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„The effects of resistance training and Vitamin D on DNA damage and age markers in elderly people"

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Abbreviations

AGEs – advanced glycation end-products

BMD – bone mineral density

BW – body weight

CBMN – cytokinesis-block micronucleus assay

CT – computer tomography

DALY – disability adjusted life years

DNA – deoxyribonucleic acid

DBP – vitamin D-binding protein

DXA – dual-energy X-ray absorptiometry

EU – European Union

NBUD – nuclear bud

MNC – mono nucleated cell

MRI – magnet resonance imaging

NPB – nucleoplasmic bridge

PBL – peripheral blood lymphocyte

PTH – parathyroid hormone

QALY – quality adjusted life years

RNS – reactive nitrogen species

RONS – reactive oxygen and nitrogen species

SAM - S-adenosyl methionine

SD – standard deviation

SNP – single nucleotide polymorphism

VDR – vitamin D receptor

VDRE – vitamin D response element

Introduction

As we age, we observe a substantial loss of muscle tissue, accumulation of fat tissue and a decrease in physical activity, leading to a negative feedback loop for the onset of non-communicable diseases, increase in DALYs and ultimately to premature death. Numerous comparisons between former athletes and the general population were able to show, that athletic individuals live a longer life, thus allowing for the assumption that physical activity can influence the process of aging positively (1,2).

Senescence and ageing display a loss of function of different cell types and the accumulation of damage, which could be physiologically, physically and/or psychologically caused by smoking, alcohol or other lifestyle factors (3).

There are multiple preventive measures to slow these processes down and keep functions that are required to live autonomously.

In 2015, 1.2 million citizens died prematurely in the EU and in 2016 790.000 people died prematurely based on unhealthy lifestyle factors, such as tobacco smoking or physical inactivity (4,5).

In this thesis, the outcomes of the “NutriAging vitamin D” project at the University of Vienna and the Comenius University in Bratislava will be presented. The results of the cytokinesis-block micronucleus cytome assay (CBMN-assay) will be discussed more detailed. The main goal of the study was to investigate the effect of different dosing routines of vitamin D supplementation on wellbeing, strength performance and DNA damage markers in healthy adults aged 65 to 85 years (6).

Furthermore, this thesis includes a chapter for novice teams that are learning the CBMN-assay and are seeking information to increase proficiency in this method and reduce potential pitfalls.

Literature review

Aging

Many physiological, molecular and cellular processes occur when organisms approach the end of their lifespan. There are several theories about why those processes are taking place.

For instance, program theories (also active/adaptive theories) where aging and death subsequently, is an evolutionary strive for rejuvenation. However, conflicting research shows, that certain genetic cascades or hormonal milieus influence aging implicitly. Damage theories propose a mechanism where constant "wear and tear" through reactive oxygen species (ROS), advanced glycation end-products (AGEs), reactive nitrogen species (RNS) or other compounds lead to deterioration of body functions, mutations in DNA through accumulation or the inability to counter these stressors.

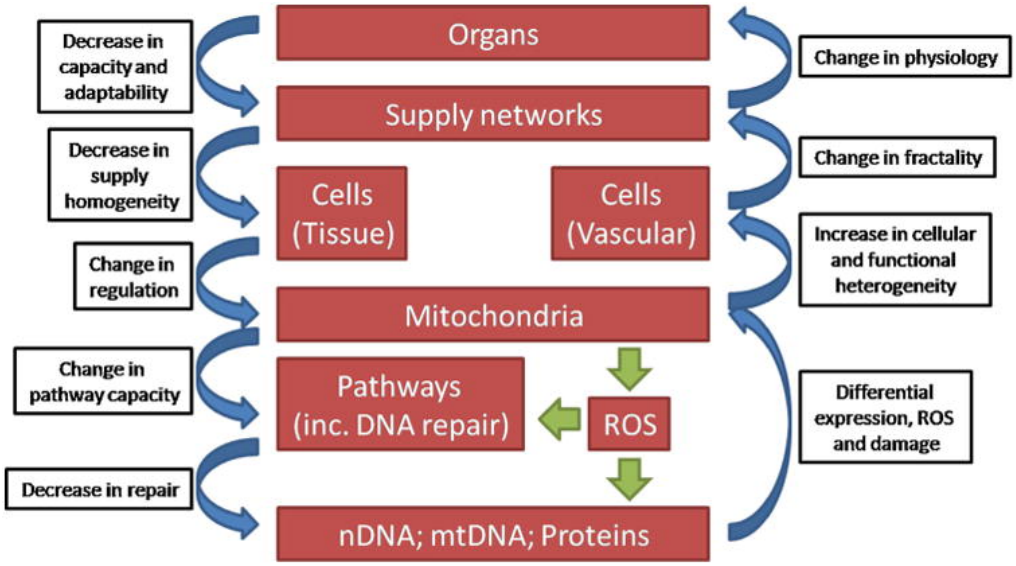

Figure 1: Changes during aging. Costa et al., 2016, adapted from Kriete et al., 2006.

Finally, there are combined theories that merged several aspects of aforementioned theories, for instance, the growing incapacity to conduct electrical processes and transport chemicals and heat. Aging is considered as a highly complex process with effects on multiple levels that all effect each other (7,8, Fig. 1).

Nonetheless, many suggested explanations for aging appear to interact with each other and there is no consensus on that matter, indicating that a more integrative approach to aging is needed (9).

Regardless, there are challenges that arise with increasing age. Certain environments or lifestyle decisions have consequences at a molecular level, that can trigger certain processes. They can cause deterioration, function loss of different cell types and the accumulation of physiologically, physically and/or psychologically damage, which could be prevented or mitigated (3).

Cessation of smoking, limiting alcohol consumption, being physically active or adhering to a diet conforming with current recommendations are all measures that are powerful lifestyle factors that seem to slow down the aging process (4).

A set of diseases and lifestyle factors relevant for this thesis are presented in the next section.

Osteoporosis

Fragility fractures due to reduced bone density and strength in the elderly is often caused by osteoporosis, a disease which is diagnosed by measuring bone mineral density (BMD) with, for example, dual-energy X-ray absorptiometry (DXA) and comparing the resulting T-score to the reference group of 20-29 year old women from the NHANES III database. If the measured T-score is below 2.5 standard deviations (SD) of the reference value, osteoporosis is diagnosed (10).

From 2017 to 2030 fractures are estimated to increase by 23% in six European countries (France, Germany, Italy, Spain, UK and Sweden), causing a severe burden for the healthcare system and a loss of 1 million quality-adjusted life years (QALYs) (11).

Primary prevention is a lifestyle according to current recommendations for nutrients and physical activity, mainly calcium and vitamin D intake and weight-bearing exercise, which all are powerful measures to prevent osteoporosis. Due to of the long onset of this condition and the fact, that peak bone mass is reached around adolescence, preventive measures have to be established and maintained throughout lifetime (12–14).

Sarcopenia

M62.50 is the ICD-10-Code for Sarcopenia and is specified with “muscle wasting and atrophy […]” (15).

Like osteoporosis, sarcopenia can increase the risk of fractures and numerous other health impairments. It is a “progressive and generalized skeletal muscle disorder that is associated with an increased likelihood of adverse outcomes including falls, fractures, physical disability and mortality” (16).

Diagnosis and severity of sarcopenia can be determined by measuring muscle strength (necessary criterion), muscle quantity/quality (confirming criterion) and physical performance (if abovementioned criteria are met, sarcopenia is severe).

Examples for determining muscle strength can be hand grip strength or the chair-rise-test, muscle quality/quantity with a CT, MRI or DXA-Scan and physical performance with measurement of gait speed, tests that were used in the NutriAging project as well.

Prevalence of sarcopenia is estimated to reach up to 33% with higher values in continuing care settings (17). Resistance training in combination with sufficient protein intake has been shown to have a powerful impact on muscle strength and are therefore a feasible measure to prevent or manage sarcopenia, even in the elderly population (18–20).

Protein

A key nutrient to maintain muscle mass and quality and therefore prevent sarcopenia, is protein, provided there is enough physical activity (21).

Since older individuals show weaker reactions in response to protein intake, it has been suggested to increase the recommended daily protein intake of 0.8 grams per kg bodyweight (g $kg^{-1\ BW}$) for individuals older than 65, especially because most of them fail to meet those requirements (22–26). The Nutrition Societies of Germany, Austria and Switzerland recommend 1 g $kg^{-1\ BW}$ for adults over 65 (25). However, a review by Franzke et al. from 2018, proposes even higher amounts may be needed for octogenarians and above (27).

Subjects in this particular study population failed to meet protein requirements as well, although the recommendation related to bodyweight possibly needs to be modified,

due to the prevalence of overweight and obesity. Other metrices, such as relative fat or lean mass or ideal body weight might be more appropriate to calculate protein intake for this specific age group (28).

Resistance training

The importance for resistance training for adults over 65 is evident in the guidelines for physical activity by the WHO: "[…] as part of their weekly physical activity, older adults should do varied multicomponent physical activity that emphasizes functional balance and strength training at moderate or greater intensity, on 3 or more days a week, to enhance functional capacity and to prevent falls." (29).
Although the effects of aging present themselves in many different ways, loss of lean tissue and strength consistently occur. In the face of muscular deterioration, strength training can be a powerful measure to prolong autonomy, if an individual and evidence-based approach to resistance training is applied (30).

Vitamin D

In 2012 an evaluation of a national representative sample of Austrians found that vitamin D status in 64% of the elderly had insufficient vitamin D levels (<50 nmol/l or <20ng/ml) and a study by Cashman et al. observed that 40.4% of sampled individuals throughout Europe had levels below <50nmol/l (31,32).
Deficiencies of vitamin D can cause impairments in bone mineralization, leading to fractures and is even thought to accelerate aging processes (33).
Particularly vitamin D administered in combination with calcium showed a decreased risk of falls in elderly persons thus decreasing the risk for fractures and frailty (34,35).

Metabolism and functions

Several compounds are considered as vitamin D while cholecalciferol (vitamin D_3) is the most important. Small amounts can be absorbed from sources of animal origin, although 100 grams of cod liver can contain up to 250 μg/ 10.000 IE of vitamin D (36). Another form of vitamin D is ergocalciferol (D_2), which can be found in yeasts and fungi

and shows the same properties, though to a lesser degree, due to the lower affinity to vitamin D-binding protein (DBP) (37,38).

The main source of vitamin D_3 is the endogenous formation of cholecalciferol from 7-dehydrocholesterol via exposure to UVB-radiation. Therefore, the definition of a vitamin does not apply to vitamin D, because of the ability to form D_3 endogenously. Once cholecalciferol is formed by the skin or ergocalciferol is absorbed by food or supplements, both enter circulation by binding to DBP, which is capable of binding all vitamin D metabolites. For bioactivation, two hydroxylation steps are necessary, the primary at C25, the second at C1. The first hydroxylation happens in the liver mainly via CYP2R1 and converts D_3 and D_2 to 25-hydroxycholecalciferol (25-OH-D_3) or 25-hydroxyergocalciferol (25-OH-D_2) respectively which are the most important circulating forms of vitamin D. In the kidneys both get converted further via CYP27B1 to the bioactive compounds 1, 25-hydroxycholecalciferol (1,25-OH_2-D_3 or calcitriol) or hydroxyergocalciferol (1,25-OH_2-D_2 or ercalcitriol) respectively (39). They serve as ligand for the vitamin D receptor (VDR), a transcription factor that binds to numerous locations in the DNA. Those locations, also called vitamin D response elements (VDREs), are expressing hundreds of genes and vitamin D expresses all of its functions this way (40).

For the sake of completeness, it has to be mentioned that not only the kidneys show activity of 1α-hydroxylase, but also prostate, breast, colon, lung, pancreatic β-cells, monocytes and parathyroid cells in a autocrine/paracrine way (41).

Vitamin D is known for its function in calcium-phosphorus homeostasis and bone health, but also plays an important role in immune function, because nearly all immune cells have the VDR present and it upregulates anti-inflammatory activity on macrophages by increasing interleukin-10 (IL-10) and decreases interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), among others. Especially vitamin D deficiency was linked to higher risks of respiratory tract infections and tuberculosis in particular and to the existence of various autoimmune diseases (42–44).

Vitamin D deficiency seems to be a critical risk factor for the onset and course of autoimmune diseases and supplementation in order to reach sufficient serum levels, may modulate disease development (45).

Vitamin D in the elderly

Bone health requires adequate vitamin D-levels throughout lifetime, since the onset of osteoporosis takes place over the course of several decades. Peak bone mass can be improved by physical activity and needs to be maintained, to prevent substantial losses in the course of aging.

A recent meta-analysis by Yao et al. showed no support for vitamin D only or vitamin D with calcium combined on fractures or hip fractures. Ongoing RCT's are not expected to change this conclusion, particularly because the dose of vitamin D administered was either too low (≤ 800 IU per day) or too infrequent (annually, quarterly or monthly) to exert any substantial effects on vitamin D levels, since 800 IU only raise 25 OHD-levels by 2.8 to 4.0 ng/ml (46) and half-life of 25(OH)D is only 2 to 3 weeks (47,48).

Kahwati et al. came to the same conclusion a year earlier and suggested, that the underlying issue of fractures might be pre-existing vitamin D deficiency or past fractures, thus highlighting the importance of maintaining recommended vitamin D levels and physical activity throughout lifetime (49).

Although vitamin D is important for bone metabolism, adequate vitamin D-levels are also important for muscle and immune function and cellular differentiation, thus supplementation or maintaining recommended levels still is necessary in old age, mainly to avoid deficiency (50,51).

Furthermore, cutaneous vitamin D production can be suppressed by lacking sun exposure due to decreased time spent outside, covering large areas of skin or the local irradiation may not be intense enough to induce vitamin D synthesis (52). Garcovich et al. even suggest limiting sun exposure in order to reduce the incidence of skin cancer (53). Applying sunscreen is able to suppress vitamin D production in theory (54), but is unlikely to have a detrimental effect of D_3 formation (55). In addition, the concentration of 7-dehydrocholesterol decreases with age, which further reduces endogenous vitamin D production (56). Other factors, such as age-related decreasing circulating levels of 25(OH)-D, are controversial and symptomatic for age at best (57), emphasizing the potential importance of vitamin D supplementation.

DNA damage and vitamin D

Exposure to sun, in particular UVB irradiation is one of the key factors for the development of skin cancer. The irradiation can trigger the formation of cyclobutane pyrimidine dimers (CPDs), products of DNA-misrepair. De Haes et al. were able to show that $1,25(OH)_2D_3$ is able to suppress the formation of CPDs in keratinocytes although the doses needed were beyond physiological doses (58).

However, while UVB-irradiation with wavelengths between 290 and 320 nm causes direct damage, UVA radiation with wavelengths between 320 and 400 nm causes damage by inducing ROS, which damages DNA, proteins and lipids as soon as endogenous antioxidative systems are depleted, leading to inflammation, immunosuppression and ultimately to cancer (59). This damage by UV exposure is able to influence the formation of MNi, while sufficient levels of vitamin D might reduce DNA damage in peripheral blood lymphocytes (60), and certain groups with conditions like type 2 diabetes mellitus might profit even more from higher vitamin D levels (61).

Manifestations of DNA damage

DNA damage can be induced endogenously by DNA interacting with ROS or exogenously by environmental, physical and chemical factors, such as UV/ ionizing radiation, toxins or high temperatures. If the damage exceeds the capacity of the major repair mechanisms, damage leads to lesions, crosslinks, single or double-strand breaks (62). Furthermore, defects in the responsible genes, that are coding for specific repair mechanisms, such as BRCA1 and BRCA2, can play a role in the repair of double-strand breaks (63).
Ultimately, DNA damage causes abnormalities at chromosome level and subsequently lead to the formation of micronuclei, nucleoplasmic bridges and nuclear buds (64).

Micronuclei

The CBMN is an assay which is used to determine the severity of DNA damage through chromosome loss, breaks or DNA misrepair complexes in human and mammalian cells. Through in vitro induction of a cell cycle in peripheral blood lymphocytes (PBL) and subsequent stop of cell division right after telophase with cytochalasin B, which inhibits spindle assembly, MNi, NPBs and NBuds can be conserved and evaluated (65)

Formation of MNi, NPB and NBuds

Micronuclei were first observed in erythrocytes by William Holly and Justin Jolly in 1890 and 1907 independently from each other and described as "*red blood corpuscles*" or "*globules rouges des mammifères*" (66). Half a century later, it was observed that ionizing radiation and chemical agents can induce the formation of micronuclei and in 1961 they were associated with vitamin deficiencies, an early indication for suboptimal DNA repair mechanisms and their consequences (67–69).

The CBMN assay is able to identify MNi and associated events by blocking cytokinesis after the first nuclear division and can assess other markers like cell death and cytostasis, extending its significance and relevance. It is a widely used protocol to examine the genotoxic potential of chemicals and it can quantify the amount of damage caused by ionizing radiation (70–72).

