Thesis

Irisin - a regulator of exercise induced effects on the "bone-muscle-unit"

submitted by

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Graz, Dezember 2020 *Eva Singer eh*

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List of abbreviations

Table 1: abbreviation list

HbA1c	Haemoglobin A1c (long term blood glucose parameter)
TSH	Thyroid stimulating hormone
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
eGFR	Estimated glomerular filtration rate
LDL	Low-density lipoprotein
Hs-CRP	High-sensitivity C-reactive protein
IL-6	Interleukine 6
CK	Creatine kinase

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Zusammenfassung

Gegenstand dieser Arbeit ist eine Studie über das im Jahr 2012 erstentdeckte Hormon Irisin. Es wird bei körperlicher Aktivität aus dem Muskel freigesetzt. Eine genauere Erforschung des Hormons könnte dazu beitragen, den positiven Einfluss von Bewegung auf den menschlichen Körper zu erklären. Frühere Studien zeigten eine Expression des Hormons in Abhängigkeit von der Intensität der körperlichen Betätigung. Um ein besseres Verständnis der Dynamik der Irisinbildung und -ausschüttung zu erhalten, wurde eine Pilotstudie mit fünf Frauen mittleren Alters, sowie eine Tierstudie mit 20 weiblichen Sprague-Dawley-Ratten durchgeführt. Menschliche und tierische Probandinnen durchliefen ein zuvor definiertes Trainingsprogramm, bei dem vor und nach den Einheiten Blutproben entnommen wurden. Bei der Studie am Menschen konnten keine statistisch signifikanten Ergebnisse akquiriert werden - hier sollte jedoch die geringe Teilnehmerzahl berücksichtigt werden. Dennoch konnten einige Korrelationen festgestellt werden: eine mäßig positive Korrelation zwischen dem Irisinspiegel und dem Body Mass Index und eine mäßig negative Korrelation zwischen dem Irisin- und Cholesterinspiegel. Sclerostin, ein Hormon welches zusätzlich untersucht wurde, und so wie Irisin auch am Knochenstoffwechsel beteiligt ist, korrelierte stark mit dem Alter der Probandinnen. Bezüglich der Tierstudie hatten Ratten aus der Trainingsgruppe im Vergleich zu nicht trainierenden Ratten eine signifikant geringere Gewichtszunahme. Dies traf selbst dann zu, wenn eine fettreiche Diät gefüttert wurde. In der Gruppe mit fettreicher Ernährung wogen die Tiere, die sich regelmäßig am Laufband bewegten, statistisch signifikant weniger als nicht trainierende Tiere ($p=0,008$). Diese Ergebnisse stimmten mit den Daten früherer Studien überein, die ebenfalls den gewichtsreduzierenden Effekt von Bewegung bestätigten. Die durchgeführte Studie konnte die detaillierte Kinetik von Irisin nicht bestimmen, jedoch wurden bestehende Informationen bezüglich Bewegung und Gewichtszunahme untermauert, sowie interessante Korrelationen von Sclerostin und dem Alter der Probandinnen entdeckt.

Abstract

Fairly new discovered in 2012, the hormone irisin could have an impact on many different areas of the human body. As it is released from the muscle during physical activity, it might help to explain the positive influence of exercise on peoples´ health. Former studies presented an expression of irisin depending on the intensity of exercise. In order to get a better understanding of the dynamics of irisin, we conducted a human pilot study with five middle-aged women as well as an animal study with 20 female Sprague-Dawley rats. Human and animal probands underwent a previously defined procedure of physical activity and blood draws were taken before and afterwards. Keeping in mind that the number of test persons was quite small, no statistically significant results could be found. Nevertheless, some correlations were detected: a moderate positive correlation between irisin levels and BMI as well as a moderate negative correlation between irisin and cholesterol levels. Sclerostin, another hormone taking part in bone metabolism was highly correlated with the age of probands. Regarding the animal study, exercising individuals gained significantly less weight compared to non-exercising ones. This even applied when a high fat diet was fed; animals of the exercising high fat diet group weighed statistically significant less than non-exercising animals (p=0,008). Those findings matched the data of former studies, which as well confirmed the weight-reducing effect of exercise. The conducted study could not determine the detailed kinetics of irisin but confirming information regarding exercise and weight gain as well as interesting correlations of sclerostin and age of probands could be found.

1 Introduction

1.1 Overview

Regular physical activity reduces the risk of cardiovascular disease and overall risk of mortality (1-3). When comparing the most active against the least active groups of a population, the risk of cardiovascular mortality can be reduced by 35% and the all-cause mortality by 33% (4). Exercise not only decreases the risk of age and lifestyle related conditions like osteoporosis, diabetes and obesity, but also slows their progression (5). There has been research on mechanisms that lead to those health benefits, but most of them are not entirely comprehended.

Concerning the mass, the muscles are the largest organ system of the human body. Combined with the skeletal system they enable us to move. Those two systems are strongly intertwined and in order to function well, they have to communicate. It is known that adult bone formation happens through moderate strain and gravitational load, hence, whenever skeletal muscles are used during physical activity. The muscles´ force is transferred onto the bones via tendons and together with the compression of movements, exercise acts as a trigger for bone formation.

1.2 Myokines and irisin

Besides physical ways of bone formation, there are also humoral inductors like parathyroid hormone and cholecalciferol, that are necessary for the regulation of the bone itself and the overall homeostasis of the body. Those hormones are released by the parathyroid and thyroid gland, not from the muscle or bone itself. (6) However, in the last decades different studies have shown that the muscle is also a secretory organ (7, 8). Additional to the mechanical axis, muscles can communicate with bones through myokines. Myokines are small molecules, most of them belong to the group of cytokines and are released by the muscle during physical activity. This leads to the assumption, that the secretory function of the muscle could as well have an influence on different bone diseases, in addition to the mechanical impact. Myokines might explain some of the benefits of physical activity. Some myokines that have already been investigated are interleukine 6 (IL-6), interleukine 15 (IL-15) and brain-derived-neurotrophic factor (BDNF). Another myokine that was found to

be released during exercise more recently is irisin, a cleaved domain of the membrane protein fibronectin type III domain containing 5 (FNDC5). (9)

1.3 Irisin

Irisin was first described by Boström et al. in 2012 and was identified as the proteolytically cleaved ectodomain of the membrane protein fibronectin type III domain containing 5 (FNDC5) (9). Human FNDC5 is mainly expressed in the skeletal muscle and other muscle containing organs like heart, tongue and rectum, but also in adipose tissue (8, 10). FNDC5 expression in rats takes place primarily in subcutaneous white adipose tissue, less in visceral white adipose tissue. Hardly any FNDC5 expression occurs in a rat's brown adipose tissue. (11, 12)

Due to the fact that this newly found ectodomain of FNDC5 functions as messenger between muscles and different other organs, it was called Irisin, after the Greek goddess Iris. Irisin consists of 112 aminoacids (excluding the signal sequence) and its precursor is encoded by the FNDC5 gene. The expression of FNDC5 is stimulated by the transcription-cofactor peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α). The aminoacid-sequence of irisin is identical in mice and humans. It was shown that plasma irisin levels ascend after endurance exercise training. (9)

Irisin was discovered combining western blot and mass spectrometry. Western blot is a molecularbiological procedure, where single proteins in a sample of many proteins can be detected. To divide the sample-proteins, heating and electrophoresis were used, followed by the actual blotting of samples onto a membrane. Then proteins could be detected by specific antibodies and antibodies with an attached detection system. (13, 14) Subsequently, mass spectrometry was used to first ionize the prepared samples and separate the molecules according to their mass-to-charge-ratio. With this method, the sequence of a polypeptide could be defined. (15)

Most following studies used enzyme-linked immunosorbent assay (ELISA) kits for detection and quantification of irisin (16-18). In these assays irisin binds onto specific antibodies on microplates, the supernatant gets removed and the plate washed in order to avoid unspecific bounded antigens. An enzyme-linked second antibody is put onto the plate, which induces a colour reaction. The colour intensity

can be detected with a photometer and the concentration of the original protein can be recalculated. (19)

To this date there are no valid reference values. Different studies used different methods and consequently came to different ranges of results. Most of the studies, including this one, are using immunoassays, but mass spectrometry is viewed as the gold standard. As per the user manual of the used immunoassay, the supposed physiological irisin levels in human vary from 0,2 to about 5 µg/ml. Reference ranges of other studies are considered in the discussion and presented in table 14 in the annexe.

Raschke et al. and Albrecht et al. doubted the validity of irisin ELISA-kits due to the fact that they came upon cross-reacting proteins. Moreover, the precursor of irisin, FNDC5, has the atypical start codon ATA, instead of ATG, which lead them to the assumption that irisin is not even translated and claimed it to be a myth. (17, 20) But further investigations using tandem mass spectrometry showed that irisin indeed exists (21, 22).

According to the studies of Boström et al., the up regulation of irisin triggered by physical activity leads to a so called “browning” of white adipose tissue, which will be described in chapter 1.5. Irisin stimulates thermogenic programmes through the expression of uncoupling-protein 1 (UCP-1) and increases cellular mitochondrial density. (9, 23)

In osteocytes and adipose tissue αV integrins, a subgroup of integrins, act as receptor for irisin. The released irisin from exercising muscle tissue docks on the αV integrins of the bone and increases the expression of sclerostin, which is able to modulate bone remodelling. (24) Although irisin triggers thermogenesis, Colaianni et al. suggested, that rather the skeleton is the actual target organ than the white adipose tissue (23).

The kinetics of irisin are not at all understood. There have been several studies that investigated the release of irisin during or after physical exercise, some of them made contradictory findings.

In the original study of Boström et al. a 65% increase of irisin was shown in mice after three weeks of wheel running. Human irisin levels were also twice as high after a ten-week exercise programme compared to baseline measurements. The mRNA levels correlated approximately with the higher irisin levels in plasma. (9) After 30

minutes of exercise irisin levels increased significantly in young healthy subjects, but stayed unaltered after eight weeks of exercise, as it was examined by Huh et al. (10).

No change in human serum irisin or FNDC5 after acute and different types of long-term exercising could be found by Pekkala et al. (18), whereas Roca-Rivada et al. confirmed the original findings with a rat model and showed a significant increase in irisin secretion of muscles after three weeks of spontaneous running. In subcutaneous and visceral adipose tissue even one week of training led to an increased irisin secretion. (11)

Irisin is augmented acutely after exercise, decreases quickly and remains unchanged. An increase can be expected after maximum workload. (25)

Concerning the heterogeneity of outcomes, a meta-analysis compared existing data in 2016. The authors came to the conclusion, that randomized controlled trials showed an association of a decrease of irisin and chronic exercise. Non-randomized studies did not lead to a representative result. (26)

The findings of a study conducted in 2017 showed rising irisin levels in muscle biopsies after high intensity interval training, but not after aerobic exercising. No relevant changes of irisin blood plasma levels were found in any of the intervention groups. (16)

Irisin correlates with beneficial metabolic parameters (27). In order to understand its impact on health and a possible way of preventing diseases, its kinetics have to be studied further. Particularly the impact of irisin on overall health and its triggers and pathways should be investigated. It seemed as if irisin has the potential to contribute to the therapy of many metabolic diseases like obesity, diabetes, osteoporosis and even Alzheimer's disease (28). On the other hand there are also some publications that question the detection methods and the suggested physiological role of irisin (17). Albrecht, Norheim et al. investigated the antibodies against irisin which are used in commercial ELISA kits using Western blot. They pointed out, that they are often cross-reacting with other proteins and therefore irisin´s importance is questionable (17). Although it is widely known and well researched that moderate physical activity is beneficial for a human's overall health (29), it is not entirely understood, how this benefits are achieved. Therefore, a rather newly discovered hormone like irisin, that is released by the muscle, which is obviously a big part of physical activity, arouses great interest.

