universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„The impact of vitamin D, calcium and strength training on the RNA and gene expression in the elderly“

verfasst von / submitted by

Simon Srienc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magister der Pharmazie (Mag. pharm.)

Wien, 2020 / Vienna, 2020

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	Univ.-Prof. Mag. Dr. Karl-Heinz Wagner
Mitbetreut von / Co-Supervisor:	

“If you don’t believe you can do it, then you’ve got no chance at all” – Arsene Wenger

Danksagung

Ich würde mich gerne bei allen bedanken, die mir bei dieser Diplomarbeit geholfen haben. Insbesondere bei Herrn Univ.-Prof. Mag. Dr. Karl-Heinz Wagner und Frau Bsc. Msc. Agnes Draxler für ihren fachlichen Beistand und für ihre Geduld bei der Arbeit mit mir.

Großer Dank gebührt auch meinen Eltern und meiner ganzen Familie, deren moralische und auch finanzielle Unterstützung es mir überhaupt erst ermöglicht haben, dieses Studium abzuschließen.

Zu guter Letzt bedanke ich mich auch bei meinen Freundinnen und Freunden, die mir den Alltag eines Pharmaziestudierenden erleichtert haben und mir geholfen haben auch einfach einmal abzuschalten.

Zahvala

Rad bi se zahvalil pri vsemi, ki so me podpirali pri delu za svojo diplomsko nalogo. Posebno pri gospodu Univ.-Prof. Mag. Dr. Karl-Heinz Wagnerju in pri gospe Bsc. Msc. Agnes Draxler, ki sta mi pomagala pri strokovnimi vprašanji in sta tudi imela veliko potrpljenje pri delu z mano.

Najlepša hvala tudi mojimi starši in moji celi družini, ki so me preko celega študija moralno in tudi finančno podpirali. Brez vas to ne bi spravil.

Zahvaljujem se tudi pri mojih prijateljicah in prijateljih, ki so mi olajšali študentski čas.

Danke euch / Hvala vam

Contents

Figures

Tables

0. Abbreviations

CBs: calbindins

CPG: cytosine followed by guanine

CGI: a cluster of CpGs, “CpG island”

DBP: vitamin D binding protein

DNMTs: DNA methyltransferases

GFR: glomerular filtration rate

HATs: histone acetyltransferases

HDACs: histone deacetylases

HDMs: histone demethylases

HMTs: histone methyltransferases

IGF-1: insulin like growth factor 1

IIS: insulin and IGF-1 pathway

IU: international units

KDM1A: lysine specific demethylase 1

lncRNA: Long non-coding RNA

miRNA: micro RNA

ncRNA: non-coding RNA

NGS: next generation sequencing

PBS: phosphate buffered saline

PBMC: peripheral blood mononuclear cells

PCR: polymerase chain reaction

PTH: parathyroid hormone

RANK: receptor activator for Nuclear Factor κ B

RANKL: receptor activator for Nuclear Factor κ B ligand

ROS: reactive oxygen species

RT-qPCR: quantitative reverse transcription polymerase chain reaction

RXR: retinoic acid X receptor

TG: thioglycerol

TJ: tight junction

TRPV: transient receptor potential vanilloid

VDR: vitamin D receptor

VDRE: vitamin D responsive element

1,25(OH)2D3: calcitriol

25 (OH) D: 25-hydroxyvitamin D (calcidiol)

1. Introduction

It is well-known, that the percentage of older people in the human population is increasing. According to the WHO, the number of people aged 60 or older will double until 2050. The driving factors behind this are advanced medical treatment and a higher awareness for a healthy lifestyle, which is resulting in higher life expectancy. Overall, older individuals are also more susceptible to medical attention, resulting in higher medical costs and a higher health care utilization (World Health Organisation WHO 2015). Vitamin D deficiency is a global health issue, particularly prevailing in the elderly (Sahota 2014). The capacity of producing vitamin D in the human skin decreases by 75% in older people compared to the young, leading to low metabolic vitamin D levels, especially during the winter time and early spring (Hill et al. 2018). Vitamin D deficiency can lead to various kinds of diseases such as osteomalacia, muscle weakness or autoimmune diseases (Holick 2004). On the other hand, an appropriate vitamin D level is associated with the reduction of diseases and improvement of physical and mental capabilities (Wimalawansa 2019). Gene regulation and expression are very important focal points to understand the process of ageing, but also for the development of age-related diseases (Booth and Brunet 2016). As a well-known gene regulator, vitamin D and subsequently the vitamin D receptor are of great interest.

With the NutriAgeing project, the Department of Nutritional Sciences in Vienna, in combination with the Comenius University in Bratislava, conducted a many faceted study, that focused on examining the impact of vitamin D, calcium and resistance training on the elderly. As a part of this project, in this thesis, I place emphasis on changes to be expected after the intervention, regarding gene expression and the RNA. Furthermore, this thesis provides a broad overview of age-related processes and vitamin D, in respect to epigenetic changes.

2. Literature review

2.1 RNA

The ribonucleic acid (RNA), is one of the main building blocks in the human body. It consists of ribose nucleotides, connected by phosphodiester bonds, thus forming strands of varying lengths. It is composed of the nucleic acids: adenine, guanine, cytosine and instead of thymine as in the DNA, it contains uracil. RNA is single stranded, but can be self-complementary, so it can develop complex three-dimensional folding structures, which stabilize the molecule. There are three different main types of RNA: tRNA, mRNA and rRNA. Those are present in all organisms and take over functions in various cellular biochemical processes, including transportation of the genetic code and production of proteins. Additionally, changes in the RNA can contribute to the development of diseases. In terms of its function, RNA can be divided into two main groups. In the first, there is coding RNA and, in the second, non-coding RNA (ncRNA). One specific type of non-coding RNA is the microRNA (miRNA), which can disrupt the gene expression process by binding to the mRNA. Thus, the miRNA plays a role in cancer, neurodegenerative and other diseases (Kunal Chatterjee 1998). Besides miRNA, there is another type of non-coding RNA – the long non-coding RNA (lncRNA). The lncRNA has a length of more than 200 nucleotides and their main functions include maintaining the cell identity and cell differentiation. In addition, both types of non-coding RNAs have the ability to interact with other RNAs, creating thereby RNA-RNA hybrids. Those hybrids then engage in controlling the gene expression and biogenesis of other RNAs. Furthermore, the RNA in cells is always covered by a host of proteins – the RNA binding Proteins (Zavolan and Gerber 2018).

2.1.1 RNA and Ageing

The mRNA expression profiling is commonly used to examine changes in the gene expression of ageing humans. Especially in the brain, many functions, such as the upregulation of stress or immune response pathways show changes in the mRNA expression pattern. In addition, a decrease in expressed genes involved in energy

metabolism and neuronal functions is also attributed to a different mRNA expression pattern. (Somel et al. 2010)

Recent discoveries have shown that miRNA could play a significant part in ageing and the development of age-related diseases. As mentioned above, miRNA has the ability to bind to the mRNA of specific genes and thus prevent their translation. A normal senescent cell will stop growing and induce the programmed cell death. In the process of senescence, many tumor suppressors and death signals may hinder the normal functions of the cell. Interestingly, the expression of various tumor suppressors such as p53 or p21, is controlled by miRNAs. Other factors contributing to the senescence of the cell include the production of reactive oxygen species (ROS), shrinking of telomeres, mitochondrial damage and the production of tumor suppressor proteins. It has been discovered, that miRNAs are playing a role in all of those pathways and mechanisms. Studies with mice have shown a higher expression of miRNAs in senescent cells and studies with lower life forms have found a correlation between high miRNA levels and a longer lifespan. (Williams et al. 2016)

2.2 Ageing

Ageing is a normal process in the life of all living organisms, eventually leading to the death of cells. This process can be accelerated through numerous diseases, such as Parkinson's disease, Alzheimer's disease and other neuropathologic diseases. In addition, cardiovascular diseases, cancer and skin diseases are linked to ageing (Williams et al. 2016). Ageing is affected by a downturn of functionality on a molecular, cellular, tissue and organismal level (Booth and Brunet 2016). In 2013 Lopez-Otin et al. identified nine hallmarks, that constitute the development of ageing, which include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. All these factors are inter-connected thus it is difficult to observe their role in the ageing process in isolation. Each hallmark had to fulfil the following criteria: 1. It should manifest during the normal process of ageing. 2. Its aggravation in experiments should accelerate ageing. 3. Its amelioration should hinder the process of ageing.

Each of the hallmarks fulfils these requirements to varying degrees. (López-Otín et al. 2013).

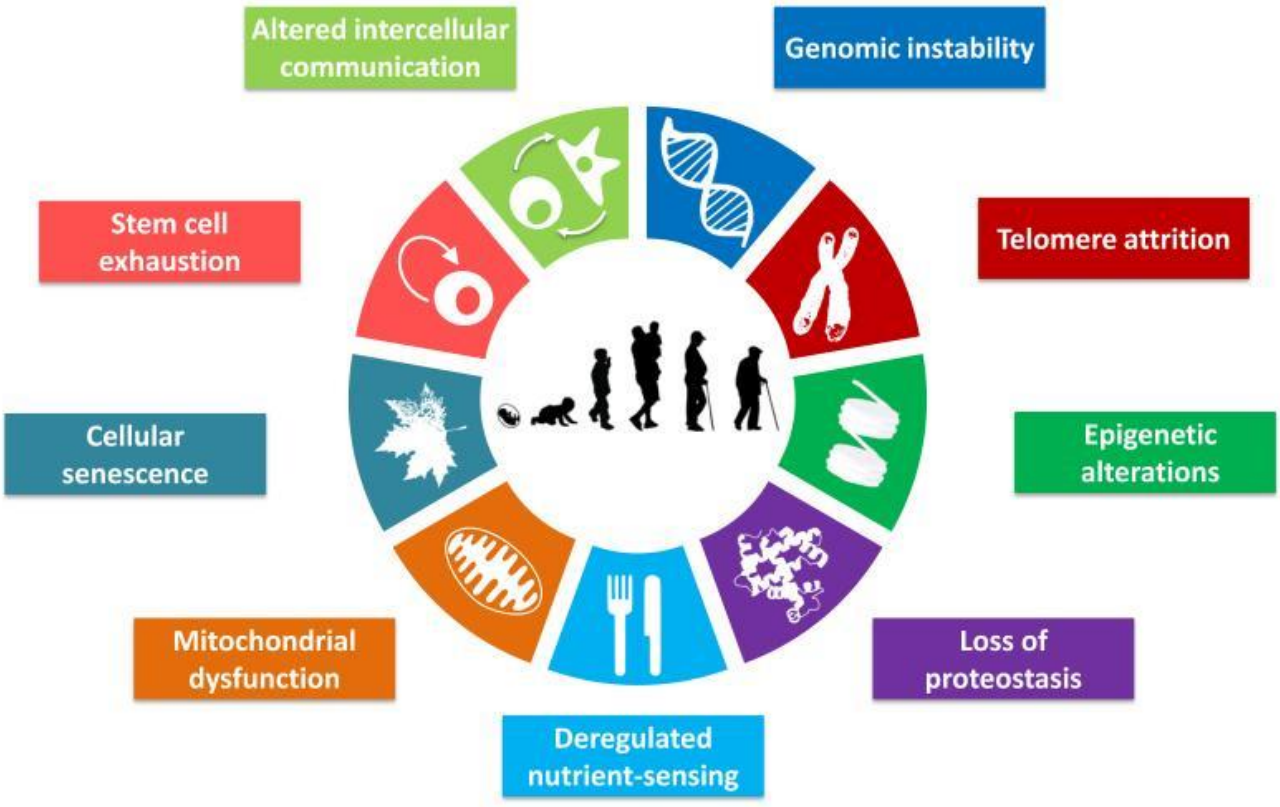

Figure 1: The nine hallmarks of ageing (López-Otín et al. 2013)

2.2.1 Genomic instability

The integrity and stability of DNA is permanently confronted by many exogenous challenges such as physical, chemical and biological ones. Additionally, endogenous threats, including DNA replication errors, spontaneous hydrolytic reactions and ROS, are a risk to the stability to the genome. Those exogenous and endogenous impacts can lead to damages such as point mutations, translocations, chromosomal gains and losses, telomere shortening, and gene disruption caused by the integration of viruses or transposons. As a consequence, the organism developed repair mechanisms for DNA damages, however these deteriorate with age. All those DNA changes can affect essential genes and transcriptional pathways, which may lead to dysfunctional cells. Damaged cells can then lead to the disorder of tissues and affect

organismal homeostasis. The damage is especially dangerous, when it affects the stem cells, particularly since they are playing an essential role in tissue renewal. All those mechanisms lead to an accumulation of genetic damage, which is a substantial factor for the development of ageing (López-Otín et al. 2013).

2.2.2 Telomere attrition

Telomeres are particularly vulnerable to age-related deterioration, because the regular replicative DNA polymerase lacks the ability to fully replicate the terminal ends of linear DNA molecules. Such a replication is accomplished by a special polymerase called telomerase, which is not expressed in most mammalian somatic cells. This circumstance leads to a progressive loss of telomere-protective sequences from the end of the chromosomes (Rebelo-Marques et al. 2018). Telomeres act similarly to DNA breaks, but cannot be detected by repair mechanisms, resulting in persistent DNA damages, which are subsequently leading to senescence and/or apoptosis of cells. In addition, telomere deficiency is correlated with premature development of diseases. (López-Otín et al. 2013)

2.2.3 Epigenetic alterations

Epigenetic alterations also belong to the nine hallmarks of ageing and are described in chapter 2.5.5.

2.2.4 Loss of proteostasis

In order to preserve the stability and functionality of their proteomes, all cells have a variety of quality control mechanisms at their disposal. Proteostasis includes both, mechanisms to stabilize the folded protein, most notably by the family of heat shock proteins, and mechanisms for degradation of proteins by proteasomes or lysosomes. These mechanisms are working in a coordinated manner, restoring the structure of misfolded proteins or simply removing and destroying them completely. These processes ensure, that the intracellular protein renewal is working sufficiently and damaged components are not being accumulated. However, with increasing age, especially the proteolytic system through proteasomes and lysosomes declines. This decline results in a harmed proteostasis, that is associated with ageing and age-related diseases, such as Parkinson’s and Alzheimer’s disease and cataracts. (López-Otín et al. 2013)

2.2.5 Deregulated Nutrient-sensing

The intracellular signalling pathway of IGF-1 and insulin signalling, together known as ‘insulin and IGF-1 signalling’ (IIS) pathway, are the most sustained age-related pathways in evolution. IGF-1 is the secondary mediator for the growth hormone. When both are reduced, the reduction leads to longevity. However, paradoxically, during the normal process of ageing as well as in longevity, the IIS pathways and growth hormones are declining. That is leading to the assumption, that a decreased IIS pathway and growth hormone level, through lessening cell growth and metabolism, minimizes the systemic damage in the organism. In conclusion, this means that the defence mechanisms to prevent ageing, are eventually becoming harmful and might lead to an exaggeration of ageing. Other important nutrient-sensing systems besides the IIS pathway, that is responsible for glucose sensing, are: the mTOR, for sensing high amino acid concentrations, the AMPK, for detecting high AMP levels and sirtuins, for detecting high NAD+ levels (López-Otín et al. 2013).

2.2.6 Mitochondrial dysfunction

During ageing of cells and organisms, the effectiveness of the respiratory chain is decreasing, leading to a leakage of electrons and a reduced ATP production. Mitochondrial dysfunction increases the production of ROS, which is further inducing damage to the cell and mitochondria. ROS have always been considered as a major agent in the process of ageing. Although, recent studies have suggested, that ROS are a trigger for the production of proliferative and survival signals in stress situations and may actually have a beneficial effect on longevity. However, in advanced age and at higher ROS levels, the ageing process worsens, leading to conflicting data regarding their role in ageing. Besides ROS, there are multiple other factors, that can lead to mitochondrial damage and contribute to ageing. These factors include increased apoptosis, inflammatory reactions, reduced biogenesis of mitochondria, mutations in the mitochondrial DNA, oxidation of mitochondrial proteins and more (López-Otín et al. 2013).

2.2.7 Cellular senescence

Cellular senescence has been described as a blockage of the cell cycle combined with stereotypic molecular changes. Cellular senescence can be triggered by telomere shortening, but also by DNA damage or the de-repression of the INK4/ARF locus. However, similarly to the production of ROS, cellular senescence is a self-protective mechanism. Its intention is to remove damaged cells and induce their elimination by the immune system. After the removal of these cells, they must be replaced, which leads to a balance between these two processes. However, at an advanced age, this system can become ineffective and errant, thus an increase in senescent and damaged cells can occur (Rebelo-Marques et al. 2018).

2.2.8 Stem cell exhaustion

The diminishing ability to regenerate tissues is a major sign of ageing. Important processes such as the haematopoiesis decline, result in anaemia and myeloid malignancies (López-Otín et al. 2013). The ability of haematopoietic stem cells to cell division decreases with age, leading to negative long-term consequences for the organism. On the other hand, the over expression of stem cells also has a detrimental effect, as stem cell niches can get exhausted. However, physical exercise can migrate stem cells to impaired tissues, leading to their engraftment and regeneration (Rebelo-Marques et al. 2018). Overall, stem cell exhaustion can be regarded as one of the main culprits for tissue and organismal ageing (López-Otín et al. 2013).

2.2.9 Altered intercellular communication

Ageing is known to alter intercellular communication on the neuroendocrine, endocrine and neuronal level. Therefore, neurohormonal signalling pathways, such as the renin-angiotensin or the adrenergic system decrease, whereas inflammatory reactions occur more frequently. The organisms ability to identify and handle pathogens or malignant cells deteriorates and the overall composition of the peri and extracellular space alter, changing the mechanical and functional attributes of all tissues. (López-Otín et al. 2013)

2.3 Calcium

It is well established, that the Ca^{2+} ion is an extremely important and essential component of the human body, with approximately 99% of it located in the skeleton. There, it helps to preserve the strength and the rigidity of the bone (Emkey and Emkey 2012). Plasma calcium homeostasis plays also a big part in life enabling processes, such as: maintenance of the skeleton, regulation of hormonal secretion, transmission of nerve impulses and vascular activities. It is mainly taken up through dietary sources or supplementation. Excessive calcium is excreted either by faeces or urine. The two main hormones, which have an impact on calcium homeostasis are parathyroid hormone (PTH) and calcitonin. When serum calcium levels are decreasing, PTH secretion increases and subsequently releases calcium from the bone. In addition, it releases calcitriol, which improves calcium absorption. However, when serum calcium levels are increasing, calcitonin is being released, which suppresses the calcium release from the bone and reduces the calcium reabsorption from the kidney tubes (Li et al. 2018). Factors such as growth, pregnancy or obesity can increase the absorption of calcium, whereas others like ageing or the menopause can reduce it (Emkey and Emkey 2012). Various studies are showing that the intake of calcium has a positive effect on the prevention of osteoporosis, especially in postmenopausal women, but also in people of different sex and age groups. Furthermore, calcium supplementation also seems to decrease the risk of cardiovascular diseases and gastrointestinal cancer. Calcium also shows an effect in reducing the number of fractures (Li et al. 2018).

Excessive intake of calcium has been associated with hypercalcemia, hypercalciuria, nephrolithiasis, constipation, vascular and soft-tissue calcification, interactions with zinc and iron, and prostate cancer (Emkey and Emkey 2012). Issues with calcium homeostasis have also been associated with bone diseases and metabolic diseases and an increased risk of epithelial cancer (Diaz de Barboza et al. 2015).

2.3.1 Intestinal Ca^{2+} absorption

The intestinal Ca^{2+} absorption is an essential process for the maintenance of Ca^{2+} absorption and bone health. Two main mechanisms are responsible for this process. The first one is the so-called transcellular pathway, which is a saturable, metabolically driven transport, mainly regulated by vitamin D. The second one is the paracellular pathway, which is a non-saturable passive transport. The transcellular pathway is predominantly used, when the Ca^{2+} intake is low, as the Ca^{2+}channel TRVP6 and the Ca^{2+} carrier Calbindin (CB), which are both essential for this pathway, are rate-limited and downregulated, when there are higher Ca^{2+} levels (Diaz de Barboza et al. 2015). In addition, the sojourn time of Ca^{2+} in the duodenum and jejunum, where the transcellular pathway mostly takes place, is short. At high Ca^{2+} levels in the blood, the paracellular transport reaches greater importance, as it takes place at the whole length of the intestines (Bronner 2003).

2.3.2 The transcellular pathway

The transcellular pathway implicates Ca^{2+} movement from the mucosal-to-serosal side of the intestinal barrier that occurs against a concentration gradient. It is regulated by nutritional and physiological factors and prevalent in the duodenum and jejunum (Diaz de Barboza et al. 2015). Vitamin D or, more precisely, the hormonal metabolite 1,25(OH)2D3 is, within nutritional and physiological factors, the main regulator of the transcellular pathway (Pérez et al. 2008).

The channels mainly responsible for the entry step of Ca^{2+} into the intestine are the epithelial Ca^{2+} channels TRPV5 and TRPV6, albeit that TRPV5 is expressed 100- to 1000- fold less than TRPV6 in the intestine (Kellett 2011). Those channels are mainly expressed in kidneys and the intestines, but also in other organs, and permeate divalent cations. Those channels have a constitutively active Ca^{2+} permeability and a high selectivity for the Ca^{2+} ion (Pérez et al. 2008).

The L-type channel $Ca_V1.3$ is also capable of active, transcellular transport of Ca^{2+} in the intestine. It appears to complement the role of TRPV6 channels. The TRPV6 channel has an important role in the phases between meals, when the membrane repolarizes and is also vitamin D dependent. Over-night or during starvation, the

apical membrane repolarizes and intestine gradually atrophies, so that Ca^{2+} lost into the lumen by desquamation must be recovered by TRPV6. Whereas the $Ca_v1.3$ channel activates, when the membrane depolarizes, therefore the combination of the two channels provides an important regulator of extracellular Ca^{2+} levels (Kellett 2011).

Calbindins (CBs) are proteins thought to be responsible for transporting Ca^{2+} from the apical side of the enterocytes to the basolateral membrane of the cell. The most important CB is the CB_{9k}. It has been proposed, that CB_{9k} not only carries the Ca^{2+} ions, but also creates a buffer and thus prevents the Ca^{2+} from reaching toxic levels, especially during phases of high Ca^{2+} influx. It must be noted, that due to controversial data from genetic studies, the exact role of CB_{9k} in the Ca^{2+} homeostasis is not yet fully known, as the calcitriol-mediated active transport of Ca^{2+} still occurs, when CB_{9k} and TRPV6 are not present. This absence during the active transport suggests that there are other channels and/or proteins involved in the transcellular pathway (Christakos et al. 2017).

2.3.3 Ca2+ pump and Na+/Ca2+ exchanger

The exit of Ca^{2+} from the enterocytes is facilitated by the Ca^{2+} pump (PMCA) and the Na+/ Ca^{2+} exchanger (NCX1). There are several subtypes of the PMCA, with PMCA1b being the main protein, which brings Ca^{2+} which brings Ca^{2+} out of the enterocyte. Whereas NCX1 is just responsible for around 20% of Ca^{2+} excretion (Diaz de Barboza et al. 2015).

2.3.4 The paracellular pathway

As mentioned above, the paracellular pathway is a passive transport mechanism, located on the whole length of the intestines. The molecules permeate the membrane through tight junctions (TJ). Transmembrane proteins, including occludin and claudins, are part of the TJs. Those proteins are synthesized in adjacent cells and in charge of closing the junctions, thus inhibiting the free movement of molecules through the paracellular space (Diaz de Barboza et al. 2015). Movement of Ca^{2+} through the TJs is a process, that depends on concentration and the electric gradient, across the epithelium (Pérez et al. 2008). Furthermore it has been

suggested, that calcitriol has an effect on the paracellular pathway, through regulating the tight junctions (Christakos et al. 2017).

2.4 Vitamin D

Vitamin D is a hormone that is essential for the development, growth and maintenance of the human skeleton. Its main function is the regulation of the calcium homeostasis (as mentioned in 2.3 Calcium) (Holick 2003). However, vitamin D is also involved in many non-calcaemic processes, including stimulation of insulin production, modulation of activated T and B lymphocyte function, effects on myocardial contractility, prevention of inflammatory bowel disease and promotion of thyroid-stimulating hormone secretion. More than 90 % of the daily vitamin D intake comes from the absorption of UVB light (290-315 nm) in the skin (Holick 2004). To a lesser extent, it can also be derived from dietary sources. Plants and fungi obtain Vitamin D2 (Ergocalciferol), whereas Vitamin D3 (Cholecalciferol) can be found in animal products, such as fish oils, oily fish, eggs and dairy products (Laird et al. 2010). The daily dietary intake only amounts to 2-4 µg (Deutsche Gesellschaft für Ernährung 2012). It is assumed that Vitamin D3 has the greater effect on 25-hydroxyvitamin D (25(OH)D) levels than Vitamin D2 (Armas et al. 2004; Tripkovic et al. 2012). In contrast to ergocalciferol, cholecalciferol has a higher conversion rate to 25(OH)D and a higher affinity to both, vitamin D binding protein (DBP) and vitamin D receptor (VDR) (Tripkovic et al. 2012).

