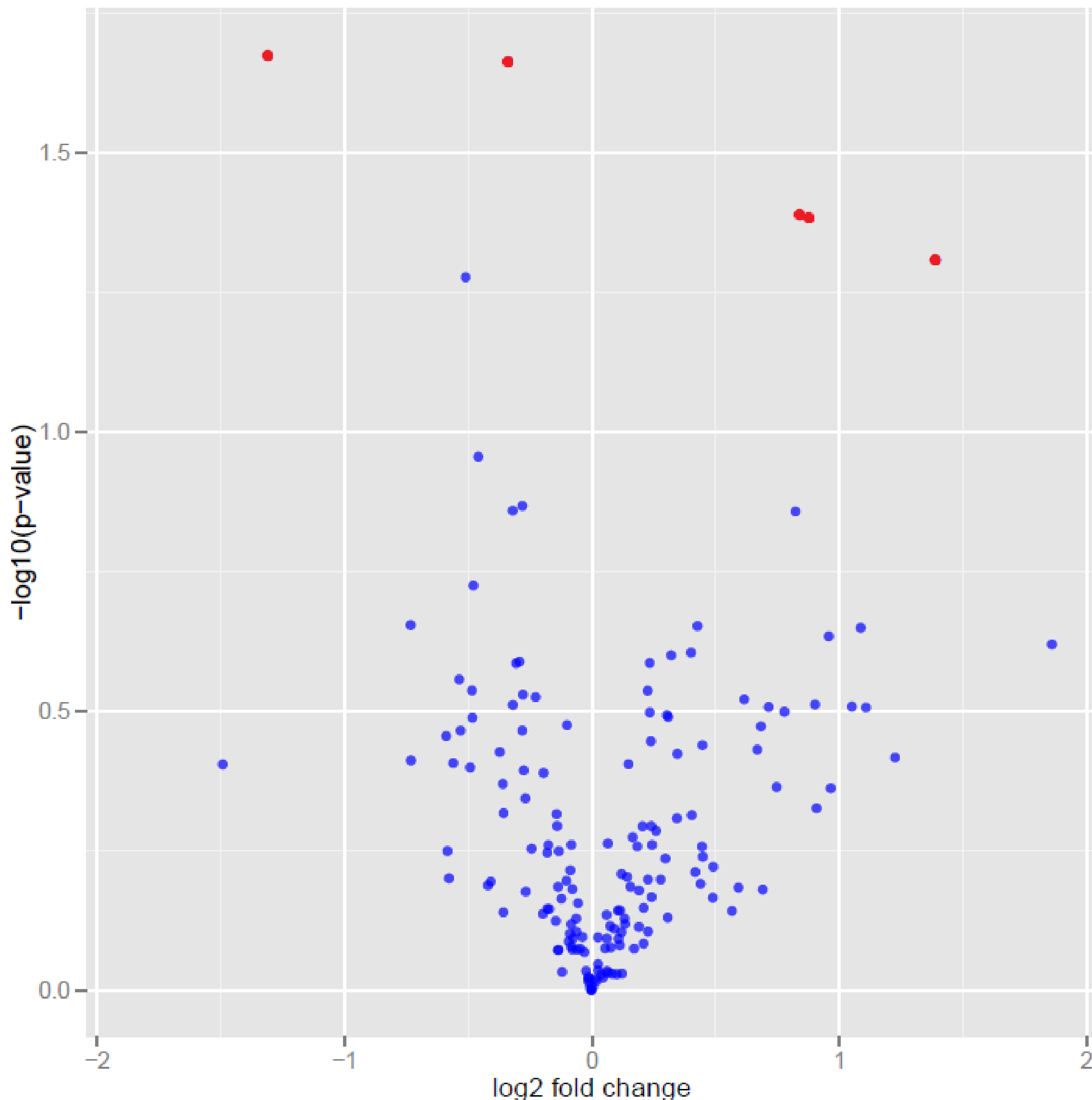

Figure 9: Differentially expressed microRNAs. On the x-axis, fold changes are plotted on a log2 scale and on the y-axis, *p*-values are plotted on –log10 scale. The 5 most significant microRNAs are marked in red. These microRNAs passed the significance level with a cutoff of $p < 0.05$. MicroRNAs on the upper right (above 1 on the log2 fold change scale) and the upper left (below -1 on the log2 fold change scale) showed both significant fold changes and passed the significance level cutoff.

MiR-450a-5p showed the strongest increase with a fold change of 2.6 ($p = 0.049$) whereas miR-30e-3p was decreased most with a fold change of -2.5 ($p = 0.021$). In between, we found miR-128-3p (fold change = 1.8, $p = 0.041$) and miR-98-5p (fold change = 1.8, $p = 0.041$) to be upregulated and miR-15b-5p to be downregulated with a fold change of -1.3 ($p = 0.022$).