1.4 Irisin, sclerostin and bone-muscle communication

It is known that bone formation happens via strain of muscles and compression of gravitation (30). An increase in muscle strength is always followed by an increase in bone strength, as Rauch et al. could show in a clinical trial (31). The strong connection between muscles and bones brought up the idea of regarding them as a “bone-muscle unit” rather than two separate systems on their own. The discovery of muscles affecting bones not only in a strictly mechanical manner, but also via secretion of hormones (8) led to a new point of view on the relationship between muscle and bone. It also widens the field of potential options for treating bone diseases like osteoporosis. In order to pursue that option, the exact paths of bone and muscle communication have to be identified.

As mentioned above, myokines are molecules that are released by the muscle and communicate with other tissues in the body. Although the first recognized target of the myokine irisin is the adipose tissue, the bone is also a possible destination. In young female athletes a positive association between irisin and bone density scores were found (32), and bone mineral density in healthy children positively correlates with irisin levels. Furthermore, bone mineral status was determined by irisin more than by alkaline phosphatase or parathyroid hormone, which are common markers for bone metabolism. (33) Therefore, irisin could potentially be used as a novel marker of bone structure or metabolism.

Colaianni et al. showed that the mass of cortical bone in mice can be positively influenced by low-dose injections of recombinant irisin (34). Using hind-limb suspended mice as a model for disuse-induced osteoporosis, as it is common in elderly or physically disabled people, recombinant irisin mitigated the negative effects of disuse on both bone and muscle (35). Keeping in mind that data was gathered on mice, this might promote the development of a therapeutic application of irisin.

In 2014 it was ascertained that levels of irisin and another hormone, sclerostin, are highly associated (36). Sclerostin is a protein that is encoded by the SOST gene, expressed in adults mainly by osteocytes and takes part in bone metabolism by inhibiting its formation (37). Sclerostin inhibits osteoprotegerin, a sham receptor for receptor activator of nuclear factor kappa B ligand (RANKL). RANKL takes part in bone resorption because it is an essential factor for osteoclastogenesis. Therefore, sclerostin inhibits bone formation indirectly, as shown in figure 1. (24)

In culture, added irisin prevented osteocytes from hydrogen-peroxide-induced apoptosis at concentrations which can be found physiologically too. In vivo, irisin treatments raised the mRNA levels of sclerostin in osteocytes and plasma levels dose-dependently. (24) Kim et al. discovered that expression of RANKL is lower in FNDC5 null mice, therefore also less bone resorption takes place. After performing ovariectomy, mice with FNDC5 deletion, which means no irisin expression, were resistant to ovariectomy-induced bone loss. Moreover, irisin levels in sham-operated mice were more than double than in mice where ovariectomy was conducted. (24)

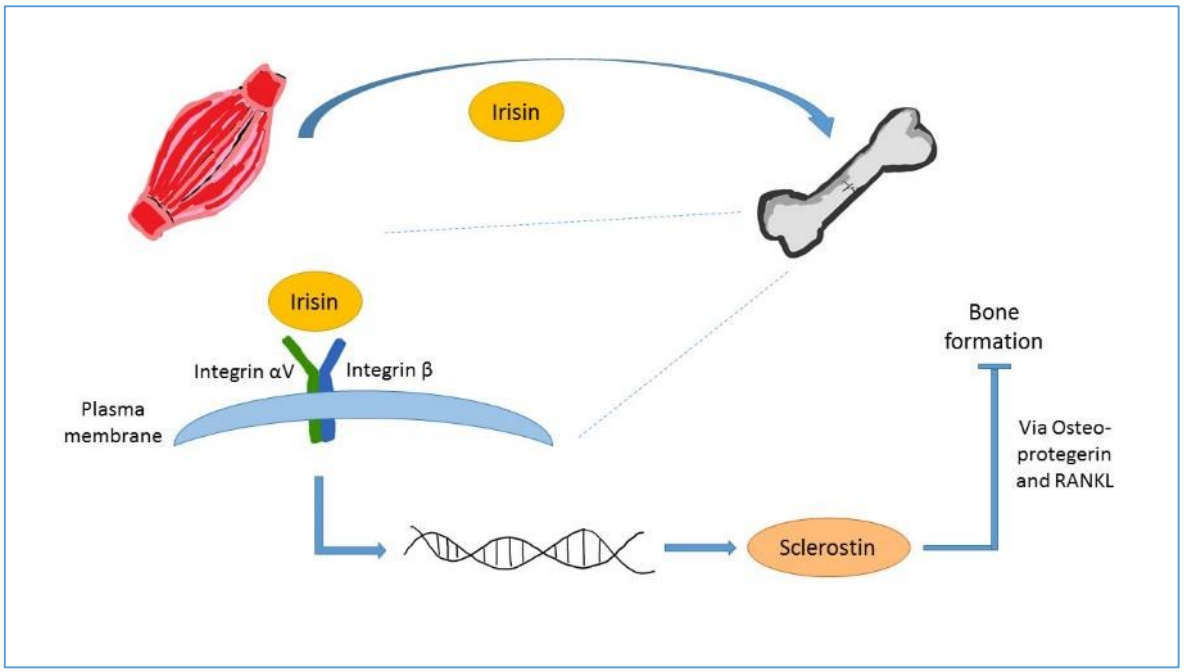

Figure 1: short overview of the expression and interaction of irisin and sclerostin

All this data show a strong connection between muscle activation and bone metabolism. In order to use this information for future therapeutic approaches, research has to be done and exact pathways and functionalities have to be discovered.

1.5 Irisin, obesity and metabolic diseases

Obesity is the excessive accumulation of fat in adipose tissue, with potential adverse effects on overall health. The world health organization defines it as a body weight of 30 or more kg/m² (38). Along with obesity often comes dyslipidaemia, insulin resistance and hypertension, which is then called metabolic syndrome. People suffering from metabolic syndrome have a higher risk of developing coronary heart disease and overall cardiovascular mortality (39).

The human body comprises two main kinds of adipose tissue: white and brown adipose tissue. Additionally, there exists a lesser-known beige (or sometimes called brite) adipose tissue. White adipose tissue stores energy through triglycerides, brown adipose tissue prevents the body from hypothermia by using thermogenesis. (40) This is possible due to a high mitochondrial density and the uncoupling of electron transport chain from energy production via uncoupling protein 1 (UCP1). This mechanism produces heat and prevents from obesity because of the increased energy expenditure.

Brown fat is especially important during infancy to keep a stable body temperature, but in most large mammals it is dissolved in adulthood (41). Normally brown adipocytes are situated in brown adipose tissue depots, whereas beige adipocytes can be found sporadically in between white adipocytes too, mainly in the subcutaneous white adipose tissue. Specific environmental causes such as cold exposure, physical exercise and long-term treatment with peroxisome proliferator-activated receptor-γ (PPARγ) agonists lead to higher levels of circulating irisin and FNDC5, which in turn stimulates UCP1 expression of beige adipocytes. (9, 41, 42) The process of emerging beige adipocytes is also called “browning of white adipose tissue” (42).

Exposure to cold temperatures showed a proportional linkage between irisin levels and shivering intensity (22). It seems paradoxical to increase a thermogenic hormone during physical activity, but Boström et al. hypothesized, that the release of irisin evolved from shivering muscle contraction in order to increase non-shivering thermogenesis in brown adipose tissue (9).

In 2014 it was shown that recombinant irisin injected into mice had a positive impact on their body weight and glucose homeostasis. To receive the recombinant irisin, the research team used cDNA of human irisin, synthesized by a commercial supplier, and transformed the yeast species Pichia pastoris via a cloned plasmid.

This yeast species is able to express the transfected proteins. Protein expression was inducted through a kit from Invitrogen, r-irisin was then purified from the supernatant through a two step-method. (43)
The suggested way of the positive effect of irisin on body weight and glucose homeostasis is via an “irisin-induced phosphorylation of the p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal–related kinase (ERK) signalling pathways” (43). Furthermore, Zhang et al. could show that recombinant irisin also stimulated betatrophin expression, which in turn stimulates pancreatic β-cell proliferation and improves glucose tolerance (43). Kim et al. could confirm this results in 2018, where they described a two-fold increase in mRNA levels of UCP1 after wild-type mice were injected with recombinant irisin. They found integrin αV complexes to operate as irisin-receptors in fat tissue and they also mediate the thermogenic gene programme that is induced by irisin. (24)
Due to its effects on the browning of adipose tissue and the potential in diabetes treatment, irisin is an auspicious therapeutic target when it comes to obesity and metabolic syndrome.

Taken together, physical activity has an important influence on peoples´ health, but the exact connections are not understood. Irisin is the cleaved ectodomain of the membrane protein FNDC 5. As it derives from muscle cells, it can also be called a myokine. Along with the exploration of other myokines, improved investigation on irisin might lead to a better understanding of how physical activity is beneficial for the human body. Several findings suggest that irisin plays a role in metabolism, bone formation and thermogenesis. Its levels are higher after exercise and it triggers thermogenic programmes (9). In animal studies, irisin had a positive impact on glucose homeostasis (43). It is highly associated with sclerostin, a protein which itself is operating in the control of bone formation. In sum, irisin is a fairly new discovered hormone which could have an impact on many different fields of the human body.

2 Hypothesis

We here hypothesize that physical activity induces the expression of irisin - a central regulator of the bone muscle unit via sclerostin thereby showing beneficial effects on obesity related pathophysiological mechanisms.

3 Aims

The aim of the present study is to determine the expression of irisin and sclerostin regarding physical activity and obesity in a human pilot study and in an experimental animal model.

4 Methods

4.1 Human pilot study

4.1.1 Study cohort and study design

To explore the effects of physical activity on the expression of irisin, a prospective interventional pilot study was conducted. Five human participants were included, and blood samples were collected at various time points before and after physical activity (figure 2).

Five healthy women aged between 45 and 65 years were searched and recruited, then underwent a physical examination, including measuring of weight, height, blood pressure, auscultation of heart and lung, electrocardiogram, neurological status and an interview about the use of medication and the presence of any acute or chronic illness. Every time when weighing the probands the same scale was used. One participant used a low dosed beta-blocker and another one had hypothyroidism, which was not in need of medical treatment. Nonetheless, these were declared as suitable for the pilot study after careful risk estimation.

Before entering the study, written and informed consent was given. The ethical committee of the Medical University of Graz approved the study (identifier: 30-168 ex 17/18).