2.4.1 Sufficient and insufficient vitamin D levels

According to the German Society for Nutrition, vitamin D deficiency is occurring at cholecalciferol levels below 20 ng/ml, which corresponds to 50 nmol/L (nmol/L = ng/ml * 2.5). Without endogenous vitamin D production, a daily intake of 20 µg vitamin D would be required to sustain the sufficient level of 20 ng/ml. (Deutsche Gesellschaft für Ernährung 2012). It is well established, that a severe vitamin D deficiency (<10 ng/ml) over a long period of time, is leading to rickets in children and osteomalacia in adults (Laird et al. 2010). Furthermore, vitamin D deficiency can lead to muscle weakness, autoimmune and cardiovascular diseases, type 2 Diabetes and mental diseases such as depression (Hoffmann et al. 2015).

Additionally, sufficient vitamin D levels can decrease the risk of various types of cancer such as prostate, breast, colon, gastric and more. However, accumulating data suggests, that cancer cells are able to weaken the antitumorigenic effects of vitamin D. With vitamin D playing an important role in several mechanisms, which support the immune system such as DNA damage repair or antioxidant defence, still a lot of research has to be done to maximize the cancer preventing effects of the hormone (Jeon and Shin 2018). More in 2.5.1 – vitamin D and cancer prevention.

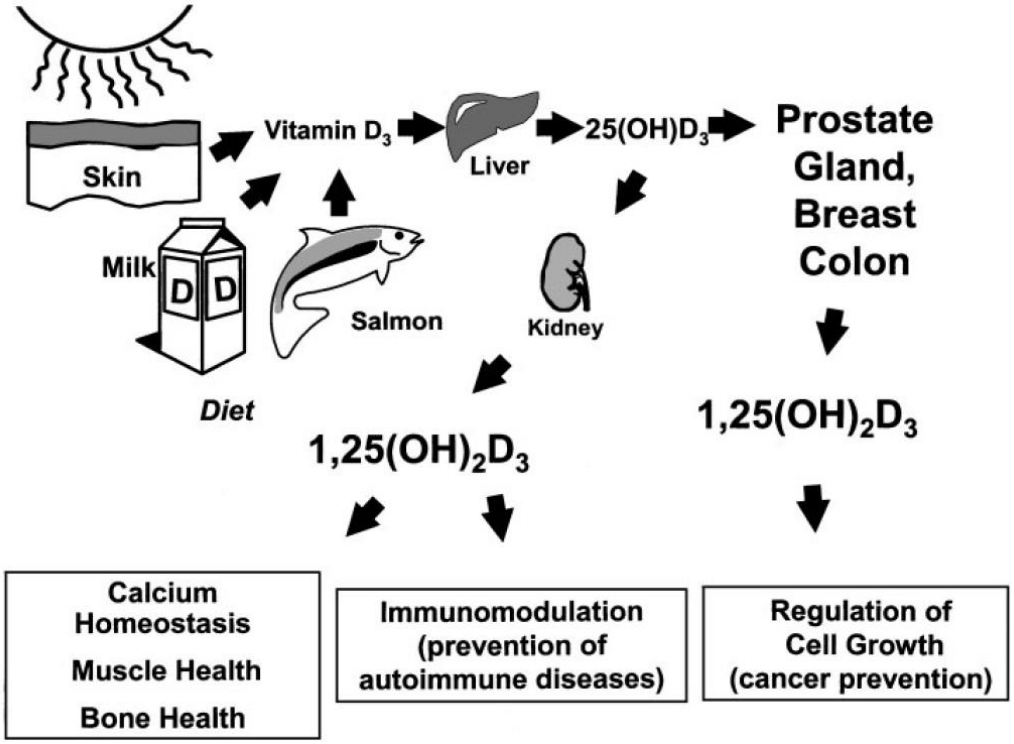

Figure 2: Production, metabolism and biologic function of Vitamin D3. UVB-light and animal products are the main sources of vitamin D. There are various organs and tissues in the human body, which can hydroxylate calcidiol into the more potent calcitriol, with the kidney being the main one. Vitamin D affects calcium homeostasis, muscle health and bone health. Additionally, it plays a significant part in the prevention of autoimmune diseases and cancer. (Holick 2003)

2.4.2 Vitamin D synthesis

UVB light penetrates the skin. In epidermal and dermal layers of the skin, light is absorbed by 7-dehydrocholesterol. The UVB rays convert 7-dehydrocholesterol into pre-vitamin D3, by cutting the bond between carbons 9 and 10. Subsequent isomerization is transforming pre-vitamin D3 into cholecalciferol (as can be seen in

figure 4). The newly formed Vitamin D3 is released from the skin and binds to the DBP in the extracellular space (Holick 1994). From there it is transported to the liver. It is reported, that an exposure to sunlight between 10 and 15 minutes in the summer, is producing the maximum amount of Vitamin D. Excessive exposure to UVB light leads to the production of the inactive photoproducts lumisterol and tachysterol, which have just a minor impact on the calcium homeostasis. This mechanism prevents Vitamin D toxicity from sun exposure (Laird et al. 2010).

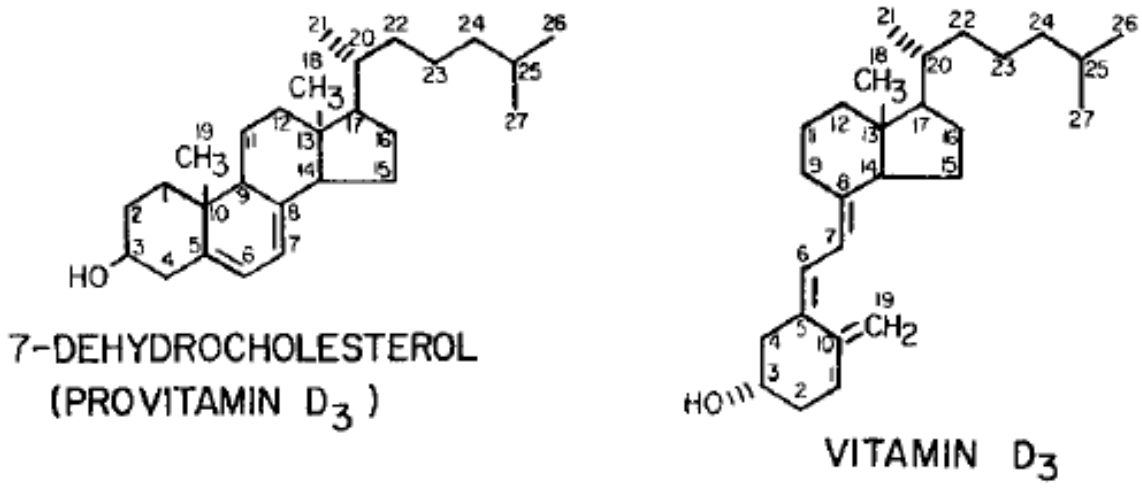

Figure 3: Vitamin D3 and its precursor, 7-dehydrocholesterol (Holick 2003)

2.4.3 Vitamin D metabolism

Within the liver, the vitamin D is hydroxylated by the 25-hydroxylase, thus forming the major circulating type 25(OH)D3, also known as calcidiol. In this form, it is binding to DBP, which is transporting 25(OH)D3 to the proximal tubule cells of the kidney. Inside the cell, 25(OH)D3 is hydroxylated by the 1-α hydroxylase to the biologically active form: 1,25(OH)2D3, also known as calcitriol. The 1-α hydroxylase has been found not just in the kidney, but also in other tissues such as colon, brain and pancreas (Laird et al. 2010). 1,25(OH)2D3 is binding to the VDR in the small intestinal cells. There, calcitriol and VDR are building a complex with the retinoic acid X receptor (RXR) in the nucleus. This complex is binding to the vitamin D responsive element (VDRE) of the TRPV6, which is enabling a greater absorption rate of calcium (Holick 2006). In addition, 1,25(OH)2D3 is interacting with the VDR in osteoblasts, which is leading to an increased expression of the receptor activator for Nuclear

Factor κ B ligand (RANKL). RANK (Receptor activator for Nuclear Factor κ B) is binding the RANKL, thus transforming a preosteoclast into an osteoclast. The osteoclast is releasing hydrochloric acid, amongst other chemicals, which is then releasing calcium from the bone into the circulation (Laird et al. 2010). As a result of that mechanism, vitamin D helps maintaining the calcium homeostasis, even, when dietary calcium levels are low (Holick 2006).

2.4.4 Vitamin D receptor

Given the essential role of Vitamin D in the calcium and phosphate homeostasis, the highest amount of the VDR is expressed in the intestine, bone, kidneys and the parathyroid glands. Interestingly, the VDR has also been found in non-calcium-regulating cell types including dermal fibroblasts and keratinocytes of skin, immune cells, selected cardiovascular cell types, and cellular components of numerous other tissues. This information is indicating, that there are many different major biological functions of vitamin D, besides the regulation of mineral homeostasis (Pike et al. 2017).

As mentioned above 1,25(OH)2D3 is binding to the VDR, which is then forming a heterodimer with RXR, thus triggering a gene response. The VDRE is known as the “classic” binding site for genes, that are induced by 1,25(OH)2D3. Through the VDR, vitamin D is regulating the vast number of genes. The binding of VDR to the relies to vitamin D and it reaches a much higher level, when 1,25(OH)2D3 is present. (Pike et al. 2014).

2.4.5 Vitamin D and ageing

Vitamin D is the main regulator of calcium absorption (see 2.3 Calcium). At an advanced age, calcium absorption decreases as a consequence of lower calcidiol and calcitriol levels in the human body. There are a number of reasons, leading to reduced circulating 1,25(OH)2D3 levels. With age, the renal function is deteriorating, prompting a decreasing hydroxylation of 25(OH)2D3 to 1,25(OH)2D3. Although dietary intake of vitamin D can improve calcium absorption, renal impairment still significantly weakens its effect. If the glomerular filtration rate (GFR) drops below 50 ml/min., both, a lower calcium absorption and increased PTH levels have been observed. Other factors, that explain lower calcium absorption with age include a

higher resistance of the bowel mucosa to vitamin D and its metabolites and decreasing oestrogen levels in women after the menopause (Hill et al. 2018). A lower vitamin D status is also attributed to a declining dermal capacity of producing vitamin D out of 7-dehydrocholsterol, as well as to lower 7-dehydrocholesterol levels in the skin of elderly people. It has been estimated, that the dermal capacity of producing vitamin D in senior citizens (65 years or older) decreases by about 75%, when exposed to the same amount of sunlight, compared to the young (Hill et al. 2018). In addition, with age and vitamin D deficiency, the expression of the VDR decreases. This decline could be a possible explanation for the increased risk of falls, fractures and muscular weakness in the elderly (Hill et al. 2018). 1,25(OH)2D3 is also known to slow down the ageing process, through decreasing oxidative stress, cell and tissue damage. On the other hand, when vitamin D levels are low, there is an increase in oxidative stress and systemic inflammation (Wimalawansa 2019).

2.5 Epigenetics

The term epigenetics describes all factors, that result in changes in the gene functionality, without changing nucleotide sequences. The most common epigenetic mechanisms are post-translational modifications, including methylation and acetylation (Carlberg 2017). During methylation, a methyl group is added to the C5 position of cytosine, thus forming 5-methylcytosine (Moore et al. 2012). Histone acetylation occurs when histone acetyl transferases (HATs) acetylate the amino side chain of lysine on histones (Marmorstein and Zhou 2014).

DNA and histones form a complex, which is called chromatin. The density of chromatin may vary in different regions, with the open euchromatin containing mostly active genes and the condensed heterochromatin mainly inactive genes. Chromatin modifying enzymes though, can make highly dense heterochromatin accessible, creating facultative heterochromatin. Methylations and acetylations can change the structure of chromatin. Histone acetylations are often involved with transcriptional activation, whereas methylations are mostly mediating chromatin repression, albeit in some cases they also activate chromatin (Carlberg 2017).

As mentioned above, the active form of vitamin D3, 1,25(OH)2D3, is the high affinity ligand of the VDR and the receptor is the only target of 1,25(OH)2D3 in the nucleus

(Carlberg 2017). Genome wide analysis showed, that VDR is predominantly binding to loci of open chromatin. When the ligand is binding to the VDR, up to 30% more chromatin is made accessible. Many nutrients, such as folic acid and vitamin B are well-known for their influence on DNA methylation. Whereas the epigenetic effect of vitamin D is mainly associated with histone acetylation. The role of vitamin D in DNA methylation is still not fully understood (Fetahu et al. 2014).

With vitamin D being a major “controller” of systemic inflammation, oxidative stress and mitochondrial dysfunction, it also plays an important role in the ageing process (Wimalawansa 2019). Additionally, 1,25(OH)2D3 is regulating different pathways with the intention of modulating cell proliferation. For instance, an elevation of the transcription factor NF-κB, is associated with many diseases such as cardiovascular diseases, Diabetes type 2, rheumatoid arthritis and others. Vitamin D has the ability to intervene in pathways related to NF-κB and block them. As NF-κB is also connected to oxidative stress and cell response to inflammatory processes and injury, 1,25(OH)2D3 has also an impact on them. Furthermore, these pathways have an impact on mitochondrial respiration, on the overproduction of ROS and connected to that, on cellular and DNA impairment (Wimalawansa 2019). In addition to calcitriol, calcidiol has an effect on the management and subsequent elimination of entering microbes, parasites and toxins (Wimalawansa 2019).

Another transcription factor of interest is the Nrf2, which takes a central part in the protection of cells against oxidative stress. When calcitriol binds to the VDR, it releases Nrf2, which then regulates gene expression, and increases the number of antioxidant genes (Wimalawansa 2019).

2.5.1 Vitamin D and cancer prevention

The positive effects of vitamin D regarding cancer prevention, can be explained through the reduction of cell proliferation and the increase of cell differentiation, after epigenetic modulation of vitamin D pathways (Wimalawansa 2019). Additionally, it is suggested, that vitamin D slows down cancer progression through anti-inflammatory, immunomodulatory, proapoptotic and antiangiogenic effects. Thus, leading to a lower cancer mortality rate. However, recent studies do not support the lowering of cancer

incidence by vitamin D. Further, there is conflicting evidence regarding the effect of vitamin D on the reduction of the cancer mortality rate. (Manson et al. 2018)

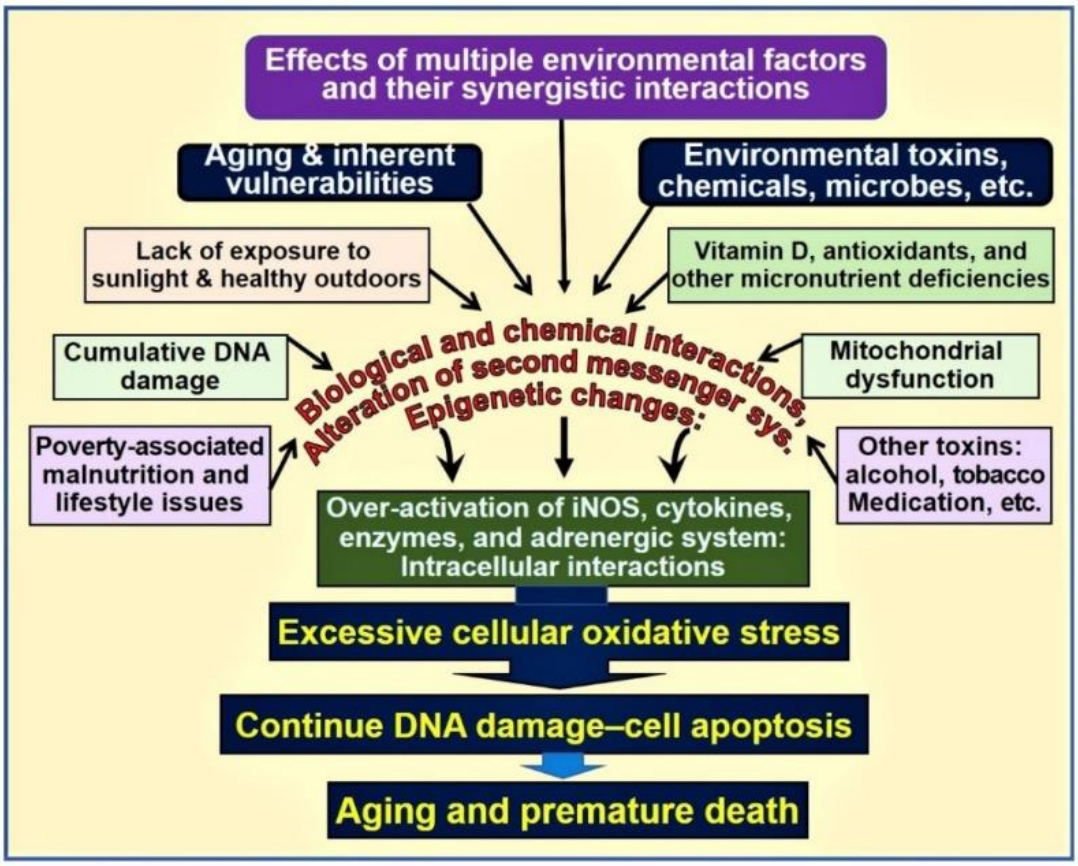

Figure 4: Chemical and biological effects, which can lead to epigenetic alterations and therefore enhance the ageing process (Wimalawansa 2019)

2.5.2 DNA methylation

The most thoroughly studied epigenetic marker is DNA methylation. It occurs most frequently, when a cytosine nucleotide is followed by guanine (CpG). Many CpGs in proximity to each other form CpG islands (CGI). DNA methylations are involved in many important biological processes, including cell cycle, differentiation and genomic imprinting. The enzymes responsible for methylation are the DNA methyltransferases (DNMTs) (Fetahu et al. 2014). The DNMTs catalyse the reaction to add the methyl group onto the C5 position of cytosine and thus form 5-methylcytosine (Moore et al. 2012). The reduction or loss of global DNA methylation, for example in the cancer epigenome, can be resulting in disturbances in the genome. However, hypermethylation of promoter regions of tumor suppressor genes is leading to a

failure of the expression of key genes. Thus, hypermethylation is affecting pathways, connected with maintenance of cellular functions, such as cell cycle, apoptosis and DNA repair. The hypermethylation of promoter regions of tumors, is also known to silence tumor suppressor genes (Fetahu et al. 2014).

Although the mechanisms behind are not entirely clear, it is documented, that 1,25 (OH)2D3 is capable of triggering DNA demethylation. It is likely that mostly passive demethylation is taking place over several cycles of DNA replication. Albeit, also active transport can happen, taking between one to four hours. Studies with different cancer cells are showing, that vitamin D can alter DNA methylation and thus regulate the aforementioned processes, associated with hypermethylation (Fetahu et al. 2014).

2.5.3 Histones, epigenetics and vitamin D

As mentioned above, histones are a part of the building blocks of chromatin. They can be modified post-translationally as well, mostly through acetylation, methylation and phosphorylation. When genes are epigenetically silenced, the hypermethylation of CGIs is often connected to the loss of acetylation and the loss or gain of methylation in specific parts of the histone. Acetylation of the histone is commonly associated with the activation of the transcription. This process is being controlled by two enzymes: the histone acetyltransferases (HATs) and the histone deacetylases (HDACs). The interaction between these two enzymes is essential in the regulation of gene expression and is also regulating different developmental processes and states of diseases (Fetahu et al. 2014). The HATs usually act as the activators of transcription, whereas HDACs are known as the repressors. Overexpression of the HDACs is associated with proliferation of cancer or tumor cells. Although, the silencing of specific HDACs, can also be leading to tumor growth inhibition. Histone methylation on the other hand, can be leading to both, gene expression and repression.

Similar to histone acetylation, the methylation is catalysed by two enzymes. On one hand there is the histone methyltransferase (HMT) and on the other the histone demethylase (HDM) (Fetahu et al. 2014). HMTs mediate histone modifications, whereas HDMs remove those modifications (Marmorstein and Zhou 2014). An

example for an HDM would be the lysine specific demethylase 1 (KDM1A), which is associated with negative outcomes of various cancer types, when it is highly expressed. The VDR has an influence on the activity of HDMs. 1,25(OH)2D3 can have an inhibiting or activating effect on the expression of HDMs. For example, vitamin D is associated with inducing the expression with specific demethylases, such as KDM6B, which is a histone mark, connected to gene repression in tumor cells (Fetahu et al. 2014).

2.5.4 The importance and role of Vitamin D in epigenetics

It is assumed that the vitamin D system is responsible for the regulation of about 3% of the human genome. Thus, keeping the vitamin D system in the right balance is critical (Fetahu et al. 2014). The liganded VDR, that are not involved in the calcium-homeostasis, have a big influence on the regulation of the expression of genes, that are responsible for cell proliferation, differentiation and apoptosis. The major hindrance in the therapeutic use of vitamin D to take advantage of these effects, is the resistance of cancer cells to vitamin D. Epigenetic mechanisms, such as promoter methylation of specific vitamin D system genes or their corepressors, could be responsible for a weakened responsiveness of VDR to 1,25(OH)2D3.

The most important enzymes for regulating vitamin D levels and signalling are: CYP2R1, CYP24A1, CYP27B1 and, of course, VDR. The major problem with these enzymes is, that they are very much susceptible to epigenetic modification. All four of the aforementioned enzymes have either CPGs or CGIs in and around their promoter regions. Thus, through DNA methylation and histone modification of these regions, the state of the chromatin can be changed from open to closed. As mentioned above (2.5 epigenetics), the VDR is binding to open loci of chromatin with greater affection. Therefore, when the chromatin is closed, the genes for the VDR, as well as the other enzymes are being repressed (Fetahu et al. 2014). Several studies have shown, that the expression of those genes is being deregulated in multiple types of cancer. Epigenetic changes are suspected to play a part in that process. Looking at the example of the VDR, which is very rarely mutated, a methylation of the promoter region is a reasonable explanation of a reduced sensitivity for vitamin D. Several

studies confirm this theory, showing highly methylated VDR genes in various cancer cells (Fetahu et al. 2014).

2.5.5 Epigenetics and Ageing

The regulation of gene expression is not only the key in determining the cell functions, but small variations can change the cells destiny. Epigenetic factors, such as transcription factor binding, DNA methylation, histone marks, nucleosome positioning and non-coding RNAs are central for the regulation of genes. Alterations in any of these factors, are leading to changes in gene expression, thus initiating epigenetic changes. Although it is still unclear what exactly is triggering these epigenetic variations, it is known that they occur with age (Booth and Brunet 2016).

Gene regulation is very sensitive, one alteration in a single transcription factor can change the function of cells or even tissues. Thus, changes in gene regulation frequently occur within the process of ageing, prompting the development of ageing phenotypes and diseases. In addition, through transcriptional networks and chromatin modifiers, a cell can react and respond to extracellular and intracellular signals. Chromatin marks are enduring and show a progressive alteration, that recurs in all cells, thus chromatin marks can serve as a memory, that helps to propagate age-induced cell dysfunction. According to recent evidence, changes in the epigenome are occurring very timely in the ageing process and could even be the cause of it (Booth and Brunet 2016).

3. Materials/ Methods/ Results

3.1 The NutriAging project

The NutriAging project is a cross-border cooperative project between the University of Vienna and the Comenius University in Bratislava. The aim of the project is to raise the awareness of seniors to better nutrition and lifestyle, thus promoting “healthy” ageing. Furthermore, the project aims to reduce the development of age-related diseases such as mental diseases, cardiovascular diseases, diabetes mellitus in the cross-border region and with that, the reduction of costs in the health sector. Additionally, the effect of selected nutrients like vitamin D, omega 3 fatty acids and proteins on the well-being of the elderly are being examined. The foundation of this thesis, was the vitamin D study, which investigated the effects of vitamin D and calcium in combination with resistance exercise on the health of the elderly (Wagner, Karl-Heinz, Muchova Jana 2019).

3.1.1 Study design

The study was designed as an intervention study and operated as a double-blind, randomized, placebo-controlled trial. The study included three groups. Group one received a placebo in form of a calcium preparation. Group two received a daily dosage of vitamin D and daily calcium. Group three received a higher dose of vitamin D once per month and daily calcium. Then, all groups participated in resistance training over the course of ten weeks. The total study duration was 17 weeks and included three major assessments: t0 at baseline, an intermediate assessment t1 after the supplementation and the final assessment t2 after the training. Additionally, three minor assessments were conducted to control the serum vitamin D level.