4 Discussion

In the present study, we were able to demonstrate a direct impact of vitamin D supplementation on circulating microRNAs in an intervention study of 25OHD compared to placebo.

We found 19 miRNAs, which passed the significance level of $p < 0.05$, to be differentially expressed. Four miRNAs, including two members of the let-7 family, miR-192-5p and miR-194-5p, showed significantly altered expression levels in samples taken both after 4 and 12 weeks of treatment with vitamin D. This indicates a relation between vitamin D effects and the extracellular microRNA trafficking.

Our findings contribute to the further understanding of vitamin D dependent physiological processes and elucidate the complex functional network of microRNAs.

As vitamin D deficiency is a widespread health problem which can be treated with a simple form of therapy, it is of high medical interest to fully understand the potentials and consequences of vitamin D supplementation (33). Furthermore, microRNAs are a topic of arising interest with increasing potential for therapeutic use (34). Thus, our work meets at the interface of basic research and clinical application.

The main strength of this study was the randomized double-blind controlled setting which offered optimal conditions for investigating the effects of oral vitamin D intake on serum microRNA levels. A special feature of this diploma thesis is the way of treatment. In comparison to in vitro studies, in which exclusively 1,25OH2D is used, this study demonstrates the effects of 25OHD on microRNAs. The in vitro treatment with 1,25OH2D produces a non-physiological increase of effective 1,25OH2D inside the cells. Thus, the tissue specific rates of conversion from 25OHD to 1,25OH2D get neglected. In this in vivo study, the local environment and, therefore, the natural rates of conversion remained unchanged, resulting in physiologic conditions to measure the impact on microRNA metabolism. This study reflects the impact of a short term increase of 25OHD on microRNAs under physiologic conditions. Additionally, the microRNA quantification was performed with high accuracy due to the high sensitivity of the "miRCURY LNA™ Universal RT microRNA PCR" (35). Furthermore, multiple steps of quality control were performed leading to high reliability of the collected data.

Nevertheless, this study suffered from some restrictions. Due to extensive costs for the microRNA quantification including RNA isolation and real time qPCR, only a limited number of samples was analysed and statistical power was lacking. Another factor was the inhomogeneity of detected miRNAs among different samples. Many miRNAs were not

present in all samples. Because of the low number of analysed individuals, this study should only be considered as an orienting investigation, presenting valuable information for further analysis of the most promising microRNAs in a bigger cohort as well as exploration of downstream targets of these microRNAs.

A study conducted by Jorde et al. at the University of Tromsø investigated the impact of vitamin D on circulating microRNAs in 2012. In two pilot studies, they treated five non-vitamin D deficient individuals each with 40.000 IU of vitamin D per week for 12 months. The twelve miRNAs with highest significance were then selected and analysed in a larger cohort. Despite some differences in the inclusion criteria, cohort characteristics, and the way of treatment, the design of this study is comparable to the present one, but the duration of therapy is far longer (1 year). (36)

In the two pilot studies, besides let-7f and miR-26a, other miRNAs were detected than we found in our study. However, these two miRNAs were not differentially expressed in our study. At least five miRNAs out of the two pilot studies were significantly altered in our analysis too, but fold changes were partly converse. (36) A further study examined levels of circulating microRNAs in 21 individuals after cholecalciferol treatment in 2017. They found diminished expression of miR-7 and miR-192 and a rise in miR-152 expression after a treatment of 16 weeks with 2.000 IU of cholecalciferol per day. (37) In our study, miR-192 was increased. These inconsistent results demonstrate the necessity of larger cohorts to be screened and the possible impact on microRNA levels by differences in the study design/supplementation periods. As there might potentially be early and late effects in vitamin D supplementation, the time course of the supplementation is crucial in the interpretation of the study findings.

Another point is the definition of vitamin D deficiency. As many factors, such as genetic determinants (38), may influence vitamin D levels, these conditions might be taken into account when setting inclusion criteria in vitamin D interventions.

Despite the restrictions mentioned above, the results of this study show trends in a vitamin D dependent microRNA metabolism. To further investigate the pathophysiologic role of microRNAs, these trends are able to determine the direction of future research. The following sections will describe potential influences of our candidate miRNAs in context of glucose metabolism and the most recent developments in cancer. Therefore, published studies will be presented. As mentioned in the methods section, changes higher than 2fold and lower than -2fold are considered to mark a significant change of expression. Some microRNAs (mir-192, miR-200 and miR-215), which will be discussed now, showed lower

fold changes but due to their outstanding role in the pathogenesis of the diseases mentioned, they will be included into the discussion. None of these fold changes were lower than ±1.5.