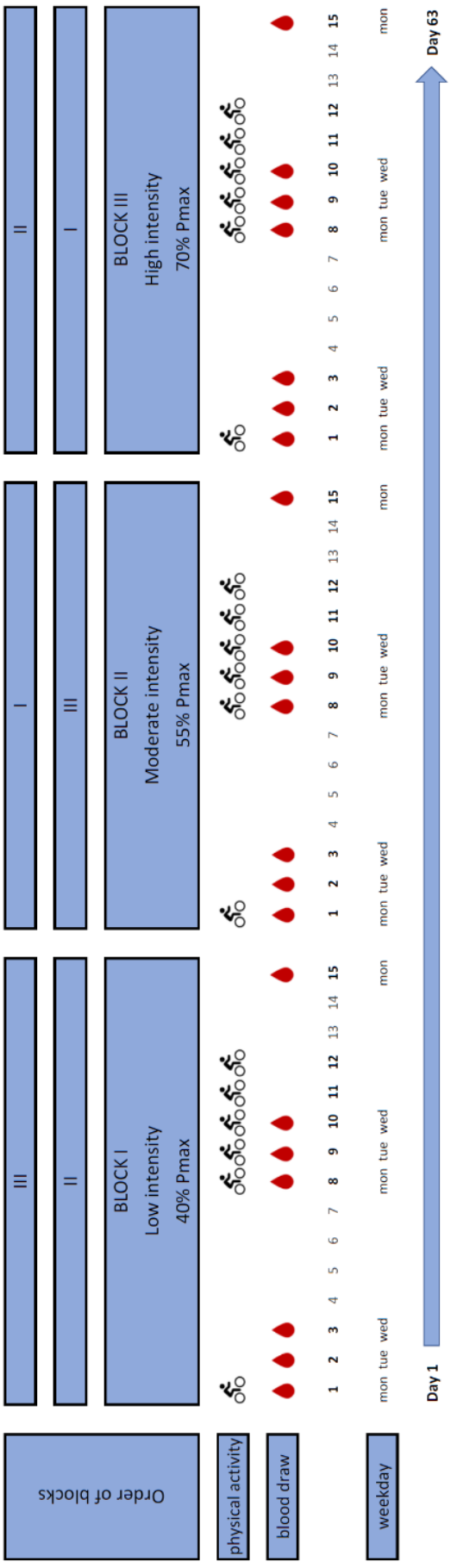

Figure 2: schematic diagram of the conduction of the study

Exclusion criteria were pregnancy, severe chronic disease, obesity (BMI >30kg/m²) and high-grade leisure time physical activity (more than two hours of sports training per week).

The participants went through three standardized blocks of ergometric exercise with different levels of intensity. Low intensity was defined as 40%, moderate intensity as 55%, and high intensity as 70% of the maximum intensity level. The following formula was used to estimate the maximum intensity level of each participant (adjusted for females):

$$P_{max,woman} = \Big(2.5 - \big(0.025 \cdot (age - 30)\big)\Big) \cdot weight \qquad (44)$$

Each block contained an isolated single exercise session and a series of daily exercise sessions. The training sessions and blood drawings were conducted as outlined in the general overview in figure 2.

To avoid the potential bias of training effect, the probands were randomly assigned to different orders of completing the blocks. Two participants started with the low-intensity-block, followed by the middle one and finished up with the high-intensity-block. Two others accomplished the blocks in reverse order, starting with the highest. One person began the trainings with moderate intensity, followed by lowest and then the highest intensity.

4.1.2 Blood collection and sample preparation

Blood samples were taken directly before, 24 hours after, 48 hours after and 148 hours after the single exercise, as well as 24 hours after the first, 24 hours after the second and 72 hours after the fifth of the serial exercises. They were drawn using the VACUETTE® serum separator blood sampling system from Greiner Bio-One. A total of 105 blood samples was taken, 21 from each participant. The sample vessels were left standing still at room temperature for at least 30 minutes to allow completion of clotting. After that, serum was maintained via centrifugation at 2300g and immediately stored in aliquots at -80 °C. Aliquots were thawn up at the time of measurements which occurred en-bloc.

4.2 Animal model

20 female Sprague Dawley rats were included in the study. In every cage three animals were kept. They had a twelve hours day and twelve hours night routine. Ten of them were fed a high-fat diet (HF), ten of them were given a standard rat diet (ND), both from ssniff Spezialdiäten GmbH, Germany. Food and water were provided ad libitum. The high-fat diet contained 60% fat, 20% protein and 20% carbohydrates, whereas the standard rat diet contained 9% fat, 24% protein and 67% carbohydrates. Initially all rats weighed about 250 grams. The rat-training consisted of 30 minutes of exercise for five days a week with a speed at 30 centimetres per second on a treadmill. At the time of blood drawing, the rats were doing the programme for 8 months. The study was approved by the national authorities for animal research.

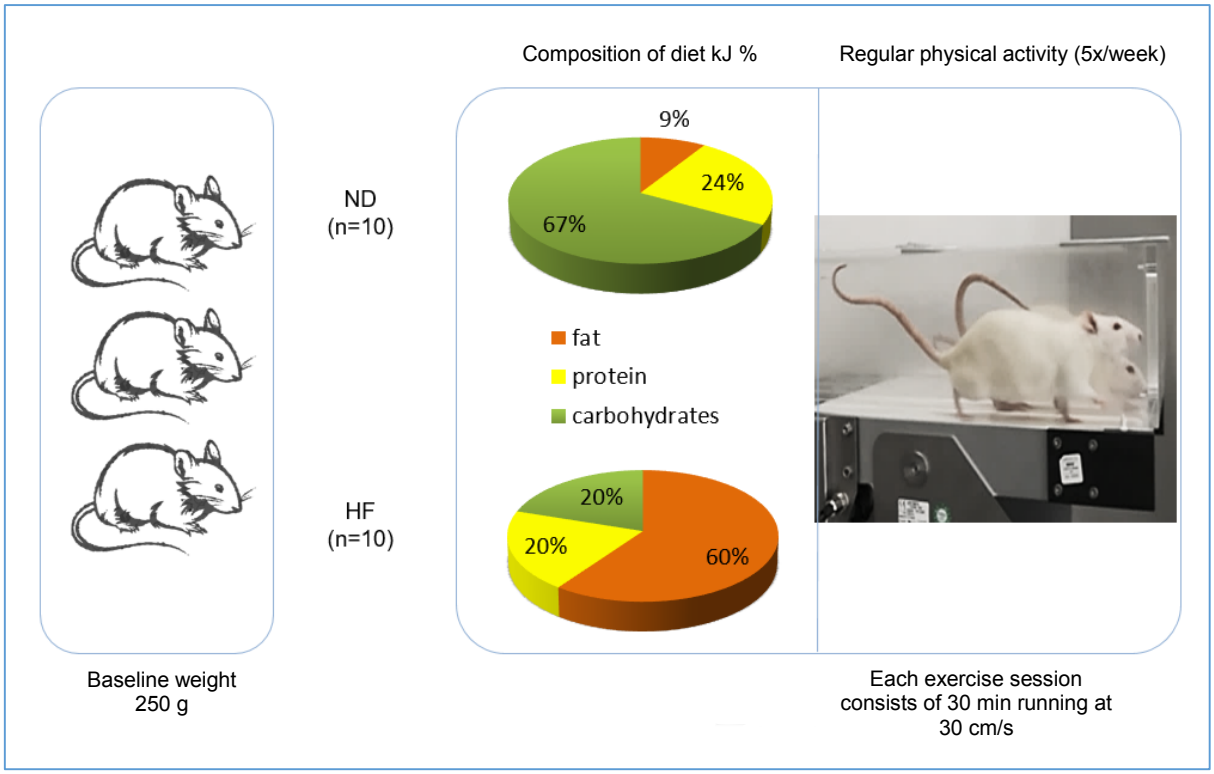

Figure 3: overview over diet and training of rats

4.2.1 Blood collection and sample preparation

On day one 600 µl of blood were taken before doing the exercise, one week after that 600 µl were taken immediately after physical activity. The blood was collected through tail vein puncture using isoflurane anaesthesia. After collecting the blood in serum separator tubes (Multivette® 600, Sarstedt), it was allowed to clot for 30 minutes at room temperature, then centrifuged at 10 000g for 5 minutes and stored at -80°C.

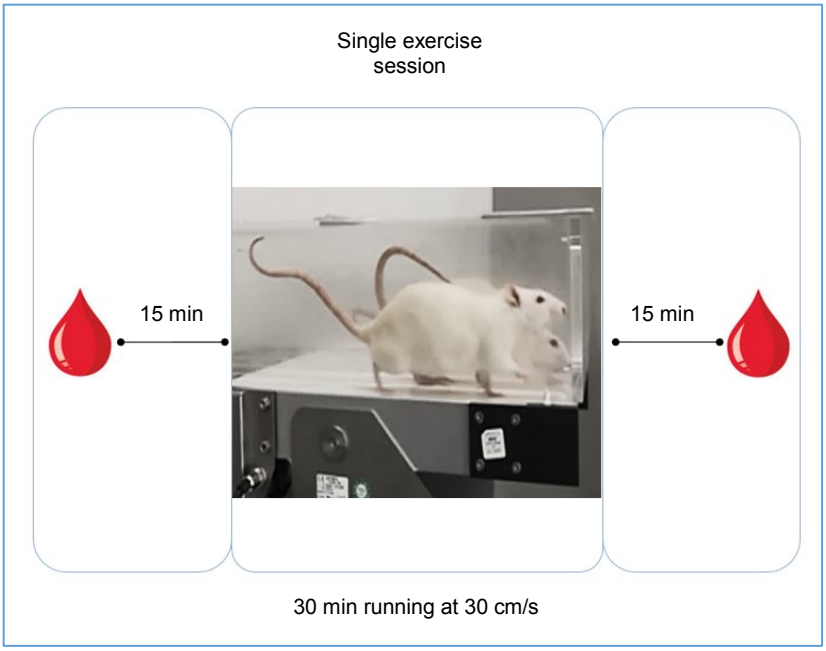

Figure 4: schedule of blood sampling of rats

4.3 Quantification of irisin

We used a competitive ELISA assay (Cat. No. AG-45A-0046YEK-KI01, AdipoGen®, Liestal, Switzerland) to quantify the concentration of irisin in the serum samples. The limit of detection (LoD) is 0.001 µg/ml, the interval of linearity reaches up to 5 µg/ml. Based on scientific literature this test is most used for the determination of irisin. According to the manufacturer, this ELISA assay is suitable for blood samples from humans as well as from rats.

The measurements were conducted as described in the manual of the kit. Taken together, 50 µl of standards and diluted serum (50 µl plus 150 µl buffer) were applied in duplicate into each well of the plate. The prepared detection antibody (12 ml buffer plus 19,2 µl antibody) was placed onto the samples, which then were incubated for

one hour at 37°C, covered with a plate sealer. The wells were then washed with an auto-washer and tapped to remove residual liquid. In the next step 100 µl horseradish-peroxidase (HRP) -linked antibody (120 µl in 12 ml buffer) were added into the wells and again incubated for one hour at 37°C, then washed and tapped as in the previous step. 100 µl of Tetramethylbenzidin (TMB) Substrate Solution were applied into each well. The plate was then left for incubation at room temperature in the dark for 20 minutes to develop a colour reaction, which was then stopped by adding 100 µl of Stop Solution. The optical dispersion of the colour reaction was measured with a spectrometer from Molecular devices at 450 nm. The intensity of colour was indirect-proportional to the irisin-concentration. A computer-based four-parametric standard curve-analysis (optical dispersion versus standard concentration) with eight standard concentration points was created to determine the irisin-concentrations of the samples. The used programme was SoftMax® Pro Software from Molecular Devices, LLC. In all samples the intra assay coefficient of variation was <15%.