MNi, NPB and NBuds develop from acentric chromosome fragments, acentric chromatid fragments or malsegregation of whole chromosomes. MNi in particular are whole chromosomes or chromosome/chromatid fragments that were not included in mitosis and are individual structures, enveloped by a nuclear membrane, left in the cell after telophase. They can originate from unrepaired double-strand breaks or defective genes, coding for repair mechanisms or enzymes of the non-homologous end joining pathway, which are susceptible for errors under certain circumstances (63,73–75).
Additionally, MN formation can be a consequence of simultaneous excision repair (for instance 8-oxo deoxyguanosine, a biomarker of oxidative stress and a trigger for mutagenesis) or unfitting bases that were integrated to the DNA (uracil); both can lead to double-strand breaks (76,77).
NPB are remnants of centromeres of dicentric chromosomes which can arise either by misrepair of broken chromosomes or telomer end-to-end fusion. These dicentric chromosomes get drawn to opposing sides of the cell during mitosis, remain intact after anaphase and then get surrounded with a nuclear membrane. They usually break after completion of cytokinesis, but the inhibition of cytokinesis, inherent to the CBMN, allows NPB to accumulate and persist (65,78).
Another nuclear event are NBuds, which have the same morphology as MNi and appear as small protrusions from the nucleus, connected with a band of nucleoplasmic material that is thinner than the actual NBud. They can be a result of amplified genes, that were transported to the periphery of the nucleus for elimination. The mechanism for removal of amplified genes constitutes via formation of double minutes, small extrachromosomal fragments of DNA and a consequence of chromothripsis. NBuds may as well contain excess DNA or terminal fragments or be a temporary result of a NPB breakage and become eventually MNi (73,79,80).

Age and sex

The frequency of MNi and related events increase with age. This may be due to defective repair mechanisms or the accumulation of DNA damage and numerous studies in different population groups throughout the world share similar observations (81–84).

The increase of DNA damage with age might reach a maximum at some point, as Bonassi et al. suggested in 1995, which was solidified with the work by Wojda et al and Franzke et al., indicating that there is a survivorship bias and that the MNi frequency peaks between the age of 60 and 70 years (85–87).

The body of evidence discovered higher counts of MNi in females, a majority of those consisting of X chromosome, which could be the effect of having twice as much X chromosomes than men. There is conflicting evidence, showing no significant effect of sex on MNI or related events and may as well be explained by intraindividual factors (84,88).

Nutrient Status and DNA damage

Nutrients with an impact on MNi formation are vitamins that are directly involved in genomic stability, such as folate and cobalamin. Folate serves as donor for single-carbon groups which is necessary for DNA synthesis and repair. In conjunction with cobalamin it is used for cytosine methylation via S-adenosyl methionine (SAM) and therefore maintaining methylation patterns. Folate deficiency can lead to incorporation of uracil, due to accumulation of dUMP, which subsequently can cause single- and double-strand breaks. If cobalamin and methionine are insufficient, the resulting lack of SAM leads to an irreversible conversion of 5,10-methylentetrahydrofolate to 5-methytetrahydrofolate which again, increases dUMP levels. Deficiencies in either of those nutrients can therefore increase the rate of DNA damage with all associated negative effects (89–91).

Vitamin D may influence MNi formation by reducing oxidative damage to DNA and preserving DNA repair mechanisms as low vitamin D levels in mothers were associated with higher MNi counts in their newborns (92–94).

Furthermore, smoking significantly increases the frequency of MNi as do other lifestyle factors such as drinking or obesity (83,95,96). Frequency of MNi are negatively correlated with Vitamin B12 levels and lower with levels >300 pmol/l and MNi are positively associated with homocysteine and are lower with levels <7.5 μmol/l. The kind of diet and relevant single nucleotide polymorphisms influence the occurrence of MNi (97).

Physiological effects of exercise

Despite the known positive effects of exercise on numerous health outcomes and life expectancy, strenuous exercise also can cause damage by increasing reactive oxygen and nitrogen species (RONS), as Aguiló et al. were able to show in elite road bikers (98).

Gargallo et al. investigated the effect of high versus moderate intensity resistance exercise in untrained older women. While moderate intensity resistance training was able to improve markers of oxidative stress, the high intensity group showed contrasting results and concurrently both groups improved physical function, regardless of intensity. This might imply the importance of carefully considered intensity prescriptions for resistance training regimen for the elderly in order to maximize adaptations following exercise, but simultaneously keep the oxidative stress levels low (99).

NutriAging D project

In the course of “the NutriAging vitamin D” project, a double-blind, randomized controlled trial was conducted in elderly persons between 65 and 85 years. It is part of the NutriAging project, a cross border venture between the Universities of Bratislava and Vienna, funded by the European Regional Development Fund. Subjects were recruited to investigate the differences of vitamin D levels before and after a 14-week intervention, the effect of resistance training on DNA damage markers and the effect of vitamin D on resistance training outcomes, as well as many other markers of wellbeing.

Starting in January 2019, participants in Vienna were recruited through newspaper articles and radio broadcasts which were then invited to information events, where the basic parameters for the intervention study were outlined. During the event, questionnaires were completed, to exclude participants not qualified for the study. Right after the event, all qualified volunteers had their initial vitamin D-levels checked participants over 75 nmol/L were excluded. All eligible participants were then invited to another event where different cognitive and performance tests were carried out. Then, participants had their blood drawn before, 4 weeks after beginning and right after completion of the intervention, which consisted of 10 weeks of resistance exercise and, dependent on the group, different vitamin D administration protocols.

Methods

Study Design

The “vitamin D-study” was conducted between January 2019 and July 2019 and was randomized, controlled and double-blind. 100 participants were recruited, of which 85 have competed the trial.
They were divided into three parallel groups and were randomly assigned to one of three groups:

1. Group 1: Control group, 400 mg calcium daily as placebo
2. Group 2: Daily dose of 20 μg (800 I.E.) vitamin D and 400 mg calcium daily
3. Group 3: Monthly megadoses of 1250 μg (50.000 I.E) vitamin D in total and 400 mg calcium daily

Blood samples were taken, physical and cognitive performance tests were conducted before (T1), after 4 weeks (T2) and after 14 weeks (T3) of intervention.

Recruitment

Subjects between the age of 65 and 85 were recruited by internet, radio and newspaper. Several events at the Centre for Sport Science and University Sports in Vienna were organized, where participants were informed about the procedure and organization of the study (Fig. 2). During those events, health questionnaires were completed in order to exclude persons which did not meet inclusion criteria at the beginning. All eligible persons were given a preliminary number and then asked to donate blood to determine vitamin D levels, as well as to share their contact information, to make an appointment for the final examination, where the inclusion criteria were investigated

Figure 2: Recruiting event at the Schmelz, January 2019

Inclusion criteria

Participants had to be between 65 and 85 years old, in good health and mental condition (mini-mental-state-test score >23), without serious health issues, not taking vitamin D-supplements and not performing regular resistance training in the past 6 months.

During the initial event, a preliminary questionnaire was distributed to exclude prospects with serious health issues or those, who would be exposed to above average sun exposure before the intervention. Participants had to agree to participate in the whole 14-week trial and were required to have vitamin D levels of 75nmol/l or lower.

378 participants were interested and 231 were examined, 100 were allowed to participate and started the trial with T1 and 85 subjects finished at T3.

Exclusion criteria

Subjects with vitamin D-levels above 75 nmol/l were excluded as well as those with:

- Osteoporosis
- Chronic kidney disease, renal stenosis
- Cardiovascular diseases (e.g. instable Angina pectoris, untreated arterial hypertension)
- Heart bypass
- Cardiac pacemaker, defibrillator
- Aortic aneurysm
- Diabetic retinopathy
- Acute tumor disease
- Regular solarium visits
- Vacation in regions with high sun exposure during the trial (especially the southern hemisphere)
- Use of blood-thinning medication (e.g. Aspirin, Eliquis, Xarelto, Pradaxa)
- Regular intake of cardiac glycosides, thiazide diuretics, glucocorticoids or antibiotics in the past 6 months
- Regular resistance training (≥1/week in the past 6 months)

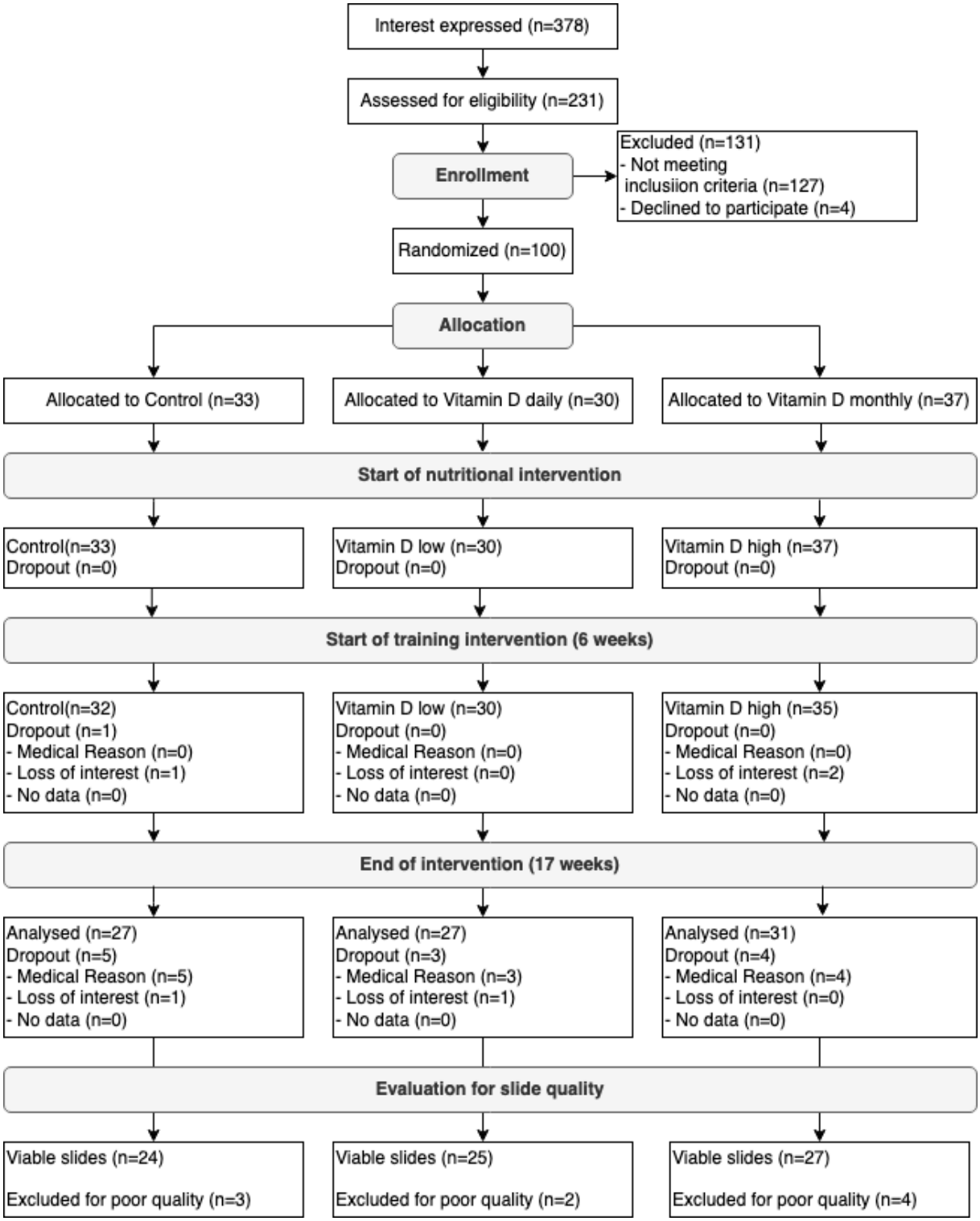

Figure 3: Flow chart of "NutriAging" project

Intervention

All participants performed 10 weeks of resistance training and were randomized into three different groups.

Group 1 (CT) was the control group and received no vitamin D supplement, but two doses of 200 mg calcium and information about healthy lifestyle, provided by the Austrian Nutrition Society.

Group 2 (VDD) received 2x 200 mg calcium a day and additionally a supplement with 20 μg (800 I.E.) of vitamin D_3 and were advised to take it daily for the duration of the trial.

Group 3 (VDM) received 2x 200 mg calcium daily and several megadoses of in total 1250 μg vitamin D (50.000 I.E) per month, partitioned in two doses of 500 μg and two 125 μg vitamin D_3-capsules, beginning with T1 (Fig. 4).

All participants were instructed to take the supplements daily and at the same time, as well as in combination with a meal.

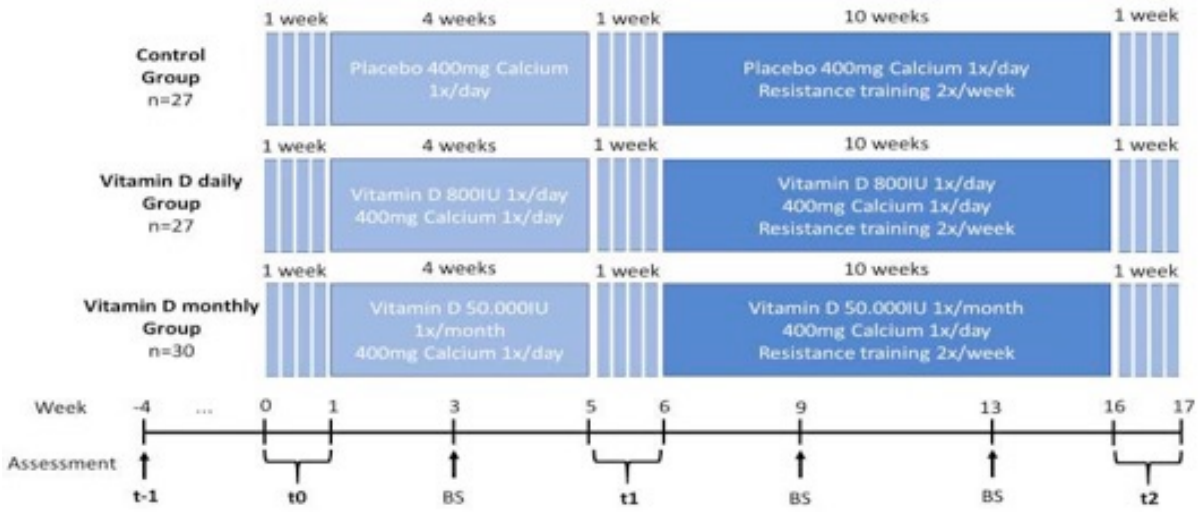

Figure 4: Allocation and study procedure (Franzke et al., 2018 unpublished)

Resistance training

All participants had to undergo a 10-week strength program with in total 20 sessions of 60-90 minutes duration. Prescribed were two sessions a week and they were at least 48 hours apart from each other and performed in small groups of a maximum of

five participants under the supervision of at least one experienced fitness trainer at selected gyms in Vienna.

Before every session, participants completed a short questionnaire about well-being, motivation and sleep quality and started with a five-minute warm-up on a treadmill or cross-trainer. Furthermore, weights and individual machine adjustments were logged for the whole duration of the intervention.

Subjects performed a full body program with four pairs of exercises where one pair would train the agonist and antagonist of a given body part (e.g. vertical push /pull). In every session both exercises were performed in a superset-manner, meaning that participants executed one set of exercise A1 and started exercise A2 immediately after (10-20 seconds) and only after completion of A2 were allowed to rest for 60-75 seconds. Cadence was prescribed with 3 seconds in the eccentric and an explosive or fast concentric phase, with no time in both isometric phases (30X0). After completion of all eight exercises, participants took five minutes for stretching or performed other measures for regeneration. Participants were then asked to rate their perceived exertion on a scale from 0 to 10 which was recorded as well.

The program design consisted of four phases (Fig. 5):

1. Introductory phase (2 weeks): to familiarize participants with the exercises, repetition and set-scheme and cadence/speed of movement, 10-20 repetitions with two sets at light intensity.
2. Adaptation phase (1-2 weeks): intensity is increased compared to previous phase, participants were supposed to choose an intensity that enabled them to perform 10-15 repetitions. To calculate intensities for the following progressive overload weeks, the 4th session encompassed a 5 repetition max-test (5 RM 1st).
3. Intensification I (2-3 weeks): with 60-70% of 5RM, participants performed 2 sets with 8-12 repetitions. As soon as they successfully executed 12 repetitions on both sets, intensity was increased by one unit (one plate, weight stack or implement) in the next session. This procedure was continued for the whole program.

4. Intensification II (2-3 weeks): Intensity was kept the same, but volume was increased by one set to 3 sets per exercise in total, if possible. The very last session included another 5 RM test (5RM last).