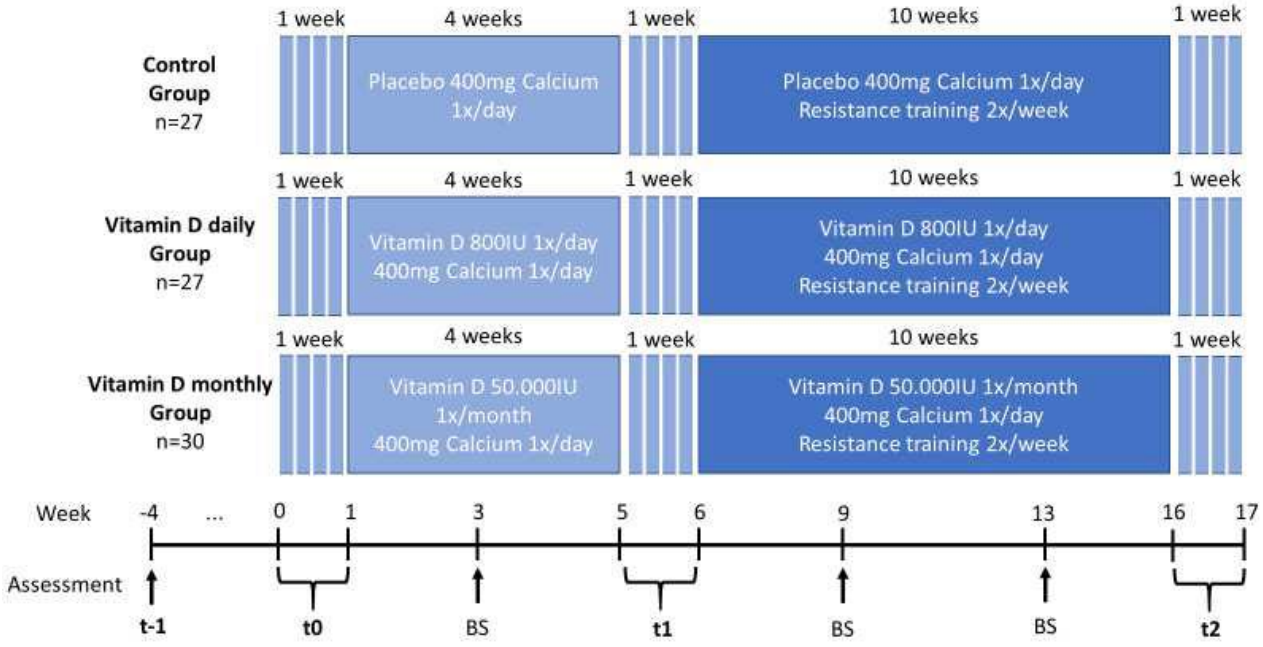

Figure 5: The design of the vitamin D study

3.1.2 General procedure

Figure 5 illustrates the flow chart of the study. At t-1 the eligibility of the candidates was assessed. After being randomized into the different intervention groups, in order to provide baseline data, the participants were examined prior to the intervention at baseline t0. Four weeks after t0 and the subsequent start of the nutritional intervention, the second major assessment was being conducted.

After t1, over the course of ten weeks, the test persons took part in the resistance exercise training and thus reaching t2. At that point, after 16 weeks of the intervention, the participants were examined again, providing the data after the intervention with vitamin D and resistance exercise. In addition to t0, t1 and t2, blood samples were taken after three, nine and thirteen weeks. Apart from different intervention groups, the subjects were also divided in different subgroups ranging from A to E. This measure was taken to ensure, that lab staff could cope with the amount of blood samples provided. Therefore, seven weeks of consecutive testing was conducted, in which the participants in group A were examined in the first week and the participants of group E in the last one, with the other groups in between in alphabetical order. Mondays and Tuesdays were mostly the days where blood

samples were taken, anthropometric tests conducted and the subjects were familiarized with the functional tests. The funcitonal tests were then carried out on Wednesday, Thursday and Friday.

After t0, the participants were provided with their supplements. Resistance training started after t1, meaning that after the 6th week the subjects received supplements and performed the guided exercise. In addition to blood donations, anthropometric testing and resistance training, also urine and faecal samples were taken. T2 signified the finish of the intervention and thus the finish of the study for the participants.

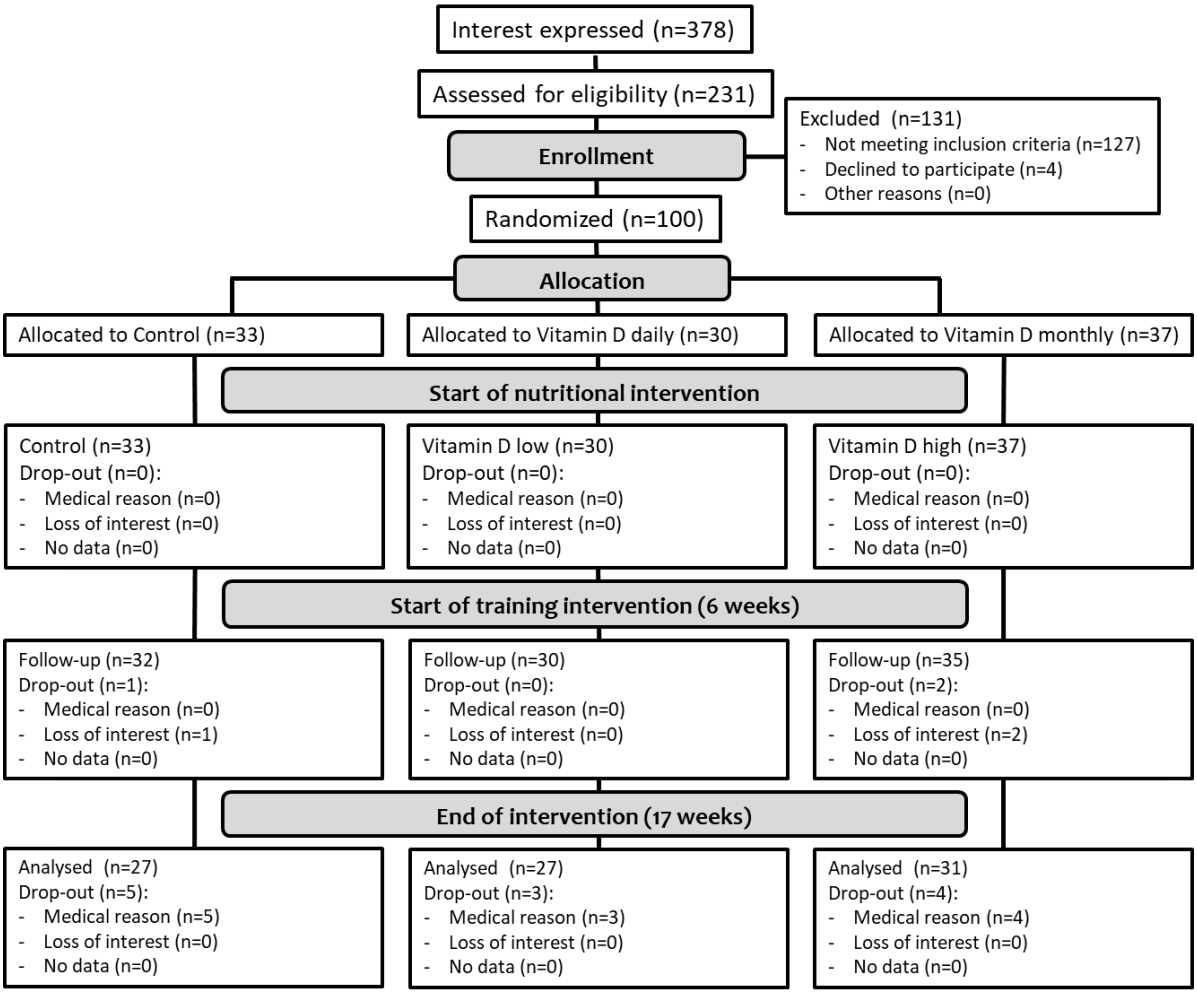

Figure 6: Flow chart of the study

3.1.3 Recruitment and participants

The participants of this study were recruited via different forms of communication and advertising. Namely, they were recruited via radio, newspapers and internet advertisements. Interested people could come to informational meetings, where they received a broad overview of the study, including inclusion and exclusion criteria. The target population of this study were community dwelling males and females between the age of 65 – 85 years, who lived independently (i.e. not in a retirement home, no life assistance by a third party, in good health condition: i.e. no walking stick, rollator etc.). Additionally, they had to have an appropriate mental state, which was achieved by a score of >23 in the mini mental state test. All inclusion criteria had to be fulfilled in order to be accepted in the study.

The exclusion criteria included vitamin D levels over 75 nmol/l, chronical illness that would contradict medical training therapy, severe cardiovascular disease (decompensated chronic cardiac insufficiency, extreme or symptomatic aortic stenosis, instable angina pectoris, untreated arterial hypertension, cardiac arrythmia), intake of diuretics (thiazides), intake of cardiac glycosides, diabetic retinopathy, osteoporosis or osteopenia with vitamin D and/or calcium substitution, disorder of the parathormone level, kidney disease, kidney stones, disturbance of the calcium levels, frailty index of three and higher, regular intake of cortisone or antibiotics (last six months), regular resistance exercise (>1x / week) in the last six months before the inclusion, missing declaration of consent, non-compliance to the study protocol: <70% of the planned vitamin D intake, <70% of the workouts. In case a person did fulfil any of the exclusion criteria above, she/he was excluded from the study.

Interested parties were also invited to promote the study to relatives and friends. Several of those informational meetings took place over the course of a few weeks. People, who were interested in the study had to inform the study management team either immediately or over the course of the following weeks via telephone or e-mail. Once people wanted to join the study, an appointment for further questions and the final determination of eligibility was set (t-1).

All participants gave their written informed consent, were informed about the procedure, benefits and risks of the study. The study was conducted according to the Austrian law (including Doctors act, CISA, Data protection act), the declaration of

Helsinki (World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects 2013) and in accordance with ICH-GCP guidelines. The ethics committee of the University of Vienna approved the study protocol (Reference number: 00405).

3.1.4 Randomization and blinding

After t-1, the study physician registered the officially eligible participants and randomly assigned them into one of the three groups, with the use of a randomization tool of the Medical University of Graz (Institute for Medical Informatics, Statistics and Documentation). Test persons were randomized according to certain parameters, including age (65 to 69.9 years; 70 to 74.9, 75 to 79.9, 80 to ≤85 years), gender (male or female) and baseline vitamin D level ((<50 nmol/l; 50-75 nmol/l). The personnel involved with testing, analysing or the intervention, as well as the participants in the study were blinded, with respect to group allocation.

3.1.5 Supplementation

Group two received a daily intake of calcium and vitamin D, in the form of two 200 mg calcium tablets and one 800 IU vitamin D tablet per day. Group three, received two daily 200mg calcium tablets and additionally, two 20000 IU and two 5000 IU capsules of vitamin D once per month, starting at t0. Group one - the placebo group, only received a supplementation of 200 mg calcium twice per day. The supplements were administered in boxes with 28 chambers, with each chamber containing their supplementation for one day. The participants were instructed to take the supplements always to their meals and at the same time of the day.

3.1.6 Resistance exercise

The resistance exercise for every group started after t1. The training phase comprised altogether of 20 guided resistance training sessions over the course of ten weeks. Every week, there were two trainings scheduled, with at least 48 hours in between. The participants were forbidden to train individually on other days. The workout started on low intensity during the first two weeks, in order to familiarize the

participants with it. After that period, the intensity increased. Machine, free weight and own bodyweight exercises were used in the workouts, with the aim to address all muscle groups. These mixed type exercises consisted of the sitting leg curl, lat pulldown, leg press, chest press, rowing, goblet box squats, the dumbbell shoulder press and the front plank with changing single leg raise.

3.2 Tools for the labwork

3.2.1 For the Lymphocyte isolation

- Leucosep-tubes, 50ml
- Sterile Plastic-Pasteur-Pipettes
- Glasspasteurpipettes
- Sterile syringes and pipettes with 20, 100, 200 and 1000 µl
- Sterile Tubes, 1,5 ml
- Falcon Tubes for the MK-Medium, 50 ml
- Polystyrene Round-Bottom Tubes, 5 ml
- Sterile filters, 0,45 µl
- Sterile Terumo Syringe without needle, 50 ml
- Sterile beakers
- Microscope slides, 125x25mm, VWR
- Cover glasses, 24x32 mm
- CountessTM automated cell counter, Invitrogen
- CountessTM Cell counting chamber slide
- Heraeus TM Megafuge 40, 1.0R, 40R Centrifuge, Thermo ScientificTM
- Shandon Cytospin 4
- ESCO CelCulture® CO2 Incubator
- Water bath

3.2.2 For the RNA extraction

- Pipette Eppendorf Research® Plus, 0.5 – 10 µl, grey
- Pipette Eppendorf Research® Plus, 2 – 20 µl, yellow
- Pipette Eppendorf Research® Plus, 20 – 200 µl, yellow
- Pipette Eppendorf Research® Plus, 100 – 1000 µl, blue
- Biotix®, uTIP, universal fit pipette tips, sizes: 1250 µl, 300 µl, 200 µl, 20µl and 10µl
- Eppendorf® Refrigerated Microcentrifuge, Model 5417R – used at 4°C
- VWR® Microcentrifuge Microstar 17R – used at room temperature
- Nanodrop One
- Thermofisher Scientific, Qubit 4 Fluorometer
- Electrophorese Chamber – PerfectBlue® GelSystem Maxi M “Revolution”
- Peqlab EV 231 Power Supply
- Thermo Scientific Barnstead, Easypure™ LF Lab Water system
- Heidolph REAX 2000 shaker
- VWR® Microcentrifuge Ministar
- Eppendorf DNA LoBind tube (1.5 ml)

3.3 Chemicals

3.3.1 For the Lymphocyte isolation

- Dulbecco’s Phosphate, Sterile 500 ml Sigma Aldrich
- Red Blood Cell Lysis
- Buffer 100ml Roche
- Dimethylsulfoxide (DMSO) 500 ml Sigma Aldrich
- Fetal Bovine Serum (FBS) 500 ml FisherScientific
- Trypan Blue stain 0,4% Thermofisher
- Sodium pyruvate solution, sterile 100 ml Sigma Aldrich
- RPMI 1640, sterile 500 ml Sigma Aldrich
- L-Glutamine, sterile 200 µl Sigma Aldrich
- Phytohaemagglutinine (PHA) 10 ml Sigma Aldrich

- Cytochalasin B Sigma Aldrich
- Diff-QuickTM Fixative Solution 1000 ml RAL Diagnostics
- Diff-QuickTM Solution I 1000 ml RAL Diagnostics
- Diff-QuickTM Solution II 1000 ml RAL Diagnostics
- Entellan® new 100 ml Merck

3.3.2 For the RNA extraction

- Promega - ReliaPrep™ RNA cell Miniprep System, 50 preps, containing:
    - Collection tubes (2x50/pk)
    - Reliaprep™ Minicolumns (50/pk)
    - 1 – Thioglycerol (900 µl)
    - Column Wash solution (CWE) (5 ml)
    - Nuclease-Free water (13 ml)
    - BL Buffer (32.5 ml)
    - Elution Tubes (50/pk)
    - RNA Wash solution (RWA) (35 ml)
    - Yellow Core Buffer (2.5 ml)
    - MnCl2, 0.09M (250 µl)
    - DNase I (lyophilized) (1 vial)
- Sigma – Aldrich, RNaseZAP™, Cleaning agent for removing RNase
- 70% Isopropanol – for cleaning the working area
- Electran® microbiology grade Propan-2-ol – for using in samples
- TAE 50x Buffer – containing:
    - EDTA disodium salt (50mM)
    - Tris (Tris(hydroxymethyl)aminomethane) (2M)
    - Acetic acid (1M)
- Sigma Aldrich Formamide (100 ml)
- Quantitas Fast DNA Marker Biolabs New England,
- (2x) RNA Loading dye, blue
- Biotium GelRead® Nucleic Acid Gel Stain

3.4 Methods

3.4.1 Lymphocyte isolation

It is important to note, that all steps, excluding the centrifugations, were carried out under sterile conditions, under the laminar airflow space.

The lymphocytes were isolated from the fresh blood samples of the participants of the NutriAging Study at each date, according to the following protocol:

Leucosep tubes (50ml) were filled with the fresh blood samples, one tube per person. The leucosep tubes were then centrifuged at 1000 x g, for 15 min, at room temperature, without brake, DEC:1.

After that, the blood plasma was transferred with sterile plastic pasteur pipettes and used for further measurements. The lymphocytes were harvested into new falcon tubes (15ml) and filled up with phosphate buffered saline (PBS). From this point on, all the tubes containing lymphocytes and the reagents were cooled on ice.

The freshly filled falcon tubes were mixed carefully by turning the tube on the cap 5 times and then centrifuged at 1300 rpm, for 10 min. at 4C°, with brake.

The supernatant was removed with a vacuum pump, until there was only 1 ml of sample left, the rest was removed carefully with a pipette, until there was only the cell pellet left at the bottom of the tube. Then the cell pellet was dispersed and the tube filled with ice cold PBS again.

After the tube was shaken 5x, it was again centrifuged at 1300 rpm, for 10 min. at 4C°, with brake.

Again, the supernatant was removed, first with the vacuum pump and thereafter with the pipette. The cell pellet was dispersed in 1ml of red blood cell lysis buffer and incubated in the dark for 20 min.

After the incubation, the sample was centrifuged at 500 x g, for 5 min., at 4C°. The supernatant was removed and the cell pellet dispersed and filled up with ice cold PBS to a volume of 5 ml and thoroughly mixed. Then the cell counting was carried out.

For the examination of the RNA, three aliquots of around 5 million cells were prepared for each test person. Two of those were extracted immediately, the remaining one was used as a back-up sample and frozen at -80C°. The back-ups were then extracted at various points during and after the study, to increase the probability of getting at least one sample with valid data for each test person.

3.4.2 RNA Extraction

Finding the best possible extraction kit for our purposes constituted an important part of this thesis. Therefore, prior to the beginning of the study, four different extraction kits and protocols have been tested. Those four extraction kits included the RNeasy Mini kit, the TRI reagent protocol, the ReliaPrep miRNA cell and tissue prep system and the ReliaPrep RNA Cell Miniprep System.

3.4.2.1 RNeasy Mini kit

After pelleting the lymphocytes, it was dispersed in 600 µl of Buffer RLT, which is a buffer containing guanidine thiocyanate and freshly added β-mercaptoethanol. Then, the sample was centrifuged and the lysate was homogenized in a QIAshredder spin column. 1 volume of 70% ethanol was added onto the column and mixed via pipetting. Up to 700 µl of the sample, were transferred into a RNeasy spin column and centrifuged at around 8000 rpm for 15 seconds. The flow through was discarded and 700 µl of RW1 buffer, containing small amounts of guanidine thiocyanate, was added onto the spin column and centrifuged. The flow-through was discarded again and 500 µl of Buffer RPE, containing freshly added ethanol, was added onto the spin column and centrifuged. After the centrifugation the flow-through was discarded again and the column was centrifuged at full speed for one minute. Then, the RNA was eluted with 50 µl of RNase-free water. (Qiagen 2019)

After the isolation of the RNA a Nanodrop-measurement and a gel-electrophoresis were carried out, to check the RNA quality (nanodrop and gel-electrophoresis are explained in detail later). The results were not satisfactory, as especially the 260/230 values were far below the necessary requirements for the quality of RNA.

Sample	Conc. µg/ml	260/280	260/230
1	106	1,99	1,40
2	96,4	2,03	1,59
3	92,3	1,96	0,41

Table 1: RNeasy Mini kit Nanodrop results. The concentration was high, after the extraction with this kit. Additionally, the 260/280 values were in the acceptable range. The 260/230 values were not satisfactory – indicating a contamination of the sample.

3.4.2.2 TRI-reagent protocol

Another protocol tested, was the TRI-reagent protocol. This protocol started with the creation of a cell pellet through centrifugation of the lymphocytes, suspended in PBS. This pellet was then lysed in 1 ml of TRI reagent by gentle pipetting. After that, 0.2 ml of chloroform per ml of TRI reagent used, were added to the sample. Then, the samples were shaken and incubated for 5 minutes at room temperature. Next, the samples were centrifuged at 12000 x g for 5 minutes at 4C°. After the centrifugation the samples were separated into three phases: a red organic phase containing protein on the bottom of the tube, an interphase with the DNA and an upper aqueous phase containing the RNA. The upper phase containing the RNA was transferred to a new collection tube and 0.2 ml of 2-propanol per ml of TRI reagent have been added. The samples were then incubated for approximately 10 minutes at room temperature and centrifugated with 12.000 x g at 4C° subsequently. The supernatant was removed and the cell pellets were dispersed and washed in 1 ml of 70% ethanol. The samples were vortexed and centrifuged at 7.500 x g and 4C°. The cell pellets were then air-dried for approximately 5 minutes. The pellets were resuspended in 50 µl of nuclease-free water. (Sigma-Aldrich Accessed: 2020)

This protocol produced better results compared to the RNeasy Mini kit. However, it proved to be difficult to carry out, with the separation of the three phases being especially tricky. In addition, a second testing showed a complete degradation of the RNA in the samples. Overall, the practicability of this protocol did not suit the requirements of the study.

Sample	Conc. µg/ml	260/280	260/230
1	41,2	1,81	1,77
2	47,0	1,7	2,28
3	28,8	1,75	1,92
4	47,9	1,78	1,95

Table 2: TRI reagent Nanodrop results. All values were in or close to the acceptable range.

Sample	Conc. µg/ml	260/280	260/230
1	384,4	1,96	2,30
2	358,8	1,95	2,23
3	422,8	1,96	2,02
4	109,3	1,83	2,05
5	96,6	1,83	2,03
6	122,9	1,88	2,02

Table 3 : Results of the second testing with the TRI reagent. The concentration was high, but the gel showed a degradation of the samples.

3.4.2.3 ReliaPrep miRNA cell and tissue prep system

After creating the cell pellets through centrifugation, the supernatant was removed and the pellets resuspended in 200 µl LBA buffer with freshly added 1-thioglycerol. Then, the samples were vortexed and subsequently 130 µl RDB were added. Again, the samples were vortexed for 10 seconds and then centrifuged for 2 minutes at 12000 x g. The clear solution was transferred to a new clean tube, to which 400 µl of 100% isopropanol was added. Then, the lysate is transferred to a ReliaPrep mini column and centrifuged at 12000 x g for 30 seconds. The liquid was discarded and the remaining homogenisate was transferred to the mini column and again centrifuged at 12000 x g for 30 seconds. After the lysate was removed, 500 µl of freshly prepared RNA wash solution have been added. Then, the samples were centrifuged again at 12000 x g for 30 seconds. The same procedure was repeated a

second time, just that it was centrifuged at 12000 x g for 2 minutes. After that, 40 µl of nuclease-free water have been added and the tubes were centrifuged for 1 minute at 12000 x g. 5 µl DNase I and 5 µl DNase 10X buffer have been added to each sample, which were then left to incubate for 5 minutes at room temperature. Subsequently, 150 µl of LBA buffer and 300 µl of 95% ethanol were added to the mixture, which was thereafter vortexed for 10 seconds. 500 µl of the sample were then transferred to a second mini column and centrifuged at 12000 x g for 30 seconds. After discarding the liquid, 500 µl of RNA wash solution have been added to each mini column. Then, they were centrifuged again at 12000 x g for 30 seconds. This step was repeated, with the caveat, that the centrifugation was taking 2 minutes. After that, 30 µl of nuclease-free water has been added and the samples were centrifuged one last time for 1 minute, eluting the RNA. (Promega 2016)

The ReliaPrep miRNA cell and tissue prep system was in many ways very similar to the ReliaPrep RNA cell miniprep system protocol, as they are both products of Promega. The ReliaPrep miRNA cell and tissue prep system has got a few more procedure steps, making it a bit more complicated and time-consuming. In addition, the results were spectacularly different, with the ReliaPrep miRNA cell and tissue prep system samples being worse in both, quantity and quality, when compared to the RNA cell mini prep system protocol.

Sample	Conc. µg/ml	260/280	260/230
1	7,7	1,58	1,22
2	10,4	1,52	1,70
3	5,8	1,50	0,54
4	7,3	1,50	0,84

Table 4: Nanodrop Results of the ReliaPrep miRNA cell and tissue prep system. All results are far from the desired values.

3.4.2.4 ReliaPrep RNA Cell Miniprep System

This protocol proved to be the best way to isolate RNA for the purposes of this study, with repeatable constantly good results, which is why it is explained more detailed.

The extraction commenced with centrifuging the washed and cooled lymphocytes, using a refrigerated centrifuge (Eppendorf® Refrigerated Microcentrifuge (Model 5417R), 3000 rpm, at 4° for 5 minutes.

The supernatant was removed and the cell pellet dispersed in 500 µl of BL+TG Buffer. This mixture was thoroughly mixed for 5-10 seconds by using a vortex mixer (Heidolph REAX 2000 shaker). The tube containing the sample was then centrifuged again for approximately 5 seconds (VWR® Microcentrifuge Ministar), to make sure there was no sample left under the lid.

Then 170 µl of 100% isopropanol were added to the sample. Again, the tube was vortexed for 5-10 seconds and the sample was centrifuged, to make sure there was no remaining part under the lid.

500 µl of this lysate was transferred onto a Reliaprep™ Minicolumn, which was previously placed in a collection tube. This tube was then centrifuged at 12,000 – 14,000 x g for 30 seconds at 20°-25C° (VWR® Microcentrifuge Microstar 17R). After the centrifugation, the collection tube was discarded and the Reliaprep™ Minicolumn placed into another collection tube.

Then the remaining sample (should be about 170 µl) were transferred onto the Reliaprep™ Minicolumn. This tube was again centrifuged at 12,000 – 14,000 x g for 30 seconds at 20°-25C° and the collection tube subsequently discarded.