4.4 Quantification of sclerostin

4.4.1 Humans

Sclerostin quantification of human blood samples was performed by the immunoassay DiaSorin LIAISON®, a chemiluminescent immunoassay (CLIA) from the company DiaSorin (Stillwater, MN, USA) on a DiaSorin LIASION XL analyzer. The given measurement range reached from 50 to 6000 pg/ml. In this assay, the specimens were at first incubated with the calibrators and the buffer, then mixed and incubated with a specific monoclonal antibody, which bounded sclerostin. After dispensing the coated magnetic particles, the third incubation took place. A washing step was carried out to remove unbound material. Then, a flash chemiluminescent reaction was induced by adding the starter reagents. The emitted light could be detected, it was proportional to the sclerostin concentration in a sample.

4.4.2 Rats

The quantification of rat sclerostin was conducted with the immunoassay Quantikine® ELISA Mouse/Rat SOST Immunoassay from R&D Systems Europe, Ltd., Abingdon OX14 3NB, UK. The immunoassay is suitable for rat- and mouse-sclerostin detection. The LoD is 1,63 pg/ml, the quantification is linear up to 1000 pg/ml. The analysis was performed as described in the manual. Briefly summarised, the blood samples were put onto a microplate which was pre-coated with a monoclonal antibody specific for mouse and rat sclerostin. Any existing sclerostin bounded to the antibody in the wells. In the next step, all unattached substances were washed away, and a sclerostin-specific polyclonal antibody was added. After another wash substrate solution, which turned the substance blue, was added, as well as stop solution, which turned the product yellow. The measured colour intensity was proportional to the bounded sclerostin.

4.5 Quantification of human routine laboratory parameter

General biochemical tests included a full blood count, fasting glucose, HbA1c (CASPAR HA 8180, Menarini Diagnostics), TSH, AST, ALT, creatinine, eGFR, triglycerides, cholesterol, LDL, HDL, hs-CRP, IL-6 (cobas® 8000 analyzer, Roche Diagnostics). All analyses were performed routinely at the CIMCL.

4.6 Statistics

Data were analysed with IBMs SPSS programme, package version 26. Results are expressed in means ± standard deviation. Depending on distribution of data, paired and unpaired (students) t-test, Mann-Whitney-U-test, Wilcoxon-test or Kruskal-Wallis-test were performed. P-value <0.05 was considered statistically significant. Correlation analysis was performed using linear regression analysis.

5 Results

5.1 Human pilot study

5.1.1 Basic anthropometric parameters and vitals

Five healthy women, aged between 50 and 57 years, participated in the study. The basic anthropometric parameters are shown in table 2. The body mass index of one participant (29,9 kg/m²) was located at the higher end of the approved range, that was defined for the study. The vitals (heart rate and blood pressure) were normal throughout the study.

Table 2: basic parameters

		Mean	Median	Min	Max	Standard deviation
N	5					
Sex	Female					
Age (a)		52	51	50	57	2,6
Height (m)		1,58	1,57	1,53	1,64	0,04
Weight (kg)		61,4	59,9	47,3	71,9	9,32
BMI (kg/m²)		24,6	23,5	20,2	29,9	3,36
Bp systolic (mmHg)		116	113	110	130	7,45
Bp diastolic (mmHg)		77	79	70	85	5,91
Heart rate (bpm)		73	70	60	91	10,15

Due to the different constitution of the five probands the calculated maximum training intensity varied from 94 to 142 watts which consequently led to a 40% load between 38 and 57 watts, a 55% load between 51 and 78 watts and a 70% load between 65 and 100 watts. The maximum intensity for each proband can be found in table 5 in 5.1.3.

5.1.2 Routine laboratory parameters

Almost all routine laboratory measurements at baseline (full blood count, fasting glucose, TSH, AST, ALT, creatinine, eGFR, triglycerides, cholesterol, LDL, HDL, hs-CRP, IL-6) were in their respective reference ranges. Only some cholesterol values were slightly above the suggested reference range. In context with the physical examinations the laboratory results at baseline approved the health of the participants.

5.1.3 Descriptive statistics of irisin

The average irisin level of all samples was 2,75 µg/ml (± 0,68). The similar median (2,66 µg/ml) indicated that there were no big outliers. With a minimum of 1,38 µg/ml and a maximum of 4,70 µg/ml, the total range was 3,32 µg/ml. Shapiro-Wilk test indicated that the irisin results were not normally distributed (p=0,005). Non-parametric tests had to be applied.

Table 3: descriptive statistics of irisin measurements

N=105	Irisin in µg/ml	Standard error
Mean	2,75	0,06
Median	2,66	
Variance	0,46	
Standard deviation	0,68	
Minimum	1,38	
Maximum	4,70	

Looking at the different blocks themselves, 35 samples were taken and examined per block. The means of irisin were 2,76 µg/ml (first block, P=40%), 2,87 µg/ml (second block, P=55%) and 2,64 µg/ml (third block, P=70%). The minima, maxima and standard deviations are listed below in table 4.

Table 4: descriptive statistics of irisin measurements in µg/ml divided into the three different intensities

	Irisin Block I (P=40%)	Irisin Block II (P=55%)	Irisin Block III (P=70%)
Mean	2,76	2,87	2,64
Standard error	0,08	0,12	0,13
Median	2,75	2,74	2,43
Standard deviation	0,52	0,72	0,77
Minimum	1,69	1,63	1,38
Maximum	4,33	4,70	4,43
Range	2,64	3,07	3,05

The individual mean irisin levels of all five test persons are shown in the following table. The mean irisin levels varied from 2,19 µg/ml to 3,78 µg/ml. Of all baseline values the minimum was 2,13 µg/ml and the maximum 3,82 µg/ml.

Table 5: mean irisin measurements in µg/ml with minima and maxima of the individual probands

Proband	Mean irisin	Mean irisin Block I	Mean irisin Block II	Mean irisin Block III	Min	Max	Maximum training intensity in W
1	3,78	3,50	3,94	3,91	3,09	4,70	100
2	2,71	2,74	2,62	2,76	2,14	3,46	80
3	2,81	2,75	3,21	2,48	2,16	3,55	100
4	2,29	2,48	2,38	2,00	1,38	2,97	65
5	2,19	2,33	2,20	2,04	1,63	2,82	70

To get an overall idea of the distribution of the irisin measurements, you can see the boxplot hereinafter. All irisin values are shown depending on the intensity and the time of blood draw.

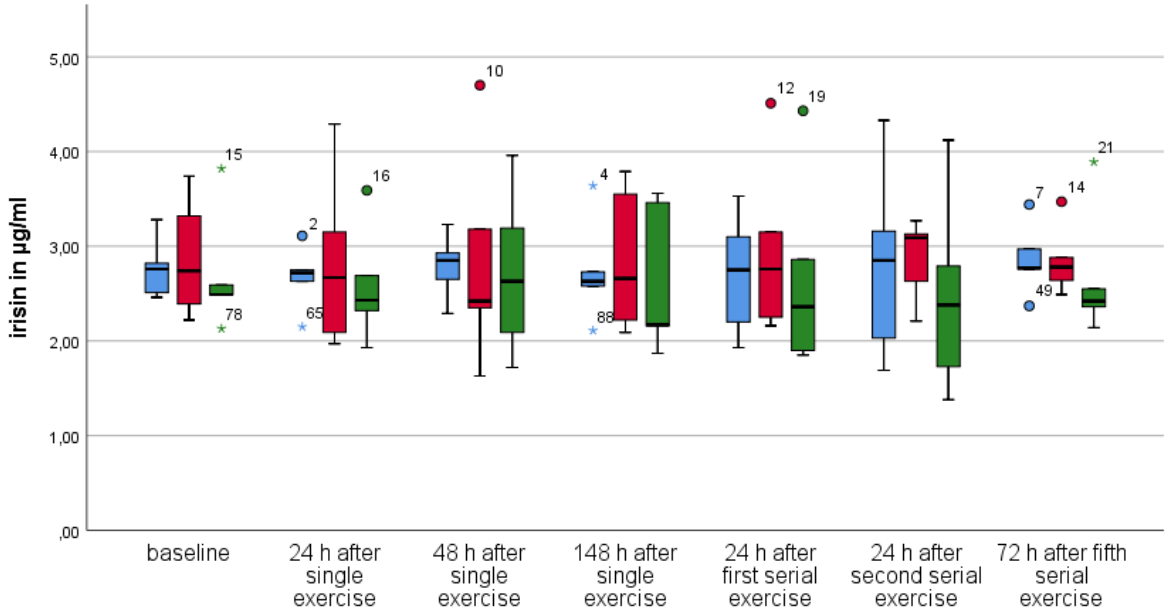

Figure 5: boxplot of all irisin measurements in each intensity block at different sampling points
Blue=40% intensity, red=55% intensity, green= 70% intensity

5.1.4 Difference in irisin levels before and after exercise

Blood samples were taken before and after exercise sessions to determine if a difference in irisin levels can be shown, as hypothesized.

5.1.4.1 Comparison of the first and seventh measurement

To investigate whether there was a training effect along the conduction of the study, every first and every last measurement of each block were compared. According to the study protocol, the first measurement was before any exercises took place, the seventh measurement was after a single and a serial exercise session. Every block was analysed by itself. The values of the first and seventh measurements in blocks I and II were normally distributed. T-test was used for further analysis. The measurements of the third block were not normally distributed, therefore Wilcoxon signed-rank test was used. In all three blocks no statistically significant difference

between the first and the seventh value could be found (block I p=0,56, block II p=0,85, block III p=0,78)

Also, the comparison of all the first and all the seventh measurements, (without separating the results into the three intensity blocks) did not show any statistically significant distinction (p=0,75).

5.1.4.2 Comparison of the first and last measurement

The comparison of all initial irisin values and all last irisin values was conducted in order to show a potential correlation between physical activity and irisin levels. Due to the study protocol the last values were measurements from different intensities. Wilcoxon-test was performed and could not show any significance (p=0,68). The results are presented in figure 6.

Figure 6: boxplots of all first (left) and all last (right) measurements of irisin

5.1.5 Comparison of different exercise intensities

In this chapter we analysed the irisin levels measured after different intensities of exercise. First, all measurements of one block were compared to the other blocks in 5.1.5.1, second, the measurements after exercise (without the baseline measurements) were compared in 5.1.5.2.

5.1.5.1 Comparison of all measurements per block

The descriptive statistics of each block are shown in 5.1.3. To recap, the mean irisin measurements of each block are listed in table 6.

Table 6: mean irisin measurements of each block

Block	Mean
P=40%	2,76 µg/ml
P=55%	2,87 µg/ml
P=70%	2,64 µg/ml

Tests on standard distribution showed that the 40% intensity block was normally distributed (p=0,438), the 55% intensity block as well, but with a very small interval to 0,05 (p=0,08) and the 70% intensity block was not normally distributed (p=0,018). Due to the tests on standard distribution and the small number of cases it was decided to perform non-parametric tests further on, although the low intensity block was normally distributed.