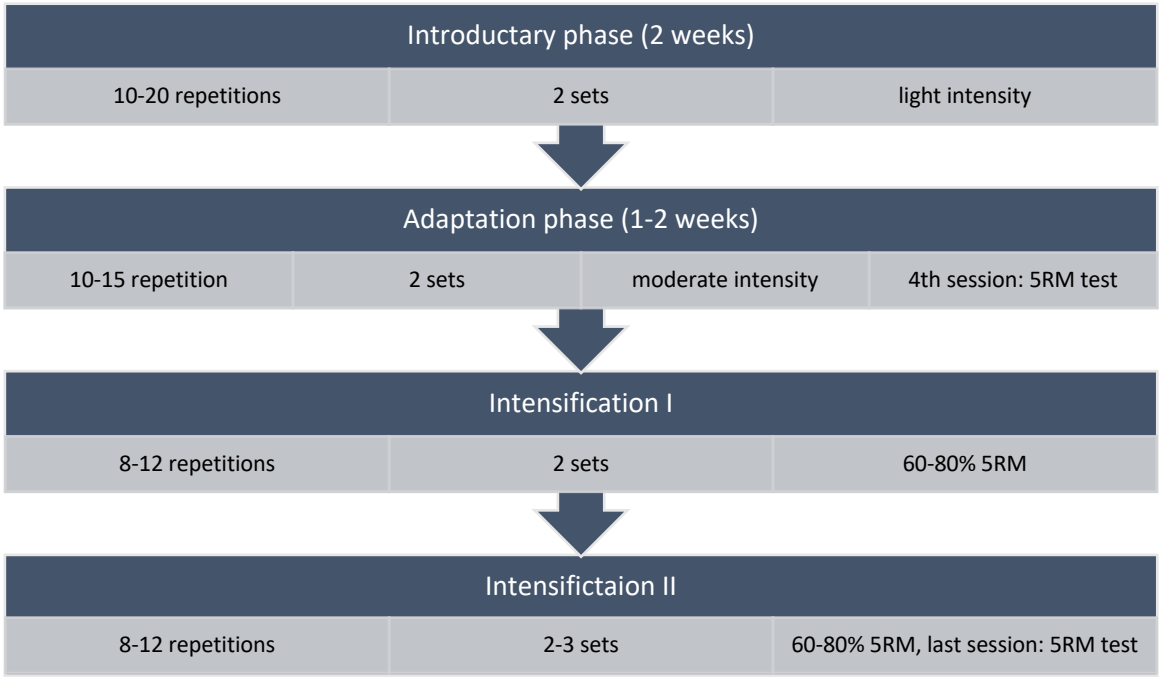

Figure 5: Training plan and course of micro and meso cycles

CBMN-Assay

Chemicals

Reagent	Quantity	Manufacturer
RPMI 1640, sterile	500 ml	Sigma Aldrich
Fetal bovine serum	500 ml	Sigma Aldrich
Sodium-L-pyruvate, sterile	50 ml	Sigma Aldrich
L-Glutamine, sterile	200 µl	Sigma Aldrich
Dulbecco's phosphate buffered saline sterile (PBS)	500 ml	Sigma Aldrich
Phytohemagglutinin (PHA-M)	20 mg	Merck
Cytochalasin B		Sigma Aldrich
Dimethylsulfoxid (DMSO)	250 ml	Sigma Aldrich
Diff Quick staining set		Medion Diagnostics
Entellan	250 ml	Merck
Trypan blue 0,4%	20 ml	Thermo Fischer
Red blood cell lysis buffer	100 ml	Roche

Table 1: List of chemicals used for the assay

Materials

Name	Size	Manufacturer
Centrifuge tubes	15, 50 ml	Greiner
Countess cell counting chamber slides		Invitrogen
Cover glasses	24 x 32 mm	VWR
Cytocentrifuge cups		
Falcon tubes	15 ml	VWR
Filter cards		Thermo Electron Coop.
Glass pipettes, sterile		
LeucoSep tube	50ml	Greiner
Metal slide holders		
Micro centrifuge tube	1,5 ml	Star Lab
Pasteur pipettes, sterile		
Pipette tips, sterile	20, 100, 200, 1000 μl	
Round bottom falcon tubes	5 ml	
Slides	125x25 mm	VWR
Staining rack		
Syringe filter	0,45 μl	VWR
Syringe without needle, sterile	50 ml	Terumo
VACUETTE blood collection tubes, LH lithium heparin	9ml	Greiner

Table 2: List of materials used for the assay

Equipment

Name	Brand
Countess™ automated cell counter	Invitrogen
HERAEUS™ Megafuge 40	Thermo Scientific
Cytospin 4	Thermo Scientific
CelCulture® CO_2 incubator	ESCO
Water bath	

Table 3: List of equipment used for the assay

Sample Preparation and Execution

Isolation of lymphocytes

Every first half of the week during the trial, four EDTA-tubes with blood were collected from 1-15 participants at the University Sports Center in Vienna and transported in a Styrofoam cooling box to the lab at the Department of Nutritional Sciences. Before arrival of the blood samples, 50 ml LeucoSep-tubes were placed in a dark room and allowed to come to room temperature. All following steps were done under aseptic conditions within a laminar flow hood.

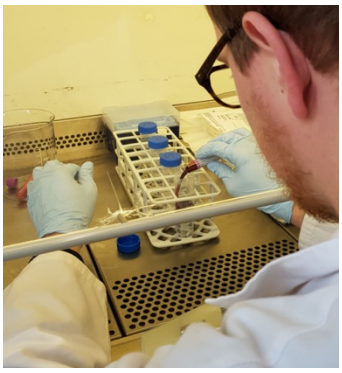

Figure 7: Transfer of blood from EDTA to Leucosep-tubes

Upon arrival of the blood samples, the four EDTA-tubes of every participant were tilted upside down five times and without touching, two EDTA-tubes were poured in one LeucoSep-tube respectively and put onto ice (Fig. 7). They were then centrifuged at 1000g for 10 minutes, without brake, which took between 25 to 30 minutes, depending on the number of tubes.

Figure 6: Plasma extraction. Lymphocytes are visible as the cloudy layer between the plasma and the clear solvent

Once the centrifuge stopped, the lymphocytes, visible as a cloudy layer in the middle of the tube, were carefully extracted with a pasteur pipette in a circular manner, while concentrating on the wall of the tube (Fig. 6), to a sterile falcon-tube and filled up with PBS to the 15 ml mark and carefully tilted upside down five times. The remaining plasma was collected as well, for further examinations by another team.

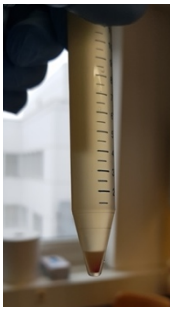

Figure 8: Lymphocytes after centrifugation

The falcon tubes were subsequently centrifuged for 10 minutes at 4°C at 1300 rpm with brake, so a pellet of lymphocytes was formed at the bottom of the tube (Fig. 8). Next, superfluous PBS was removed with a glass-pipette, connected to an electrical pump, until only 1 cm of PBS was left in the tube. With 1 ml of fresh PBS the pellet was suspended using a 1000 μl micropipette and then was filled up again to the 15 ml mark with fresh PBS for another run in the centrifuge with the same setting as before.

After removing the supernatant again, the pellet was suspended with 1 ml of Red Blood Cell Lysis Buffer and was put on ice in the dark for 20 minutes.
Thereafter, the tubes were centrifuged at 500 g for 5 minutes at 4°C. The supernatant was discarded and the remaining pellet resuspended with 1 ml of PBS, filled up to the 5 ml mark with cold PBS and gently mixed with the same pipette.

Cell count

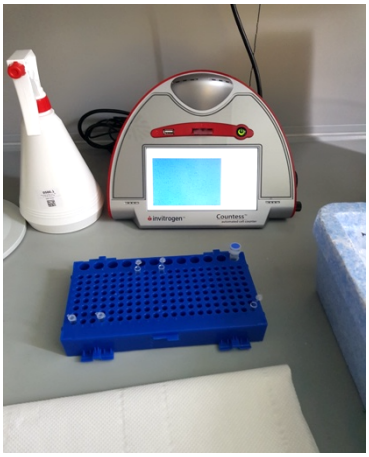

Figure 9: Setup for cell count

For every participant, 20μl sample were gently suspended with 60μl PBS in a 1,5ml tube. 10 μl of this suspension were taken to another tube with 10 μl of Trypan blue and carefully mixed with a 20 μl micropipette. 10 μl of this mixture were then pipetted into a chamber which was put into a Countess cell counter. If needed, cells were focused to a degree where membranes would be distinguishable and cytoplasm would appear brighter than the surrounding matrix (Fig. 9). Cell count, viability and dead cells were recorded. If a cell count did not yield the desired number of 7,5 x 10^5 living cells, the cell count was repeated with a fresh suspension. Aliquots were calculated and then prepared to ensure a sufficient number of cells for CBMN and comet assay, DNA and RNA-analysis. Remaining lymphocytes were saved and frozen at -80°C. The freezing medium consisted of FBS, which was thawed and warmed up to 37°C in a pre-warmed water bath, and DMSO. Depending on the amount of FBS in the falcon tube, 10% were added as DMSO (e.g. for 13 ml FBS, 1.3 ml DMSO were added) and mixed.
After passing on the aliquots to the respective teams or for freezing, the aliquot for the CBMN assay was suspended in 1.5 ml Eppendorf-tubes, centrifuged at 3000 rpm at 4°C for 4 minutes and ultimately transferred to round-bottom falcon tubes.

CBMN-Assay

Day 1: Isolation and preparation

During the last steps of lymphocyte isolation, the CBMN assay could commence with preparing the culture medium. With a 50 ml syringe that was equipped with a 0,45 μm filter, 30 ml of RPMI were filtered and moved to a 50 ml falcon tube and another 10 ml to a 15 ml falcon tube which was left under the flow hood for day 2. Together with a falcon tube of previously aliquoted FBS, the 30 ml of RPMI were placed into a 37°C water bath and allowed to reach that temperature. Previously prepared L-glutamine and sodium pyruvate were taken out of the freezer too and allowed to thaw at room temperature.

Once the reagents were up to 37°C, the RPMI was mixed with 3,3 ml FBS, 330 μl L-glutamine and 330 μl sodium pyruvate. Of that mixture, about 3 ml were transferred separately to a 15 ml falcon tube and placed in a refrigerator for day 2.

The duplicate aliquots were combined with 750 μl of the warmed culture medium. Three two-fold tubes (6 tubes) were arranged in a rack and one by one 15 μl of PHA was added, while the exact time was recorded. Lids were placed loosely onto the tubes and the rack was then placed into an incubator with a humidified atmosphere, 37°C and 5% CO_2. Additional samples were stimulated at least 20 minutes later than the rack before and the samples were left for 44 hours to incubate.

Day 2: Addition of Cyt-B

Following steps were done with PSE and under a laminar flow hood. 10 to 15 minutes before stopping cell division with Cyt B, it was taken out of the freezer, allowed to thaw and 100 μl Cyt B were diluted in 900 μl of culture medium. Then, the first rack was taken out of the incubator and placed under the hood. As soon as exactly 44 hours after addition of PHA have passed, 56.2 μl from the top of the medium were removed and 56.2 of Cyt B-solution were added. Samples were then returned to the incubator for another 28 hours.

Day 3: Harvesting and fixing

All materials for fixing and staining were prepared. All slides were wiped with alcohol before use. Four slides for every participant, two slides per tube respectively, were set up and labelled. All steps were performed under non-sterile conditions.

The first two slides 1A and 1C would receive the sample of the first, the latter two slides 2B and 2D, the sample of the second tube of one participant. All necessary slides were prepared beforehand, depending on the number of tubes in the incubator. Twelve of those prepared slides were then put into metal holders with a filter card and a cytocentrifuge cup in the same sequence as the samples in the rack.

Precisely 28 hours after stopping cell division with Cyt B, 200 µl supernatant of every tube were removed and 46 µl DMSO added to the sample and gently mixed with a 1000 µl micropipette. 120 µl of sample solution were then pipetted into the cytocentrifuge cup. Once all twelve slides were loaded, they were centrifuged at 600 rpm for five minutes at high acceleration. Then the slides were set aside to dry for 10 minutes at room temperature. At the same time, Quick Fix, Diff-Quick 1 and Diff-Quick 2 were filled into glass (Fig. 10). Once the slides were dried, the slides were put back-to-back into a staining rack and placed into the first container with Diff-Quick for 10 minutes, then it was immersed ten times into Diff-Quick 1, eight times into Diff-Quick 2 (Fig. 10), rinsed with tap water and lastly rinsed with demineralized water. Slides were removed from the holder and left to dry face down. After inspection of the dyeing process, slides were left overnight at room temperature in a dark space. If inspection of slides through a light microscope did not yield satisfactory results, the dyeing process was repeated as an attempt to achieve a better outcome, those slides would carry the letter E and F and possibly G and H, granted enough sample was left in the tube.

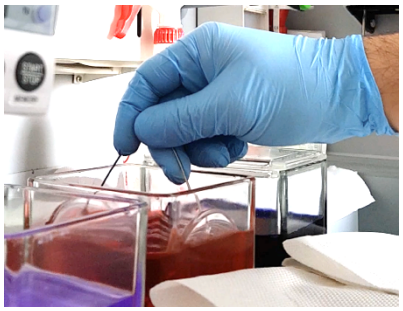

Figure 10: Submerging the slides into Diff-Quick 2

Day 4: Covering and storage

The next day, cover slips were glued onto the slides underneath a fume hood. Slides were placed on tissue paper and 1-2 drops of Entellan were placed next to the spot and a cover glass was slowly put onto the spot, trying to avoid air bubbles. As soon as the glue has set, slides were organized in microscope slide boxes.

Scoring criteria

In accordance with criteria proposed by Fenech et al. (65) mono-, bi- and multinucleated cells, apoptotic and necrotic cells and micronuclei (MNi), nucleoplasmic bridges (NPB) and nuclear buds (NBuds) were scored.
Per spot, 500 binucleated cells (BN) should be counted to reach 2000 cells in total for every participant. While counting to 250 BN, all aforementioned components were scored, from 251 to 500 only MNi, NPB and NBuds were counted.
On the basis of Fenech's scoring criteria, our team agreed on several additional criteria for respective cell components to ensure equivocal differentiation between scorers (65):

Mono-, bi- and multinucleated cells

> Cells have to be intact with undamaged cytoplasm and one, two or more nuclei respectively, with normal nucleus morphology. The nucleus and nuclei have to be approximately the same size and staining intensity and should not be merged or overlap each other except if the membrane of both nuclei was clearly distinguishable. Nucleus/nuclei has to be surrounded by cytoplasm. They could contain one or more MNi or NBuds, bi- and multinucleated cells could contain one or more NPB.

Apoptotic cells

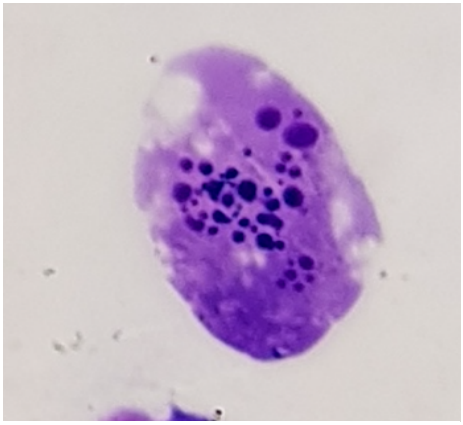

Figure 11: Ongoing apoptosis, with visible condensation and beginning lesions

Cells undergoing apoptosis were scored if condensation within cell compartments of the cell and nucleus with intact membranes is observable. Cells that contain several round and small nuclear bodies that are darker than the surrounding cytoplasm while having an intact membrane were counted as apoptotic cells in the early stage. In that stage, cell and nuclear membrane may be intact or allow for condensation of nuclear content. Late-stage apoptotic cells would already show lesions within the membrane of the nucleus but are not pale and bright as necrotic cells (Fig. 11). Apoptotic cells should be of similar size of intact adjacent cells.

Necrotic cells

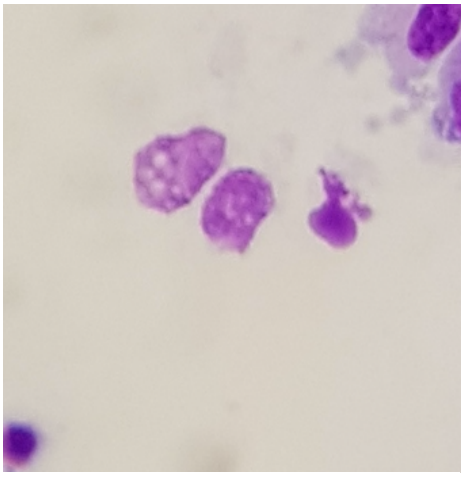

Figure 12: A necrotic cell with pale cytoplasm, no visible membrane and beginning fragmentation

Necrosis was identified by pale cytoplasm in comparison with viable cells. Depending on the stage, necrotic cells can show damaged membranes with multiple vacuoles and a mostly intact nucleus (early) or leakage of cytoplasm into the surrounding matrix and fragmentation of the nucleus and disintegration of nuclear structures. They should be mostly round (Fig. 12).

They were scored if intact cells were adjacent. Necrotic cells at the outer edge of the spot were not counted, as well as large areas of necrotic cells.

BNs for scoring MNi, NPBs and NBuds

Binucleated cells must contain two nuclei which should have an intact morphology, be of the same size and color and ideally not be in contact with each other. If so, boundaries of both cells should be clearly visible.