After the Reliaprep™ Minicolumn was placed into a new collection tube, 500 µl of RNA wash solution were added. The sample was then centrifuged at 12,000 – 14,000 x g for 30 seconds at 20°-25C° and the collection tube once more discarded. The Reliaprep™ Minicolumn was transferred into a new collection tube.

In a new, sterile tube the DNase I incubation mix was prepared. The DNase I Incubation mix consists of 24 µl yellow core buffer, 3 µl 0.09M $MnCl_2$ and 3 µl of DNase I Enzyme per sample. The mix had to be prepared fresh every time it was used. 30 µl of the freshly prepared incubation mix was applied directly onto the Reliaprep™ Minicolumn membrane. The sample was then incubated for 12 minutes at room temperature.

After the incubation 200 µl of Column wash solution were applied onto the Reliaprep™ Minicolumn. The column was subsequently centrifuged at 12,000 –

14,000 x g for 15 seconds at 20°- 25C°. Afterwards the collection tube was discarded and the Reliaprep™ Minicolumn transferred into a new one.

500 µl of RNA was solution were applied onto the Reliaprep™ Minicolumn and the sample was centrifuged at 12,000 – 14,000 x g for 30 seconds at 20°- 25C°. The collection tube was discarded and the Reliaprep™ Minicolumn transferred to a new one again.

This time 300 µl of RNA wash solution were applied onto the Reliaprep™ Minicolumn and the centrifugation was made at full speed for 2 minutes, in order to remove any kind of chemical residue. The collection tube was discarded and the Reliaprep™ Minicolumn was transferred into an Eppendorf DNA LoBind tube (1.5 ml).

The next step was the elution of the RNA with 60 µl of nuclease Free water and centrifugation at 12,000 – 14,000 x g for 1 minute at 20°- 25C°. After centrifugation, the samples were immediately put on ice, where another 15 µl of nuclease free water was applied onto the Reliaprep™ Minicolumn. These columns were then centrifuged one last time at 12,000 – 14,000 x g for 1 minute at 20°- 25C°. The obtained 75 µl of RNA solution was then divided into three parts: 12 µl for immediate further measurements, 5 µl for the determination of RIN values and 58 µl for later measurements. (Promega 2012)

The Nanodrop results can be found in the appendix.

3.5 Quantity and quality assessment of RNA

The quantity and quality of the RNA was assessed in three different ways – via the Thermofischer™ Scientific Nanodrop™ One, via Agarose Gel electrophoresis and via the Thermofisher Scientific™ Qubit 4 Fluorometer. Prior to the study, also the Qubit RNA IQ Assay has been investigated.

3.5.1 Thermofischer™ Scientific Nanodrop™ One

The Thermofischer™ Scientific Nanodrop™ one is a UV/VIS spectrophotometer, which is designed for micro-volume analysis of quantity and purity of nucleic acids. The device uses the Beer-Lambert equation to correlate the absorbance with the concentration. It gives information about nucleic acid concentration, the absorbance

at 260/280nm and the absorbance at 260/230nm. The A260/A280 and A260/A230 values are purity ratios and should be for RNA at about 2 for the former and between 1.8 and 2.2 for the latter. Any deviation of the aforementioned values would indicate an impurity in the sample, likely to be caused by contaminants or residual solvents and reagents in the sample. (Thermo Fischer Scientific 2017)

2 µl of fresh sample were used for this measurement.

3.5.2 Agarose Gel Electrophoresis

Gel electrophoresis is a common tool to separate DNA, RNA or Proteins based on their electric charge. An electric field is applied to the electrophoretic chamber, containing the gel and the running buffer. The negatively loaded RNA is therefore migrating from the cathode on one end of the chamber to the anode on the other end. The structure of the gel then enables smaller molecules to move faster and bigger molecules to move slower, thus creating a pattern. Markers, containing molecules with different but known sizes are used as a reference for the samples (Kara Rogers).

- *Preparation of the Gel*

  The gel was made of 1g Agarose, which was suspended in 100 mL od 1x TAE Buffer. This solution was melted in the microwave. Then 5 µl of GelRead® and 5 mL of Formamide were added to the hot melted solution. GelRead® is a sensitive nucleic acid dye, used to replace the highly toxic ethidium bromide, as staining for RNA]. Formamide is used to disrupt the secondary structure of the RNA, which subsequently leads to better analysis of the sample (Aranda et al. 2012). The liquid agarose mixture was then cast into a form.
  After cooling down, the gel was loaded with 7 µl blue (2x) RNA Loading dye and 5 µl of the purified RNA samples respectively. In addition, two bands of the Quantitas Fast DNA marker 100 bp – 2000 bp have been loaded onto the gel. This marker is running in the gel in addition to the samples, in order to quickly assess the size of the RNA particles. The gel ran for 90 minutes at 80V and room temperature.

For larger quantities of fresh samples, a 3g Agarose gel, with 15µl GelRead® and 15 mL of Formamide was used.

To detect the image a Biorad Gel Doc XR+ Gel Documentation System with Image Lab Software was used.

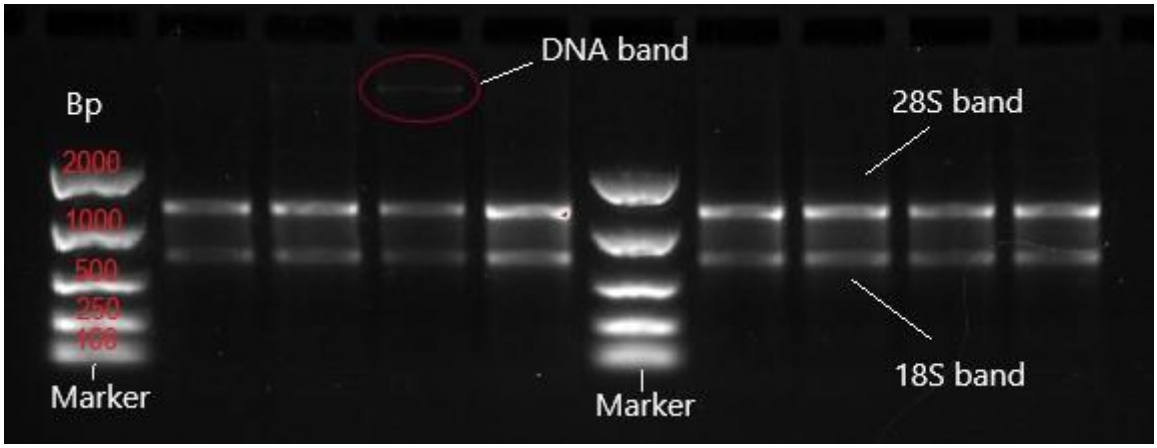

Figure 7: RNA Quality Assessment via gel electrophoresis

- *Interpretation of the image*

The 28S and 18S bands are clearly visible for each sample. The ratio of 28S to 18S RNA should be approximately 2:1, which can be estimated in the image above. There is a DNA band detectable for one sample, indicating that the DNAse treatment did not work in that sample. All the other bands are DNA free, which is another indicator, that the RNA extraction worked well. Prior to further measurements, the sample with the DNA band must be treated with DNase again and in addition, the Nanodrop and RNA Broad range measurements must be repeated.

3.5.3 Thermofisher Scientific™ Qubit 4 Fluorometer

The Thermofisher Scientific™ Qubit 4 Fluorometer is a, compared to spectrophotometrical methods, more accurate way of determining the concentration of extracted RNA in a sample. Through using a specific dye for RNA assays, only RNA molecules are binding fluorescent particles, which are then captured by the fluorometer. (Thermo Fisher Scientific Accessed: 2020)

Figure 8: The Thermofisher scientific Qubit 4 Fluorometer

3.5.4 RNA Broad Range assay

In order to measure the RNA concentration, a Qubit® RNA Broad Range (BR) Assay has been carried out. First, 200 µl of a Qubit working solution had to be made for each sample. That solution consisted of the RNA BR reagent and the RNA BR buffer – mixed in the ratio of 1:200. In order to calibrate the fluorometer, two standards had to be prepared with 190 µl of the working solution and 10 µl of the standard. Afterwards, the sample solutions were made, consisting of 198 µl of the working solution and 2 µl of the sample. All standards and samples had to be vortexed for 2 seconds and subsequently left to incubate for about 2 minutes. Then, the measurement could take place. The results of the Qubit RNA BR assay can be found in the appendix. (Thermo Fisher Scientific 2015)

3.5.5 RNA IQ assay

Prior to the begin of the study, the RNA IQ assay has been tested. The procedure is very similar to the RNA BR assay, with the exemption, that three RNA IQ standards are used to calibrate the fluorometer. (Thermo Fischer Scientific 2018) For the step by step instructions, see paragraph *RNA Broad Range assay.* The RNA IQ assay is usually used to divide the RNA into smaller and larger RNA parts, with smaller RNA occurring primarily in samples, which show signs of degradation. For the purposes of this study, this assay was too time-consuming and deemed surplus to requirements, especially as the integrity of the RNA was examined with the gel electrophoresis. Furthermore, the RNA IQ assay is using too much sample.

At this point it must be emphasized, that the next step, which is PCR has not and will not be conducted by the writer of this thesis. Although, this thesis provides an overview over different methods of PCR and programs used to analyse the genes.

3.6 The principle of PCR

The polymerase chain reaction or PCR in short, is a tool to copy and amplify nucleic acids or parts of them. The PCR needs a template, in form of DNA or RNA and two primers, for the copying process. There are three basic steps in the PCR. First, the nucleic acid must be denatured at 90 - 96C°. Then the annealing starts, in which the primers bind to their complementary bases of the now single stranded nucleic acids. The third step is the replication of the nucleic acid strand by the polymerase, started by the primer. The result are two new helices, for every nucleic acid strand, composed of one original strand and complemented by one newly assembled strand. The more PCR cycles are running, the more material is generated (Powledge 2004). Once the PCR is completed, the product is separated by size in form of an agarose gel electrophoresis and made visible through a fluorescent dye. This, so called end-point detection lacks sensitivity and can also be varying from run to run (Ishmael and Stellato 2008).

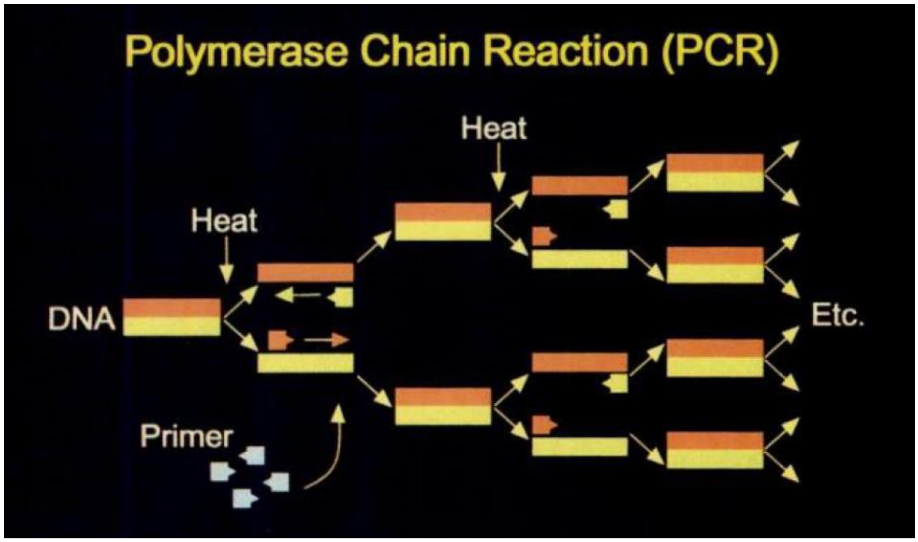

Figure 9: The mechanism of PCR. First, the denaturation using heat. Second, the binding of the primers to their complementary bases. Third, the replication of the DNA strand through the polymerase. (Wrobel 1995)

3.6.1 Real time RT qPCR

The quantitative reverse transcription real time polymerase chain reaction is, because of its simplicity and sensitivity, one of the most widely used methods to detect and measure small amounts of nucleic acids (Bustin et al. 2009). RT-qPCR is a combination of three steps: (a) the reverse transcriptase (RT)-dependent conversion of RNA into cDNA, (b) the amplification of the cDNA using the PCR and (c) the detection and quantification of amplification products in real time. Real time RT-qPCR uses fluorescent dyes, which bind to the DNA and in doing so, enable the amplification and detection of the PCR Reaction in one single tube. The assay measures the fluorescent signal, which is proportional to the amount of DNA, that has been produced during each PCR cycle (Nolan et al. 2006). The visualization of the PCR can be separated into four phases (see figure 10): a linear ground phase, an exponential phase, a linear phase, and a plateau phase. During the first few cycles of the PCR, products are not yet detectable – this is the linear ground phase. The exponential phase describes the phase, in which the product doubles after every circle, thus the fluorescent dye binds and makes it detectable. In the linear phase the reaction slows down. In the plateau phase, the reaction has used up all the reagents and no more products are generated (Ishmael and Stellato 2008).

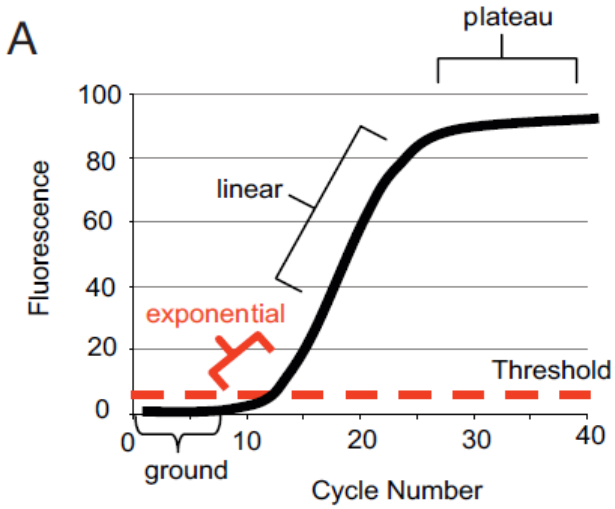

Figure 10: Visualization phases of PCR (Ishmael and Stellato 2008)

Individual reactions are characterized by the PCR cycle at which fluorescence first rises above a threshold background fluorescence, a parameter known as the threshold cycle (Ct). The more target DNA there is, the lower the Ct will be, as the point of fluorescence will be reached faster. This correlation between fluorescence and amount of amplified product permits accurate quantification of target molecules over a wide dynamic range (Nolan et al. 2006).

Although the RT-qPCR does also pose too many problems – some of which are explained in the MIQE guidelines below.

3.6.2 The MIQE Guidelines

The Minimum Information for publication of Quantitative Real-Time PCR Experiments (in short MIQE) provide general guidelines to help the interpretation and evaluation of the RT-qPCR results. It cites the most common technical deficiencies, that lead to poor assay results as the following: (a) inadequate sample storage, sample preparation or variable nucleic acid quality, which can lead to varying results; (b) the selection of inadequate reverse-transcription primers and primers for the PCR, leading to inefficient assays; (c) unsuitable data and statistical analyses, producing deceitful results (Bustin et al. 2009).

The guidelines state, that when working with RNA, it is substantial to document how samples have been stored and if they have been processed immediately or frozen to be processed later. Exact quantification of the RNA yields is also very important, as it is desirable to use the same amount of RNA, when comparing samples. According to the MIQE it is also vital to check the sample for RNA degradation not just via spectrophotometrical analysis, but also at least via gel electrophoresis. The steps for reverse transcription and qPCR must also be very well documented (Bustin et al. 2009). The RNA structure can also have a great impact on the efficiency of reverse transcription and the qPCR, which is why RNA folding should be taken into consideration. The sequences should be checked with nucleic acid software (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1-2.3.cgi).
The use of no template controls (NTCs) is very helpful and should be included in every plate of samples, as they can detect PCR contamination and false products.
Additionally, positive controls of nucleic acids extracted from experimental samples are useful for monitoring assay variation over time. The most important characteristics for assessing the assay performance are the following: PCR efficiency, linear dynamic range, limit of detection, and precision (Bustin et al. 2009). The MIQE has been considered in this thesis in order to secure reliable data and interpretations.

3.6.3 Microarrays

The principle of microarrays is quite simple: known DNA sequences are printed on microscope slides. Then two samples of either DNA or RNA are prepared with two different fluorescent dyes. They are subsequently mixed and hybridized with the DNA spots. Sequences, that aren't hybridizing with the DNA sequences, are washed away. After hybridization, the fluorescence is measured with a specific microscope, that can assess the strength of fluorescence of every dye on every spot separately. That measurement is determining the ratio and thus the relative abundance, of the sequence of each specific gene of the DNA or RNA samples. The microarray is a relatively cheap and universal way to measure gene expression (Brown and Botstein 1999). The major disadvantages of the microarray assay are, that the sensitivity is not very high and the results are varying, thus not providing a satisfying level of

reproducibility (Groen 2001). This is also one of the main reasons, why we did not consider it for our studies.

3.6.4 Next generation sequencing

As a successor of the Sanger sequencing (also known as the first generation), Next generation sequencing (NGS) first occurred in 2008 and has evolved at a rapid pace ever since. First, the selected cDNA template is being enriched. That procedure can be accomplished by amplification methods or by hybridization capture methods. The enriched cDNA is then fragmented by physical methods (e.g. sonic waves). The fragmentation is necessary, because the target DNA has to have a base pair length of between 150 and 500 for library sequencing (Hui 2014). The next steps are ligation of the target to a primer and clonal amplification of the library. After a subsequent sequencing reaction, billions of reads are generated. Each read contains the sequence, typically ~100 bases in length, of a single template clone. A highly complex bioinformatical process is necessary for obtaining and interpretation of the data. Short reads, which lead to difficulties with assembling and mapping to the reference sequences, belong to the common problems associated with NGS. Also, sequencing errors are prevalent, albeit rapid improvements are made in that field (Hui 2014).

3.6.5 RNA seq.

The high throughput transcriptome sequencing has become the preferred method to determine gene expression levels (Costa-Silva et al. 2017). The transcriptome provides insight into the transcriptional structure of genes, including their star sites, their 5’ and 3’ ends, splicing patterns and their post-transcriptional modifications. In addition, the quantity of the gene expression levels under different conditions can be determined. Thus, understanding the transcriptome can be helpful for understanding cell development and diseases (Wang et al. 2009). The process of RNA-seq. starts with the fragmentation of the RNA into smaller cDNA sequences, which are then sequenced from a high throughput platform. Second, the newly generated sequences are outlined to a genome or transcriptome. Then, the expression level for each gene is estimated. Fourth, the produced data is normalized and the differentially expressed

genes are registered. The last step is putting the received data into a biological context. As the RNA seq. is becoming very commonly used, many different tools and software are being developed, to process the obtained data set. Compared to the microarray technology, the RNA seq. shows a greater amount of reproducibility of data. Furthermore, RNA seq. allows the sequencing of unknown transcripts. One of the challenges of RNA seq. is the big amount of data the method produces and how to process it in an effective manner, without errors in image analysis (Costa-Silva et al. 2017).

3.6.6 NormFinder

The “NormFinder” is an algorithm, designed to find the optimal gene for normalization, amongst a set of candidates. It lists the candidate genes in order of their expression stability in a given sample set and given experimental design. “NormFinder” can analyse expression data obtained through any quantitative method. For this study RT-PCR was used. The input data needs to be on a linear scale. The program requires a minimum of 3 genes and a minimum of 2 samples per group. Analysis of 5-10 candidate genes and at least 8 samples per group is recommended (Lindbjerg Andersen et al. 2004-2010).

A gene for normalization or reference gene is essential for the normalization of target-gene expression and thus for the accuracy of the RT-qPCR. A reference gene should be constantly expressed and not influenced by experimental or pathological conditions (Jo et al. 2019).

“NormFinder” is providing a stability value for each gene. Furthermore, it is highlighting the best gene with the lowest value. In addition, the program is choosing the best combination of two genes for a two gene normalization factor, as well as the best combination of two genes. The extended output is also providing information about the standard error of the stability value and both, the estimated intra- and intergroup variations of each gene and/or group (Lindbjerg Andersen et al. 2004-2010).

4. Discussion

Before the discussion it must be once again emphasized, that the analysis of the RNA samples of the NutriAging study is still ongoing, therefore there will be no results regarding the gene expression presented in this thesis. For this reason, a literature survey has been conducted, which is summarizing similar studies.

Muscle cell studies

There is a widespread consensus that vitamin D and physical exercise has a positive effect on the well-being of the elderly. Antoniak and Greig performed a systematic review with studies, which comprised randomised controlled trials including men and women, aged ≥65 in 2017. The analysis included 7 studies and 792 participants. The studies were divided into two groups: the first group compared vitamin D supplementation and exercise training to just exercise training. The second group compared vitamin D supplementation and exercise training with vitamin D supplementation alone. The study supports the hypothesis, that resistance exercise and vitamin D supplementation improves the muscle strength in the elderly. However, vitamin D supplementation alone, showed no such effect. Antoniak and Greig also highlighted the fact, that few studies have investigated the effect of both resistance training and vitamin D supplementation on musculoskeletal health (Antoniak and Greig 2017).

Glendenning et al. conducted 2012 in a randomised, double-blind, placebo-controlled, prospective, parallel study, with 686 women aged 70 or older. The participants received supplements with 150.000 IU of vitamin D every three months, over the course of 9 months. It has to be noted, that the women only received written lifestyle advise on maintaining physical activities and the intake of 1300 mg of calcium. The study concluded, that the intake of high-dose vitamin D every three months, has no effect on the frequency of falls in elderly women (Glendenning et al. 2012). Furthermore, Sanders et al. came to the same conclusion in 2010. Their study with 2256 community-dwelling women, aged 70 or older, which received 500.000 IU of vitamin D once annually for between 3 and 5 years, even resulted in an increased risk for falls and fractures (Sanders et al. 2010).

Girgis et al. investigated the gene expression in muscle cells. In their study from 2014 they examined the effect of vitamin D on the expression of VDR and its target genes in their studies with male mice. Primary cells were taken from 3-week-old, male mice and the cells were then cultured. Myotubes were generated and treated with 1,25(OH)2D3. Their RNA was isolated, using the Qiagen RNeasy mini kit. The gene expression was examined with a real-time qPCR. The outcome showed, that the treatment with vitamin D resulted in a higher expression of VDR and its target gene CYP24A1, as well as CYP27B1 (Girgis et al. 2014).

Similar to Girgis et al., Pojednic et al. investigated the effect of vitamin D on muscular gene expression, but in human samples. Therefore, Pojednic et al. conducted a study, which included two parallel examinations in 2015. One looked at cell culture primary myoblasts in young adults, the other however, examined muscle biopsies of older men and women (Pojednic et al. 2015). Muscle fibres are composed of myoblasts (Perry and Rudnick 2000). For the study, cell line preparation muscle biopsies were obtained from three young adults. The myoblasts were then treated with one of three different doses of 1,25(OH)2D3 over the course of 18 hours. The doses were 1 pmol/L, 10 pmol/L or 1 nmol/L. After the treatment, the RNA was isolated using the Trizol method and the gene expression was measured with a real-time qPCR. The results showed, that the treatment with vitamin D, dose-dependent, significantly increased the expression of VDR and its target CYP24A1 gene in the myoblasts, compared to untreated control cells. (Pojednic et al. 2015). These results confirmed the findings of Girgis et al. a year earlier.

The muscle biopsies of the elderly were obtained from two clinical studies, conducted earlier. Although it must be noted, that the participants in one of those studies only received in resistance training, without supplementation of vitamin D. The participants of the other clinical study examined received a vitamin D supplementation of 4000 IU per day over the course of 16 weeks. The RNA was isolated using the Aurum Total RNA Fatty and Fibrous Tissue RNA Extraction Kit. The gene expression was measured with real-time qPCR. The results showed a consistent increased expression of VDR in the human muscle cell of the elderly (Pojednic et al. 2015).

In 2017 Hassan-Smith et al. conducted a study with 116 healthy individuals of different age groups and sexes, examining the role of different metabolites of vitamin D on skeletal muscle strength and more interestingly, also on gene expression in the

muscle. It must be noted, that only the vitamin D status of the participants was measured, and they received no additional vitamin D supplementation. Only 14% had a sufficient vitamin D status (>30ng/ml). Muscle biopsies were taken from the vastus lateralus and real-time qPCR was performed for the analysis of 92 muscle genes, such as mTOR, SIRT3, myogenin or GHR. In contrast to Pojednic et al. and Girgis et al., this study found no detectable expression of CYP24A1 and CYP27B1 in the muscle biopsies. Although the expression of VDR was detectable, the study found no link between the participant age and different expression rates (Hassan-Smith et al. 2017). The data showed negative correlation between the VDR expression and the serum 25(OH)D3 concentration, but fat mass influenced the expression positively. Reviewing the muscle genes, only 4 showed positive correlations with serum concentrations of 1,25(OH)D3. Those four genes were ACACA, PPP3R2, PSMA2 and SREBF1. Meanwhile 24 genes including VDR, myogenin and SIRT3 appear to have a good link with serum 25(OH)D3. Interestingly, none of the 4 genes, which have a positive connection to 1,25(OH)D3, show the same for 25 (OH)D3. The 24 genes play a part in hormonal/intracellular signalling, cell stress, proteosomal activity, protein translation, amino acid metabolism, muscle contraction, myogenesis and mitochondrial function (Hassan-Smith et al. 2017).