4.1 MiR-326, let-7 and miR-194 in the context of diabetes mellitus

Three of the significant candidate miRNAs in our study have been discussed to affect glucose metabolism in the literature. MiR-326 has been found to play a role in the pathogenesis of diabetes type 2. Interestingly, miR-326 was strongly decreased after 12 weeks of treatment with vitamin D in our study. In a case-control study by Santovito et al., levels of circulating microRNAs in 18 individuals with diabetes type 2 and 12 healthy controls were investigated by real time qPCR. Several microRNAs showed significant alterations in their expression level. MiR-326 showed the strongest upregulation in diabetic patients. Vice versa, the levels of plasma adiponectin were significantly lowered. Adiponectin, a peptide hormone indirectly proportional to human fat mass, increases the sensitivity to insulin. Computer analysis demonstrated adiponectin as a plausible target of miR-326, supporting the inverse correlation found in this study. (8)

A study by Sebastiani et al. investigated microRNA levels in lymphocytes in patients suffering from T1DM. In these cells, miR-326 was increased significantly. The study suggests miR-326 to play a role in autoimmune mediated β-cell destruction. These findings may correlate with expression levels of miR-326 in blood, which makes miR-326 a potential future biomarker of autoimmunity. (39)

Furthermore, Du et al. reported about high miR-326 expression levels in TH-17 cells with regard to multiple sclerosis. As TH-17 cells are participating in autoimmunity, miR-326 is suspected to accelerate autoimmune processes. (40)

As presented in the results section, the expression level of miR-326 was highly decreased after 12 weeks of vitamin D treatment. With a fold change of -3.5, this microRNA revealed the strongest down regulation in the present study, indicating a distinct negative impact of vitamin D on miR-326. Comparing the decrease of miR-326 to the results of the studies mentioned above, an anti-diabetic effect of vitamin D can be hypothesized. The reduction of miR-326 by oral vitamin D intake may be a supporting factor to rebalance the disrupted equilibrium in diabetic microRNA metabolism. With a possible direct impact on adiponectin (8), vitamin D supplementation may affect insulin sensitivity via miR-326. Additionally, downregulation of miR-326 may have direct effects on autoimmunity and β-cell destruction.

In two studies about glucose metabolism in mice, let-7, which was the first microRNA to be discovered in humans, was found to promote diabetic conditions. Frost and Olson reported about the down regulation of insulin response resulting in bad glycemic control after an artificial increase of let-7 levels. Vice versa, a blocking of let-7 by antimiRs led to an anti diabetic effect. (41) The second study demonstrated a diabetogenic effect of let-7 on muscular tissue in mice. (42) In our results, two members of the let-7 family were significantly downregulated by vitamin D treatment. Let-7e-5p was decreased 2.6fold and let-7a-5p was decreased 1.5fold. This reduction in expression levels could refer to a possible positive impact of vitamin D on glucose homeostasis. However, further investigation is needed in humans.

Another microRNA, which was reported to show altered expression levels in context of diabetes, is miR-194. In 2016, Latouche et al. investigated microRNA levels in muscle biopsies of type 2 diabetic humans and rats by micro array profiling. In both species miR-194 was reduced in diabetic individuals. (9) In our study, miR-194 was increased 2.2fold, indicating a possible upregulation of miR-194 by oral vitamin D intake. However, at present the exact mechanism of the alterations in miR-194 metabolism in skeletal muscle remains unclear.

Furthermore, miR-194 has been put in the context of diabetes in a cell culture model simulating diabetic conditions. Micro-array profiling revealed miR-194 to be significantly higher expressed during toxic stimulation of β-cells by interleukin 1β. Again, these results should be compared to our findings with caution due to the differences between in vivo and in vitro expression. (43)

To sum up, our results indicate an antidiabetic effect of oral vitamin D intake by influencing specific microRNAs linked to the pathophysiology of both diabetes type 1 and 2. Both because of antidiabetic effects and the high fold change we have seen in our results, miR-326 seems to link vitamin D and diabetes. Because it possibly targets adiponectin, miR-326 might affect insulin sensitivity.

4.2 Differentially expressed microRNAs in the context of cancer

Numerous microRNAs have been found to be deregulated in various types of cancer. Some of them might have tumor suppressive effects. Hereinafter selected microRNAs presented in the results section with promising effects in the field of carcinogenesis will be discussed.