Micronuclei (MNi)

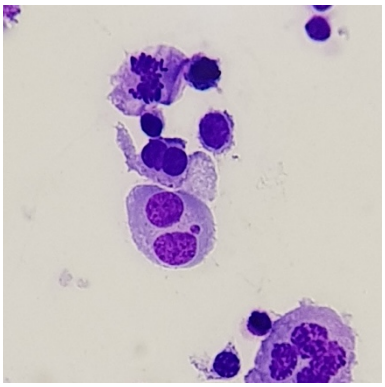

Figure 13: Micronucleus in a binucleated cell

MNi should not be smaller than 1/16th and not bigger than 1/3rd of the diameter of the main nuclei (1/256th to 1/9th of area). MNi can touch the main nuclei, but not overlap with the membrane of the nuclei, the boundary should be clearly visible. The color can be similar or darker than the nuclei, differentiation between particles of dye was done by putting the spot under question in and out of focus. A MNi would persist through all stages of focus. MNi should have a round to oval form (Fig. 13)

Nucleoplasmic bridge (NPB)

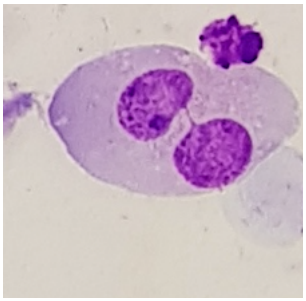

Figure 14: Nucleoplasmic bridge

Connections between two nuclei, considered as nucleoplasmic bridges, should not be wider than 1/4th the diameter of the main nuclei and be of the same staining intensity as the main nuclei. In rare cases, multiple bridges are observable, they were counted as one (Fig. 14).

Nuclear Bud (NBud)

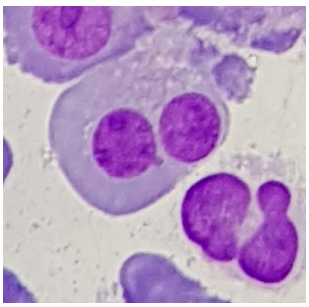

Figure 16: A nuclear bud protruding from one of the nuclei

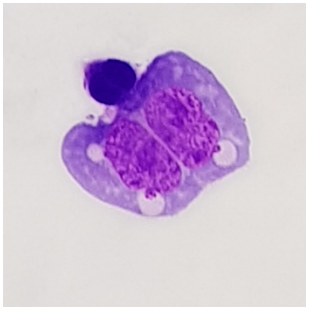

Figure 15: Multiple NBuds protruding from the nuclei, surrounded by a vacuole

A NBud consists of nuclear material with a connection to the main nucleus that should be thinner than the main diameter of the bud, a clearly visible incision. Staining intensity is the same as in MNi and sometimes are situated within a vacuole. If a MNi touches the nucleus and the boundary is not clearly visible, a bud was scored (Fig.16). Sometimes multiple NBuds are observable, then every NBud was counted (Fig. 15).

Statistical Analysis

Data analyses were performed with IBM SPSS Statistics 26. First, distribution of anthropometric data and several MNi-related values for the whole sample, separated by intervention group and sex, are shown. Those analyses only include participants who completed the full 14 weeks of the study.

Tests for group and/or time differences only included slides that met the inclusion criteria of at least 500 cells per participant. A Shapiro-Wilk-Test was able to show, that most of the data are not normally distributed, hence for comparisons between means of MNi-parameters an ANOVA with repeated measures and Friedman-test was performed. Although normal distribution could not be assumed, ANOVA still shows robust results (100) and was therefore used. After exclusion for poor slide quality, 74 participants were included in the analysis. Effects of time and group were tested, as well as time and group combined.

Significance was set at $\alpha=0.05$ (*p=<0.05, **p=<0.01, ***p=<0.001)

Results

Subject characteristics

Sex	CT	VDD	VDM	Total
Female	9	10	10	29
Male	18	17	21	56
Total	27	27	31	85

Table 4: Distribution throughout intervention groups

In total, 85 subjects completed the 14 week-long trial, 27 each in the control (CT) and the vitamin D daily (VDD) group, and 31 in the vitamin D monthly (VDM) group. There were almost twice as many male than female subjects, a distribution that continued throughout all groups (Tab. 4).

Age was distributed equally between groups with mean age of 70.3 years in control, 70.6 years in VDD and 70.9 years in VDM respectively. There was more variation within than across groups (Tab.6, Fig. 17).

Group	Mean [KI] age (years)	SD	Median	Min	Max
Control	70.30 [68.37; 72.24]	4.89	68.83	64.92	84.92
VDD	70.62 [68.81; 72.44]	4.59	68.75	65.25	79.17
VDM	70.87 [69.20; 72.54]	4.63	69.71	65.00	80.92

Table 5: Age characteristics throughout intervention groups

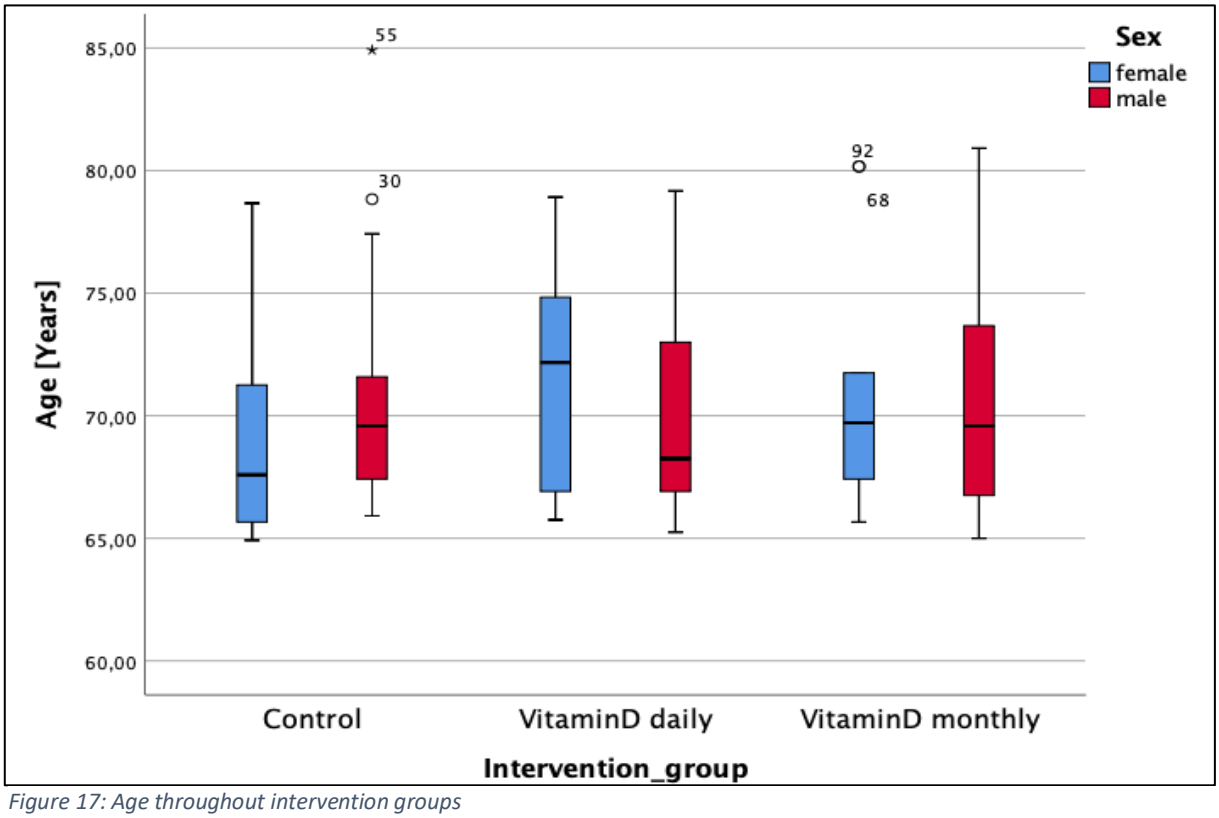

Figure 17: Age throughout intervention groups

Anthropometric data

BMI data shows that mean and median BMI in all groups was above 25 kg/m^2, so more than 50% of subjects were at least overweight, some subjects even showed severe obesity with BMI values over 40 kg/m^2, if the classification for adults 20 years and older is applied (Tab. 6, Fig. 18). However, a higher BMI in older individuals seems to have protective effects and values below 23 kg/m^2 were associated with higher mortality in community-based adults aged ≥ 65 years (101). BMI values between 23 kg/m^2 and 29 kg/m^2 in older adults show the lowest mortality rates and should be considered as the acceptable range (102).

Weight status between and within groups was heterogenous (Fig. 18). Although the majority of participants was overweight or obese, most of their waist-to-hip ratios remained under the critical cutoffs at 0.85 for women 1 for men (Fig. 19), which may be an indicator of weight accumulation in other locations than the waist. All waist-to-hip ratios were significantly different between sexes in all groups. There were no significant differences for BMI between sexes or intervention groups.

[kg/m^2]	Mean [KI] BMI (kg/m^2)	SD	Median	Min	Max	Sig.
Control	26.16 [24.24; 28.08]	4.86	25.67	19.32	42.57	0.11
VDD	28.29 [26.46; 30.13]	4.63	27.00	21.14	38.94	
VDM	27.20 [25.45; 28.95]	4.87	26.86	19.74	42.56	

Table 6 :BMI characteristics throughout study groups

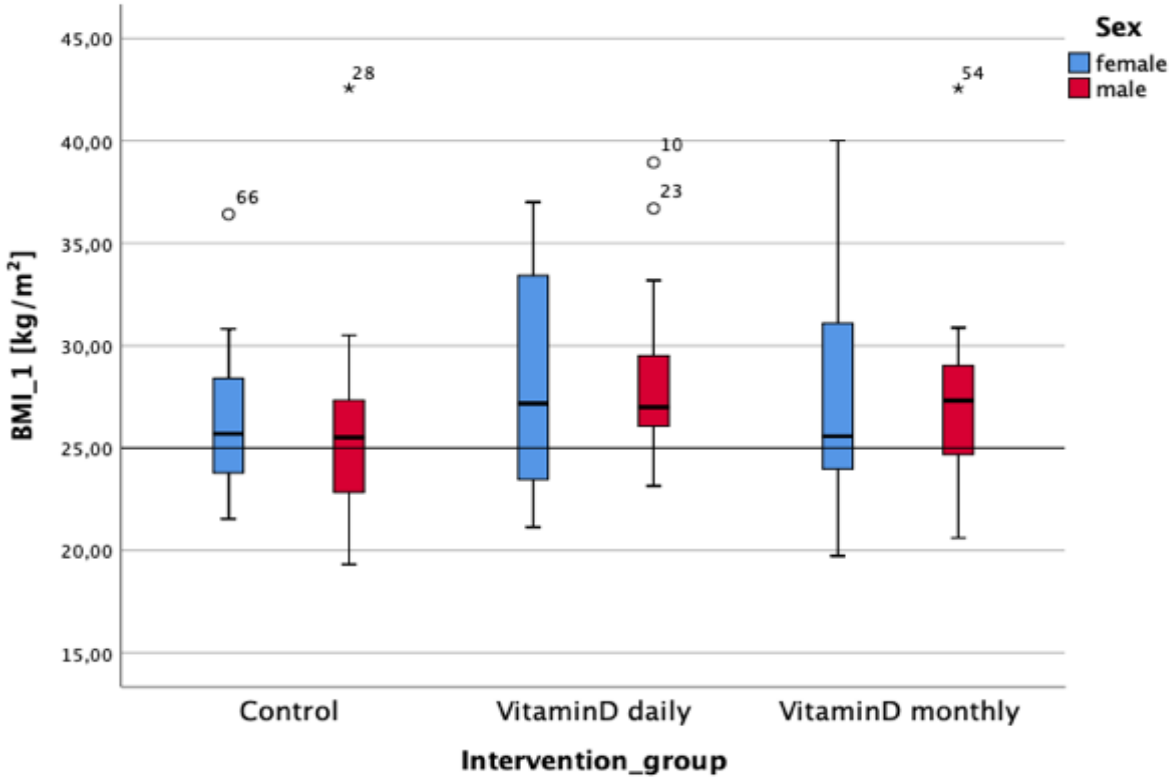

Figure 18: Distribution of BMI by intervention group and sex

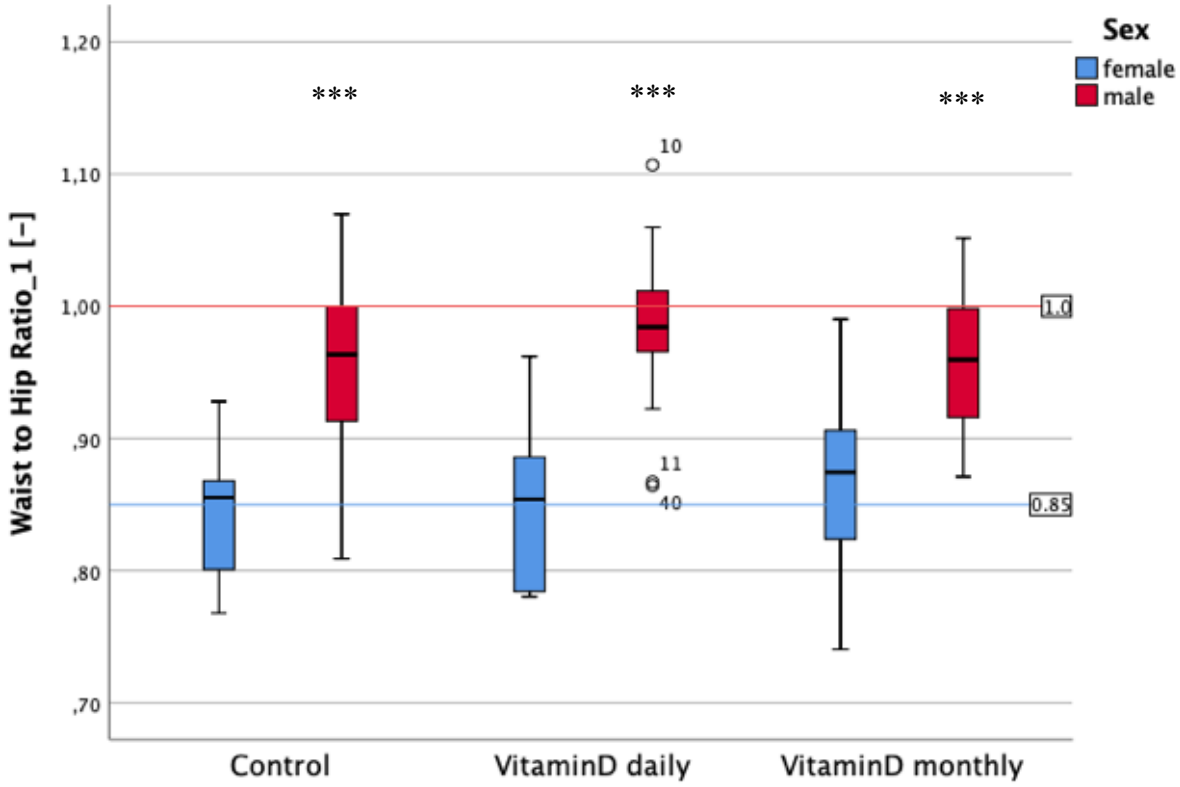

Figure 19: Distribution of waist to hip ratios by intervention groups and sex, cutoffs at 0,85 for female and 1 for male subjects are included, differences between sexes were p=<0.001.

Protein intake and vitamin D status

As shown in figure 20, protein intake in all groups for a majority of participants is insufficient, according to the recommendations of the nutrition societies of Germany, Austria and Switzerland (103). More than 50% of participants regardless of sex or intervention group did not meet the required amounts of protein intake, therefore weight of the subjects needs to be considered. Intakes were significantly different between sexes only in control group (p=0.04), no significant differences between groups.

Vitamin D levels show a similar pattern, as most of the participants are below the threshold of the recommended level of 25 ng/ml serum 25(OH)D, which can be expected at the end of winter. There were no significant differences between sexes for Vitamin D and no differences between groups (Fig. 21).