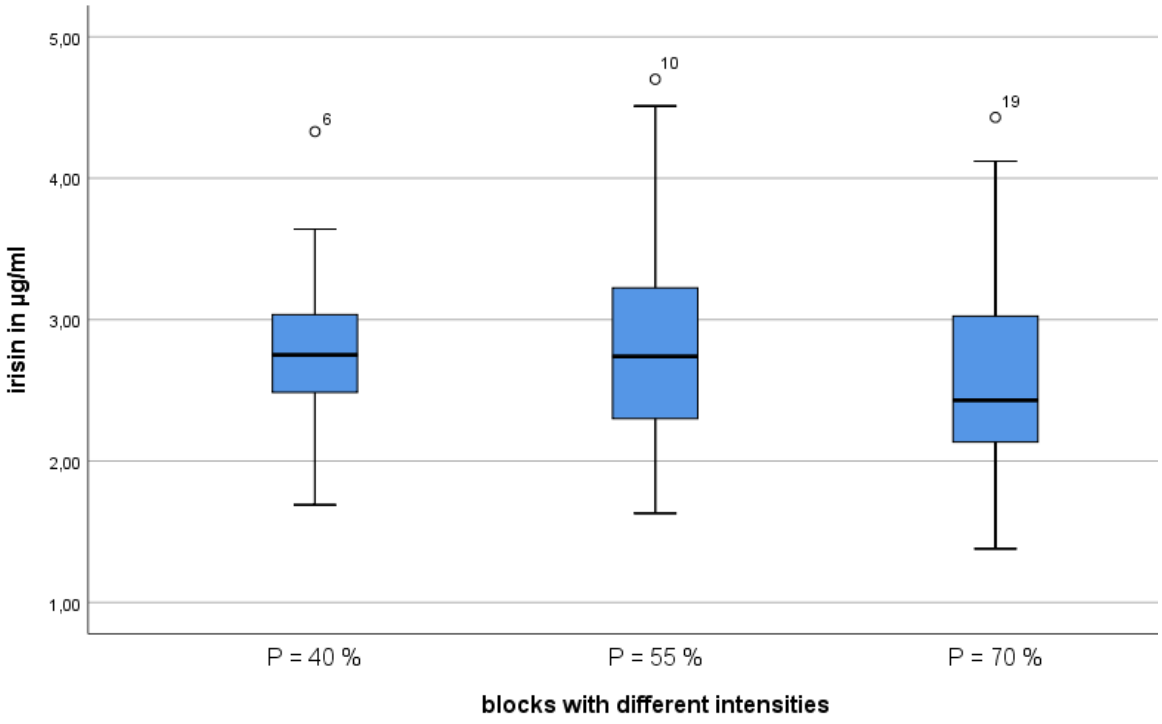

Figure 7: boxplots of irisin levels split into the three defined intensities

The boxplot (Figure 7) shows the three groups of different intensity next to each other. To check if there was a difference between the individual groups, Mann-Whitney-U test was performed. It showed no statistically significant difference ($p>0,05$). The most significant, but not statistically significant variation could be shown between block I and III ($p=0,121$) as one could assume by the different heights of the median values in the boxplots. The significance level of the different blocks can be found below in table 7.

Table 7: overview of the significance level of the different intensity blocks

Block	Mann-Whitney-U test
I versus II	p=0,756
I versus III	p=0,121
II versus III	p=0,136

Figures 8 to 10 show the irisin results of each participant for the three blocks of different exercise intensity.

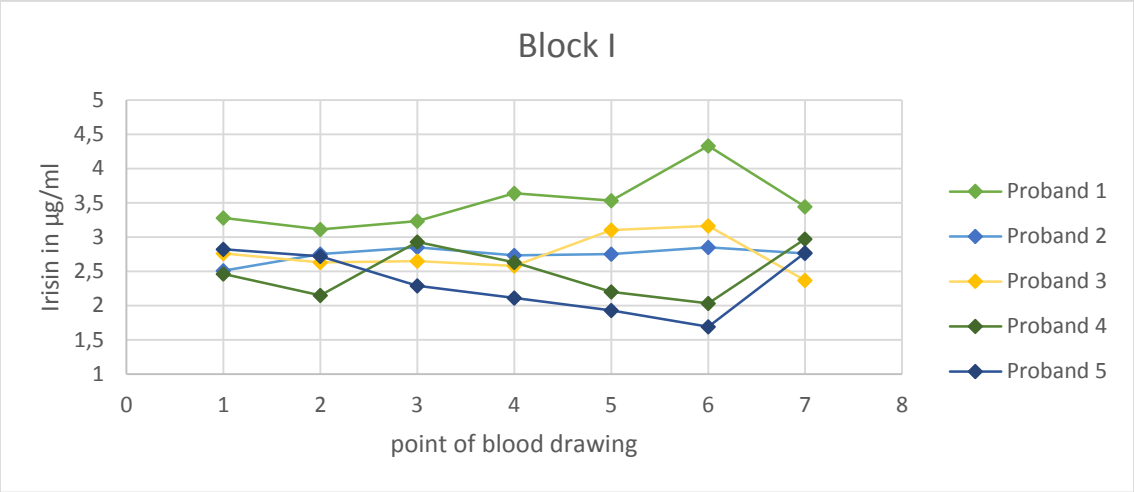

Figure 8: irisin results of each participant at each sampling point in the first block

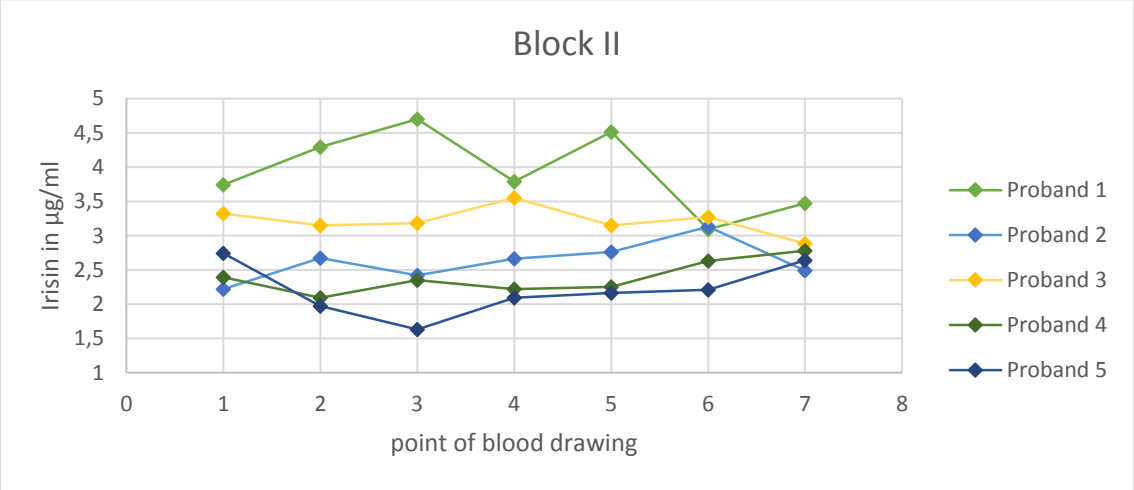

Figure 9: irisin results of each participant at each sampling point in the second block

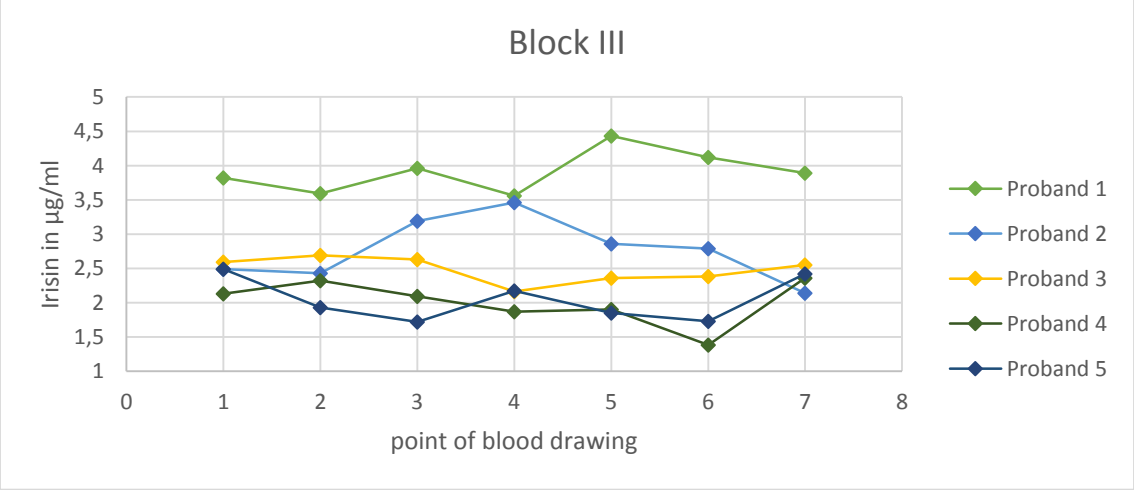

Figure 10: irisin results of each participant at each sampling point in the third block

5.1.5.2 Comparison of measurements per block without baseline values

The first and fourth irisin measurement could be labelled as baseline values of the single and the serial exercise sessions. In this chapter the irisin levels of each block were compared without those baseline measurements.

Shapiro-Wilk test indicated that all three blocks were normally distributed ($p>0,05$) and parametric tests could be used further on. All blocks were compared to each other, but no statistical significance was revealed.

Table 8: overview of the significance level of the different intensity blocks without baseline values

Block	T-test
I versus II	p=0,576
I versus III	p=0,486
II versus III	p=0,275

5.1.6 Comparison of individual irisin levels

The irisin measurements of each proband were analysed individually. Interestingly, the first test person had higher levels than all the other ones. The results are pictured in figure 11 below. Analysing the irisin results for each proband via Kruskal-Wallis-test, the individual irisin results were statistically significant different ($p<0,05$).

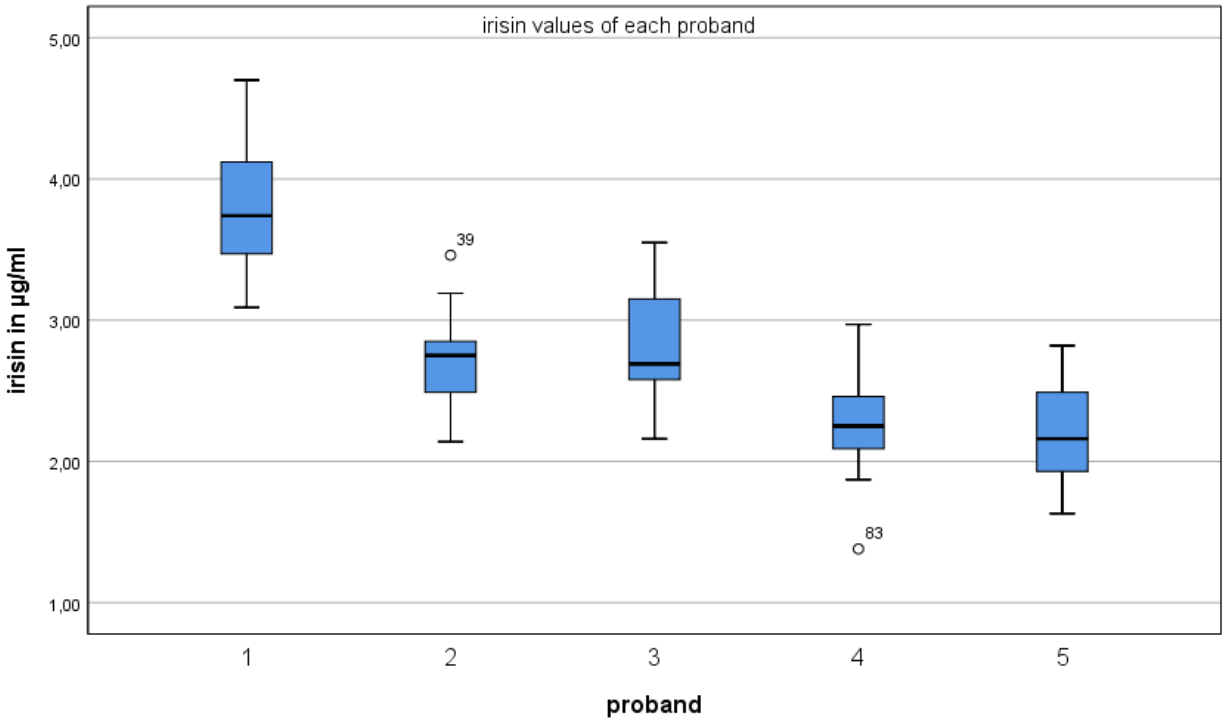

Figure 11: boxplots of irisin measurements per proband

5.1.7 Relative irisin measurements

To highlight eventual differences between the participants irisin results were put in a valid relation to one another. The percentages of the irisin values were calculated for each participant and each block with the baseline value resembling 100%. Figures 12 to 14 show the differences between irisin percentages at each time point. We set the baseline values at zero for better visualization of the dynamics.