Similar to Hassan-Smith et al., Hangelbroek et al. conducted a study, looking at the effect of calcidiol (25(OH)D3) on the transcriptome of the skeletal muscle. Muscle biopsies were obtained from 10 subjects, which were taking a daily dose of 400 IU of calcidiol and 12 subjects from the placebo group (Hangelbroek et al. 2019). The subjects were taking part in another trial, carried out by Vaes et al., and were supplemented for 6 months. This was a randomized double-blind intervention study, with men and women aged 65 or older, with a vitamin D status of between 25 and 50 ng/ml and a BMI of between 20 and 35 kg/m² (Vaes et al. 2018). Hangelbroek et al. isolated the RNA from the biopsies, using Qiagens RNeasy mini kit. A qPCR was performed specifically for the VDR gene and a Microarray analysis has been conducted as well. The outcomes showed that increased 25(OH)D levels did not result in higher expression of the VDR in the skeletal muscle cell. In fact, the effect of calcidiol on the VDR expression was very low to not significant and the receptor is generally being expressed at very low levels in the skeletal muscle cell. In addition,

the supplementation with calcidiol showed little to no effects on the expression of VDR target genes, with the exception of the insulin-like growth receptor 1 (IGF-1). Hangelbroek et al. explained these findings with the still ongoing discussion, whether VDR is expressed in mature human skeletal muscle cells in sufficient levels at all. They are coming to the conclusion, that VDR is predominantly present in developing muscle cells and after muscle injury. Therefore, supplementation with 25(OH)D3 may have a greater effect, when the muscle is injured (Hangelbroek et al. 2019).

To summarize the studies examining the impact of vitamin D on muscle strength present, that vitamin D alone has no benefit for the muscle strength of elder people, as Antoniak and Greig suggested. According to Glendenning et al. a vitamin D supplementation alone has no impact on the risk of falls, whereas the study of Sanders et al. even showed an increase of falls and fractures after the vitamin D supplementation. Regarding gene expression in muscle cells, Girgis et al. and Pojednic et al. produced similar results in their studies with mice and humans respectively. The expression of VDR and their target genes CYP24A1 and CYP27B1 increased after vitamin D supplementation in the muscle cells. This is a finding that Hassan-Smith et al. confirmed, as they did not observe a significant increase in the expression of CYP24A1, CYP27B1 or VDR in the muscle cells without the supplementation of vitamin D. Furthermore Hangelbroek et al. came to the conclusion, that the VDR is expressed at very low levels in skeletal muscle cells.

Study	Subjects	Supplement-ation	Exercise	Examination	Results
Systematic review Antoniak and Greig, 2017	792 participants; Men and women; 65 years or older	Yes	Yes	The effect of combined resistance exercise training and vitamin D3 supplementation on musculoskeletal health and function	Improvement of muscle health through combination of vitamin D supplementation and resistance exercise. No such effects through vitamin D alone.

Glendenning et al. 2013; randomised, double-blind, placebo-controlled, prospective, parallel study	686 women, aged 70 or older	150.000 IU, three times over the course of nine months, 1300 mg of calcium	No – although the women got written lifestyle advice regarding physical activities	Effects of high dosages of cholecalciferol on falls, mobility and muscle strength in older postmenopausal women	No significant effect on the frequency of falls has been noted
Sanders et al. 2010, randomized, placebo-controlled trial	2256 women, aged 70 or older	500.000 IU of cholecalciferol once annually for 3-5 years	No	The effect of annual high doses of cholecalciferol on falls and fractures in older women	The risk for falls and fracture increased
Girgis et al. 2014	Murine quadriceps muscle samples	1 IU of vitamin D/g from weaning; treatment of muscle cells with calcitriol or ethanol during cell cultivation	No	Expression of VDR and their target genes in skeletal muscles of mice	The expression of VDR increased, as well as the expression of its target genes CYP24A1 and CYP27B1
Pojednic et al.; 2015	Cell culture and muscle biopsies	Yes	No	The effect of vitamin D on the expression of VDR and its target genes	Significant increase of the VDR expression, as well as the expression of CYP24A1
Hassan-Smith et al., 2017	116 healthy volunteers; 79 women, 37 men, aged 20-74	No	No	The effects of calcidiol and calcitriol on human skeletal muscle function and gene expression	Negative correlation between serum 25 (OH) D levels and the VDR; positive correlation between 4 genes and calcitriol; positive correlation

					between 24 genes and calcidiol
Hangelbroek et al. 2019/Vaes et al. 2018; double- blind, placebo-controlled trial	Muscle biopsies of 10 individuals treated with vitamin D and 12 persons with placebo; all test persons were 65 or older	400 IU of calcidiol daily for six months	No	The (non)-effect of calcidiol on the skeletal muscle transcriptome in frail and older adults	Increased 25(OH)D levels do not lead to a higher expression of the VDR in the skeletal muscle. In fact, the general expression of the VDR in skeletal muscle cells is low. Additionally, other VDR target genes were not impacted by the supplementation, with the exception of the IGF-1.

Table 5: Summary of studies, investigating the effect of vitamin D on muscle cells

Whole blood studies

Hossein-nezhad et al. carried out a study in 2013 where they examined the broad gene expression in white blood cells, before and after supplementation with vitamin D. Eight healthy, white, male and female participants, aged 18 or older finished the study. At the randomized, controlled, double blind trial, the individuals got a supplementation of either 400 IU or 2000 IU of vitamin D daily for two months. Prior to supplementation, four subjects were 25(OH)D3 deficient (< 20ng/ml) and the other four were either insufficient or sufficient (> 20ng/ml). Total RNA was isolated with the QIAGEN RNeasy kit and it was then reverse transcribed with whole transcript cDNA synthesis kit. A microarray analysis was performed, to examine the broad gene expression. In addition, a real-time qPCR has been used for four genes, including CD83, TNFAIP3, KLF10 and SBDS. The results showed, that there was no

significant difference in gene expression between the group, that got 400 IU and the group that got 2000 IU supplemented (Hossein-nezhad et al. 2013). Although, there had been a significant difference in gene expression between the baseline and after the two months of supplementation for both groups. 291 genes have been found, to either increase or decrease in expression, after the vitamin D treatment. Of the 291 genes, 81 showed a significant decrease and 209 showed a significant increase in expression. Furthermore, the results showed a significant difference in gene expression for 66 out of the 291 genes, between the deficient and the insufficient/sufficient group. The analysis of the biological functionality of the 291 genes showed, that the genes were involved in many different pathways in the human body. The most relevant functions include transcriptional regulation, apoptosis, response to stress and DNA repair, metabolic functions, immune response and cell cycle activity. Looking at the qPCR genes, CD83, showed a decrease in expression, resulting in better immune health and thus a reduced risk of autoimmune diseases, such as multiple sclerosis. In addition, TNFAIP3 showed a decreased expression too. The gene is also associated with multiple diseases, such as Morbus Crohn, Lupus erythematous, rheumatoid arthritis and type 1 Diabetes (Hossein-nezhad et al. 2013).

In their sub-study in 2017, Pasing et al. explored changes in the human transcriptome upon vitamin D supplementation. In the course of the randomized controlled trial “Prevention of type 2 diabetes with vitamin D supplementation in subjects with reduced glucose tolerance”, this sub-study examined 94 subjects, between the ages of 25 and 86, who received a dosage of 20.000 IU of cholecalciferol weekly, over the course of five years. The participants were comprised of 47 people in the intervention group and 47 people in the placebo group. Total RNA was then isolated from fasting blood samples, using the Qiagens PAXgene blood RNA isolation system. To examine the gene expression, a microarray was performed. According to Pasing et al., no significant difference in the general expression of genes between the intervention group and the placebo group could be detected. Although, looking at 141 genes regulated by vitamin D, six genes were found with a changed expression, between these two groups, namely: *FPR2, CD52, IL1R2, GNG10, RPS26* and *FOLR3* (Pasing et al. 2017). The authors explained, that the small difference between the two study groups could be in part due to the already sufficient serum concentration of the subjects at baseline (~ 61 ng/ml). Furthermore,

the long intervention time, could lower the response of the gene expression to the vitamin D intake. Interestingly, the study has found a greater difference in gene expression, when comparing sex. There were 58 genes that were significantly more expressed in women, than in men (Pasing et al. 2017). Pasing et al. assume, that the higher serum concentrations of both, the DBP and 25(OH)D in women, might contribute to this difference. Additionally, the 20 participants with the highest and the 20 participants with the lowest baseline serum concentration, were compared to each other. There were 198 genes found to be differently expressed between those groups. Comparing these two groups with all 94 subjects, Pasing et al. conclude that a certain vitamin D threshold exists, above which a vitamin D supplementation may have no additional effect (Pasing et al. 2017).

In their Post-hoc analysis of the styria vitamin D hypertension trial in 2017, Trummer et al. investigated the potential correlation between vitamin D levels and IGF-1 expression. In the hypertension study there were 175 of 200 participants included in the analysis. The subjects eligible, were 18 or older, had to have arterial hypertension and a vitamin D level of below 30 ng/ml. The participants of the single-centre, placebo controlled, randomised, double-blind study, received a daily dose of 2800 IU of 25(OH)D3 or a placebo, over the course of 8 weeks. The serum concentrations of cholecalciferol, calcitriol and IGF-1, were measured with chemiluminescence immunoassays. The IGF-1 was chosen because it is essential for many different pathways including cell differentiation and apoptosis. Various previous studies have hinted at a possible interaction between the IGF-1 and vitamin D levels. Trummer et al. found out, that the intervention with vitamin D, did not have any effect on the expression of IGF-1. Although, the serum levels of calcitriol did rise after the intervention. The authors explained this increase of 1,25(OH)2D3, with the alleged renal induction of 1-alpha-hydroxylase through IGF-1. This induction is documented also in previous studies. Thus, IGF-1 and 1,25(OH)2D3 are positively correlated (Trummer et al. 2017).

In their intention-to-treat, double-blind, placebo-controlled, dose-finding, randomized clinical trial, Berlanga-Taylor et al. investigated the impact of 25(OH)D on the whole genome. In addition, a correlation between vitamin D and plasma cytokines, such as IFN-γ, IL-10, IL-8, IL-6 and TNF-α has been examined in this study. For the examination, 305 community-dwelling individuals, aged over 65 years were

supplemented with either 4000 IU, 2000 IU or placebo daily for 12 months. The mean 25(OH)D level at baseline was approximately 50 nmol/L. The RNA was extracted with the PAXgene Blood RNA kit. The gene expression was measured through microarrays. Contrary to Hossein-Nezhad et al., Berlanga-Taylor et al. did not observe any significant long-term changes in the whole genome expression after the intervention. Furthermore, Berlanga-Taylor et al. backed up the results of Pasing et al., who also did not observe any significant difference between the gene expression of the intervention and the placebo group. In addition, Berlanga-Taylor et al. did also not find any significant changes in the plasma levels of the aforementioned cytokines. It must be noted, that the biological samples were only taken at baseline and after the intervention. Therefore, it cannot be ruled out, that a short-term change in the gene expression might have occurred in the short term of the intervention. Additionally, no specific genes have been examined in this study (Berlanga-Taylor et al. 2018).

Carlberg et al. investigated in their placebo-controlled trial from 2013 primary vitamin D target genes and their role in determining benefits of a vitamin D supplementation. 71 participants, men - 60 years or older and women - 65 years or older, with impaired glucose tolerance, were selected for this trial. They received either 40 µg, 80 µg of vitamin D or placebo, daily for 5 months during winter. Peripheral blood mononuclear cells (PBMCs) and adipose tissues were taken from the participants. The RNA was isolated with the TRIzol method, further purification was conducted with miRNeasy mini kit columns. After that, a qPCR has been carried out. CD14 and THBD have been chosen as target genes, as they are known for their long response, following a supplementation with 1,25(OH)D. Interestingly, none of the samples showed a significant increase in the expression of CD14 and THBD after the intervention. The authors then split the intervention group into two parts: those who responded better to vitamin D and those who did not. The first half (i.e. the better responding half) showed a significant increase in the expression of CD14 and THBD after the intervention, whereas the other half did not show an increase. Carlberg et al. explained this with the hypothesis, that the lower half has either a restricted response to vitamin D supplementation or a very efficient vitamin D response, so that they are already saturated with very low levels of vitamin D. The choice of CD14 and THBD as biomarkers for vitamin D was confirmed, as the upper half of the participants showed a significant decrease in the expression of IL-6 after the vitamin D intervention as

well. IL-6 is known to play a part in inflammatory reactions. Thus, a downregulation of IL-6 combined with an upregulation of CD14 and THBD, is indicating a benefit of the vitamin D supplementation (Carlberg et al. 2013).

In their study in 2018, which was part of the VitDBol study series, Neme et al. investigated in vivo changes to the transcriptome of white blood cells after vitamin D intake. Five individuals – four 22-year old women and a 51-year old man, received a vitamin D bolus of 2000 µg and blood samples were taken at baseline and 24 hours later. Prior to the supplementation, the subjects showed a vitamin D status between 39,7 nmol/L and 106,5 nmol/L. PBMCs were harvested and the RNA was isolated with the Direct-zol mini prep kit. Furthermore, RNA-seq has been carried out. Examining 15.040 generally expressed genes, the authors found 702 genes, which were significantly impacted by the vitamin D bolus. Of those 702 genes, 501 were upregulated and 201 were downregulated. A subsequent KEGG pathway analysis showed, that those genes were mainly responsible for protein translation, cellular differentiation and cellular growth control. Neme et al. compared the results with similar studies and found that 181 target genes overlapped with the genes they found. Those genes included well-known target genes such as CD14, FBP1 or NINJ. Comparing the results of Neme et al. with the aforementioned study of Hossein-nezhad et al. from 2013, only 13 of the genes, including JUN, JUNB and JUND, could be found on the list of the 702 genes. Similar to Carlberg et al., Neme et al. ranked the 5 participants based on their response to the vitamin D on their individual gene expression. The participant who responded best, showed a 2,01-fold, whereas the participant who responded the worst showed a 1,53-fold decrease in gene expression. Surprisingly, the study revealed a negative correlation between serum calcidiol and calcitriol levels and the expression of the 702 genes (Neme et al. 2019).

Kariuki et al. conducted a similar study in 2016, where they investigated the effects of calcitriol on the transcriptional and cellular response in Afro Americans. But contrary to the trial of Neme et al. they investigated those effects in vitro. For the purpose of this study, blood was taken from 88 healthy Afro Americans, which were aged between 19 and 62 years. Subsequently, PBMCs were isolated. Those PBMCs were treated in vitro with 1,25(OH)2D3 for 6 hours. Then, the RNA was isolated via the RNeasy Plus mini kit and low-level microarrays have been performed. Kariuki et al. examined 11,897 genes, of which 720 showed a significant response to the

treatment with calcitriol (Kariuki et al. 2016). Neme et al., found that 78 genes overlapped with the 702 genes including CEBPB, CD14, CDK6 and SIRT6 (Neme et al. 2019). Of the 720 genes found to react to calcitriol in this study, many genes showed an involvement in immune response pathways such as TREM1 signalling and granulocyte and T-helper cell differentiation. This finding once again underlines the importance of vitamin D in the modulation of the immune system. Furthermore, a higher activation of the VDR/RXR pathway has been registered, including genes such as CD14 or CYP24A1 (Kariuki et al. 2016).

To summarize the studies with whole blood samples, there were conflicting results regarding the gene expression and the intake of vitamin D. Hossein-nezhad et al. found a significant change in the expression of 291 genes after vitamin D supplementation, with a majority of genes involved in the immune response. This observation was backed up by Carlberg et al., Neme et al. and Kariuki et al. This change in gene expression and its relationship to the immune response could possibly explain the influence of vitamin D on various diseases. On the other hand, Pasing et al. found only six genes differently expressed and Berlanga-Taylor et al. found no significant change in the gene expression. Pasing et al. explained this with the already high vitamin D levels of the participants at baseline, indicating that a vitamin D threshold exists, above which there may be no additional effect of a vitamin D supplementation.

Study	Subjects	Supplement-ation	Exercise	Examination	Results
Hossein-nezhad et al., randomized, placebo-controlled, double-blind trial; 2013	Eight humans; aged 18 or older; male and female	400 IU or 2000 IU daily for two months	No	Influence of vitamin D supplementation on genome wide expression of white blood cells	Significant difference in gene expression between baseline and the end of the intervention; no difference between the two supplementation groups; 291 genes were impacted by the

					vitamin D intervention
Pasing et al.; randomized, controlled sub-study of "Prevention of type 2 diabetes with vitamin D supplementation in subjects with reduced glucose tolerance"	94 people between the ages of 25-86; male and female	20.000 IU of cholecalciferol or placebo, once weekly, over the course of five years	No	Changes in the human transcriptome upon vitamin D supplementation	No significant differences between supplementation an placebo group; only 6 of 141 genes examined were expressed significantly different; a significant difference occured between male and female test subjects in 58 genes
Trummer et al.; Post hoc analysis of the styria vitamin D hypertension trial in 2017	175 people; aged over 18; vitamin D status of below 30 ng/ml; with hypertension	2800 IU of calcidiol or placebo; once daily for eight weeks	No	The impact of a vitamin D intervention on the expression of IGF-1	No significant difference in the expression of IGF-1 before and after the intervention
Berlanga-Taylor et al. intention-to-treat, double-blind, placebo-controlled, dose-finding, randomized clinical trial; 2018	305 community-dwelling individuals; aged over 65;	4000 IU, 2000 IU of vitamin D or placebo; daily for 12 months	No	Genomic response to vitamin D supplementation	No significant long-term changes in the whole genome expression; No significant changes in cytokine plasma levels
Carlberg et al.; placebo-	71 participants;	40 µg vitamin D, 80 µg of	No	Can vitamin D target genes	No initial increase of target genes

controlled trial; 2013;	Men 60 years or older; Women 65 years or older; impaired glucose tolerance	vitamin D or placebo; daily over the course of five months		indicate the benefits of vitamin D supplementation	THBD and CD14 after the supplementation; after splitting the intervention group 50/50 into better responders and lower responders, the better responders showed an increase in the expression of THBD and CD14; overall, the target genes THBD and CD14 indicated a benefit of the vitamin D supplementation
Neme et al.; 2018	Five individuals; Four 22-year old women and one 51-year old man; Vitamin D levels ranging from 39 nmol/L to 106 nmol/L	One-time vitamin D bolus of 2000 µg	No	In vivo changes of the transcriptome after vitamin D supplementation	702 of 15,040 genes responded significantly to the vitamin D intake; Surprisingly a negative correlation between the expression of the 702 genes and serum calcidiol and calcitriol levels have been found
Kariuki et al.; 2016	88 healthy Afro Americans; Between 19 and 62 years	Treatment of PBMCs with calcitriol for six hours	No	In vitro changes in transcriptional and cellular response to calcitriol in afro americans	720 of 11,897 genes showed a significant interaction with calcitriol. 78 of those 720 genes

					overlapped with the genes found by Neme et al.. The majority of those 720 genes played a part in immune response and VDR/RXR pathways.

Table 6: Summary of studies involving whole blood samples and gene expression

5. Conclusio

The most important part of this thesis was finding the right kit for extracting RNA from lymphocytes. After trying four different kits and protocols, the ReliaPrep RNA Cell Miniprep system proved to be the best method for extracting RNA. The kit produced constantly good, repeatable results. In addition, the extraction was very practicable, taking a reasonable amount of time, with the included DNase treatment, proving to be a big advantage too. Furthermore, the effect of vitamin D on the expression of genes was investigated in this thesis. Vitamin D supplementation has an undisputable benefit to the health of individuals with insufficient vitamin D levels. Although, the mechanisms on a gene expression level still remain not entirely understood, an increasing number of studies has investigated this topic over the past few years. However, to my knowledge, none has examined the effects of a vitamin D supplementation and strength exercise on the gene expression in white blood cells, making the NutriAging study the first investigating it. This thesis provides a preview on what to expect of this study from a gene expression point of view, however the papers reviewed produced conflicting results. Additionally, most of the studies differed considerably from the NutriAging study either in the length of the intervention or in the number of participants, making a prediction on the outcomes even harder.

6. Abstract

Vitamin D is known to play a significant role in ageing and disease processes. Thus, vitamin D deficiency, especially in older people, may be accelerating these developments. The NutriAgeing project is examining the impact of the combination of vitamin D, calcium and strength training on the well-being and health of community-dwelling elder people. As a part of this project, this thesis is investigating the anticipated effect of such an intervention on the RNA and gene expression, through the analysis of previous studies in this scientific field. Although, the results of these studies provided conflicting evidence regarding gene expression after the supplementation with vitamin D. Additionally, finding the right RNA extraction kit constituted an important part of this thesis. For that purpose, four different kits including the RNeasy Mini kit, the TRI reagent protocol, the ReliaPrep miRNA cell and tissue prep system and the ReliaPrep RNA Cell Miniprep system have been tested. The ReliaPrep RNA Cell Miniprep system proved to be the best way to extract RNA. Furthermore, the processes of lymphocyte isolation, as well as the analysing methods, such as the PCR applied in this study, are described.

6. Zusammenfassung

Es ist allgemein bekannt, dass Vitamin D eine wichtige Rolle in diversen Alters- und Krankheitsprozessen spielt. Deshalb liegt der Verdacht nahe, dass ein Vitamin D Mangel, vor allem bei älteren Menschen, genau diese Prozesse beschleunigen kann. Das NutriAgeing Projekt untersucht daher die Auswirkungen einer Intervention mit Vitamin D, Calcium und Krafttraining auf das Wohlbefinden und die Gesundheit von Seniorinnen und Senioren, die nicht institutionalisiert sind. Als Teil dieses Projektes, wurde in dieser Diplomarbeit der zu erwartende Effekt einer solchen Intervention auf die RNA und Genexpression untersucht, in dem eine Literaturrecherche durchgeführt wurde. Allerdings lieferten die Ergebnisse dieser Analyse keine klaren Ergebnisse, nachdem manche Studien einen signifikanten Unterschied in der Genexpression beobachteten und andere nicht. Ein weiterer Fokus dieser Diplomarbeit lag auf der Suche des richtigen Kits zur Extraktion der RNA. Dafür wurden vier verschiedene Kits und Protokolle getestet. Diese umfassten das RNeasy Mini kit, das TRI reagent protocol, das ReliaPrep miRNA cell and tissue prep system und das ReliaPrep RNA Cell Miniprep system, wobei das Letztgenannte sich als am besten erwies. Des Weiteren werden in dieser Arbeit einzelne Schritte wie z.B. die Lymphozyten Isolation und die Grundanalysemethoden, wie sie in der Studie angewendet wurden, beschrieben.

Bibliography

Antoniak, Anneka Elizabeth; Greig, Carolyn A. (2017): The effect of combined resistance exercise training and vitamin D 3 supplementation on musculoskeletal health and function in older adults: a systematic review and meta-analysis. In: *BMJ Open* 7 (7), e014619. DOI: 10.1136/bmjopen-2016-014619.

Aranda, Patrick S.; LaJoie, Dollie M.; Jorcyk, Cheryl L. (2012): Bleach gel: a simple agarose gel for analyzing RNA quality. In: *Electrophoresis* 33 (2), S. 366–369. DOI: 10.1002/elps.201100335.

Armas, Laura A. G.; Hollis, Bruce W.; Heaney, Robert P. (2004): Vitamin D2 is much less effective than vitamin D3 in humans. In: *The Journal of clinical endocrinology and metabolism* 89 (11), S. 5387–5391. DOI: 10.1210/jc.2004-0360.

Berlanga-Taylor, Antonio J.; Plant, Katharine; Dahl, Andrew; Lau, Evelyn; Hill, Michael; Sims, David et al. (2018): Genomic Response to Vitamin D Supplementation in the Setting of a Randomized, Placebo-Controlled Trial. In: *EBioMedicine* 31, S. 133–142. DOI: 10.1016/j.ebiom.2018.04.010.

Booth, Lauren N.; Brunet, Anne (2016): The Aging Epigenome. In: *Molecular cell* 62 (5), S. 728–744. DOI: 10.1016/j.molcel.2016.05.013.

Bronner, Felix (2003): Mechanisms of intestinal calcium absorption. In: *Journal of cellular biochemistry* 88 (2), S. 387–393. DOI: 10.1002/jcb.10330.

Brown, P. O.; Botstein, D. (1999): Exploring the new world of the genome with DNA microarrays. In: *Nature genetics* 21 (1 Suppl), S. 33–37. DOI: 10.1038/4462.

Bustin, Stephen A.; Benes, Vladimir; Garson, Jeremy A.; Hellemans, Jan; Huggett, Jim; Kubista, Mikael et al. (2009): The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. In: *Clinical chemistry* 55 (4), S. 611–622. DOI: 10.1373/clinchem.2008.112797.

Carlberg, Carsten (2017): Molecular endocrinology of vitamin D on the epigenome level. In: *Molecular and cellular endocrinology* 453, S. 14–21. DOI: 10.1016/j.mce.2017.03.016.

Carlberg, Carsten; Seuter, Sabine; Mello, Vanessa D. F. de; Schwab, Ursula; Voutilainen, Sari; Pulkki, Kari et al. (2013): Primary Vitamin D Target Genes Allow a Categorization of Possible Benefits of Vitamin D3 Supplementation. In: *PLoS ONE* 8 (7). DOI: 10.1371/journal.pone.0071042.