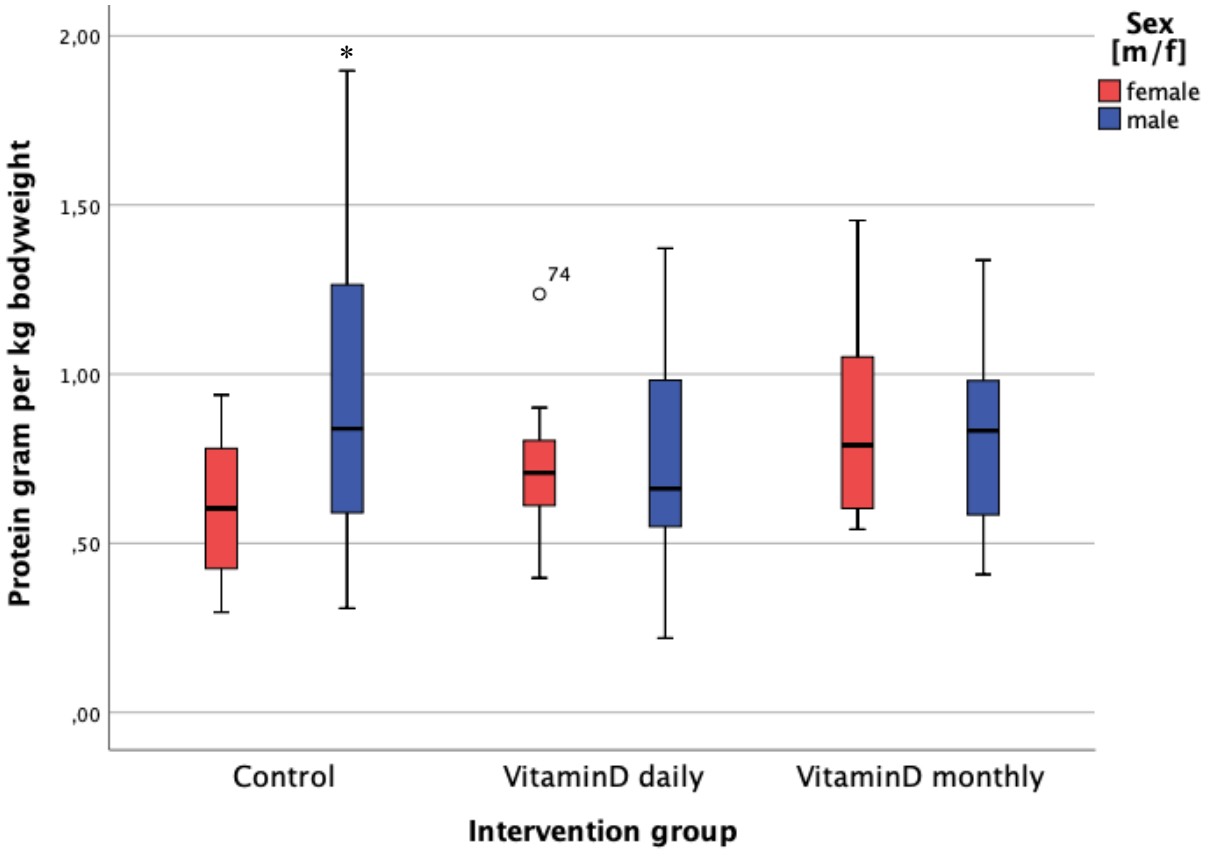

Figure 20: Protein intake relative to bodyweight in kg divided by intervention group, dashed line marks the recommended 1 g per kg bodyweight

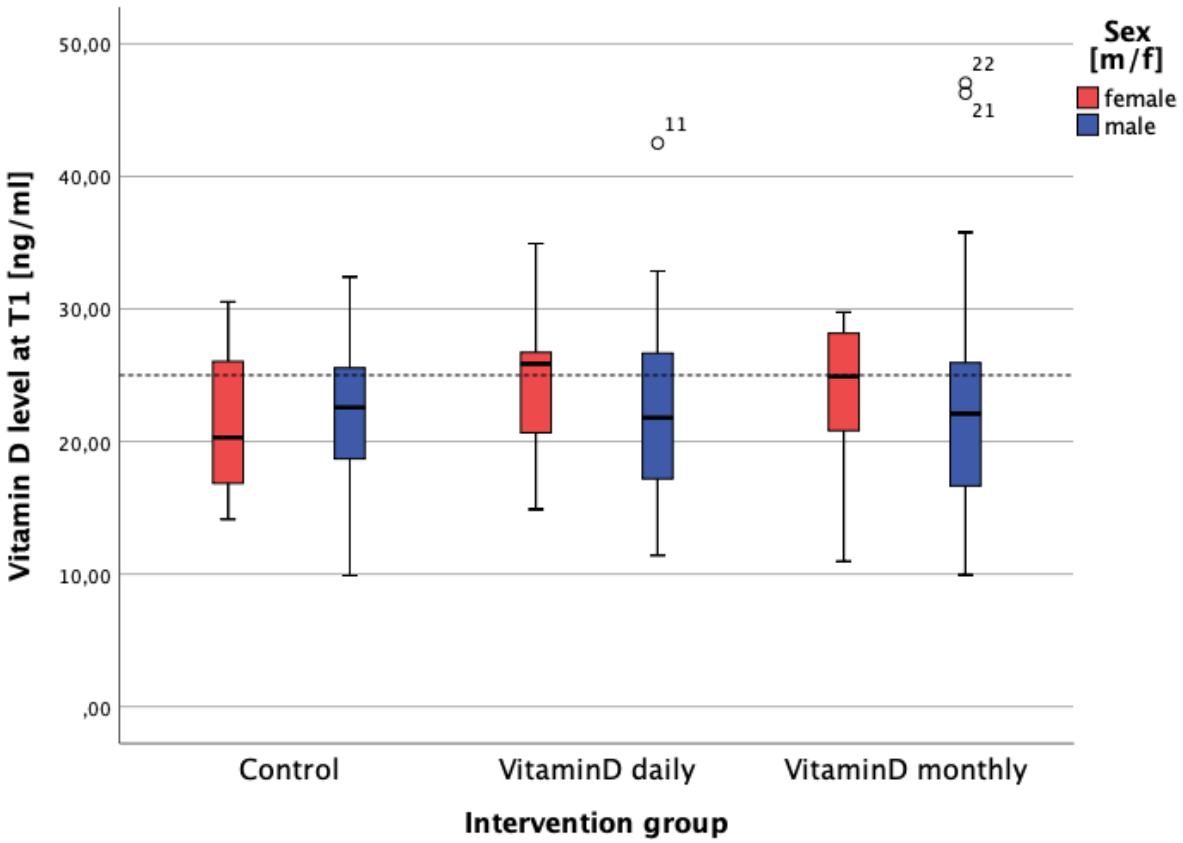

Figure 21: Vitamin D status according to intervention group, the dashed line shows the threshold for insufficiency at 25 ng/ml

CBMN parameters at baseline and within the intervention

The following section will show differences between time points, tables are shown only when significant differences could be detected.

An overview of MNi frequencies and other CBMN parameters at baseline (T1) are shown in table 7, all scored values are within the expected range given by Fenech et al. (65). Table 8 shows the mean frequencies of total sum of MNi (∑MNi) and other CBMN parameters, as well as minimum and maximum values for all participants, table 9 and 10 show values for female and male subjects respectively.

[Per 1000 Bn cells]	Mean ± SD	Min	Max	Anticipated
BN cells with MNi	10.79 ± 5.2	2	40	-
Total number of MNi	11.93 ± 5.88	2	45	0-30
NPBs	1.04 ± 1.68	0	6	0-10
NBuds	2.65 ± 1.68	0	8	0-5
Apoptotic cells	6.08 ± 5.5	0	28	
Necrotic cells	3.92 ± 3.13	0	20	
NDI (%)	1.82 ± 0.12	1.35	2.07	1.3-2.2
Apoptotic cells (%)	0.41 ± 0.38	0	1.95	0-7
Necrotic cells (%)	0.26 ± 0.20	0	1.26	0-9

Table 7: CBMN parameters at baseline

Group		∑MNi T1	∑MNi T2	∑MNi T3	NPB T1	NPB T2	NPB T3	NBuds T1	NBuds T2	NBuds T3
CT	Mean	11.47	10.37	14.33	1.2	0.84	0.67	2.77	3.52	2.96
	SD	4.72	6.66	6.96	1.13	0.78	0.84	1.46	1.93	2.32
	Min	5	2	6.5	0	0	0	0.5	0	0
	Max	24	35.5	38	4	2.5	3.5	7	8	9.5
VDM	Mean	12.53	10.69	13.36	1.22	0.6	0.88	2.72	4	2.94
	SD	8.03	6.09	5.07	1.24	0.57	0.71	2.14	2.28	1.83
	Min	4	1	6	0	0	0	0	1	1
	Max	44.5	31.5	30	5.5	2	2.5	8	9	7.5
VDD	Mean	11.84	10.88	16.59	0.73	0.84	0.61	2.49	3.69	2.59
	SD	4.65	4.58	7.64	0.83	0.78	0.74	1.45	2.56	1.32
	Min	2	3	7	0	0	0	0	0.5	0
	Max	21.5	21	37	3	3	2.5	5.5	13	6
Total	Mean	11.93	10.65	14.82	1.04	0.77	0.72	2.65	3.72	2.82
	SD	5.88	5.75	6.72	1.08	0.72	0.76	1.68	2.26	1.83
	Min	2	1	6	0	0	0	0	0	0
	Max	44.5	35.5	38	5.5	3	3.5	8	13	9.5

Table 8: Frequencies of micronucleus and other CBMN parameters according to intervention group and time point

Group		∑MNi T1	∑MNi T2	∑MNi T3	NPB T1	NPB T2	NPB T3	NBuds T1	NBuds T2	NBuds T3
CT	Mean	9	9	9	9	9	9	9	9	9
	SD	14.78	17.44	18.39	1.39	0.94	0.94	2.72	4.06	3.78
	Median	4.21	7.74	8.61	1.19	0.85	1.24	1.06	1.79	2.82
	Min	7	7	9	0	0	0	2	2	1
	Max	20	36	38	4	2	4	5	7	10
VDM	Mean	9	9	9	9	9	9	9	9	9
	SD	16.61	13.67	14.78	1.39	0.39	1.17	2.67	2.83	2.44
	Median	11.16	8.02	2.80	1.76	0.49	0.79	2.37	1.41	2.08
	Min	8	5	11	0	0	0	0	1	1
	Max	45	32	19	6	2	3	8	5	8
VDD	Mean	7	6	7	7	6	7	7	6	7
	SD	13.36	11.75	18.14	0.64	0.17	0.93	2.5	3.25	2.57
	Median	5.64	4.31	9.11	0.69	0.26	0.98	1.53	1.57	0.67
	Min	2	6	9	0	0	0	1	1	2
	Max	18	19	33	2	1	3	5	5	4
Total	Mean	25	24	25	25	24	25	25	24	25
	SD	15.04	14.6	17.02	1.18	0.54	1.02	2.64	3.4	2.96
	Median	7.56	7.27	7.14	1.32	0.67	0.98	1.69	1.63	2.15
	Min	2	5	9	0	0	0	0	1	1
	Max	45	36	38	6	2	4	8	7	10

Table 9: Frequencies of micronucleus and other CBMN parameters according to intervention group and time point for female participants

Group		∑MNi T1	∑MNi T2	∑MNi T3	NPB T1	NPB T2	NPB T3	NBuds T1	NBuds T2	NBuds T3
CT	Mean	14	14	15	14	14	15	14	14	15
	SD	9.14	7.25	11.9	0.82	0.64	0.5	2.86	3.82	2.47
	Median	2.37	3.48	4.50	0.58	0.66	0.46	1.54	1.92	1.89
	Min	5	2	7	0	0	0	1	2	0
	Max	14	13	21	2	2	2	6	8	8
VDM	Mean	16	12	16	16	12	16	16	12	16
	SD	9	7.71	12.56	1.19	0.5	0.72	2.38	4.63	3.22
	Median	5.36	4.09	5.92	1.08	0.43	0.63	1.95	2.65	1.68
	Min	4	1	6	0	0	0	1	2	1
	Max	27	17	30	4	1	2	7	9	7
VDD	Mean	18	20	20	18	20	20	18	20	20
	SD	11.39	10.15	16.05	0.86	1.08	0.5	2.56	4.2	2.6
	Median	3.85	4.54	7.24	1.00	0.78	0.63	1.47	2.97	1.49
	Min	7	3	7	0	0	0	0	2	0
	Max	21	21	37	3	3	3	6	13	6
Total	Mean	48	46	51	48	46	51	48	46	51
	SD	9.94	8.63	13.74	0.96	0.79	0.57	2.58	4.2	2.75
	Median	4.17	4.26	6.30	0.92	0.70	0.58	1.64	2.57	1.67
	Min	4	1	6	0	0	0	0	2	0
	Max	27	21	37	4	3	3	7	13	8

Table 9:Frequencies of micronucleus and other CBMN parameters according to intervention group and time point for male participants

Int. group	Valid slides	In %
CT	24	31.6 %
VDD	25	32.9 %
VDM	27	35.5%
Total	76	100 %

Table 10: Number of subjects with sufficient BNs by intervention group at all three time points

Due to poor slide quality, some participants had to be excluded from the analysis. Only those with at least 500 BNs per time point were included. This reduced the total number of exploitable datasets to 76, with 24 subjects in CT, 25 in VDD and 27 in VDM respectively (Tab. 10).

Normal distribution for the total sum of MNi (∑MNi) could not be established, neither for sex, nor for intervention group, so non-parametric tests for comparisons were chosen.

Pairwise comp.	Test statistic	SE	Adj. Sig.
∑MNi T2 - ∑MNi T1	0.299	0.173	0.252
∑MNi T2 - ∑MNi T3	-1.022	0.173	**<0.001**
∑MNi T1 - ∑MNi T3	-0.724	0.173	**<0.001**

Table 11: Pairwise comparisons for all participants across T1-T3

A Friedman-test for all participants showed differences between T2 and T3 (p=<0.001) and T1 and T3 (p=<0.001), but not for T1 and T2 (p=0.252) (Tab. 11)

Pairwise comp.	Test statistic	SE	Adj. Sig.
∑MNi T2 - ∑MNi T1	0.453	0.216	0.106
∑MNi T2 - ∑MNi T3	-1.291	0.216	**<0.001**
∑MNi T1 - ∑MNi T3	-0.837	0.216	**<0.001**

Table 12: Pairwise comparisons for males across T1-T3

When considering sex, no differences between any points in time were detectable in women (p=0.97), but there was a difference between T2 and T3 (p=<0.001) and T1 and T3 (p=<0.001) in men, but not between T1 and T2 (p=0.106) (Tab. 12).

Pairwise comp.	Test statistic	SE	Adj. Sig.
∑MNi T2 - ∑MNi T1	0.205	0.302	1.000
∑MNi T2 - ∑MNi T3	-0.886	0.302	**0.010**
∑MNi T1 - ∑MNi T3	-0.682	0.302	0.071

Table 13: Pairwise comparisons for control group across T1-T3

When dividing by intervention group, statistical significance could be shown in the control group for a difference between T2 and T3 (p=0.01). The difference between T1 and T3 (p=0.071) and T2 and T1 (p=1.000) were not significant (Tab. 13).

Pairwise comp.	Test statistic	SE	Adj. Sig.
∑MNi T2 - ∑MNi T1	0.214	0.309	1.000
∑MNi T2 - ∑MNi T3	-0.857	0.309	**0.016**
∑MNi T1 - ∑MNi T3	-0.643	0.309	0.112

Table 14: Pairwise comparisons for VDD group across T1-T3

Pairwise comp.	Test statistic	SE	Adj. Sig.
∑MNi T2 - ∑MNi T1	0.458	0.289	0.337
∑MNi T2 - ∑MNi T3	-1.292	0.289	**<0.001**
∑MNi T1 - ∑MNi T3	-0.833	0.289	**0.012**

Table 15: Pairwise comparisons for VDM group across T1-T3

The VDD group only showed significant differences between T2 and T3 (p=0.016, Tab. 14), the VDM group showed significant differences between T2 and T3 (p=<0.001) and between T1 and T3 (p=0.012, Tab. 15)

Frequencies of MNi (total and cells with MNi), in female subjects were significantly higher, compared to men, range between 1.24 and 1.7 and are near the expected coefficient of 1.4 (Fig. 22, Tab. 16).