4.2.1 MiR-192 and miR-194 in osteosarcoma

Osteosarcoma is the most common malignant bone tumor, affecting both children and adolescents. The proliferating cells are able to form bone matrix and spread metastases especially into the lungs (44).

Two studies published in 2018 investigated the role of miR-192 in osteosarcoma cell lines. In the first study, expression levels of miR-192 in 25 tissue samples taken by surgery from osteosarcoma patients were identified by RT-qPCR. In these tissues, levels of miR-192 were significantly lower than in controls. After transfection of miR-192 to cell lines, cell growth was reduced, and the cells showed lower rates of invasion into a specific gel. Furthermore, a wound healing assay revealed reduced capacity of wound healing. These results indicate a reduced growth rate and changes in cellular motility, crucial factors of tumor progression and metastasis. In addition, cells died faster after exposure to cisplatin. A relation between these effects and the ubiquitin specific protease 1 [USP1], which was increased in osteosarcoma tissue and decreased after transfection of miR-192, seems to be possible. Repressing USP1, miR-192 modifies the cell cycle, DNA repair and many other effects dependent from ubiquitination. (45)

In our results, miR-192 was increased 1.6fold after 12 weeks of vitamin D treatment. Although the expression levels between blood and osteosarcoma tissue cannot be compared one to one, the enhancement of miR-192 might points to a possible protective effect of vitamin D against carcinogenesis. If there is a connection between the extracellular levels of miR-192 (as we measured in serum in healthy individuals) and the osteosarcoma cells, which possibly release miR-192 into the blood, this microRNA could be a possible biomarker for osteosarcoma in the future. If - in turn - extracellular miR-192 molecules could be internalized into osteosarcoma cancer cells, slowing down the cellular proliferation and motility, miR-192 and also vitamin D could be used in new pharmacologic targeting in osteosarcoma.

The second study supporting such a hypothesis presented similar effects of miR-192. In 22 osteosarcoma tissues again miR-192 was significantly decreased. In this study, Agomirs were used to provoke miR-192 expression. Comparable to the results just mentioned, the rate of proliferation was lowered, including higher rates of apoptosis. Again, also cell motility was changed with higher rates of invasion and migration. In this case, a further target of miR-192 was identified. Matrix metalloproteinase-11 [MMP-11], which can to degrade extracellular matrix, was expressed at high levels in the osteosarcoma tissues and expressed at low levels after miR-192 enhancement. Due to this inverse correlation

amongst other targets, the downregulation of MMP-11 might be responsible for the tumor-suppressive effects of miR-192. (46)

Those findings further highlight the role of miR-192 in the progression of osteosarcoma. If the effect of vitamin D on miR-192 gets confirmed in a larger cohort, new perspectives on vitamin D as a possible anti-tumor drug might open up.

Another well investigated microRNA in fields of osteosarcoma is miR-194. A study by Han et al., analysed 107 tissue samples from osteosarcoma surgeries. They found miR-194 to be less expressed in osteosarcoma cell lines. In this study, endogenous production of miR-194 was provoked by infecting the cells with a virus. This led to slowdown in tumor progression with lower rates of proliferation. They found protein levels of Cadherin 2 and insulin like growth factor-1 receptor to be reduced after miR-194 enhancement. This indicates a tumor suppressive effect of miR-194 by downregulating these proteins. (47)

In our results, miR-194 was reduced 2.2fold after 12 weeks of vitamin D treatment. Again, the direct impact of circulating levels of miR-194 on bone tissue remain unclear, but the tumor suppressive characteristics of miR-194 are promising in terms of cancer therapy.

The crosstalk of vitamin D and miR-192/miR-194 and its possible tumor suppressive effects need to be further elucidated.

4.2.2 MiR-192, miR-194, miR-200, miR-215, and let-7e in colorectal cancer

Colorectal cancer is the most investigated cancer type in terms of microRNAs. As mentioned in the introduction, many microRNAs have been identified to play major roles in regulating pathways in cancer progression. As colorectal cancer is one of the most common cancer types in the western world, the scientific interest in finding new therapeutic options is enormous.

A few microRNAs, which have been reported to have interesting effects on colorectal cancer cells, have shown significant changes in their expression levels after vitamin D treatment. As already stated miR-192 and miR-194 were significantly altered with fold changes of 1.6 and 2.2 after 12 weeks of treatment.