Christakos, Sylvia; Veldurthy, Vaishali; Patel, Nishant; Wei, Ran (2017): Intestinal Regulation of Calcium: Vitamin D and Bone Physiology. In: *Advances in experimental medicine and biology* 1033, S. 3–12. DOI: 10.1007/978-3-319-66653-2_1.

Costa-Silva, Juliana; Domingues, Douglas; Lopes, Fabricio Martins (2017): RNA-Seq differential expression analysis: An extended review and a software tool. In: *PLoS ONE* 12 (12), e0190152. DOI: 10.1371/journal.pone.0190152.

Deutsche Gesellschaft für Ernährung (2012): Neue Referenzwerte für Vitamin D. Online verfügbar unter https://www.dge.de/presse/pm/neue-referenzwerte-fuer-vitamin-d/, zuletzt geprüft am Accessed: 10.02.2020.

Diaz de Barboza, Gabriela; Guizzardi, Solange; Tolosa de Talamoni, Nori (2015): Molecular aspects of intestinal calcium absorption. In: *World journal of gastroenterology* 21 (23), S. 7142–7154. DOI: 10.3748/wjg.v21.i23.7142.

Emkey, Ronald D.; Emkey, Gregory R. (2012): Calcium metabolism and correcting calcium deficiencies. In: *Endocrinology and metabolism clinics of North America* 41 (3), S. 527–556. DOI: 10.1016/j.ecl.2012.04.019.

Fetahu, Irfete S.; Höbaus, Julia; Kállay, Enikő (2014): Vitamin D and the epigenome. In: *Frontiers in physiology* 5, S. 164. DOI: 10.3389/fphys.2014.00164.

Girgis, Christian M.; Mokbel, Nancy; Cha, Kuan Minn; Houweling, Peter J.; Abboud, Myriam; Fraser, David R. et al. (2014): The vitamin D receptor (VDR) is expressed in skeletal muscle of male mice and modulates 25-hydroxyvitamin D (25OHD) uptake in myofibers. In: *Endocrinology* 155 (9), S. 3227–3237. DOI: 10.1210/en.2014-1016.

Glendenning, Paul; Zhu, Kun; Inderjeeth, Charles; Howat, Peter; Lewis, Joshua R.; Prince, Richard L. (2012): Effects of three-monthly oral 150,000 IU cholecalciferol supplementation on falls, mobility, and muscle strength in older

postmenopausal women: A randomized controlled trial. In: *J Bone Miner Res* 27 (1), S. 170–176. DOI: 10.1002/jbmr.524.

Groen, Albert K. (2001): The pros and cons of gene expression analysis by microarrays. In: *Journal of Hepatology* 35 (2), S. 295–296. DOI: 10.1016/s0168-8278(01)00156-8.

Hangelbroek, Roland W. J.; Vaes, Anouk M. M.; Boekschoten, Mark V.; Verdijk, Lex B.; Hooiveld, Guido J. E. J.; van Loon, Luc J. C. et al. (2019): No effect of 25-hydroxyvitamin D supplementation on the skeletal muscle transcriptome in vitamin D deficient frail older adults. In: *BMC geriatrics* 19 (1), S. 151. DOI: 10.1186/s12877-019-1156-5.

Hassan-Smith, Zaki K.; Jenkinson, Carl; Smith, David J.; Hernandez, Ivan; Morgan, Stuart A.; Crabtree, Nicola J. et al. (2017): 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 exert distinct effects on human skeletal muscle function and gene expression. In: *PLoS ONE* 12 (2), e0170665. DOI: 10.1371/journal.pone.0170665.

Hill, Tom R.; Granic, Antoneta; Aspray, Terence J. (2018): Vitamin D and Ageing. In: *Sub-cellular biochemistry* 90, S. 191–220. DOI: 10.1007/978-981-13-2835-0_8.

Hoffmann, Michelle R.; Senior, Peter A.; Mager, Diana R. (2015): Vitamin D supplementation and health-related quality of life: a systematic review of the literature. In: *Journal of the Academy of Nutrition and Dietetics* 115 (3), S. 406–418. DOI: 10.1016/j.jand.2014.10.023.

Holick, M. F. (1994): McCollum Award Lecture, 1994: vitamin D--new horizons for the 21st century. In: *The American journal of clinical nutrition* 60 (4), S. 619–630. DOI: 10.1093/ajcn/60.4.619.

Holick, Michael F. (2003): Vitamin D: A millenium perspective. In: *Journal of cellular biochemistry* 88 (2), S. 296–307. DOI: 10.1002/jcb.10338.

Holick, Michael F. (2004): Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. In: *The American journal of clinical nutrition* 80 (6 Suppl), 1678S-88S. DOI: 10.1093/ajcn/80.6.1678S.

Holick, Michael F. (2006): Resurrection of vitamin D deficiency and rickets. In: *The Journal of clinical investigation* 116 (8), S. 2062–2072. DOI: 10.1172/JCI29449.

Hossein-nezhad, Arash; Spira, Avrum; Holick, Michael F. (2013): Influence of vitamin D status and vitamin D3 supplementation on genome wide expression of white blood cells: a randomized double-blind clinical trial. In: *PLoS ONE* 8 (3), e58725. DOI: 10.1371/journal.pone.0058725.

Hui, Pei (2014): Next generation sequencing: chemistry, technology and applications. In: *Topics in current chemistry* 336, S. 1–18. DOI: 10.1007/128_2012_329.

Ishmael, Faoud T.; Stellato, Cristiana (2008): Principles and applications of polymerase chain reaction: basic science for the practicing physician. In: *Annals of Allergy, Asthma & Immunology* 101 (4), S. 437–443. DOI: 10.1016/S1081-1206(10)60323-7.

Jeon, Sang-Min; Shin, Eun-Ae (2018): Exploring vitamin D metabolism and function in cancer. In: *Experimental & Molecular Medicine* 50 (4). DOI: 10.1038/s12276-018-0038-9.

Jo, Jihoon; Choi, Sunkyung; Oh, Jooseong; Lee, Sung-Gwon; Choi, Song Yi; Kim, Kee K.; Park, Chungoo (2019): Conventionally used reference genes are not outstanding for normalization of gene expression in human cancer research. In: *BMC bioinformatics* 20 (Suppl 10), S. 245. DOI: 10.1186/s12859-019-2809-2.

Kara Rogers: Gel electrophoresis. In: Encyclopaedia Britannica. Online verfügbar unter https://www.britannica.com/science/gel-electrophoresis.

Kariuki, Silvia N.; Maranville, Joseph C.; Baxter, Shaneen S.; Jeong, Choongwon; Nakagome, Shigeki; Hrusch, Cara L. et al. (2016): Mapping Variation in Cellular and Transcriptional Response to 1,25-Dihydroxyvitamin D3 in Peripheral Blood Mononuclear Cells. In: *PLoS ONE* 11 (7), e0159779. DOI: 10.1371/journal.pone.0159779.

Kellett, George L. (2011): Alternative perspective on intestinal calcium absorption: proposed complementary actions of Ca(v)1.3 and TRPV6. In: *Nutrition reviews* 69 (7), S. 347–370. DOI: 10.1111/j.1753-4887.2011.00395.x.

Kunal Chatterjee, Yao Wan (1998): RNA. Encyclopaedia Britannica. Online verfügbar unter https://www.britannica.com/science/RNA, zuletzt aktualisiert am 2018.

Laird, Eamon; Ward, Mary; McSorley, Emeir; Strain, J. J.; Wallace, Julie (2010): Vitamin D and bone health: potential mechanisms. In: *Nutrients* 2 (7), S. 693–724. DOI: 10.3390/nu2070693.

Li, Kelvin; Wang, Xia-Fang; Li, Ding-You; Chen, Yuan-Cheng; Zhao, Lan-Juan; Liu, Xiao-Gang et al. (2018): The good, the bad, and the ugly of calcium supplementation: a review of calcium intake on human health. In: *Clinical interventions in aging* 13, S. 2443–2452. DOI: 10.2147/CIA.S157523.

Lindbjerg Andersen, Claus; Gundesen Christian; Adolf, Katja (2004-2010): Documentation for NormFinder.

López-Otín, Carlos; Blasco, Maria A.; Partridge, Linda; Serrano, Manuel; Kroemer, Guido (2013): The Hallmarks of Aging. In: *Cell* 153 (6), S. 1194–1217. DOI: 10.1016/j.cell.2013.05.039.

Manson, JoAnn E.; Cook, Nancy R.; Lee, I-Min; Christen, William; Bassuk, Shari S.; Mora, Samia et al. (2018): Vitamin D Supplements and Prevention of Cancer and Cardiovascular Disease. In: *The New England journal of medicine* 380 (1), S. 33–44. DOI: 10.1056/NEJMoa1809944.

Marmorstein, Ronen; Zhou, Ming-Ming (2014): Writers and Readers of Histone Acetylation: Structure, Mechanism, and Inhibition. In: *Cold Spring Harbor Perspectives in Biology* 6 (7). DOI: 10.1101/cshperspect.a018762.

Moore, Lisa D.; Le, Thuc; Fan, Guoping (2012): DNA Methylation and Its Basic Function. In: *Neuropsychopharmacology* 38 (1), S. 23–38. DOI: 10.1038/npp.2012.112.

Neme, Antonio; Seuter, Sabine; Malinen, Marjo; Nurmi, Tarja; Tuomainen, Tomi-Pekka; Virtanen, Jyrki K.; Carlberg, Carsten (2019): In vivo transcriptome changes of human white blood cells in response to vitamin D.

In: *The Journal of steroid biochemistry and molecular biology* 188, S. 71–76. DOI: 10.1016/j.jsbmb.2018.11.019.

Nolan, Tania; Hands, Rebecca E.; Bustin, Stephen A. (2006): Quantification of mRNA using real-time RT-PCR. In: *Nature Protocols* 1 (3), S. 1559–1582. DOI: 10.1038/nprot.2006.236.

Pasing, Yvonne; Fenton, Christopher Graham; Jorde, Rolf; Paulssen, Ruth Hracky (2017): Changes in the human transcriptome upon vitamin D supplementation. In: *The Journal of steroid biochemistry and molecular biology* 173, S. 93–99. DOI: 10.1016/j.jsbmb.2017.03.016.

Pérez, Adriana V.; Picotto, Gabriela; Carpentieri, Agata R.; Rivoira, María A.; Peralta López, María E.; Tolosa de Talamoni, Nori G. (2008): Minireview on regulation of intestinal calcium absorption. Emphasis on molecular mechanisms of transcellular pathway. In: *Digestion* 77 (1), S. 22–34. DOI: 10.1159/000116623.

Perry, R. L.; Rudnick, M. A. (2000): Molecular mechanisms regulating myogenic determination and differentiation. In: *Frontiers in bioscience : a journal and virtual library* 5, D750-67. DOI: 10.2741/perry.

Pike, J. Wesley; Lee, Seong Min; Meyer, Mark B. (2014): Regulation of gene expression by 1,25-dihydroxyvitamin D3 in bone cells: exploiting new approaches and defining new mechanisms. In: *BoneKEy reports* 3, S. 482. DOI: 10.1038/bonekey.2013.216.

Pike, J. Wesley; Meyer, Mark B.; Lee, Seong-Min; Onal, Melda; Benkusky, Nancy A. (2017): The vitamin D receptor: contemporary genomic approaches reveal new basic and translational insights. In: *The Journal of clinical investigation* 127 (4), S. 1146–1154. DOI: 10.1172/JCI88887.

Pojednic, Rachele M.; Ceglia, Lisa; Olsson, Karl; Gustafsson, Thomas; Lichtenstein, Alice H.; Dawson-Hughes, Bess; Fielding, Roger A. (2015): Effects of 1,25-dihydroxyvitamin D3 and vitamin D3 on the expression of the vitamin d receptor in human skeletal muscle cells. In: *Calcified tissue international* 96 (3), S. 256–263. DOI: 10.1007/s00223-014-9932-x.

Powledge, Tabitha M. (2004): The polymerase chain reaction. In: *Advances in physiology education* 28 (1-4), S. 44–50. DOI: 10.1152/advan.00002.2004.

Promega (2012): ReliaPrep RNA Cell Miniprep System. Online verfügbar unter https://at.promega.com/Resources/Protocols/Technical%20Manuals/101/ReliaPrep%20RNA%20Cell%20Miniprep%20System%20Protocol/?fq=Relia%20prep%20RNa%20cell, zuletzt aktualisiert am 11/12.

Promega (2016): ReliaPrep miRNA cell and tissue prep system. Online verfügbar unter https://at.promega.com/products/nucleic-acid-extraction/rna/reliaprep-mirna-cell-and-tissue-miniprep-system/?catNum=Z6210#protocols, zuletzt aktualisiert am 11/2019, zuletzt geprüft am Zugegriffen am 03.02.2020.

Qiagen (2019): RNeasy Mini Handbook. Online verfügbar unter https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en, zuletzt aktualisiert am Zugegriffen am: 30.01.2020.

Rebelo-Marques, Alexandre; Sousa Lages, Adriana de; Andrade, Renato; Ribeiro, Carlos Fontes; Mota-Pinto, Anabela; Carrilho, Francisco; Espregueira-Mendes, João (2018): Aging Hallmarks: The Benefits of Physical Exercise. In: *Frontiers in Endocrinology* 9. DOI: 10.3389/fendo.2018.00258.

Sahota, Opinder (2014): Understanding vitamin D deficiency. In: *Age and Ageing* 43 (5), S. 589–591. DOI: 10.1093/ageing/afu104.

Sanders, Kerrie M.; Stuart, Amanda L.; Williamson, Elizabeth J.; Simpson, Julie A.; Kotowicz, Mark A.; Young, Doris; Nicholson, Geoffrey C. (2010): Annual High-Dose Oral Vitamin D and Falls and Fractures in Older Women. In: *JAMA* 303 (18), S. 1815. DOI: 10.1001/jama.2010.594.

Sigma-Aldrich (Accessed: 2020): Product Information - TRI reagent. Online verfügbar unter https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/t9424bul.pdf, zuletzt aktualisiert am Accessed: 30.01.2020.

Somel, Mehmet; Guo, Song; Fu, Ning; Yan, Zheng; Hu, Hai Yang; Xu, Ying et al. (2010): MicroRNA, mRNA, and protein expression link development and

aging in human and macaque brain. In: *Genome Research* 20 (9), S. 1207–1218. DOI: 10.1101/gr.106849.110.

Thermo Fischer Scientific (2017): Nanodrop One User Guide. Online verfügbar unter https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FMSD%2Fmanuals%2Fnanodrop-one-user-guide-EN-309102.pdf&title=TmFub0Ryb3AgT25lIFVzZXIgR3VpZGUgW0VOXQ==, zuletzt aktualisiert am Accessed: 29.01.2020.

Thermo Fischer Scientific (2018): Qubit 4 fluorometer user guide. Online verfügbar unter https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0017209_Qubit_4_Fluorometer_UG.pdf&title=VXNlciBHdWlkZTogUXViaXQgNCBGbHVvcm9tZXRlcg==, zuletzt aktualisiert am Accessed: 03.02.2020.

Thermo Fisher Scientific (2015): User Guide: Qubit RNA assay kits. Online verfügbar unter https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FQubit_RNA_BR_Assay_UG.pdf&title=VXNlciBHdWlkZTogUXViaXQgUk5BIEJSIEFzc2F5IEtpdHM=, zuletzt aktualisiert am Accessed: 31.01.2020.

Thermo Fisher Scientific (Accessed: 2020): Qubit fluorometric quantification. Online verfügbar unter https://www.thermofisher.com/at/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/fluorometers/qubit.html, zuletzt aktualisiert am Accessed: 31.01.2020.

Tripkovic, Laura; Lambert, Helen; Hart, Kathryn; Smith, Colin P.; Bucca, Giselda; Penson, Simon et al. (2012): Comparison of vitamin D2 and vitamin D3 supplementation in raising serum 25-hydroxyvitamin D status: a systematic review and meta-analysis. In: *The American journal of clinical nutrition* 95 (6), S. 1357–1364. DOI: 10.3945/ajcn.111.031070.

Trummer, Christian; Schwetz, Verena; Pandis, Marlene; Grübler, Martin R.; Verheyen, Nicolas; Gaksch, Martin et al. (2017): Effects of Vitamin D

Supplementation on IGF-1 and Calcitriol: A Randomized-Controlled Trial. In: *Nutrients* 9 (6). DOI: 10.3390/nu9060623.

Vaes, Anouk M. M.; Tieland, Michael; Regt, Margot F. de; Wittwer, Jonas; van Loon, Luc J. C.; Groot, Lisette C. P. G. M. de (2018): Dose-response effects of supplementation with calcifediol on serum 25-hydroxyvitamin D status and its metabolites: A randomized controlled trial in older adults. In: *Clinical nutrition (Edinburgh, Scotland)* 37 (3), S. 808–814. DOI: 10.1016/j.clnu.2017.03.029.

Wagner, Karl-Heinz, Muchova Jana (2019): The NutriAging project. University of Vienna, Comenius University Bratislava. http://www.nutriaging.eu/#scr_top. Online verfügbar unter http://www.nutriaging.eu/#scr_top.

Wang, Zhong; Gerstein, Mark; Snyder, Michael (2009): RNA-Seq: a revolutionary tool for transcriptomics. In: *Nature reviews. Genetics* 10 (1), S. 57–63. DOI: 10.1038/nrg2484.

Williams, Justin; Smith, Flint; Kumar, Subodh; Vijayan, Murali; Reddy, P. Hemachandra (2016): Are MicroRNAs True Sensors of Ageing and Cellular Senescence? In: *Ageing research reviews* 35, S. 350–363. DOI: 10.1016/j.arr.2016.11.008.

Wimalawansa, Sunil J. (2019): Vitamin D Deficiency: Effects on Oxidative Stress, Epigenetics, Gene Regulation, and Aging. In: *Biology* 8 (2). DOI: 10.3390/biology8020030.

World Health Organisation WHO (2015): World report on ageing and health.

World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects (2013). In: *JAMA* 310 (20), S. 2191–2194.

Wrobel, S. (1995): Serendipity, science, and a new hantavirus. In: *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 9 (13), S. 1247–1254. DOI: 10.1096/fasebj.9.13.7557014.

Zavolan, Mihaela; Gerber, André P. (2018): Mirroring the multifaceted role of RNA and its partners in gene expression. In: *FEBS letters* 592 (17), S. 2825–2827. DOI: 10.1002/1873-3468.13230.

8. Appendix

Datum der Messung	Zellzahl	Proband (Termin 1)	RNA (ng/µl) Nanodrop	RNA (ng/µl) Fluorometer	OD260/280 (above 1.8, around 2)	OD260/230 between 2 a	Agarose-F	Dna Bande
11.02.19		D001	60,1	45,4	1,96	0,55	2	-
11.02.19		D002	21,9	14,3	1,91	0,97		-
11.02.19		D003	8	6,33	1,84	0,59		-
11.02.19		D004	46,4	40,3	1,99	1,29	2	-
11.02.19		D005	88,3	too low	1,97	1,76	2	-
11.02.19		D006	18,8	21,9	1,91	1,03	2	-
11.02.19		D007	63,7	39	1,98	0,88	2	-
11.02.19		D008	76,9	68,7	1,98	1,71	2	-
11.02.19		D009	87,4	62,5	1,96	1,51	3	-
18.02.19	5000000	D010	115,7	71,8	1,98	2,12	2	-
18.02.19	5000000	D011	105,3	75,9	2,01	2,15	2	-
18.02.19	5000000	D012	128,7	94	2,03	2,14	2	-
18.02.19	5000000	D013	116,2	80,8	2,01	2,1	2	-
18.02.19	5000000	D014	102,2	72,8	2	2,09	2	-
18.02.19	5000000	D016	65,8	111	2,04	1,92	2	-
18.02.19	5000000	D017	108	153	2,02	2,2	2	-
18.02.19	3000000	D018	67,4	107	2,05	2,19	2	-
18.02.19	5000000	D019	124,3	205	2,07	2,2	2	-
18.02.19	5000000	D020	96,5	140	2,04	2,07	2	-
18.02.19	5000000	D021	109,1	158	2,02	2,06	2	-
18.02.19	5000000	D022	117	166	2,05	2,22	1	-
25.02.19	4000000	D015	58,3	47,6	2,09	1,71	0	-
25.02.19	4000000	D023	52,6	39,2	2,09	2,04	0	-
25.02.19	4000000	D024	91,9	75,7	2,08	2,1	1	-
25.02.19	4500000	D027	64,9	49,2	2,09	1,68	1	-
25.02.19	4000000	D030	54,3	43,1	2,08	1,49	1	-
25.02.19	5000000	D031	68,9	54,5	2,07	1,88	1	-
25.02.19	5000000	D033	69,2	54,5	2,09	2,07	1	-
25.02.19	5000000	D034	49,1	37,9	2,15	2,4	1	-
25.02.19	5000000	D035	78,8	65,6	2,12	1,99	1	-
25.02.19	5000000	D036	90	74,9	2,12	2,23	1	-
25.02.19	5000000	D037	76,9	61,9	2,14	1,31	1	-
26.02.19	5000000	D026	82,5	72,4	2,07	2,16	2	-
26.02.19	5000000	D028	73,7	65,9	2,03	1,66	2	-
26.02.19	5000000	D029	75	61,3	2,04	2,05	2	-
26.02.19	5000000	D038	155,5	123	2,04	2,11	2	-
04.03.2019	5000000	D031	117,6	106	2,03	1,97	2	-
04.03.2019	5000000	D032	93	84,2	2,03	2,08	2	-
04.03.2019	5000000	D039	160,7	109	1,96	2,05	2	-
04.03.2019	5000000	D041	59,2	49,6	2,03	2,13	2	-
04.03.2019	2000000	D042	48,6	too low	2,02	2,13	2	-
04.03.2019	3000000	D043	62,2	51,3	2,02	1,24	2	-
04.03.2019	3000000	D045	73,9	69,3	2,02	1,85	2	-
05.03.2019	5000000	D046	28,5	25,8	2,12	1,94	2	-
05.03.2019	5000000	D047	36,5	30,2	2,08	1,55	2	-
05.03.2019	5000000	D048	78,3	92,7	2,07	1,58	2	-
05.03.2019	5000000	D052	107,3	126	2,07	2,15	2	-
11.03.2019	5000000	D040	61,6	21	1,96	2,08	3	-
11.03.2019	5000000	D044	79,9	83	2,04	1,93	1	-
11.03.2019	5000000	D053	99,2	105	2,04	2,13	1	-
11.03.2019	5000000	D056	108,2	113	2,05	2,24	1	-
11.03.2019	5000000	D057	110,2	95,4	2,01	2,07	1	-
11.03.2019	5000000	D058	81,9	74,7	2,04	2,25	1	-
11.03.2019	5000000	D059	66,1	57,3	2,01	2,27	1	ja
11.03.2019	4000000	D060	40	33,2	2,03	1,95	1	-
11.03.2019	4000000	D061	34,1	31,5	1,98	1,61	1	-
11.03.2019	4000000	D063	34,7	33,8	2	1,89	1	-
11.03.2019	5000000	D065	46,4	47,5	2,03	1,73	1	-
11.03.2019	5000000	D068	61,7	49,9	2	2,06	1	-
11.03.2019	4000000	D070	37,7	27,9	2,02	2,06	1	-
11.03.2019	2300000	D072	33,4	32,5	2,08	0,92	1	-
12.03.2019		D064	56,3		2,03	1,95	1	-
18.03.2019	5.000000	D049	51,9	53,2	2,04	2,15	1	-
18.03.2019	5.000000	D050	79	79,7	2,03	1,98	1	-
18.03.2019	5.000000	D051	45	46,8	2,05	2,01	1	-
18.03.2019	3.000000	D066	46,5	49,5	2	1,96	1	-
18.03.2019	5.000000	D081	61,9	65,3	2,03	1,85	1	-
18.03.2019	5.000000	D084	44,4	46,4	2,04	2,11	1	-
19.03.2019	5.000000	D071	50,8	53,2	2,01	2,04	1	-
19.03.2019	3.500000	D073	43,1	42,3	2,03	2,16	1	-
19.03.2019	5.000000	D074	62,8	61,3	2,02	2,04	1	-
19.03.2019	5.000000	D075	110,3	101	2,03	2,16	1	-
19.03.2019	5.000000	D076	43,1	43	2,02	0,69	1	-
19.03.2019	3.000000	D077	56,6	53,7	2,02	2,12	1	-
19.03.2019	5.000000	D078	79,4	77,4	2,02	1,21	1	-
19.03.2019	5.000000	D079	77,1	75,6	2,05	2,14	1	-
19.03.2019	5.000000	D080	96,5	93,8	2,07	1,81	1	-
19.03.2019	4.000000	D082	53,2	52,6	2,06	2,03	1	-
19.03.2019	5.000000	D083	58,1	59	2,06	2,04	1	-
19.03.2019	3.000000	D085	37,7	35,6	2,08	2,11	1	-
19.03.2019	5.000000	D086	60,8	61,5	2,09	1,91	1	-
25.03.2019	5.000000	D055	49,4	27,7	2,03	1,05	1	-
25.03.2019	4.000000	D067	82,1	43,2	2,04	2,06	1	-
25.03.2019	5.000000	D091	63,6	27,9	1,99	2,02	2	ja
25.03.2019	5.000000	D092	38,8	20,6	2,03	2,16	1	-
26.03.2019	2.000000	D025	22,6	14,9	1,99	1,85	1	-
26.03.2019	5.000000	D062	50,3	57	2,04	1,59	1	-
26.03.2019	5.000000	D087	57,2	62,5	2,04	1,96	0	-
26.03.2019	5.000000	D088	18,8	19	2,03	1,57	1	-
26.03.2019	5.000000	D089	57,4	62,6	2,03	1,93	1	-
26.03.2019	5.000000	D090	37,3	45,2	2,01	1,58	1	-
26.03.2019	2.500000	D093	57,1	58,7	2,01	2,07	1	-
26.03.2019	4.000000	D094	63	62,5	2,05	2,08	1	-
26.03.2019	5.000000	D095	69	78,7	2,05	1,63	1	-
26.03.2019	4.000000	D096	64,6	70,9	2,03	1,95	1	-
29.03.2019		D054	36,8	35,8	2,05	1,93	1	-
29.03.2019		D097	18,4	17,4	2,09	1,22	1	-
29.03.2019		D098	34,3	42,1	2,08	2,17	1	-
29.03.2019		D099	20,8	19,7	2,07	1,86	1	-
29.03.2019		D100	26,6	25,4	2,05	1,43	1	-
29.03.2019		D101	33	31,6	2,09	1,81	1	-