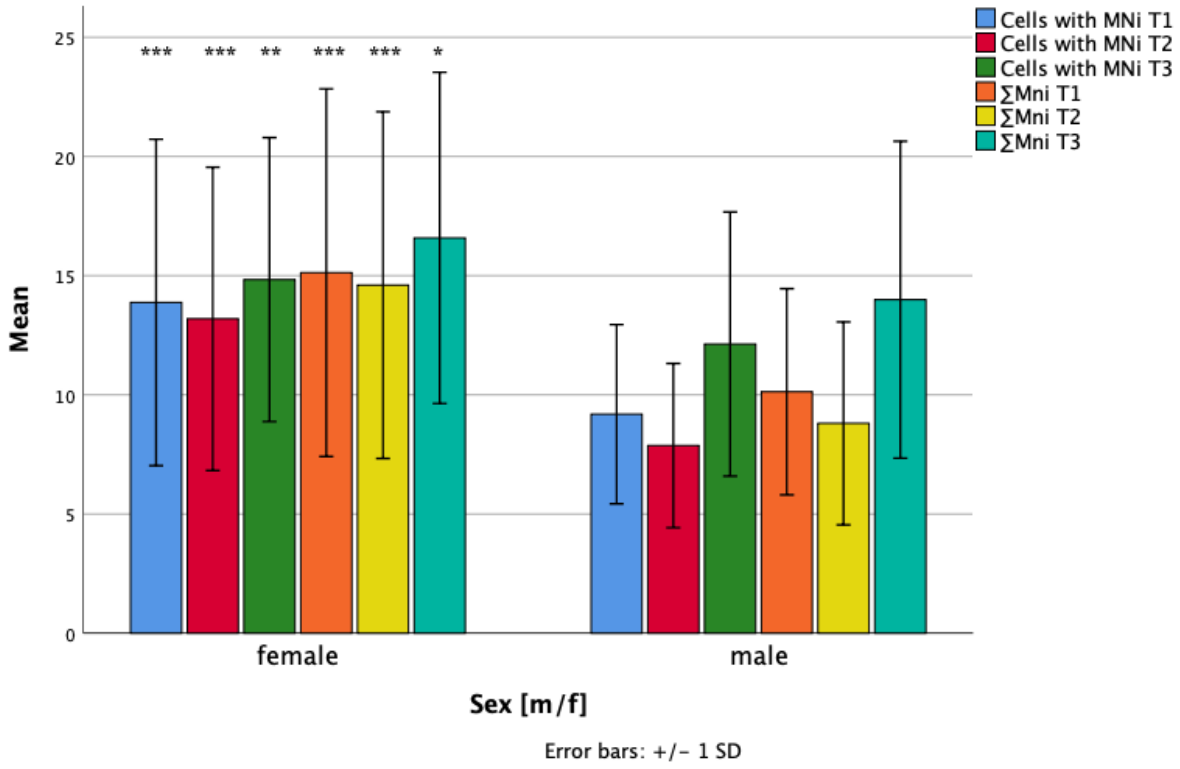

*Figure 22: Mean values of cells with MNi and ∑MNi by sex, sig. differences between sexes are highlighted**

Parameter	Mean ± SD female	Mean ± SD male	Coefficient f/m	Sig.
Cells w/ MNi T1	13.78 ± 6.72	9.00 ± 3.64	1.53	**<0.001**
Cells w/ MNi T2	13.19 ± 6.36	7.76 ± 3.43	1.70	**<0.001**
Cells w/ MNi T3	15.22 ± 6.14	12.00 ± 5.23	1.27	**0.009**
∑ MNi T1	15.04 ± 7.56	9.94 ± 4.17	1.51	**<0.001**
∑ MNi T2	14.60 ± 7.23	8.63 ± 4.26	1.69	**<0.001**
∑ MNi T3	17.02 ± 7.14	13.74 ± 6.30	1.24	**0.016**
NPB T1	1.18 ± 1.32	0.96 ± 0.92	1.23	0.695
NPB T2	0.54 + 0.67	0.79 ± 0.70	0.68	0.107
NPB T3	1,02 ± 0.98	0.57 ±0.58	1.79	0.074
NBUD T1	2.64 ± 1.69	2.58 ± 1.64	1.02	0.939
NBUD T2	3.40 ± 1.63	4.20 ± 2.57	0.81	0.367
NBUD T3	2.96 ± 2.15	2.75 ± 1.67	1.08	0.960
Apo T1 (%)	0.36 ± 0.34	0.45 ± 0.41	0.81	0.358
Apo T2 (%)	0.47 ± 0.52	0.60 ± 0.52	0.79	0.110
Apo T3 (%)	0.49 ± 0.36	0.52 ± 0.35	0.94	0.744
Nec T1 (%)	0.34 ± 0.29	0.27 ± 0.19	1.25	0.666
Nec T2 (%)	0.38 ± 0.29	0.30 ± 0.23	1.27	0.265
Nec T3 (%)	0.27 ± 0.22	0.27 ± 0.21	0.97	0.736

Table 16: Mean values of CBMN parameters for all groups, coefficient between female and male subjects and p-values of comparison of mean ranks between sexes.

Table 16 shows an overview of CBMN parameters across all time points for all participants, regardless of intervention group. There were significant differences between sexes at all time points for cells with MNi and total number of MNi. Other CBMN parameters were not significantly different.

Females had higher MNi frequencies by circa 1.5, 1.7 and 1.3 (cells with MNi) for respective time points, values similar to those of Nefic and Handzic, which reported a factor between male and female participants of 1.4 (81).

The following section will discuss the differences between time points for male and female participants for each intervention group.

Control group

	Mean	SD	Min	Max	Sig.
∑MNi T1	11.35	4.21	5	20	**0.004**
∑MNi T2	11.24	7.41	2	36	**0.004**
∑MNi T3	14.33	6.96	7	38	0.061
NPB T1	1.04	0.89	0	4	0.212
NPB T2	0.76	0.74	0	2	0.380
NPB T3	0.67	0.84	0	4	0.326
NBuds T1	2.80	1.35	1	6	0.806
NBuds T2	3.91	1.83	2	8	0.770
NBuds T3	2.96	2.32	0	10	0.239
NDI T1	1.81	0.12	1.46	2.04	**0.002**
NDI T2	1.86	0.11	1.61	2.02	**0.004**
NDI T3	1.72	0.15	1.46	2.07	0.410
NCDI T1	1.82	0.12	1.46	2.05	**0.003**
NCDI T2	1.87	0.11	1.62	2.03	**0.011**
NCDI T3	1.74	0.15	1.46	2.07	0.585
Apo [%] T1	0.35	0.29	0	1.01	0.758
Apo [%] T2	0.48	0.37	0.11	1.54	0.343
Apo [%] T3	0.54	0.45	0.04	1.55	0.445
Nec [%] T1	0.31	0.25	0	0.91	0.900
Nec [%] T2	0.32	0.19	0.09	0.72	0.094
Nec [%] T3	0.24	0.18	0.06	0.68	0.700

Table 17: Frequencies of CBMN related values for all time points in control group, p-values show difference between sexes

Table 17 shows an overview of all CBMN related values of the control group for all time points and differences between sexes.

At T1 and T2 frequencies of MNi were different between sexes, as well as the NDI and NCDI.

Pairwise comp.	Test statistic	SE	Adj. Sig.
∑MNi T2 - ∑MNi T1	0.205	0.302	1.000
∑MNi T2 - ∑MNi T3	-0.886	0.302	**0.010**
∑MNi T1 - ∑MNi T3	-0.682	0.302	0.71

Table 18: Pairwise comparisons of CT group across T1-T3 for ∑MNi

Pairwise comp.	Test statistic	SE	Adj. Sig.
Cells with MNi T2 – Cells with MNi T1	0.250	0.302	1.000
Cells with MNi T2 – Cells with MNi T3	-0.977	0.302	**0.004**
Cells with MNi T1 – Cells with MNi T3	-0.727	0.302	**0.048**

Table 19: Pairwise comparisons of CT group across T1-T3 for cells with MNi

Pairwise comp.	Test statistic	SE	Adj. Sig.
NDI T2 -NDI T1	0.591	0.302	0.150
NDI T2 - NDI T3	0.909	0.302	**0.008**
NDI T1 - NDI T3	-0.318	0.302	0.874

Table 20: Pairwise comparisons of CT group across T1-T3 for NDI and NCDI (values are identical)

Comparing total number of MNi yielded one significant difference across time points, between T2 and T3 (p=0.01, Tab. 18) as well as cells with MNi, where there was a slightly significant difference between T1 and T3 (p=0.048) and between T2 and T3 (p=0.004, Tab. 19). Values of NPBs and NBuds were not different from each other, NDI and NCDI differed between T2 and T3 (p=0.008, Tab. 20), percentage of apoptosis and necrosis did not differ across time points.

Vitamin D daily group

	Mean	SD	Min	Max	Sig.
∑MNi T1	11,74	8,57	4	45	0,083
∑MNi T2	10,26	6,64	1	32	0,066
∑MNi T3	13,36	5,07	6	30	0,218
NPB T1	1,26	1,33	0	6	0,761
NPB T2	0,45	0,45	0	2	0,592
NPB T3	0,88	0,71	0	3	0,167
NBuds T1	2,48	2,06	0	8	0,758
NBuds T2	3,86	2,34	1	9	0,062
NBuds T3	2,94	1,83	1	8	0,357
NDI T1	1,80	0,15	1,35	2,01	**0,032**
NDI T2	1,80	0,15	1,35	2,09	0,268
NDI T3	1,74	0,16	1,37	2,16	0,279
NCDI T1	1,80	0,15	1,35	2,01	**0,031**
NCDI T2	1,81	0,15	1,35	2,1	0,250
NCDI T3	1,75	0,16	1,38	2,16	0,267
Apo [%] T1	0,41	0,39	0,05	1,65	0,756
Apo [%] T2	0,70	0,65	0,07	2,49	0,786
Apo [%] T3	0,47	0,31	0,12	1,33	0,998
Nec [%] T1	0,34	0,25	0,04	0,94	0,175
Nec [%] T2	0,38	0,34	0	1,2	0,100
Nec [%] T3	0,22	0,14	0,03	0,56	0,979

Table 21: Frequencies of CBMN related values for all time points in VDD group, p-values show difference between sexes

Table 21 shows an overview of all CBMN related values of the VDD group for all time points and differences between sexes.

Only NDI and NCDI at T1 were significantly different between sexes (p=0.032 and p= 0.031 respectively).

Pairwise comp.	Test statistic	SE	Adj. Sig.
∑MNi T2 - ∑MNi T1	0.214	0.309	1.000
∑MNi T2 - ∑MNi T3	-0.857	0.309	**0.016**
∑MNi T1 - ∑MNi T3	-0.643	0.309	0.112

Table 22: Pairwise comparisons of VDD group across T1-T3 for ∑MNi

Pairwise comp.	Test statistic	SE	Adj. Sig.
Cells with MNi T2 – Cells with MNi T1	0.286	0.309	1.000
Cells with MNi T2 – Cells with MNi T3	-0.857	0.309	**0.016**
Cells with MNi T1 – Cells with MNi T3	-0.571	0.309	0.192

Table 23: Pairwise comparisons of VDD group across T1-T3 for cells with MNi

Pairwise comp.	Test statistic	SE	Adj. Sig.
%Nec T2 - %Nec T1	0.524	0.309	0.269
%Nec T2 - %Nec T3	0.762	0.309	**0.041**
%Nec T1 - %Nec T3	0.238	0.309	1.000

Table 24: Pairwise comparisons of VDD group across T1-T3 for percentage of necrosis

For the vitamin D daily group a difference between time points could be shown between T2 and T3 for total number of MNi, as well as cells with MNi (both p=0.016, Tab. 22, 23). Differences in the number of NPB, NBuds, NDI, NCDI and percentage of apoptosis were not significantly different, but there was a difference in the percentage of necrosis between T3 and T1 (p=0.041, Tab. 24).

Vitamin D monthly group

	Mean	SD	Min	Max	Sig.
∑MNi T1	11.94	4.39	2	21	0.419
∑MNi T2	10.52	4.46	3	21	0.452
∑MNi T3	16.59	7.64	7	37	0.596
NPB T1	0.80	0.91	0	3	0.543
NPB T2	0.87	0.79	0	3	**<0.001**
NPB T3	0.61	0.74	0	3	0.310
NBuds T1	2.54	1.46	0	6	0.936
NBuds T2	3.98	2.72	1	13	0.319
NBuds T3	2.59	1.32	0	6	0.946
NDI T1	1.82	0.13	1.5	2.06	0.890
NDI T2	1.83	0.13	1.51	2.08	0.452
NDI T3	1.77	0.11	1.55	2	0.889
NCDI T1	1.82	0.13	1.52	2.07	0.849
NCDI T2	1.84	0.13	1.52	2.09	0.577
NCDI T3	1.79	0.11	1.56	2.01	0.854
Apo [%] T1	0.48	0.46	0.07	1.95	0.078
Apo [%] T2	0.50	0.52	0.03	2.71	0.122
Apo [%] T3	0.52	0.29	0.05	1.08	0.681
Nec [%] T1	0.23	0.17	0	0.63	0.898
Nec [%] T2	0.27	0.23	0	0.83	0.557
Nec [%] T3	0.35	0.28	0	1.26	0.847

Table 25: Frequencies of CBMN related values for all time points in VDD group, p-values show difference between sexes

Table 25 shows an overview of all CBMN related values of the VDM group for all time points and differences between sexes.
Only NPB at T2 were significantly different between sexes (p=<0.001).

Pairwise comp.	Test statistic	SE	Adj. Sig.
∑MNi T2 - ∑MNi T1	0.458	0.289	0.337
∑MNi T2 - ∑MNi T3	-1.292	0.289	**<0.001**
∑MNi T1 - ∑MNi T3	-0.833	0.289	**0.012**

Table 26: Pairwise comparisons of VDM group across T1-T3 for ∑MNi

Pairwise comp.	Test statistic	SE	Adj. Sig.
Cells with MNi T2 – Cells with MNi T1	0.354	0.289	0.660
Cells with MNi T2 – Cells with MNi T3	-1.083	0.289	**0.001**
Cells with MNi T1 – Cells with MNi T3	-0.729	0.289	**0.035**

Table 27: Pairwise comparisons of VDM group across T1-T3 for cells with MNi

Differences in total number of MNi and cells with MNi differed significantly between T2 and T3 (p<0.001 and p=0.001 respectively) and between T1 and T3 (p=0.012 and p=0.035 respectively, Tab. 26, 27).
NPB, NBuds, percentage of apoptosis and necrosis, NDI and NCDI were not different across time points.

Vitamin D values

Vitamin D [ng/ml]	∑ of squares	F	Adj. Sig.
T1	4348.3	0.748	0.477
T2	2315.25	3.040	0.054
T3	5593.276	5.311	**0.007**

Table 28: ANOVA for vit. D levels between groups

Although normal distribution could not be shown, ANOVA with repeated measures is robust, even if requirements for normal distribution are not met (100,104). It revealed a significant difference between groups at T3 (p=0.007), but not for T2 (p=0.054) and T1 (p=0,477) (Tab. 28, Fig. 23). Table 29 shows the distribution of vitamin D values across time points for every intervention group and comparison between groups. An ANOVA with repeated measures showed differences at T2 and T3 between CT-group and VDM (T2: p=0.45, T3: p=0.06, Tab. 29)

Time point	Reference group	Mean value [ng/ml]	Intervention group	Mean Difference	SE	Sig.
T1	CT	20.4 ±+5.74	VDD	-1.88	2.21	0.673
			VDM	-2.58	2.17	0.461
	VDD	22.62 ± 7.57	CT	1.88	2.21	0.673
			VDM	-0.71	2.14	0.942
	VDM	23.33 ± 9.23	CT	2.58	2.17	0.461
			VDD	0.71	2.14	0.942
T2	CT	21.84 ± 5.49	VDD	-2.44	1.55	0.262
			VDM	-3.70	1.52	**0.045**
	VDD	24.28 ± 6.09	CT	2.44	1.55	0.262
			VDM	-1.26	1.50	0.679
	VDM	25.55 ± 4.61	CT	3.70	1.52	**0.045**
			VDD	1.26	1.50	0.679
T3	CT	25.03 ± 9.90	VDD	-2.73	2.34	0.476
			VDM	-7.36	2.29	**0.006**
	VDD	27.79 ± 7.03	CT	2.73	2.34	0.476
			VDM	-4.63	2.27	0.110
	VDM	32.40 ± 8.64	CT	7.36	2.29	**0.006**
			VDD	4.63	2.27	0.110

Table 29: Comparison of Vit. D levels between intervention groups by time point

Normal distribution for Vitamin D levels divided by intervention group could only be shown for the VDM-group at T1 (Shapiro-Wilk: p=0.019), all time points and intervention groups showed non-normal distribution.

A Friedman's two-way-analysis showed a difference between all time-points with p-values ≤0.002 for all participants (Tab. 30).

Pairwise Comparisons

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
Vitamin D level at T1 [ng/ml]-Vitamin D level at T2 [ng/ml]	-,518	,153	-3,375	,001	,002
Vitamin D level at T1 [ng/ml]-Vitamin D level at T3 [ng/ml]	-1,141	,153	-7,440	,000	,000
Vitamin D level at T2 [ng/ml]-Vitamin D level at T3 [ng/ml]	-,624	,153	-4,065	,000	,000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is ,05.