A study in 2011, in which 107 human colorectal cancer tissues were screened for microRNA alterations, discovered decreased expression levels of miR-192, miR-194, and miR-215. (48) Interestingly, miR-215 was increased 1.7fold after 4 weeks of vitamin D treatment in our study. All three microRNAs, which were significantly decreased in the tissues taken by radical resection of colorectal carcinoma patients, were increased by

vitamin D treatment. As noted, the extent of direct reciprocal influence in-between circulating microRNAs and those in cancer tissue is unclear. However, an interference of microRNA levels in blood on specific types of tissue including cancer tissues cannot be ruled out. In this case, an increase of miR-192, miR-194, and miR-215 might have possible antiproliferative effects on colorectal cancer cells.

Another finding of the study was that the resected tumors with lowest expression levels of miR-192, miR-194 and miR-215 were the biggest. Furthermore, an upregulation of miR-194 caused a decrease of proliferation in colorectal cell lines. (48)

Two further studies discovered the role of miR-192/194 in colorectal cancer. The first one profiled microRNA levels in 40 cancerous tissues and stool samples. Levels of miR-194 were significantly lowered both in cancerous tissues and in stool samples. These findings could be interpreted as further evidence for tumor-suppressive effects of miR-194. This conclusion is supported by lower proliferation rates and high rates of cell death after artificial enhancement of miR-194 in a cell culture model and in xenograft mice. As a possible reason for this tumor suppressive effect, a down regulation of AKT2 signaling was hypothesized. (49) The second study was able to show an interconnection between miR-192 expression and metastasis formation in mice. Besides reduced cell viability, a boost in miR-192 expression caused decreased incidence of hepatic metastases. Concordantly to the results of the studies by Chiang and Zhao, in this study low expression levels of miR-192 were found in a cell culture model of colorectal cancer as well. (50)

All those results indicate a tumor-suppressive effect of both miR-192 and miR-194 in colorectal cancer. As these microRNAs have been reported to be differentially expressed in many other cancer types, consequent research needs to be done to fully understand the impact of these potential tumor suppressive microRNAs on cancer cells. A potential interference between miR-192/194 and vitamin D could be a promising possibility to influence microRNAs by oral medication.

A further microRNA, frequently dysregulated in cancer, is let-7. In the context of colorectal cancer, let-7 gets discussed contradictorily. Many authors classified let-7 as tumor-suppressive. (13) In our study, two members of the let-7 family were significantly decreased after 12 weeks of treatment. Let-7e was decreased 2.6fold and let-7a 1.5fold. Considering these results, vitamin D has potential pro-oncogenic effects. Due to that, vitamin D dependent alterations in microRNA levels have to be investigated in larger cohorts. In addition, a study of the year 2018 found high levels of let-7e in 84 tissues of patients suffering from rectal cancer. Highest fold changes were found in samples of

patients with metastases. Furthermore, high levels of let-7e were found along with high rates of cellular motility in a cell culture model. Thus, let-7e possibly drives metastasis. (51)

Taking these results into account, a vitamin D dependent reduction of circulating let-7e could affect rectal carcinogenesis in a positive way, preventing metastasis.

To sum up, in the present study we exhibited four microRNAs to be differentially expressed after treatment with vitamin D, which were reported to have influence on the pathogenesis of cancer. In particular, miR-192 and miR-194 were highly decreased in colorectal tissues. Our results showed an opposite effect on circulating microRNAs. Though, the effects on these microRNAs need to be investigated in a larger cohort. However, if a direct exchange of microRNAs in-between blood and cancer tissue can be proven, a supportive anti-tumor therapy by oral vitamin D intake could be possible.

4.3 Conclusion

In this diploma thesis, a relevant impact of oral vitamin D intake on microRNA metabolism was demonstrated. Despite the low random number of individuals, the results point out the direction for further research. From a clinical point of view, especially the functional interplay of circulating microRNAs in blood and the crosstalk of microRNAs within different tissues need to be further elucidated. Thus, new perspectives to describe disease specific profiles in blood will be set up. Besides the opportunity to use microRNAs as biomarkers, they may come up as therapeutic targets in the near future. The inhibition of specific microRNAs by expression vectors, anti-miRNA oligonucleotides, and small molecule inhibitors of microRNA is topic of intense research. Also, a gain of function by either local or systemic administration of specific microRNAs seems to be possible (52). Especially in cancer therapy, the tumor-suppressive effects of microRNAs are worth mentioning. However, the interplay of microRNAs in the complex pathophysiology of cancer and metabolic diseases has to be taken into account to avoid unwanted effects. Changing the microRNA metabolism by oral vitamin D intake in order to prevent pathophysiologic processes, even at smaller doses of vitamin D as used in the present study, can change a number of microRNA regulations. In this context, vitamin D might be used as an additional supportive therapy augmenting standard medication. In conclusion, vitamin D might have the potential to significantly affect important microRNA pathways and may serve as a model to influence these complex interactions.

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