Table 7: T1 results

Sample X T1	RNA (ng/µl) Nanodrop	RNA (ng/µl) Fluorometer	OD260/280 (above 1.8, ar	OD260/230 b	Agarose-For	Dna Bande a
D001 X	59,3	30,5	1,95	1,62	2	-
D002 X	109,4	109	2	1,97	3	-
D003 X	11,4	9,13	1,82	1,1		-
D004 X	40,1	16,5	1,91	1,75	2	-
D005 X	65	65,9	2,01	1,83	2	-
D006 X	41,7	36,7	1,96	1,16	2	-
D007 X	47	40,2	2,03	1,9	2	-
D008 X	94,8	58,9	2,02	1,88	2	-
D009 X	41,5	34	2,01	1,66	2	-
D010x	112,4	89,8	2,03	2,18	2	-
D011x	129	84,4	2,01	2,04	2	-
D012x	73,1	58,6	2,05	2,13	3	-
D013x	67,4	51	2,06	2,08	2	-
D014x	85,1	141	2,05	1,93	2	-
D016x	77,2	63,6	2,07	2,19	2	-
D017x	60,3	47,1	2,1	2,1	2	-
D018x	68,8	48,7	2,06	1,97	2	-
D019x	103,9	77,9	2,04	2,26	2	-
D020x	97,2	66,8	2,05	2,22	3	-
D021x	78,6	51,1	2,07	2,04	2	-
D022x	156	81,7	1,99	2,3	3	-
D015x	70,4	56,2	2,12	2,37	1	-
D023x	132	102	2,09	2,33	1	-
D024x	113,4	92,1	2,12	2,35	1	-
D027x	124,3	88,5	2,11	2,12	2	-
D030x	72,1	56,5	2,17	2,39	1	-
D031x	103,5	83,9	2,12	2,04	1	-
D033x	92,7	61,1	2,15	1,93	1	-
D034x	62,1	51,1	2,2	2,46	1	-
D035x	63,6	50	2,2	2,3	1	-
D036x	104,1	84,1	2,14	2,21	1	-
D037x	223,6	73,8	1,96	2,36	3	-
D026x	141,2	114	2,04	2,19	2	-
D028x	89,9	75,4	2,04	2,01	2	-
D029x	102,5	85,5	2,06	2,1	2	-
D038x	147,4	108	2,03	2,13	2	-
D031x	83,7	75,6	1,99	2,09	2	-
D032x	121,71	112	2,03	1,73	2	-
D039x	180,2	164	2,01	2,2	2	-
D041x	122	108	2,02	1,97	2	-
D042x	59,6	47,4	1,98	1,91	2	-
D043x	110,9	91	1,99	1,84	2	-
D045x	100,8	92	2	1,98	2	-
D046x	34	16,9	2,03	1,21	2	-
D047x	48,7	41,1	2,03	1,93	2	-
D048x	45	41,2	2,1	1,78	2	-
D052x	46,8	43,8	2,07	1,94	2	-
D040x	42,6	41,2	2,08	1,03	1	-
D044x	46,2	43,5	2,08	1,86	1	-
D053x	38,2	39,1	2,17	1,71	1	-
D056x	68,3	61,8	2,03	1,35	1	-
D057x	82,2	86,7	2,04	2,29	1	ja
D058x	46,1	41,5	2,08	2,33	1	-
D059x	35,5	36,6	2,03	1,85	1	-
D060x	63,9	64	2,04	2,26	1	-
D061x	36,4	35,5	2,01	1,85	1	-
D063x	42,6	41,9	2,02	2,27	1	-
D065x	41,2	40,6	2, 04	2,18	1	-
D068x	42,2	31,4	2,04	1,7	1	-
D070x	32,6	27,9	2,04	2,44	1	-
D072x	24,2	40,8	2,02	1,78	1	-
D064x	48,9		2,03	1,78	1	-
D049x	44,1	44,9	2,02	1,95	1	-
D050x	53	57,1	2,03	1,88	1	-
D051x	30,8	29,5	1,98	1,31	1	-
D066x	47,2	51,8	2,02	1,82	1	-
D081x	59,9	64,5	2,04	2,01	1	-
D084x	47,9	49,5	2,05	1,95	1	-
D071x	42,9	45,9	2,12	0,93	0	-
D073x	30,4	38,6	2,15	1,73	0	-
D074x	48,3	49,4	2,16	2,07	0	-
D075x	37,4	32,7	2,12	1,68	0	-
D076x	29,4	29,6	2,16	1,9	0	-
D077x	39,4	37,5	2,14	1,44	0	-
D078x	89,2	85,1	2,1	2,21	1	-
D079x	43,4	39,4	2,15	2,26	1	-
D080x	76,7	72	2,11	2,2	1	-
D082x	30,7	45,4	2,1	1,87	1	-
D083x	60,7	58,6	2,11	2,05	1	-
D085x	38	25,4	2,11	2,13	0	-
D086x	62,8	55,8	2,08	1,96	1	-
D055x	58,8	35,1	2,06	1,99		-
D067x	68,4	42,1	2,09	1,52		-
D091x	65,5	31,8	2,01	2,08		-
D092x	34,4	19,8	2,15	1,78		ja
D025x	14,3	17,2	2,25	0,8	1	-
D062x	52	58,8	2,17	2,03	1	-
D087x	37,4	50,1	2,13	1,56	0	-
D088x	39	47	2,16	2,04	0	-
D089x	28,5	39,9	2,19	1,76	0	-
D090x	49,3	62,1	2,11	1,87	1	-
D093x	66,1	48,4	1,99	2,07	3	-
D094x	51,4	55,7	2,1	1,83	1	-
D095x	45,9	47,3	2,08	2,18	1	-
D096x	44,3	53	2,14	1,95	1	-
D054x	37,6	35	2,04	1,97	1	-
D097x	24,3	22,3	2,08	2,1	1	-
D098x	44,7	30,1	2,04	2,18	1	-
D099x	44,5	42,1	2,06	1,91	1	-
D100x	27,5	27,4	2,05	2,1	1	-
D101x	40,5	38,2	2,12	1,9	1	-

Table 7: T1 X results

D010	50,6	48,5	2,1	2,23	1	-
D011	51,4	52,2	2,06	2,15	1	-
D012	56,5	51,2	2,08	2,1	1	-
D013	35	32,9	2,14	2,18	1	-
D014	43,5	40,7	2,06	2,17	1	-
D016	70,8	69,9	2,02	2,13	1	-
D017	33,3	35,3	2,01	1,95	1	-
D018	23,9	22,9	2,13	1,51	1	-
D019	34,3	31,7	2,14	0,75	1	-
D020	42,2	43,6	2,04	1,79	1	-
D021	35,4	34,2	2,13	0,93	1	-
D022	29,8	32	2,07	1,9	1	ja
D015	32,5	32,8	2,08	0,83	1	-
D023	45,9	48,7	2,07	1,83	1	-
D024	45,9	46,6	2,11	1,73	1	-
D027	45,6	35,7	2,08	1,7	1	-
D030	77,9	70,1	2,06	2,26	1	-
D031	53,1	50,2	2,04	2,27	1	-
D033	54,3	47,9	2,05	2,04	2	-
D034	23,4	21,1	2,06	2,15	1	-
D035	71,4	61	2,05	2,34	2	-
D036	75,4	63	2,01	2,33	2	-
D037	46,5	42,6	2,09	2,11	1	-
D026	30,7	27,7	2,15	2,03	1	-
D028	60	56,6	2,07	2,21	1	-
D029	95,3	83,2	2,04	2,18	2	-
D038	87,2	70,1	2,02	2,09	2	-
D031	58,3	48,8	2,05	2,25	1	-
D032	64,8	53,7	2,06	2,24	2	-
D039	24,4	23,1	2,2	2,1	1	-
D041	48,5	43,2	2,09	2,17	1	-
D042	25,7	25,9	2,11	1,88	1	-
D043	54,3	49,3	2,08	2,24	1	-
D045	47,3	40,5	2,02	2,23	1	-
D046	54,2	38,3	1,96	2,29	2	-
D047	65,8	52,2	2	1,72	2	-
D048	53,9	52	2,04	2,24	2	-
D052	35,6	36,1	2,07	1,61	1	-
D040	55	52,1	2,01	2,17	1	-
D044	79,2	72,8	2,05	2,24	1	-
D053	76,8	70,7	2,02	1,6	2	-
D056	38,5	34,6	2,03	2,09	1	-
D057	81,7	82,4	2,07	2,15	1	-
D058	105,6	46,9	1,92	2,21	2	ja
D059	76,1	59,9	2,03	2,17	2	-
D060	20,1	20,4	2,2	2,14	1	-
D061	95,3	30,3	1,89	2,16	1	-
D063	30,2	28	2,01	2,1	1	-
D065	31,7	28,2	2,02	2,17	1	-
D068	45,1	39,2	2,02	2,08	1	-
D070	39	36,6	2,04	1,99	1	-
D072	20,2	18	2,11	1,88	1	-
D064	39,9	35,8	2,02	2,07	1	-
D049	62,3	59,7	2,05	2,22	1	-
D050	45	38,8	2,02	2,18	2	-
D051	46,1	40,9	2,04	2,23	1	-
D066	48,8	41,4	2,1	2,25	1	-
D081	31,7	26,3	2,06	2,22	1	ja
D084	52,8	46,4	2,07	2,15	1	-
D071	49,8	41,8	2,08	2,23	1	-
D073	17,9	15,4	2,1	2,08	1	-
D074	45,1	37,1	2,09	2,12	1	-
D075	88,3	82,2	2,03	2,24	1	-
D076	25,6	21,5	2,1	2,1	1	-
D077	42,7	35,7	2,08	2,05	1	-
D078	80,4	68,2	2,06	1,87	1	-
D079	71,1	59,1	2,06	2,19	1	-
D080	45,5	41,2	2,06	2,19	1	-
D082	37,9	32,7	2,06	1,93	1	-
D083	49,4	37,7	2,09	2,05	1	-
D085	29,2	25,8	2,05	2,11	1	-
D086	47,4	42,5	2,08	2,17	1	-
D055	50,2	48	2,01	2,32	2	
D067	76	65,2	2,03	2,16	1	-
D091	47,5	42,8	2,04	2,18	1	-
D092	34,7	243	2,03	2,11	1	-
D025	14,9	14,7	1,88	1,32	1	-
D062	43	39,4	2,07	2,17	1	-
D087	75,5	64,5	2,01	2,16	1	-
D088	58,3	52	2,06	1,88	1	-
D089	35,5	29,9	2,02	1,86	1	-
D090	71	59,3	2,03	2,05	1	-
D093	35	32,9	2,02	1,94	1	-
D094	31,3	28,2	2,06	2,14	1	-
D095	72,1	61	2,03	1,98	1	ja
D096	40,5	37,6	2,08	2,11	1	-
D054	36,1	32,9	2,04	2,17	1	-
D097	32,7	28,6	2,09	2,14	1	-
D098	47,3	46	2,07	2,07	1	-
D099	35,7	35,8	2,05	1,94	1	-
D100	33,1	30,4	2,08	1,99	1	-
D101	32,5	30,8	2,17	1,28	1	-

Table 8: T1 back up results

Datum der Messungen	Zellzahl	Proband (Termin 2)	RNA (ng/µl) Nanodrop	RNA (ng/µl) Fluorometer	OD260/280 (abov	OD260/230 between 2 and 2.	Agarose-Formamid Gel	Dna Bande auf Gel
18.03.2019	4.000000	D001	24,7	30,7	2	1,58	1	-
18.03.2019	5.000000	D003	16	-	2,2	1,34	1	-
18.03.2019	4.000000	D004	23,8	26,6	2,12	0,78	1	-
18.03.2019	5.000000	D005	29,6	34	2,09	1,85	1	-
18.03.2019	5.000000	D007	21,4	25,3	2,12	1,08	1	-
18.03.2019	5.000000	D008	39,7	38,7	2,04	1,43	2	ja
18.03.2019	5.000000	D009	23,2	32,7	2,2	2,04	1	-
19.03.2019	3.000000	D002	49,1	45,7	2,07	1,95	1	-
19.03.2019	4.000000	D006	71,4	68,9	2,08	1,93	1	-
25.03.2019	5.000000	D010	54,2	29,5	2,05	1,44	1	-
25.03.2019	5.000000	D012	85,4	47,4	2,04	2,11	1	-
25.03.2019	5.000000	D013	74,9	45,6	2,03	2,14	1	-
25.03.2019	5.000000	D014	41,3	24,4	2,03	1,51	1	-
25.03.2019	5.000000	D016	47,7	25,7	2,01	1,87	1	-
25.03.2019	5.000000	D017	70,4	41,6	2,04	1,83	1	-
25.03.2019	2.000000	D018	46,9	25,9	2,05	1,83	1	-
25.03.2019	5.000000	D019	50	27,22	2,06	2,04	1	-
25.03.2019	5.000000	D022	81,5	47,1	2,06	2,2	1	-
26.03.2019	5.000000	D011	57,9	65,6	2,05	2,02	1	-
26.03.2019	5.000000	D020	40,5	53,1	2,08	1,03	1	-
26.03.2019	5.000000	D021	27,7	33,7	2,07	1,78	1	-
01.04.2019	5.000000	D015	41,8	47,9	2,02	0,48	1	-
01.04.2019	5.000000	D023	38,3	37,6	2,01	0,6	1	-
01.04.2019	5.000000	D024	46,6	44,3	2,03	2,2	1	-
01.04.2019	5.000000	D027	61,9	58,9	2,02	1,89	1	-
01.04.2019	5.000000	D029	50,9	45,3	2,06	1,71	1	-
01.04.2019	4.000000	D030	44,5	37,6	2,03	2,11	1	-
01.04.2019	5.000000	D033	36,9	34,1	2,02	1,84	1	-
01.04.2019	5.000000	D034	39,3	37,3	2,04	1,05	1	-
01.04.2019	3.000000	D036	26,6	23,1	2,06	2,04	1	-
01.04.2019	5.000000	D037	35,3	31,3	2,06	1,59	1	-
01.04.2019	5.000000	D038	71,8	61,3	2,05	2,08	1	-
02.04.2019		D026	42,2	19,4	1,99	0,44	1	-
02.04.2019		D028	70,5	46,2	2,04	2,2	1	-
02.04.2019		D035	79,8	59,3	2,04	2,16	1	-
08.04.2019	5.000000	D031	35	30,8	2,05	2,19	1	-
08.04.2019	5.000000	D042	34,6	33,5	2,05	2,04	1	-
08.04.2019	5.000000	D045	66,6	58,7	2,04	1,57	1	-
08.04.2019	5.000000	D048	58,4	54,9	2,05	2,1	1	-
09.04.2019		D039	106,7	107	2,05	2,22	1	-
09.04.2019		D041	71	54	2,03	2,2	1	-
09.04.2019		D043	63,1	65,9	2,04	2,02	1	-
09.04.2019		D046	52,6	50,4	2,03	2,11	1	-
09.04.2019		D047	49,7	48,5	2,02	2,02	1	-
09.04.2019		D052	54,2	49,2	2,02	2,18	1	-
15.04.2019	5.000000	D056	60,5	60,8	2,08	2,03	1	-
15.04.2019	5.000000	D057	59,8	55,6	2,06	2,18	1	-
15.04.2019	5.000000	D059	36,4	36,3	2,07	2,2	1	-
15.04.2019	5.000000	D060	58,4	54,9	2,05	2,22	1	-
15.04.2019	5.000000	D061	72,7	68,2	2,04	2,18	1	-
15.04.2019	5.000000	D063	51,6	48,8	2,02	2,21	1	-
15.04.2019	5.000000	D065	56,7	56,3	2,04	2,07	1	-
15.04.2019	5.000000	D068	45,3	45,7	2,05	2,18	1	-
15.04.2019	5.000000	D070	41,5	42,3	2,02	2,15	1	-
15.04.2019	4.000000	D072	60,9	62,8	2,03	2,21	1	-
16.04.2019	5.000000	D040	47,3	49,6	2,02	2,07	1	-
16.04.2019	5.000000	D053	53,9	46	2,02	2,09	1	-
16.04.2019	5.000000	D064	65,8	60,8	2,03	2,13	1	-
23.04.2019	5.000000	D032	117,9	108	2,02	2	1	-
23.04.2019	5.000000	D058	39,8	37,7	2,03	2,08	1	-
23.04.2019	4.000000	D066	85,5	74,8	2,01	2,04	1	-
23.04.2019	5.000000	D071	125,7	115	2,02	2,23	1	-
23.04.2019	5.000000	D073	117,6	56,3	1,9	2,3	2	-
23.04.2019	5.000000	D076	94,3	85,3	2,03	2,25	1	-
23.04.2019	5.000000	D078	68,3	67,2	2,09	2,17	1	-
23.04.2019	5.000000	D079	59,7	61,7	2,05	2,14	1	-
23.04.2019	5.000000	D080	58,9	59,4	2,06	2,03	1	-
23.04.2019	5.000000	D082	45,5	44,2	2,11	2,19	1	-
23.04.2019	4.000000	D086	69,6	62,6	2,08	1,43	1	-
23.04.2019	5.000000	D092	34	32,4	2,17	2,12	1	-
24.04.2019	5.000000	D044	74,3	62,1	2,03	2,19	1	-
24.04.2019	5.000000	D050	96,7	86,5	2,04	2,13	1	-
24.04.2019	5.000000	D051	89,3	72,9	2,08	2,24	1	-
24.04.2019	5.000000	D074	133,8	115	2,03	2,25	2	-
24.04.2019	5.000000	D077	71,7	54,7	2,05	2,14	1	-
24.04.2019	5.000000	D081	57,2	46,4	2,12	2,18	1	-
24.04.2019	5.000000	D084	45,1	29,2	2,11	2,11	1	-
24.04.2019	5.000000	D085	29,8	22,8	2,14	1,81	1	-
29.04.2019	4.000000	D025	31,1	25,9	2,07	2,09	1	-
29.04.2019	5.000000	D049	78,9	61,5	2,05	2,25	1	-
29.04.2019	5.000000	D055	48,9	45,1	2,03	2,19	1	-
29.04.2019	5.000000	D062	42,3	38,6	2,02	2,13	1	-
29.04.2019	5.000000	D067	48,2	40,9	2,05	2,06	1	-
29.04.2019	5.000000	D091	58,1	50,7	2,03	2,19	1	-
29.04.2019	5.000000	D095	24,1	20,2	1,96	2,12	1	-
29.04.2019	5.000000	D096	46,7	39,4	2,03	1,94	1	-
29.04.2019	5.000000	D099	55,8	-	2,05	2,21	1	-
29.04.2019	5.000000	D100	49,2	-	2,04	2,19	1	-
29.04.2019	5.000000	D101	48,4	45	2,09	2,3	1	-
30.04.2019	5.000000	D054	70,5	61,2	2,07	1,54	1	-
30.04.2019	5.000000	D087	60,3	40,4	2,05	2,16	1	-
30.04.2019	5.000000	D089	33,7	25,8	2,07	1,61	1	-
30.04.2019	5.000000	D090	55	43,6	2,05	2,23	1	-
30.04.2019	4.000000	D093	73	67,6	2,02	2	1	-
30.04.2019	5.000000	D094	57,4	44,5	2,05	2,17	1	-
30.04.2019	5.000000	D097	53,5	47,4	2,05	1,68	1	-
30.04.2019	5.000000	D098	37,2	26,5	2,06	2,46	1	ja

Table 9: T2 results

Sample X T1	RNA (ng/µl) Nanodrop	RNA (ng/µl) Fluorometer	OD260/280 (above	OD260/230 betwe	Agarose-For	Dna Bande a
D001x	62	48,8	2,07	1,59	2	ja
D003x	32,9	32,8	2,17	1,49	1	-
D004x	40,8	44,3	2,15	1,93	1	-
D005x	51,8	57,4	2,15	1,86	1	-
D007x	57,4	62,9	2,2	2,14	1	-
D008x	80,2	85,7	2,12	1,43	1	-
D009x	23,6	63,3	2,2	1,93	1	-
D002x	57,3	58,1	2,09	1,81	1	-
D006x	43,6	43,7	2,1	2,14	1	-
D010x	49,1	29,2	2,12	1,41	1	-
D012x	50	26,7	2,12	1,8	1	-
D013x	46,2	25,2	2,12	2,05	1	-
D014x	31	15,9	2,23	1,56	0	-
D016x	55,1	31,5	2,13	2,14	1	-
D017x	61,7	33,4	2,1	1,65	1	-
D018x	24,5	13,1	2,23	1,74	0	-
D019x	40,3	20,5	2,14	2,05	1	-
D022x	55	28,6	2,09	2,17	1	-
D011x	36,4	46,6	2,12	1,82	1	-
D020x	55,5	64,3	2,12	2,07	1	-
D021x	34,9	41,8	2,12	1,88	1	-
D015x	38,1	43,5	2,1	2,09	1	-
D023x	48,1	41,6	2,09	2,16	1	-
D024x	37,6	35,9	2,13	1,28	1	-
D027x	50,5	48,7	2,11	2,01	1	-
D029x	32,5	29,1	2,16	2,11	1	-
D030x	35,7	36,2	2,15	1,97	1	-
D033x	33,2	30,8	2,15	1,89	1	-
D034x	64,8	66,7	2,09	2,19	1	-
D036x	34,7	35,7	2,22	2,16	1	-
D037x	36	31,7	2,15	2	1	-
D038x	81,1	72,5	2,11	2,21	1	-
D026x	33,5	27,9	2,04	2,01	1	-
D028x	124,6	101	2,04	2,16	1	-
D035x	34,8	27,8	1,99	2,1	1	-
D031x	28,6	27,3	2,03	1,91	1	-
D042x	45,9	41,1	2,04	1,83	1	-
D045x	45,8	41	2,05	1,89	1	-
D048x	33,1	28,7	2,03	1,94	1	-
D039x	77,5	76,8	2,04	1,95	1	-
D041x	37,2	35,7	2,03	1,99	1	-
D043x	61,7	55,8	2,02	1,95	1	ja
D046x	46	48,1	2,02	2,14	1	-
D047x	56,9	51,7	2,01	1,94	1	-
D052x	59,9	43,3	2,02	2,09	1	-
D056x	51	50,6	2,05	2,02	1	-
D057x	55,9	57,5	2,04	2,13	1	-
D059x	41,6	41,5	2,08	2,04	1	-
D060x	37,9	35,5	2,04	2,17	1	-
D061x	26,9	24,1	2,07	1,65	1	-
D063x	43,6	-	2,03	2	1	-
D065x	56,6	-	2,03	1,84	1	-
D068x	48,6	49,8	2,02	1,78	1	-
D070x	45,4	44,1	2,02	2,04	1	-
D072x	37,3	37,1	2,04	1,97	1	-
D040x	70,5	61,5	2,02	2,12	1	-
D053x	50,4	54	2,06	1,14	1	-
D064x	52,9	47,3	2,04	2,18	1	-
D032x	65	-	1,98	1,94	1	-
D058x	65,3	53,5	1,97	2,05	1	-
D066x	36	40,7	1,99	1,75	1	-
D071x	71,4	62,3	2,01	2,17	1	-
D073x	44,6	36,4	1,98	1,99	1	ja
D076x	77	66,4	2	2,24	1	-
D078x	54,1	55,1	2,03	1,55	1	-
D079x	63,3	60,8	2,05	2,09	1	-
D080x	39,8	37,8	2,09	2,21	1	-
D082x	49,3	48,5	2,09	2,28	1	-
D086x	65,9	66,3	2,08	2,27	1	-
D092x	26	34,6	2,18	2,06	1	-
D044x	50,5	39,7	2,1	1,99	1	-
D050x	70,7	53,5	2,07	2,05	1	-
D051x	65,2	43,8	2,02	2,13	1	-
D074x	64,2	53,6	2,07	1,24	1	-
D077x	56,1	44,7	2,05	2,01	1	ja
D081x	23,9	18,7	2,15	1,48	1	-
D084x	46	30,2	1,99	1,73	1	-
D085x	33,3	24,8	2,02	1,88	1	-
D025x	36,3	28,2	2,08	1,76	1	-
D049x	61,7	55,9	2,04	2,17	1	-
D055x	72,5	62	2,07	2,23	1	-
D062x	38,4	31,9	2,16	2,03	1	-
D067x	44,2	41,4	2,11	2,29	1	-
D091x	50	43,5	2,11	2,09	1	-
D095x	61,9	54,8	2,12	2,27	1	-
D096x	36,4	32,4	2,17	2,42	1	-
D099x	64,8	55,6	2,11	2,05	1	-
D100x	56,1	46,1	2,09	2,15	1	-
D101x	66,7	63,2	2,11	1,93	1	-
D054x	58,6	51,6	2,07	1,51	1	-
D087x	47,5	-	2,07	0,88	1	-
D089x	24,8	25,2	2,08	1,35	1	-
D090x	47,8	35,5	2,06	2,07	1	-
D093x	28,6	25,2	2,11	2,07	1	-
D094x	57,9	43,9	2,06	1,85	1	-
D097x	56,6	43,2	2,04	2,09	1	-
D098x	32,6	30,6	2,1	2,36	1	-