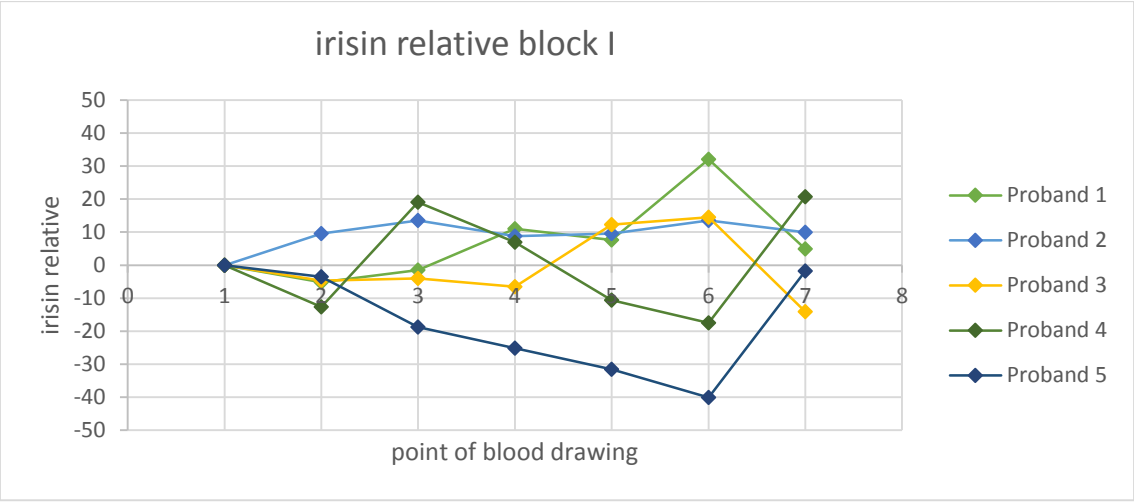

Figure 12: relative irisin values of the first block of all five probands

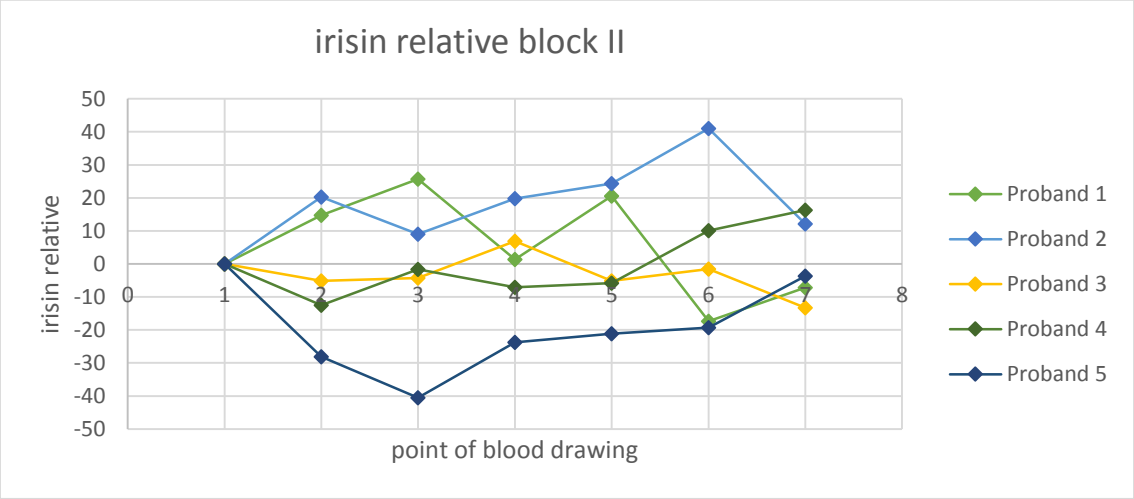

Figure 13: relative irisin values of the second block of all five probands

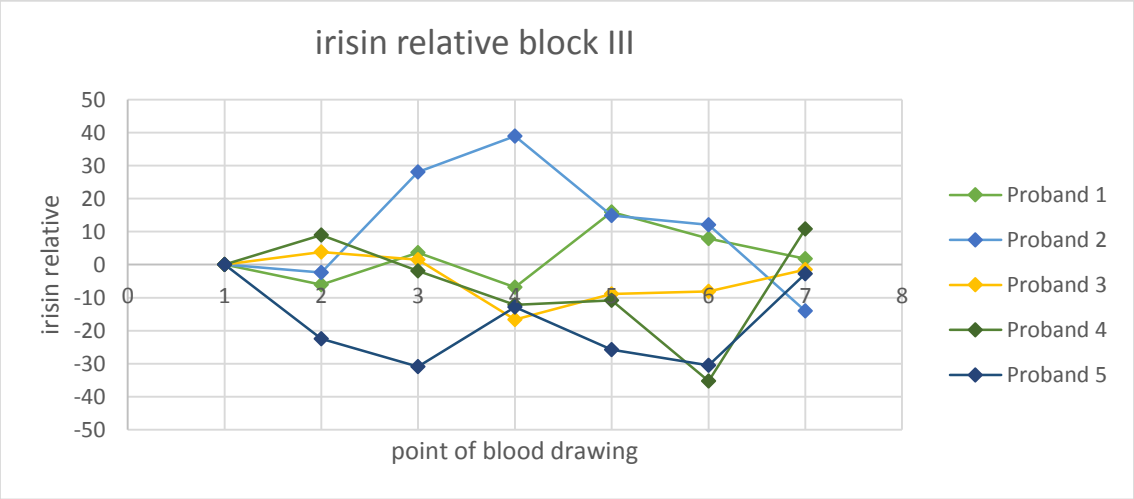

Figure 14: relative irisin values of the third block of all five probands

The relative irisin changes were also analysed for each test person individually. Relative irisin levels before and after physical activity were compared, but they increased and declined with no cohesive or repetitive pattern, no matter which block was looked at.

Table 9: overview of the rising (↑) and falling (↓) tendency of percentage irisin results per block per proband; the baseline and the last measurement were compared

	Block I	Block II	Block III
Proband 1	↑	↓	↑
Proband 2	↑	↑	↓
Proband 3	↓	↓	↓
Proband 4	↑	↑	↑
Proband 5	↓	↓	↓

5.1.8 Comparison of relative irisin levels after different intensities of exercise

Tests on standard distribution showed that the percentage irisin results of all three intensities were normally distributed. The boxplot (Figure 15) shows the three groups of different intensity next to each other. To check if there was a difference between the individual groups, T-test was performed. It showed no statistically significant variation (p>0,05). The significance level of the different blocks can be found below in table 10.

Table 10: overview of the significance level of the relative differences of the various intensity blocks

Block	T-test
I versus II	p=0,95
I versus III	p=0,45
II versus III	p=0,44

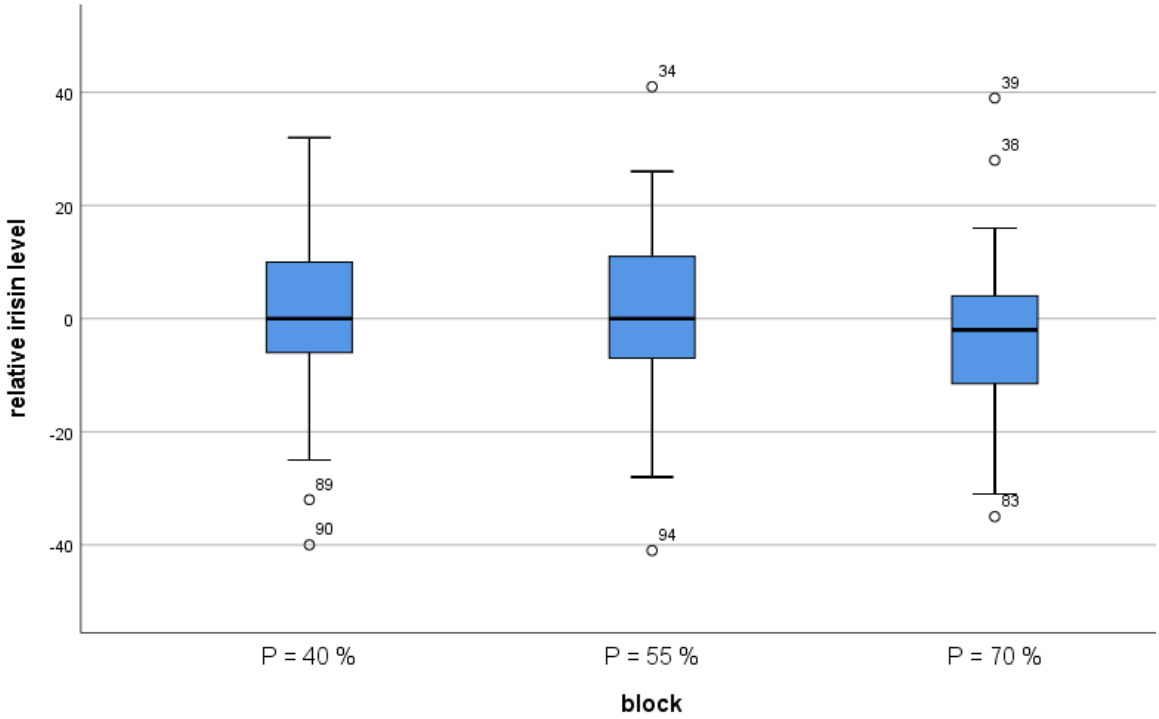

Figure 15: boxplots of the relative irisin levels divided by the three different intensities

5.1.9 Correlation of irisin levels and hours of fasting

To check if there was a correlation between irisin levels and the hours of fasting, linear regression was performed. The duration of fasting did not correlate with the concentration of irisin as figure 16 supposes and the correlation coefficient of Pearson (R=0,072) confirms.