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Table 30: Pairwise comparisons of Vit.D levels across T1-T3 for all participants

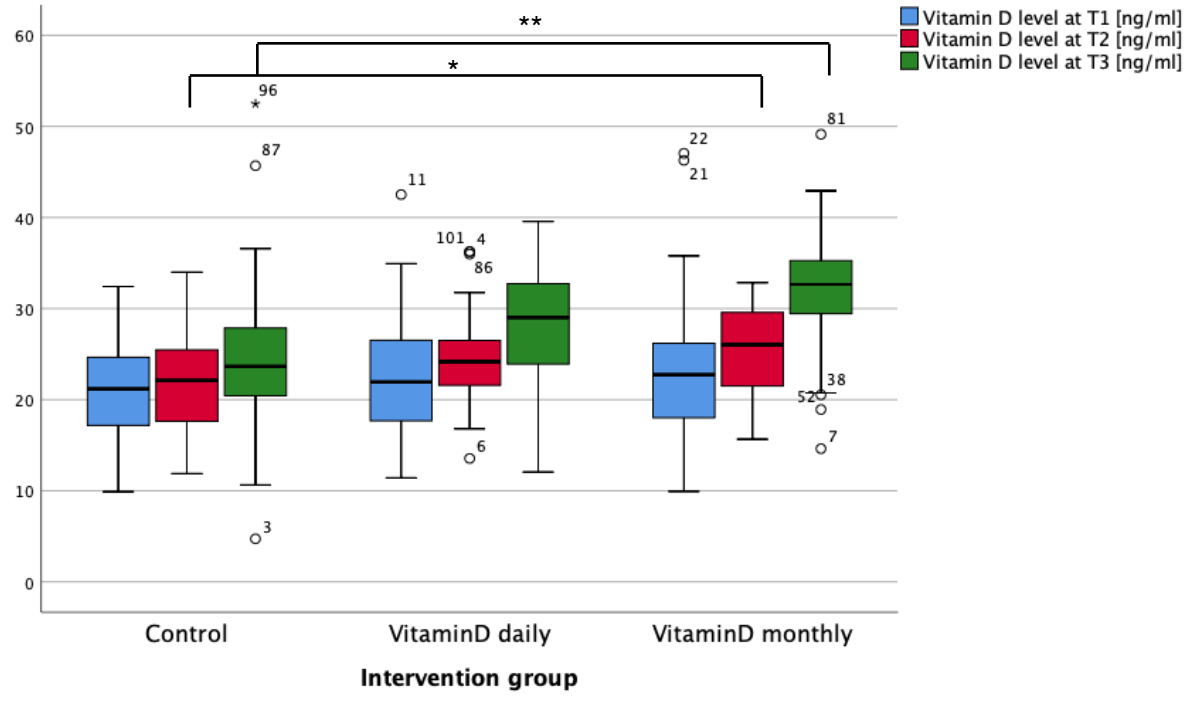

*Figure 23: Vitamin D concentrations by intervention group, significant differences are*highlighted (*p=≤0.05, **p=<0.01)*

functional/anthropometric parameters

CBMN parameters were positively associated with higher BMI and overall poor health conditions. In order to confirm those observations, correlations between various CBMN parameters (∑MNi, MNi in cells, NPBs and NBuds), functional parameters (6-minute-walking-test, handgrip strength and chair rise test) and anthropometric values (BMI, total muscle mass and relative fat mass in kg) were evaluated for every point in time. Significant correlations were observed between ∑MNi and MNi in cells with 6MWT at T2 (-0.324, p=0.006 and -0.303, p=0.011 respectively, Tab. 31). Stronger, correlations were found for ∑MNi and MNi in cells with handgrip strength in the dominant hand for T1 (-0.371, p=<0.001, -0.402, p=<0.001) and T2 (-0.45, p=<0.001, -0.476, p=<0.001 respectively, Tab. 32).

∑ MNi	Time point	T1	T2	T3
–	ρ	-0.2	**-0.324**	-0.066
6MWT	Sig.	0.089	**0.006**	0.576
MNi in cells	Time point	T1	T2	T3
–	ρ	-0.202	**-0.303**	-0.101
6MWT	Sig.	0.086	**0.011**	0.390
NBUDs	Time point	T1	T2	T3
–	ρ	-0.082	-0.134	-0.008
6MWT	Sig.	0.492	0.269	0.944
NPB	Time point	T1	T2	T3
–	ρ	-0.152	0.065	-0.2
6MWT	Sig.	0.198	0.594	0.086

Table 31: Correlation between 6-minute walking test (6MWT) and CBMN parameters

∑ MNi – HG-Test	Time point	T1	T2	T3
	ρ	**-0.371**	**-0.45**	-0.141
	Sig.	**<0.001**	**<0.001**	0.576
MNi in cells – HG-Test	Time point	T1	T2	T3
	ρ	**-0.402**	**-0.476**	-0.194
	Sig.	**<0.001**	**<0.001**	0.094
NBUDs – HG-Test	Time point	T1	T2	T3
	ρ	0.027	0.041	-0.013
	Sig.	0.819	0.733	0.912
NPB – HG-Test	Time point	T1	T2	T3
	ρ	-0.173	0.158	-0.084
	Sig.	0.143	0.190	0.471

Table 32: Correlation between hand grip-test (HG-Test) and CBMN parameter

∑ MNi – CRT	Time point	T1	T2	T3
	ρ	**-0.28**	-0.22	-0.22
	Sig.	**0.016**	0.073	0.054
MNi in cells – CRT	Time point	T1	T2	T3
	ρ	**-0.27**	-0.22	**-0.23**
	Sig.	**0.022**	0.062	**0.044**
NBUDs – CRT	Time point	T1	T2	T3
	ρ	-0.21	-0.10	-0.05
	Sig.	0.074	0.404	0.696
NPB – CRT	Time point	T1	T2	T3
	ρ	-0.05	-0.09	-0.20
	Sig.	0.652	0.475	0.090

Table 33: Correlation between hand grip-test (HG-Test) and CBMN parameters

Correlations between CBMN parameters and the chair rise test only yielded significant results with ∑MNi and MNi in cells at T1 (-0.28, p=0.016 and -0.27, p=0.022 respectively), as well as T3 for MNi in cells (-0.23, p=0.044, Tab. 33).

When correlating anthropometric values with CBMN parameters, no significant results could be seen for BMI (Tab. 34), but total muscle mass (MM) yielded negative correlations at T1 for ∑MNi and MNi in cells (-0.305, p= 0.01 and -0.345, p=0.003) and T2 (-0.346, p=0.003 and -0.368, p=0.002 respectively, Tab. 35, Fig. 24).

∑ MNi – BMI	Time point	T1	T2	T3
	ρ	0.202	0.072	0.044
	Sig.	0.087	0.551	0.704
MNi in cells – BMI	Time point	T1	T2	T3
	ρ	0.168	0.048	0.049
	Sig.	0.155	0.692	0.676
NBUDs – BMI	Time point	T1	T2	T3
	ρ	0.042	0.011	-0.092
	Sig.	0.722	0.929	0.429
NPB – BMI	Time point	T1	T2	T3
	ρ	0.031	-0.065	0.145
	Sig.	0.796	0.592	0.211

Table 34: Correlations between CBMN parameters and BMI

∑ MNi – MM	Time point	T1	T2	T3
	ρ	**-0.305**	**-0.346**	-0.047
	Sig.	**0.01**	**0.003**	0.685
MNi in cells – MM	Time point	T1	T2	T3
	ρ	**-0.345**	**-0.368**	-0.077
	Sig.	**0.003**	**0.002**	0.506
NBUDs – MM	Time point	T1	T2	T3
	ρ	0.027	0.153	0.078
	Sig.	0.82	0.207	0.501
NPB – MM	Time point	T1	T2	T3
	ρ	-0.032	0.093	0.032
	Sig.	0.794	0.444	0.785

Table 35: Correlations between CBMN parameters and total muscle mass

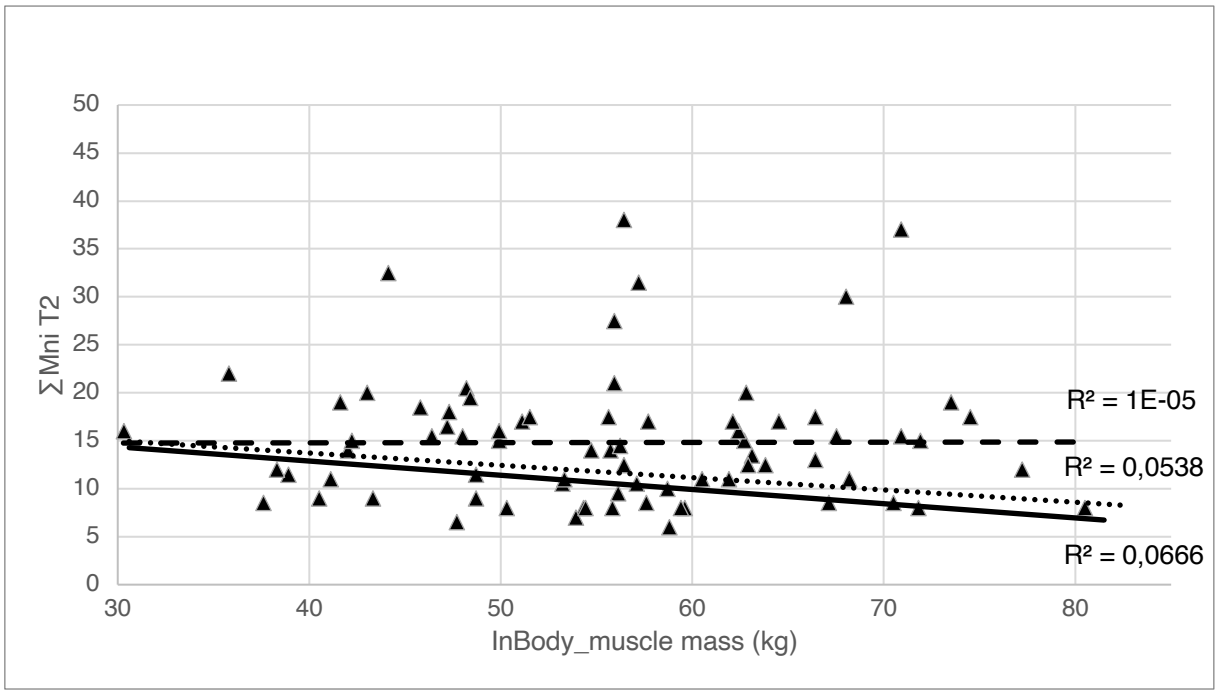

Figure 24: Associations of ∑MNi and absolute muscle muss. Steady line represents T2, dotted: T1, dashed: T3

Similar to correlations with total muscle mass, relative fat mass (FM) was positively correlated with ∑MNi and MNi in cells at T1 (0.446, p=<0.001 and 0.427, p=<0.001) and T2 (0.345, p=0.003 and 0.34, p=0.004, Tab.36, Fig.25).

∑ MNi	Time point	T1	T2	T3
–	ρ	**0.446**	**0.345**	0.128
FM	Sig.	**<0.001**	**0.003**	0.269
MNi in cells	Time point	T1	T2	T3
–	ρ	**0.427**	**0.34**	0.14
FM	Sig.	**<0.001**	**0.004**	0.229
NBUDs	Time point	T1	T2	T3
–	ρ	0.101	0.047	0.018
FM	Sig.	0.403	0.698	0.88
NPB	Time point	T1	T2	T3
–	ρ	0.065	-0.062	**0.263**
FM	Sig.	0.59	0.611	**0.022**

Table 36: Correlations between CBMN parameters and relative fat mass

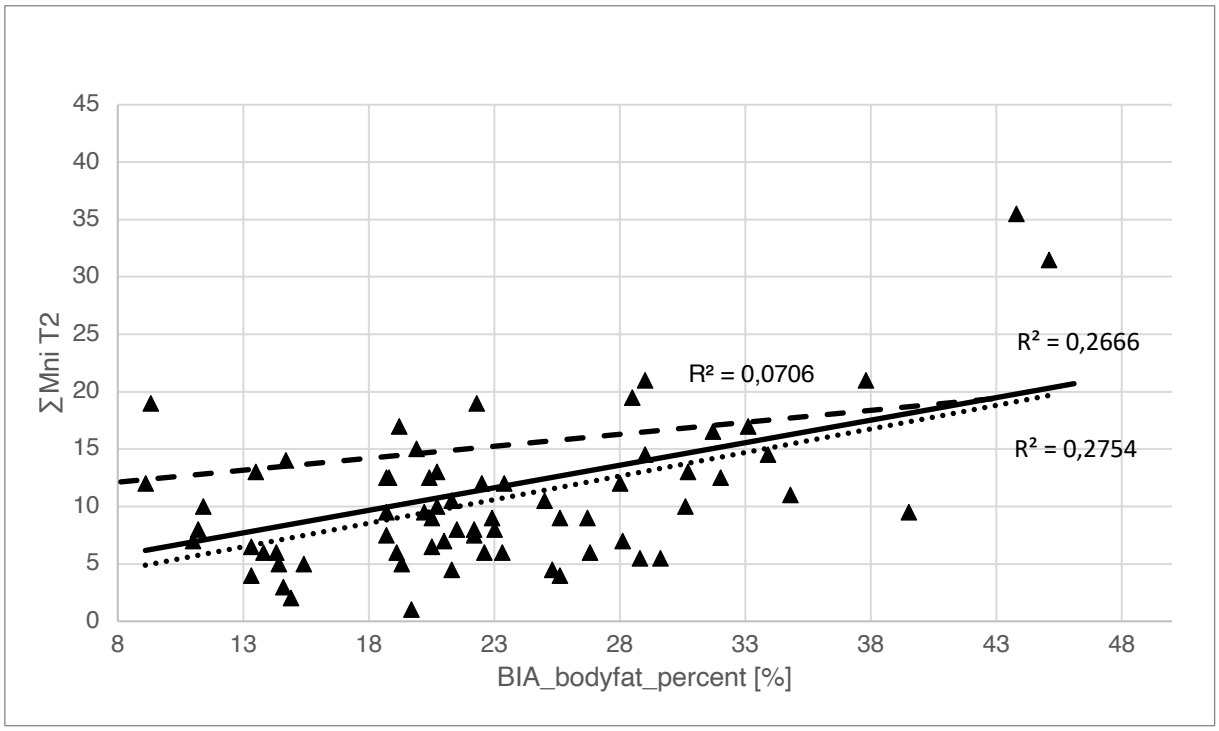

Figure 25: Associations of ∑MNi and relative fat mass. Steady line represents T2, dotted: T1, dashed: T3

Strength parameters

All participants underwent strength training for 12 weeks. All participants were able to improve their strength values, based on the four movements incorporated (Tab. 37, Fig. 28):

- leg press
- leg curl
- chest press
- lat pulldown

Participants performed a 5-repetition maximum test (5RM) prior to beginning the training intervention (5 RM 1st) at T2 and performed a 5 RM at the end of the 12-week training intervention at T3 (5 RM last). Table 37 shows the results of the 5 RM tests before and after the training program, divided by intervention group and for all four main exercises.

Figure 26 shows the results of the 5 RM tests before and after the training program, divided by sex and intervention group for the leg press only.

Intervention group	CT		VDD		VDM		Total	
5RM [kg]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
5 RM leg curl 1st	40.28	12.75	42.76	11.12	41.11	12.30	41.30	12.04
5 RM leg curl last	51.42	18.64	49.45	12.93	51.30	10.31	50.76	14.13
5 RM lat pulldown 1st	39.59	9.09	42.58	9.99	40.41	10.14	40.77	9.73
5 RM lat pull down last	48.64	11.83	51.73	12.56	48.99	10.27	49.73	11.47
5 RM leg press 1st	93.02	35.79	107.00	33.87	106.01	29.14	101.92	33.10
5 RM leg press last	138.20	45.67	141.47	34.73	132.23	35.68	137.02	38.77
5 RM chest press 1st	29.86	10.23	35.10	14.29	30.54	9.75	31.67	11.52
5 RM chest press last	37.10	12.52	42.25	15.54	40.91	12.70	40.13	13.61

Table 37: Strength values for intervention groups and all participants for the four main movements of the exercise program at the first 5 RM and last 5 RM test

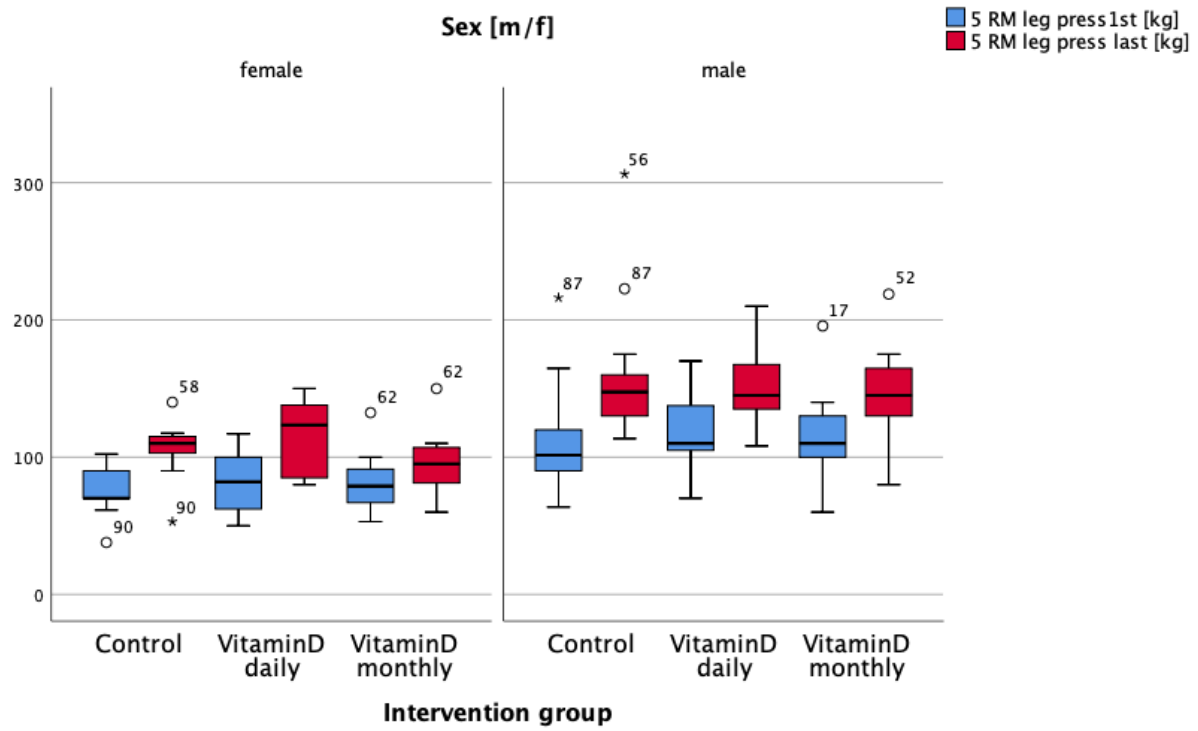

Figure 26: Distribution of absolute leg press strength values in kg at first and last 5 RM test for intervention groups by sex

Strength values showed no normal distribution, therefore non-parametric tests were used for comparisons. The highest values were achieved in the leg press, thus they were used for comparisons.