Table 10: T2 X results

Sample Z T1	RNA (ng/µl)	RNA (ng/µl) Fluoro	OD260/280 (above 1	OD260/230 betwee	Agarose-Formamid	Dna Bande a
D001	41,9	41,3	2	1,86	1	-
D003	26,2	25,5	2,17	2,22	1	-
D004	34,9	31,1	2,03	2,05	1	-
D005	57,2	49,6	1,98	1,32	1	-
D007	41,3	35,8	1,97	2,52	1	-
D008	37,9	32,2	1,97	2,53	1	-
D009	58	45,8	2,02	2,17	1	-
D002	48,7	44,1	2,11	2,11	1	-
D006	108,8	89,4	2,04	2,28	2	-
D010	51,1	50,4	2,01	2,26	1	-
D012	40	48,4	2,01	1,8	1	
D013	58,9	55,1	2,04	2,23	1	-
D014	48,6	47,3	2,05	2,17	1	
D016	35,6	36,2	1,98	2,21	1	
D017	47,2	43,8	2,01	2,1	1	
D018	37,1	37,7	2,07	2,07	1	-
D019	45,6	50	2,03	1,88	1	-
D022	47,6	52,9	1,99	2,14	1	
D011	33,6	31,3	2,05	1,91	1	-
D020	50	44,4	2,05	1,36	1	-
D021	31,9	26,6	2,04	2,23	1	-
D015	36,6	28,2	2,02	2,08	1	-
D023	36,6	38,2	2,13	2,16	1	-
D024	149,9	40,5	1,86	2,33	3	-
D027	51,3	51,4	2,06	2,11	1	-
D029	121,1	36,6	1,87	2,24	3	-
D030	130,4	32,6	1,86	2,33	3	-
D033	100,6	28,2	1,87	2,28	3	-
D034	49,8	46,3	2,05	2,21	1	-
D036	44,5	42,9	2,04	2,17	1	-
D037	45	38,7	1,97	2,04	1	-
D038	77,4	18,3	1,84	2,35	3	-
D026	176,5	46,8	1,86	2,11	3	-
D028	78,6	82,6	2,05	2,2	1	-
D035	38,8	36,2	2,02	2,21	1	-
D031	42	41,8	2,09	2,2	1	-
D042	27,2	64,4	2,11	2,13	1	-
D045	44,3	46,2	2,1	1,77	1	-
D048	48,2	46,2	2,06	2,19	1	-
D039	44,8	31,9	2,06	2,04	1	-
D041	41,4	27,3	2,08	1,72	1	-
D043	64	47,7	2,06	2,22	1	-
D046	17,6	18,2	2,14	1,81	1	-
D047	66,5	67,2	2,05	2,27	1	-
D052	91,3	-	2,03	2	1	-
D056	50,1	49,6	2,09	2,24	1	-
D057	81,2	71,7	2,06	2,19	1	-
D059	41,8	44,3	2,1	2,13	1	-
D060	37,3	36,2	2,14	1,94	1	-
D061	30,9	30,7	2,12	2,24	1	-
D063	27,8	27,5	2,04	1,58	1	-
D065	40,2	42,9	2,07	1,78	1	-
D068	50	54,5	2,09	1,6	1	-
D070	46,6	46,6	2,06	1,94	1	-
D072	32,8	32,8	2,09	1,65	1	-
D040	29,9	41,3	2,05	2	1	-
D053	45,6	49,8	2,05	2,21	1	-
D064	47,1	51,6	2,1	2,1	1	-
D032	87,3	85,4	2,03	2,25	1	-
D058	59,2	58,8	2,07	2,12	2	-
D066	53,7	57,3	2,08	2,27	1	-
D071	50,7	47,2	2,07	1,88	1	-
D073	38,2	37,4	2,1	1,24	1	-
D076	155,8	74,1	1,91	2,35	3	-
D078	79,4	73,5	2,01	2,19	1	-
D079	69	74,6	2,06	2,05	1	-
D080	57,6	54,7	2,09	2,18	1	-
D082	37,7	40,3	2,01	0,58	1	-
D086	41,8	44,7	1,95	1,97	1	-
D092	33,9	37,3	1,91	1,91	0	-
D044	56,4	70,8	2	1,58	1	-
D050	57,6	57,2	2,09	2	1	-
D051	97	95,3	2,03	2,29	1	-
D074	42,1	35	2,06	1,44	2	ja
D077	71,9	83,8	1,99	2,23	1	-
D081	56,7	61	2,01	2,11	1	-
D084	61	68,7	1,96	2,21	1	-
D085	46,7	46,4	1,94	1,8	1	-
D025	27,9	28,4	2,18	2,22	1	-
D049	37,9	36,6	2,14	2,15	1	-
D055	37,3	36,9	2,13	1,4	1	-
D062	46,9	52,2	2,04	1,72	1	-
D067	35,4	34	2,12	1,87	1	-
D091	39,2	43,1	1,98	1,84	0	-
D095	81,6	85,8	1,99	1,9	2	-
D096	17,9	20,1	2,01	0,47	1	-
D099	26,2	31,2	2,05	2,18	1	-
D100	68,1	77,8	2,01	2,16	1	-
D101	64,1	71,1	2,03	1,49	1	-
D054	50,6	53,9	1,96	1,97	1	-
D087	41,3	40,1	1,97	1,08	1	ja
D089	45,9	49,5	1,96	1,68	1	-
D090	30,4	36,1	1,92	1,68	1	-
D093	46,6	46,5	1,98	2,01	0	-
D094	54,9	62,6	1,98	1,89	0	-
D097	53,9	59	2,02	2,1	1	-
D098	36	39	2,01	2,26	1	-

Table 11: T2 back up results

Datum der N	Zellzahl	Proband (Termin	RNA (ng/µl) Nanodrop	RNA (ng/µl) Fluorometer	OD260/280 (abov	OD260/230 I	Agarose-For	Dna Banden
03.06.2019	5.000000	D002	30	30,9	2,07	1,86	1	-
03.06.2019	5.000000	D003	36,8	38,1	2,15	2,35	1	-
03.06.2019	5.000000	D004	46,8	47	2,11	2,33	1	-
03.06.2019	5.000000	D006	45,3	44,7	2,13	2,22	1	-
03.06.2019	5.000000	D007	54,9	57,7	2,11	2,19	1	-
11.06.2019	5.000000	D010	38,6	-	2,05	1,64	1	-
11.06.2019	5.000000	D011	50,5	-	2,04	0,37	1	-
11.06.2019	5.000000	D013	27,5	-	1,91	1,42	1	-
11.06.2019	5.000000	D017	68,7	70,8	2,03	1,19	1	-
11.06.2019	5.000000	D018	29,2	32,4	1,98	2,24	1	-
11.06.2019	5.000000	D019	59,7	63	2,06	2,25	1	-
11.06.2019	5.000000	D021	37	37,3	2,04	1,49	1	-
11.06.2019	5.000000	D022	41,9	41,2	2,07	2,32	1	-
12.06.2019	5.000000	D014	40	41,5	2,03	2,25	1	-
17.06.2019	5.000000	D016	56,5	56,7	2,05	1,89	1	-
17.06.2019	5.000000	D023	33	33,9	2,04	1,54	1	-
17.06.2019	5.000000	D024	23,4	22	2,01	1,13	1	-
17.06.2019	5.000000	D027	24,6	25,2	2,07	2,15	1	-
17.06.2019	5.000000	D030	40,3	39,3	2,07	1,81	1	-
17.06.2019	5.000000	D034	49,4	49	2,06	2,19	1	-
17.06.2019	5.000000	D036	13,9	13,9	2,25	1,49	1	-
17.06.2019	5.000000	D038	388,6	29,8	2,02	1,05	1	ja
18.06.2019	5.000000	D028	59,9	61,1	2,08	2,07	1	-
18.06.2019	5.000000	D033	30,8	32,7	2,06	2,01	1	ja
18.06.2019	5.000000	D035	58,7	65,5	2,08	2,21	1	-
19.06.2019		D015	31,8		2,16	2,27	1	-
24.06.2019	5.000000	D029	40,4	40,2	1,98	0,82	1	ja
24.06.2019	5.000000	D031	44,5	46	1,99	1,9	1	-
24.06.2019	5.000000	D042	62,5	59,2	2,02	1,5	1	-
24.06.2019	5.000000	D046	51,8	51,9	1,99	2,02	1	-
24.06.2019	5.000000	D047	36,5	33,9	1,98	1,22	1	-
24.06.2019	5.000000	D048	56,4	54,3	2	2,17	1	-
25.06.2019	5.000000	D020	55,8	56,8	2,05	2,18	1	-
25.06.2019	5.000000	D041	38,5	39,1	2,01	1,97	1	-
25.06.2019	5.000000	D043	55,8	55,3	2,07	1,61	1	-
25.06.2019	5.000000	D052	75,5	64,7	1,99	1,91	1	-
01.07.2019	5.000000	D056	37	48,6	2,02	2,12	1	-
01.07.2019	5.000000	D060	50,9	69	1,99	2,14	1	-
01.07.2019	5.000000	D061	52,9	64,5	2,01	1,98	1	-
01.07.2019	5.000000	D065	57,3	67,9	1,99	2,22	1	-
01.07.2019	5.000000	D068	43,8	49,5	2	2,18	1	-
01.07.2019	5.000000	D070	66,9	76	2	2,26	1	-
01.07.2019	5.000000	D086	51,6	65,9	2,02	2,15	1	-
02.07.2019	5.000000	D040	78,7	84,2	2	2,22	1	-
02.07.2019	5.000000	D057	63,4	73,6	2,01	2,18	1	-
02.07.2019	5.000000	D063	52,6	55,9	2,01	2,02	1	-
02.07.2019	5.000000	D064	44,1	47,4	2	2,09	1	-
08.07.2019	5.000000	D032	50,3	57,4	2,04	2,13	1	-
08.07.2019	5.000000	D044	58,4	64,5	2,02	2,17	1	-
08.07.2019	4.000000	D050	15,2	18,5	2,05	1,13	1	-
08.07.2019	5.000000	D051	-	-	-	-	1	-
08.07.2019	5.000000	D059	48,6	53	2,04	0,7	1	-
08.07.2019	4.000000	D066	-	-	-	-	1	-
08.07.2019	5.000000	D076	35,8	40,5	2,09	1,48	1	-
08.07.2019	4.000000	D078	64,1	70	2,05	2,12	1	-
08.07.2019	4.000000	D084	58,1	64,1	2,06	2,22	1	-
04.07.2019		D053	28,4	33,7	2,17	2,2	1	-
09.07.2019	5.000000	D058	46,2	51	2,07	2,28	1	-
09.07.2019	5.000000	D073	31,4	35,2	2,09	1,44	1	-
09.07.2019	4.000000	D074	42,6	41,9	2,05	1,86	1	-
09.07.2019	5.000000	D077	66,4	69	2,06	1,87	1	-
09.07.2019	5.000000	D080	28,9	32,8	2,07	1,51	1	-
09.07.2019	4.000000	D081	29,1	32,5	2,05	1,41	1	-
09.07.2019	5.000000	D082	59,9	63,9	2,04	1,93	1	-
09.07.2019	5.000000	D085	41,3	45,8	2,1	1,69	1	ja
15.07.2019	3.500000	D025	58,8	59,4	2,02	1,46	1	-
15.07.2019	5.000000	D055	74,4	79,8	2,03	1,69	1	-
15.07.2019	5.000000	D062	79	80	2,01	2,19	1	-
15.07.2019	5.000000	D067	67,2	65,4	2,03	2,16	1	-
15.07.2019	5.000000	D091	28,4	24,5	2	1,85	1	-
15.07.2019	5.000000	D092	38,1	37,4	2	2,04	1	-
15.07.2019	5.000000	D094	187,1	45,8	1,86	2,37	3	?
15.07.2019	5.000000	D095	121,4	38,8	1,85	2,16	3	?
15.07.2019	5.000000	D096	167,3	50,5	1,89	2,58	3	?
15.07.2019	5.000000	D099	222,6	81,4	1,89	2,36	3	?
15.07.2019	5.000000	D100	285,2	81,5	1,88	2,34	3	?
15.07.2019	5.000000	D101	116	47,4	1,89	2,3	3	-
16.07.2019	5.000000	D054	68,6	67,4	1,99	2,06	1	-
16.07.2019	5.000000	D079	71,5	64,6	1,99	1,96	1	-
16.07.2019	3.500000	D087	54,3	53,2	2,02	1,51	1	-
16.07.2019	5.000000	D089	54	48,7	1,99	2,06	1	-
16.07.2019	5.000000	D090	52	52,8	2	1,44	1	-
16.07.2019	4.000000	D093	76,5	78,3	2,01	2,22	1	-
16.07.2019	5.000000	D096 (2. Probe =	43,2	44,4	2,01	2,18	1	-
16.07.2019	5.000000	D097	60,3	51,9	1,98	2,11	1	-
16.07.2019	5.000000	D098	70,4	72	2,03	1,79	1	-
19.07.2019	5.000000	D095 (2. Probe =	43,4	41,2	2,01	1,7	1	-
19.07.2019	5.000000	D100 (2. Probe =	1,95	47,8	1,95	2,2	1	-
19.07.2019	5.000000	D101 (2. Probe =	1,98	54,6	1,98	2,09	1	-

Table 12: T3 results

Proband (Tern	RNA (ng/µl) Nanodrop	RNA (ng/µl) Fluoror	OD260/280 (abo	OD260/230 betwe	Agarose-Formami	Dna Banden
D002x	57	54,7	2,11	2,27	1	-
D003x	57,2	61,4	2,11	1,48	1	-
D004x	84,8	68,8	2,03	1,94	1	ja
D006x	25,5	27	2,17	1,25	1	-
D007x	39,3	37,5	2,13	2,18	1	-
D010x	52,5	54,8	2,05	2,23	1	-
D011x	54,8	53,9	2,05	2,22	1	-
D013x	39,3	40,6	2,06	2,24	1	-
D017x	46,2	52,6	2,1	1,9	1	-
D018x	47,9	47,6	2,08	1,72	1	-
D019x	30	33	2,08	1,96	1	-
D021x	31	29,8	2,06	0,54	1	-
D022x	34	32,6	2,06	0,93	1	-
D014x	41	46,7	2,03	1,75	1	-
D016x	52,6	53,3	2,1	1,35	1	-
D023x	41,1	39,5	2,04	2,22	1	-
D024x	17,8	18,5	2,14	1,97	1	-
D027x	24	25,7	2,2	1,89	1	-
D030x	43,7	47,1	2,11	1,74	1	-
D034x	43,6	48,3	2,13	2,12	1	-
D036x	25,7	25,3	2,18	1,96	1	-
D038x	34,4	42,6	2,11	1,45	1	-
D028x	68,7	72,4	2,06	1,86	1	-
D033x	40,3	40	2,05	1,99	1	-
D035x	33,9	35,9	2,07	2,09	1	-
D015x	36,4		2,13	2,2	1	-
D029x	58,3	62,2	2,01	1,97	1	-
D031x	55,4	53,4	2,01	2,08	1	-
D042x	75,7	73	2,03	1,88	1	-
D046x	18,4	19,2	2	1,82	1	-
D047x	50,5	48,7	2,01	1,86	1	-
D048x	43,3	43,1	2,01	2,14	1	-
D020x	76,5	76,6	2,02	2,2	1	-
D041x	55,1	54,1	2,01	1,96	1	-
D043x	46,3	46,1	2,05	1,83	1	-
D052x	48,9	47,1	2,04	2,07	1	-
D056x	51,9	69,2	2	2	1	-
D060x	38,9	50,1	1,97	1,9	1	-
D061x	41,3	48,6	2	2,01	1	-
D065x	66,8	80,7	2	2,14	1	-
D068x	62,9	78,4	1,99	1,84	1	-
D070x	74,5	88,1	1,99	1,97	1	-
D086x	47,5	56,4	1,99	1,38	1	-
D040x	37,6	37,7	1,94	1,62	1	-
D057x	59,2	61,2	2	2,1	1	-
D063x	60,3	64,6	1,98	1,77	1	-
D064x	38,7	42,5	1,96	2,11	1	-
D032x	50,1	57,1	2,07	2,08	1	-
D044x	35,4	38,4	2,07	1,3	1	-
D050x	32,4	34,2	2,1	2,13	1	-
D051x	35,7	41,5	2,16	1,65	1	-
D059x	28,4	34,5	2,15	1,92	1	-
D066x	46	51,2	2,12	1,87	1	-
D076x	39	42,7	2,15	1,41	1	-
D078x	27,5	31,3	2,17	1,68	1	-
D084x	21,4	21,1	2,34	1,28	1	-
D053x	51,5	57,5	2,13	2,18	1	-
D058x	37,1	40,1	2,06	2,16	1	ja
D073x	20,5	24,3	2,11	2,17	1	-
D074x	26,4	29,6	2,08	1,28	1	-
D077x	37,4	44,6	2,1	1,59	1	-
D080x	40,6	44,2	2,11	2,16	1	-
D081x	30,5	34,6	2,14	1,98	1	-
D082x	61,5	60,8	2,08	2,27	1	ja
D085x	30,6	38,3	2,21	1,71	1	ja
D025x	55,8	64,2	2,1	2,08	1	-
D055x	52	50,8	2,08	2,25	1	-
D062x	76,5	75,7	2,05	2,09	1	-
D067x	69,3	64	2,08	1,63	1	-
D091x	48,7	44,7	2,12	0,88	1	-
D092x	24,4	24,1	2,23	2,07	1	-
D094x	103,9	47,1	1,89	2,16	3	-
D095x	79	35,3	1,93	2,17	3	-
D096	173,8	47,2	1,87	2,32	3	-
D099x	124,5	71,7	1,95	1,51	3	-
D100x	145,9	62,2	1,92	2,13	3	-
D101x	151,2	59,4	1,91	2,27	3	-
D054x	62,3	56,3	2,02	1,97	1	-
D079x	56,3	53,4	2,04	1,9	1	-
D087x	38,9	34,7	2,11	1,74	1	-
D089x	50,7	42	2,06	1,87	1	-
D090x	41,2	38,3	2,07	2,11	1	-
D093x	42,9	40,6	2,09	1,89	1	-
D096x (2.Prob	17,8	21,3	2,2	2,13	1	-
D097x	37,1	36,5	2,08	2,09	1	-
D098x	56,1	49,1	2,05	2,27	1	ja
D095x (2. Prob	53	40,3	1,99	2,28	1	-
D100x (2. Prol	44	38,5	2	1,4	1	ja
D101x (2. Prob	54,6	54,9	2,01	2,24	1	-

Table 13: T3 X results

Proband (Termin 3)	RNA (ng/µl) Nar	RNA (ng/µl) Fluo	OD260/280 (	OD260/230 b	Agarose-For	Dna Banden
D002	70,8	-	2,02	1,8	1	-
D003	57	-	2,02	1,73	1	-
D004	38,1	36,7	1,99	2,2	1	-
D006	22,2	22,2	1,94	2,18	1	-
D007	42,2	39,4	1,97	2,16	1	-
D010	35,4	38,8	2,04	1,97	1	-
D011	62,2	70,5	2,02	1,69	1	-
D013	31,1	35,6	1,97	0,92	1	-
D017	53,2	52,8	1,99	1,93	1	-
D018	47,8	53,3	2,02	1,18	1	-
D019	42,8	51	2,02	2,23	1	-
D021	42,7	47,7	2,02	1,9	1	-
D022	50,4	56,7	2,04	2,2	1	-
D014	73,7	73,5	2,01	2,23	1	-
D016	34,4	42,6	2,01	1,71	1	-
D023	33,7	34,9	1,98	1,97	1	-
D024	23,4	23	1,95	1,68	1	-
D027	28,1	27,6	1,96	2,02	1	-
D030	46,4	41,6	2,07	2,15	1	-
D034	31,6	31,4	1,99	2,09	1	-
D036	18,4	17,9	2,01	1,7	3	-
D038	30,1	34,2	2,01	2,2	1	-
D028	33,3	40,1	2,04	2,18	1	-
D033	42,8	39,1	2,01	2,16	1	-
D035	44	41,5	2,02	2,05	1	-
D015	60,4	71,2	1,98	1,83	1	-
D029	64,7	65,6	2,04	1,86	1	-
D031	41,8	39	2,03	1,66	1	-
D042	187,7	91	1,91	2,23	3	-
D046	28,4	27,9	2,02	2,06	1	
D047	41,8	45,8	2,06	0,55	3	-
D048	57,8	57,7	2,04	1,9	1	
D020	83,5	73,9	1,99	2,25	1	-
D041	284,7	106	1,91	2,29	3	-
D043	238,7	115	1,92	2,28	3	-
D052	19,7	17,8	2,02	1,83	3	-
D056	32,9	31,2	2,06	2,01	1	
D060	35,1	29,1	1,99	2,14	1	
D061	27,4	26,6	1,99	2,14	1	
D065	41,8	39,9	1,99	2,2	1	
D068	38,9	37	2,12	2,1	1	
D086	74,2	32,8	1,96	2,28	2	
D070	34,5	31,5	1,98	2,19	1	
D040	264,2	124	1,93	2,32	3	-
D057	47	48,1	2,02	2,12	1	
D063	42,7	40,3	2,07	2,19	1	
D064	44	46,6	2,04	2,19	1	
D032	33,7	33,6	2	2,01	1	-
D044	32,8	30,1	1,92	2,14	1	
D050	27	24	1,94	2,2	1	
D051	44,6	36,6	2	2,18	1	
D059	36,2	33,9	2,01	2,18	1	
D066	43,2	42,2	2,01	2,1	1	
D076	27,8	24,3	1,99	2,14	1	
D078	37,2	35,3	1,97	2,17	1	
D084	38,8	38,8	1,99	2,05	1	
D053	53,4	43,9	2,01	2,16	1	
D058	36,2	39,6	2,03	1,92	1	
D073	17,1	14,8	2,37	2,15	1	
D074	32	27,9	1,95	2,12	1	
D077	67,3	61,8	2,02	2,18	1	
D080	18,2	18,3	1,99	2	1	
D081	31,1	29	1,95	1,99	1	
D082	32,9	31,7	2,03	2,17	1	
D085	35,9	36,2	2,01	2,13	1	
D025	51,5	43,2	1,98	1,85	1	
D055	46,6	47,1	2	2,22	1	
D062	53	53,6	2,01	1,49	1	
D067	41,3	39,9	2,02	2,15	1	
D091	31,1	30	1,95	1,94	1	
D092	31	29,7	1,92	2	1	
D094	56,3	54,9	1,95	2,18	1	
D095	44,4	44,8	1,96	2,1	1	
D096	41	41,2	1,95	2,17	1	
D099	70,7	55,7	72,4	1,98	1	
D100	72,4	64,7	1,98	2,22	1	
D101	60,8	62,5	1,98	2,18	1	
D054	36,6	39,3	2	2,08	1	
D079	34,6	33,9	2,02	1,9	1	
D087	25,7	28	2	2,12	1	
D089	71,4	40,4	1,91	2,21	3	
D090	39,6	37,5	1,99	2	1	
D093	41,2	36,5	2	2,17	1	
D096	23,3	24,2	2,02	2,04	1	
D097	35	34,1	1,98	2,15	1	
D098	16,7	14,9	1,99	1,94	1	ja
D095o	36,5	33,9	2,01	2,11	1	
D100o	51	45,2	2,02	1,82	2	
D101o	46	38,8	2,03	2,11	1	
D095A	37	44,6	1,99	2,03	1	
D100A	43,1	49,7	2,05	1,83	1	
D100B	42,6	40,9	1,96	2,1	2	
D101A	67,9	53,9	1,92	2,21	2	
D101B	71,9	59,7	1,9	2,12	2	
D101C	45,6	39,3	1,97	2,2	1	

Table 14: T3 back up results