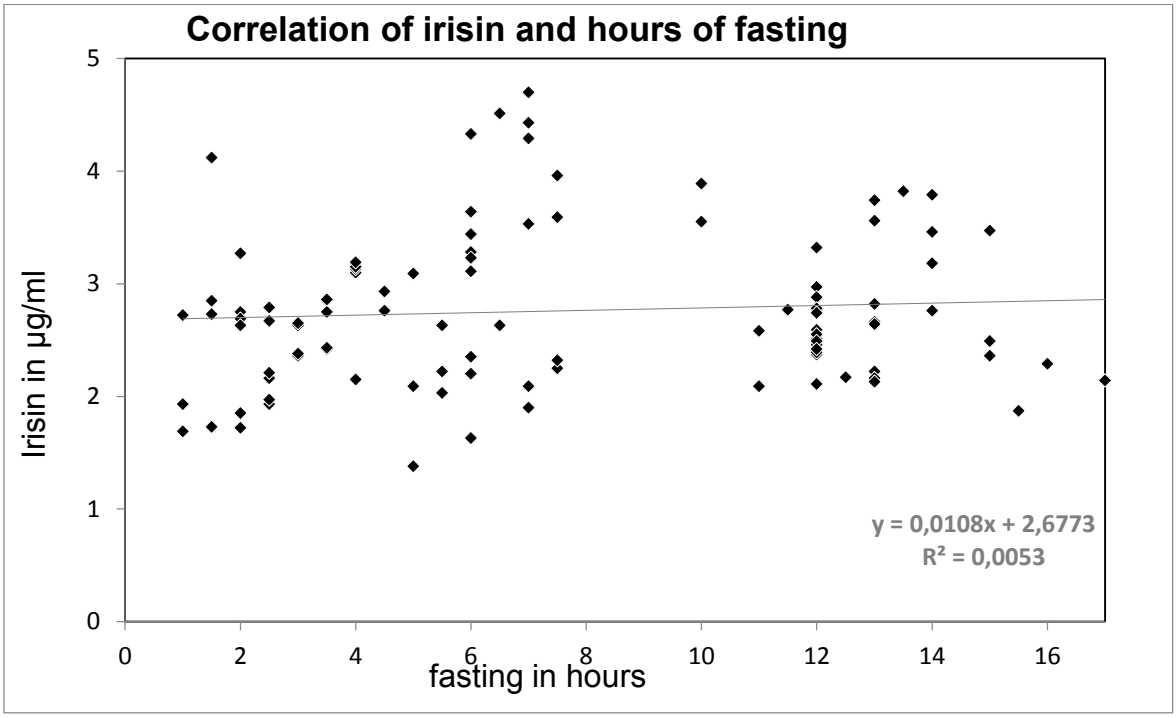

Figure 16: correlation of irisin and the hours of fasting

5.1.10 Correlation of irisin and laboratory parameters

To determine whether there was a correlation between irisin results and various laboratory and anthropometric parameters, linear regression was used. First, the mean irisin level of each proband as well as the BMI, which stayed the same during the study, were put in the diagram. Pearson´s coefficient of correlation (R= 0,63) indicated a moderate positive correlation.

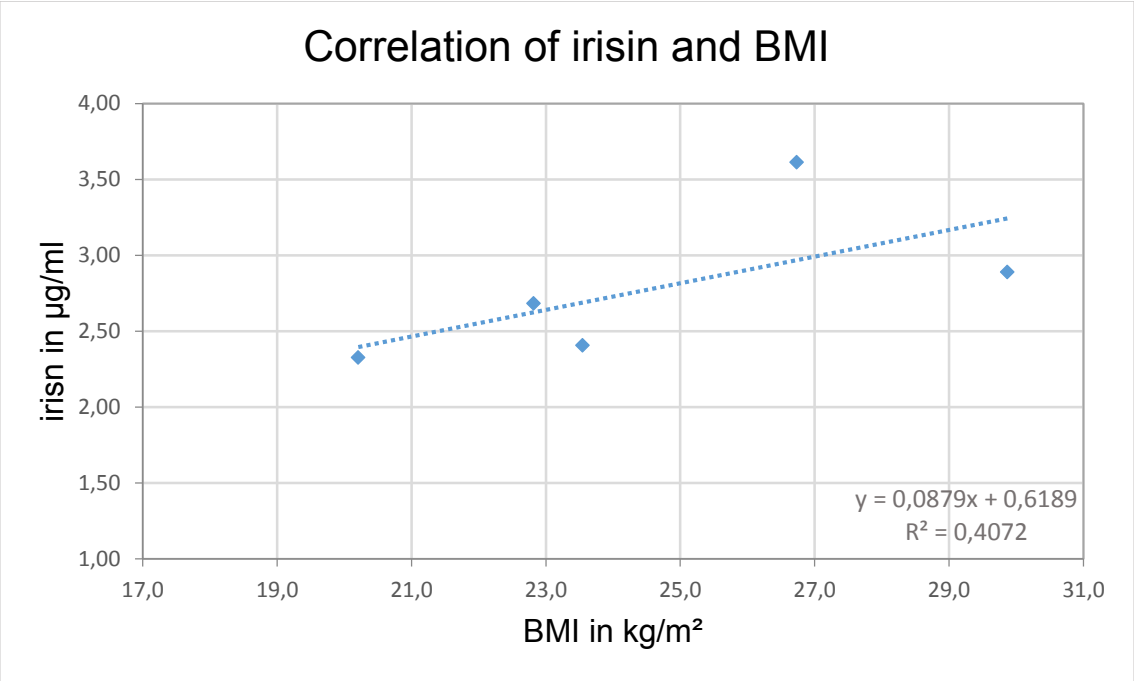

Figure 17: correlation of irisin and the body mass index

Furthermore, it was tested, if there was a correlation between irisin levels and the age of probands. As you can see below in figure 18, no correlation (R= -0,1) could be found.

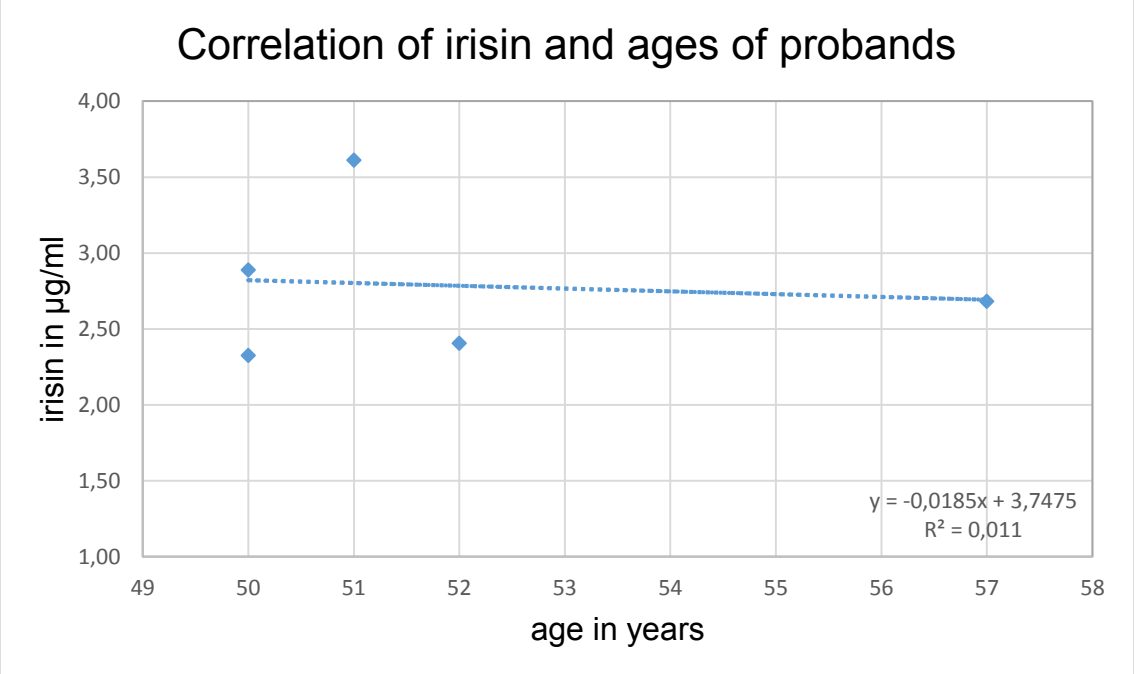

Figure 18: correlation of irisin and the age of probands

No strong, but a moderate negative correlation between irisin values and cholesterol levels could be found (R= -0,62).

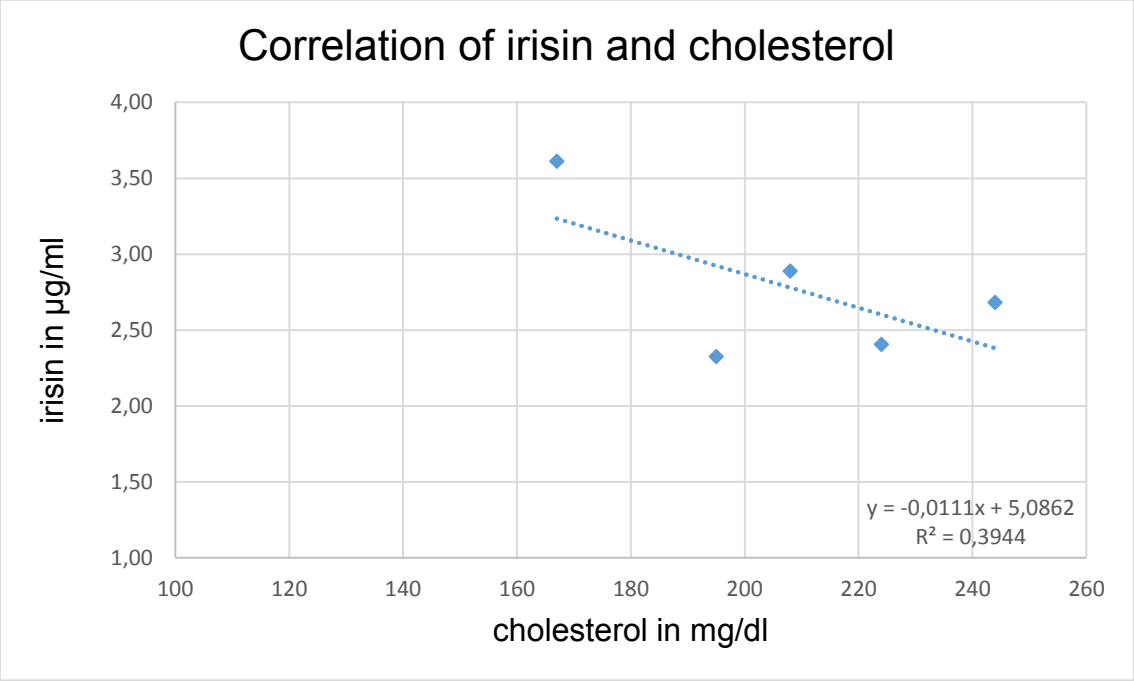

Figure 19: correlation of irisin and cholesterol levels

Considering Pearson´s coefficient of correlation (R= -0,25), no correlation could be found between the body mass index and cholesterol levels.