A Wilcoxon signed rank test revealed differences between first and last 5RM rest for all participants (p=<0.001), CT-group (p=<0.001), VDD-group (p=<0.001) and VDM-group (p=<0.001, Tab. 38)

When dividing by sex and intervention group, women overall were able to improve their performance significantly (p=<0.001). When dividing by intervention group, the CT-group (p=0.008) and VDD-group (p=0.012) were able to improve significantly, but not VDM-group (p=0.091), which may be due to the low number of subjects in this group (Tab. 39).

Intervention group	CT		VDD		VDM		Total	
5RM [kg] 1st - last	n	Sig.	n	Sig.	n	Sig.	n	Sig.
Test for difference	9	0.08	8	**0.012**	8	0.091	25	**<0.001**

Table 38: Comparison of 1st and last 5RM leg press test in women by intervention group

Men overall were able to improve significantly (p<0.001) as well as men in CT-group (p<0.001), VDD-group (p=0.001) and VDM-group (p<0.001, Tab. 40).

Intervention group	CT		VDD		VDM		Total	
5RM [kg] 1st - last	n	Sig.	n	Sig.	n	Sig.	n	Sig.
Test for difference	18	**<0.001**	15	**0.001**	21	**<0.001**	54	**<0.001**

Table 39: Comparison of 1st and last 5RM leg press test in men by intervention group

A linear model only found significant influence of sex, but not intervention group or total sum of MNi on leg press performance after 12 weeks of resistance training (Tab. 40).

Tests of between-subjects effects					
Dependent variable: 5 RM leg press last [kg]					
Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	36584.55[a]	4	9146.14	7.96	**<0.001**
Intercept	138616.31	1	148616.31	129.34	**<0.001**
Sex	35298.72	1	35298.72	30.72	**<0.001**
Intervention group	3141.48	2	1570.74	1.37	0.262
MN_MN_total3	1377.523	1	1377.52	1.20	0.277
Error	78135.63	68	1149.05		
Total	1468139.68	73			
Corrected total	114720.17	72			
a. $R^2 = 0.319$ (Adjusted $R^2 = 0.279$					

Table 40: Linear model by intervention group, sex and ∑MNiT3

Discussion

Throughout intervention groups, the age of the sample population was distributed homogenously with similar mean and median age, as well as BMI, with mean values above 25 and median values consistently below the averages, indicating a skew to the right. Maximum values for BMI were 42.5, 39 and 42.5 for CT, VDD and VDM respectively, hence the majority of the subjects were at least overweight, with some subjects being severely obese. Specifically, 22,2% of the control group, 30% of the vitamin D daily group and 40% of vitamin D monthly group were obese. This alone possibly favors the formation of micronuclei as a recent meta-analysis by Franzke et al. suggests, although more data is needed to confirm this assumption (105).

Looking at waist to hip ratios though, a majority of the subjects remained under the critical values of 0.85 for women and 1 for men. This indicates weight accumulation in other places than the waist which could be an effect of accumulation of fat in muscle tissue or water retention due to insufficient protein consumption, as the actual protein consumption per kg bodyweight did not meet the recommended intake.

At the beginning, Vitamin D showed insufficient serum levels, as more than half of the participants had levels below 25 ng/ml.

Initial numbers of MNi, NPBs and NBuds are in the anticipated range with values in women higher than in men, an observation that was seen by numerous other studies (83,81,86) and remained significant through all time points.

At T2 and T3, differences in total number of MNi for all participants increased significantly when comparing T2 with T3 and T1 with T3 ($p=<0.001$ both). When divided by intervention group, only the VDM group showed a similar pattern ($p=<0.001$ and 0.012 respectively), the VDD group only showed significant difference between T2 and T3 ($p=0.016$), as well as the CT group ($p=0.010$) which indicates, that the training intervention could have a stronger effect on MNi occurrence than just a nutritional intervention. Although vitamin D is supposed to have antioxidative properties, hence a suppressing effect on genomic damage, it had no such effect on the occurrence of MNi in both intervention groups or could not prevent the increase of MNi when resistance training started for all groups at T2.

Other parameters, such as NPB, NBud, NDI, NCDI, %Apo and %Nec increased or decreased heterogeneously throughout groups or did not change significantly. This

may be due to the fact, that scoring those parameters is difficult. Scoring of apoptotic and necrotic cells was critical, since they were either hard to differentiate from another or could not be counted due to large fields of necrotic cells or cells that looked like apoptotic cells but did not meet all criteria defined above. The reason for this can be due to an imperfect staining process or difficulties during the incubation process.

Nevertheless, significant reductions were seen in the CT group for NDI between T2 and T3 ($p=0.008$), whereas the VDD group had a significant decrease in the percentage of necrosis between T2 and T3 ($p=0.041$). The VDM group showed no significant changes in the parameters listed above.

Vitamin D serum levels are presented in figure 25, significant differences from CT group were only found in the VDM group at T2 and T3. This may be due to the higher doses administered and the lower likelihood of forgetting the supplement in the VDD group. Median and average serum vitamin D levels were higher after every blood draw, which could be the effect of seasonal change during that year, since the intervention started in late winter and early spring and ended in summer.

Functional parameters, such as 6MWT, HG test and CRT showed a few correlations with the frequencies of MNi. The strongest correlation was found between cells with MNi and HG-test with -0.45 at T2 ($p=<0.001$), followed by ∑MNi and 6MWT with -0.324 at T2 ($p=0.006$). Concurrently, BMI showed no significant correlation with any CBMN parameter, but total muscle mass (MM) showed a weak negative correlation with ∑MNi of -0.305 at T1 ($p=0.01$) and -0.346 at T2 ($p=0.003$) and MNi in cells (-0.345, $p=0.003$ at T1 and -0.368, $p=0.002$ at T2) and simultaneously relative fat mass (FM) showed a weak positive correlation with ∑MNi of 0.446 ($p=<0.001$) at T1 and 0.345 ($p=0.003$) at T2, along with cells with MNi at T1 with 0.427 ($p<0.001$) and T2 with 0.34 ($p=0.004$). Interestingly, a weak correlation with FM and NPB of 0.263 was found at T3 ($p=0.002$).

In summary, these correlations allow for the assumption, that more athletic subjects tended to have fewer MNi at baseline. As soon as the training intervention started at T2, the correlations of those parameters became non-significant and frequencies of MNI and related events increased, which may be due to the effects of the resistance exercise.

An additional blood draw two to four weeks after the training intervention could have been a valuable data point, since the acute damage from the resistance exercise would

not be present, but the benefits still would persist for a couple of weeks and therefore could be able to reduce MNi formation below initial levels.

Although the resistance exercise may have caused an increase in chromosomal damage, the potential benefits could outweigh this fact by increasing functionality and self-reliability of the subjects. Regardless of intervention group, a large part of participants improved in strength related parameters. Women tend to have less upper body strength than men (106) and therefore the exercise with the highest values, the leg press was chosen for comparisons between groups and time points. The data showed that all groups were able to increase their 5RM significantly ($p=<0.001$), when divided by sex, women in the VDM group were not able to significantly increase their leg press 5RM ($p=0.091$). Confounders for increased strength and the small number of subjects ($n=8$) indicate that the vitamin D intervention might not be the reason for this finding. A linear model showed only a significant influence of sex on 5RM, but not the intervention group or ∑MNi which was expected, since men seem to gain more absolute muscle mass than women in a given time period (107).

An additional time point T4, two to four weeks after the training intervention where the benefits of strength training still remain (108), but the acute molecular responses were already decreased, could have been a valuable supplementary data point to investigate the benefits of strength on DNA damage markers and would contribute to the decision if resistance training can outweigh the potential DNA damage (109,110).

Summary

The increasing average age in Europe and other developed countries is accompanied by an increasing prevalence of sarcopenia, osteoporosis and other age-related diseases, which impair life quality, independence and cognitive abilities.

It is therefore all the more important to implement preventive measures for individuals older than 65, for instance a balanced diet, sufficient protein and vitamin D intake, as well as physical activity in the form of resistance and endurance training.

During the 14-week long trial vitamin D supplementation did not have a significant effect on strength parameters in individuals aged 65-85, but a monthly dose of 50.000 I.E. was able to increase the vitamin D level significantly. Mean vitamin D levels of previously deficient participants increased after 14 weeks of intervention and a monthly dose of 50.000 I.E. to sufficient levels, compared to a daily dose of 800 I.E. or the control group.

As soon as the training intervention started for all participants after 4 weeks, markers of chromosomal damage increased in all subjects, which was measured with the CBMN-assay, there was no difference between groups.

This was offset by the positive effects of the resistance exercise, a majority of subjects was able to increase strength related parameters in upper and lower body after 10 weeks, which had a positive effect on physical and cognitive performance.

The trial showed, that RPE based training was feasible in this age group.

Zusammenfassung

Das zunehmende Durchschnittsalter in Europa und anderen entwickelten Ländern bringt eine wachsende Anzahl an Menschen mit Sarkopenie, Osteoporose und anderen altersbedingten Krankheiten mit sich, welche die Lebensqualität, die Selbstständigkeit und auch kognitive Fähigkeiten beeinflussen.

Umso wichtiger sind in der Altersgruppe der über 65-Jährigen Präventionsmaßnahmen wie ausgewogene Ernährung, ausreichende Proteinzufuhr und Vitamin D Versorgung, sowie regelmäßige Bewegung in Form von Kraft und Ausdauerübungen.

Die 14-wöchige Studie mit einer Vitamin D Supplementation konnte keinen signifikanten Einfluss verschiedener Vitamin D-Gaben auf Kraftparameter in 65-85-Jährigen Proband*innen bewirken, aber es konnte gezeigt werden, dass eine monatliche Gabe von 50.000 I.E. Vitamin D in der Lage war, den Vitamin D-Spiegel signifikant zu steigern. Der Durchschnitt, der zuvor mangelhaft versorgten Proband*innen war nach 14 Wochen und einer Gabe von 50.000 I.E. ausreichend mit Vitamin D versorgt, im Vergleich mit einer täglichen Supplementation mit 800 I.E oder der Kontrollgruppe.

Sobald die Trainingsintervention für alle Proband*innen nach 4 Wochen startete, stiegen bei allen Gruppen die Marker für Genomschädigungen, die mit dem CBMN-Protokoll untersucht wurden, es konnte kein Unterschied zwischen den einzelnen Gruppen festgestellt werden. Aufgewogen wurde dieser Umstand jedoch mit den positiven Effekten des Krafttrainings, der Großteil der Proband*innen konnte seine Kraftwerte nach 10 Wochen in Ober-und Unterkörper steigern, was einen positiven Beitrag auf die körperliche und geistige Leistungsfähigkeit hatte. Die Studie hat gezeigt, dass ein Training, welches auf der wahrgenommenen Anstrengung basiert, in dieser Altersgruppe durchführbar ist.

Troubleshooting and suggestions for new teams

During the trial, unforeseen issues occurred that had to be addressed. As temperatures started to rise during spring of 2019, keeping blood samples at adequate temperatures became relevant. The location of blood draw and blood analysis were about 5 kilometers apart and had to be transported during rush hour in Vienna, so at times transition times to the lab would vary from 20 minutes to 1,5 hours.
About halfway through the trial, problems with contamination arose, noticeable as yeast on the finished slides. Despite careful observance of hygiene and keeping everything as sterile as possible, those issues persisted for several weeks. During that period of time, adjustments in the protocol and serious efforts were made to eliminate critical points of potential contamination. Eventually, we were able to reconstitute adequate slide quality.

In retrospect, there were certain measures that would have increased slide quality and workflow. The following paragraph is a suggestion for a timeline and should serve as guide for new teams that are adopting the CBMN assay.

Suggested timeline:

Week 1: Getting to know the protocol and establishing a routine in the necessary steps are crucial for a successful execution of the protocol. The protocol by Fenech (65) should be read before the team starts to do the first attempts in the lab. While reading the protocol, the particular steps of execution should be focused upon. After that, the protocol should be executed 2-3 times to get used to the routine and workflow.

Week 2: After knowing the steps of the protocol, it is important to understand, why the protocol is done how it is done. It is crucial to attain the ability, to judge the quality of a slide as early as possible, only then the team can react to suboptimal slides. The protocol by Fenech should now be read beyond the execution steps, focusing on scoring and scoring criteria and then choosing 6-9 slides with good quality and counting just the visible area in the microscope. This should take, depending on the quantity of

cells, max. five minutes per slide. The results then should be compared with every individual team member. Discussing questionable cells and deciding how to count in accordance with the examples in the paper is a very important step and is a measure of quality control and the basis of future calibration. This should be done at least once a week.

Week 3: It should be decided, who will do what during the several steps of the protocol. This week shall serve as trial week, experimenting with different points of time to introduce the Cyt. B (10 minutes earlier, 10 minutes later than intended), dilution/manipulation of cell concentrations before staining and subsequent evaluating quality carefully, for instance:

- One sample gets treated like always
- One vial with the same sample is treated with Cyt B 10 minutes earlier, one 10 minutes later. Staining of all three of those is done the same.
- Another sample is diluted before staining, another vial of the same sample is treated as always and one is concentrated.

The 2007 protocol should be read completely by now and all steps fully understood. Further readings are highly advised and only increases the understanding of the protocol and the steps involved, thus the ability to produce high quality slides.

Week 4: After calibrating the scoring for two weeks on only the visible area of a slide, the capacity should be increased, by scoring one row horizontally and comparing/evaluating the results. One horizontal row may take 15 minutes at first and will decrease with further practice.

Week 5: After practicing scoring for a whole month, a complete slide with 500 binucleated cells should now be scored, comparing and evaluating the results. In the beginning, a whole slide can take up to 30 minutes and one to two slides per team member is a good start for practice. Further increases of scored slides should be aimed for.

Week 6: Contingency plans should be set up and critical control points should be identified, for instance keeping protocol about room temperature, slide

quality, sudden case of sickness of a team member, failure of crucial equipment (incubator), ensuring plenty of pipette tips ready to use, etc. Refer to recommendations for further measures to install.

Recommendations:

After completion of the study, several procedures turned out worthy to be kept and some things, that would have been a “nice to have” during the protocol.

First of all, assigning fixed roles to the individual team members during the protocol proved to be time efficient and keep the extent of random errors to a minimum.

Second, careful documentation of all necessary steps is a crucial step to retrace possible mistakes and account for them. In order to distinguish between external vs. internal factors, a reference sample, (e.g. of one of the team members) should be included, running each week alongside with the trial samples to control for errors during the protocol. This allows to decide if poor samples are the result of external factors or is based on the quality of the samples themselves.

Third, the scoring of the cells is the most tedious part of the protocol and a sufficient amount of time should be allocated to that. Every team member has to find its own strategy of how to go about large numbers of slides. Depending on the time resources of the individual scorer, a steady approach of counting a small number of slides twice or three times a day has the advantage of being less tiring for the eyes but is quite time-consuming. The number of possible slides counted depends on the quality of the slides and varies between 4 and 10 slides per sitting in our study. Breaks of 10-20 minutes between sittings are advised. During those breaks, eye exercises like switching focus from close to afar, fresh air, exposure to daylight and movement/stretching of the neck and shoulder area decreased the exhausting effect of counting. Special attention should be devoted to the wrists. The constant holding of a certain hand position and monotonic movements while moving the slide with the microscope increases the risk of tendonitis, thus stretching and strengthening the wrists and forearms is recommended.

Within a day, up to 40 slides could be counted, although the average was about 10. The time to count to 500 decreases over time and one slide will take about 15-25 minutes, depending on the quality of the slide.

In retrospect, we highly advise to start scoring the cells during the execution of an ongoing trial or as early as possible.

Further valuable steps within the protocol that need highlighting:

- Cups and metal slide holders should only be washed by hand, rinsed thoroughly with water, ethanol and finally with distilled water and then left to be air-dried. Residuals of detergents from the dishwasher my cause loss of cytoplasm. During staining, one person would be responsible for quick rinsing of the cups used, because at times there were not enough cups for all of the samples.

- After mixing a new batch of PHA, it should be tested for stimulating activity before using it on the subject samples.
  When adding the PHA at day one, it should be distributed carefully throughout the whole sample to ensure full stimulation

- The incubator should be cleaned thoroughly after every full session of the protocol, to minimize the possibility of contamination by the incubator.

- Using the autoclave on every piece of equipment that is able to autoclavable, especially glassware.

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