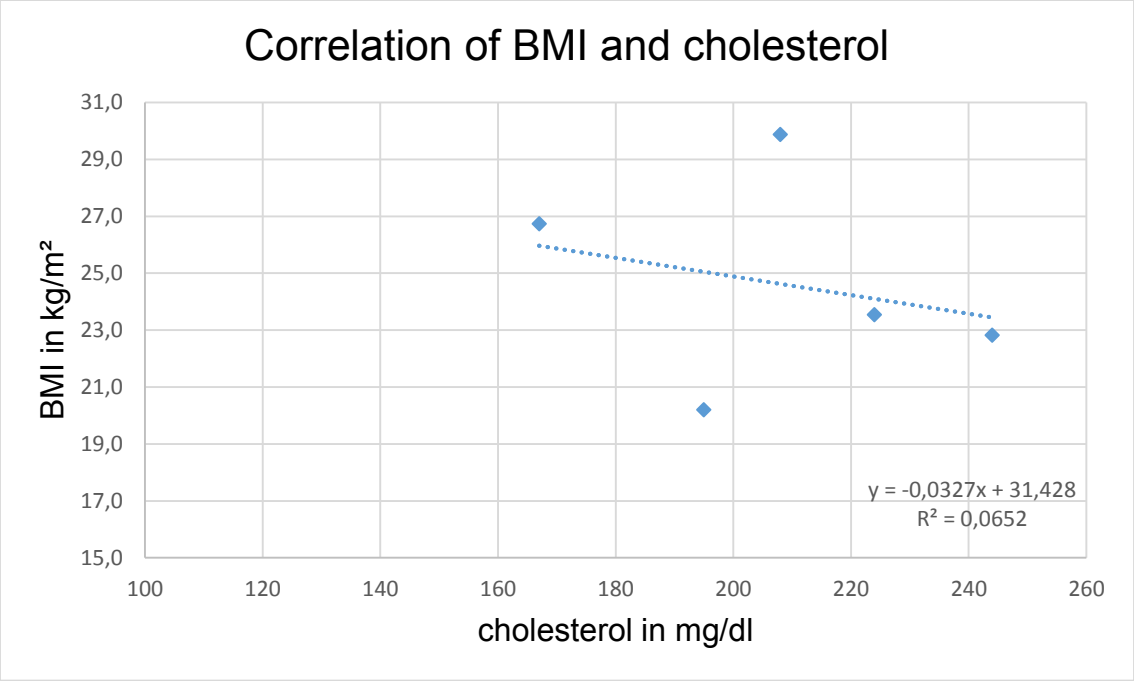

Figure 20 : correlation of the body mass index and cholesterol levels

As described in 1.3, irisin is associated with the protein sclerostin. In this study linear regression showed a poor model fit for irisin and sclerostin (R= -0,33), but a good model fit (R=0,97) for sclerostin values and the age of the test persons.

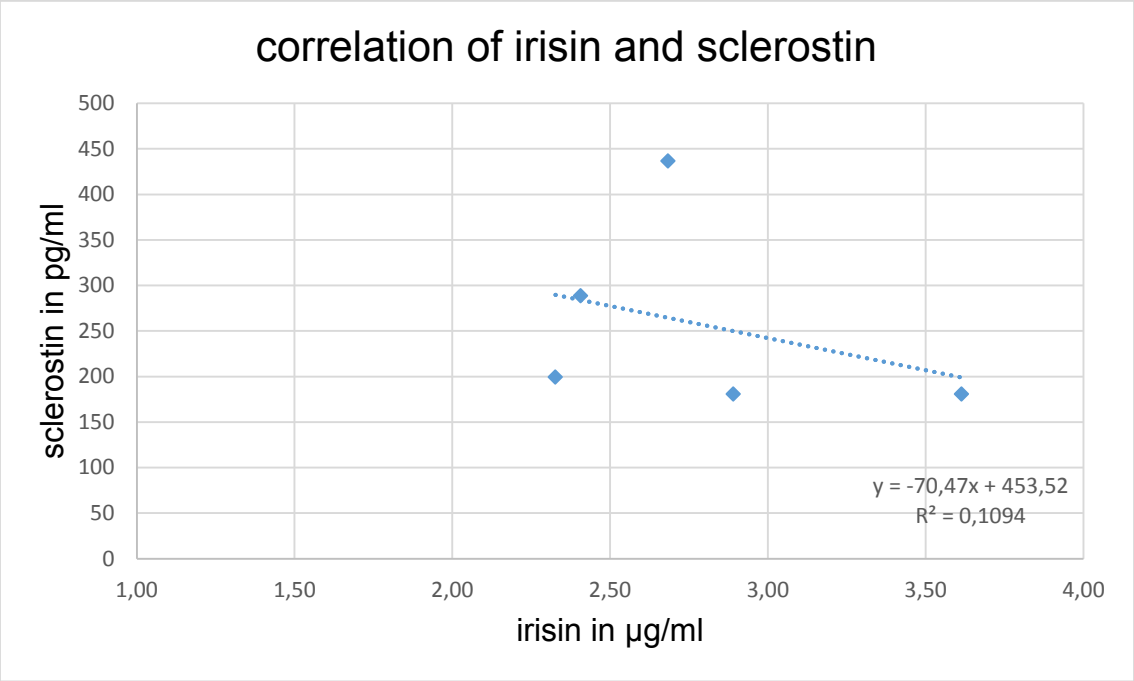

Figure 21: correlation of irisin and sclerostin

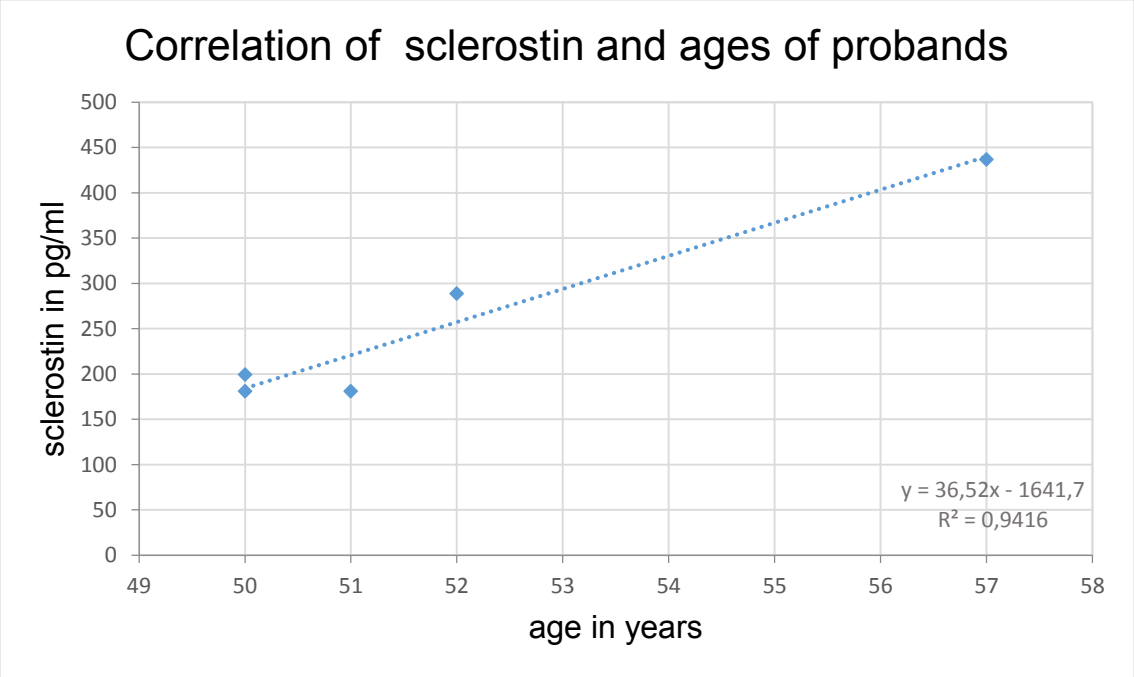

Figure 22: correlation of sclerostin and age of probands

5.2 Animal study

In addition to the human model, a part of the study also investigated the effects of physical activity in female Sprague-Dawley rats.

5.2.1 Body weight

Both diet groups showed a gain in body weight. After eight months, exercising rats with a normal diet weighed on average 382 grams (+53%), whereas exercising rats with a high fat diet weighed averaged 463 grams (+85%). Compared to non-exercising animals, regular physical activity resulted in a smaller gain of body weight in both diet groups. Comparing the control high fat diet group to the control normal diet group, a significant difference in body weight could be shown (p<0,001). Nevertheless, the body weight of exercising high fat diet rats was significantly lower than the body weight of non-exercising high fat diet rats (p=0,008). The mean body weight of the groups with their standard deviation, minima and maxima are shown in table 11. The percentage rise of the body weight was based on the first measurement which was 250 grams.

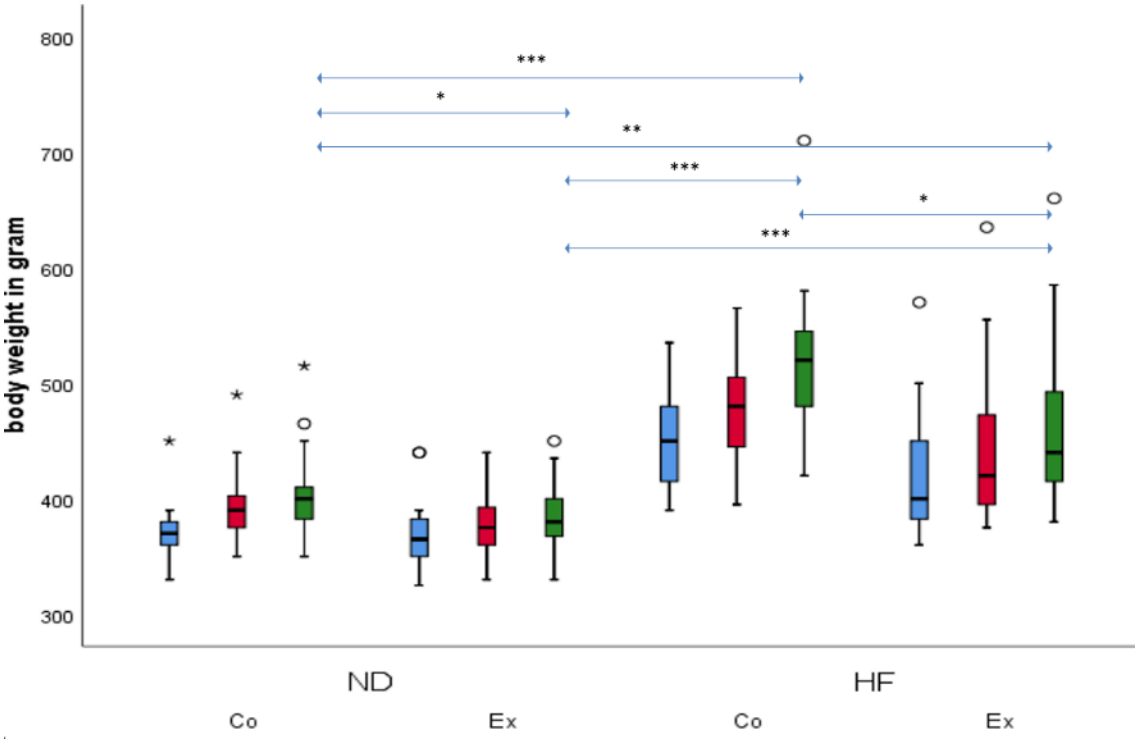

*Figure 23: body weight in gram measured after 4 (blue boxplot), 6 (red boxplot) and 8 (green boxplot) months in normal diet and high fat diet animals, with exercising and non-exercising subgroups; * p<0.05, ** p<0.01, *** p<0.001*

Table 11: bodyweight in gram after eight months; means, minima, maxima and weight gain in percent (based off of 250 grams at the beginning) of the control normal diet group (coND), exercise normal diet group (exeND), control high fat diet group (coHFD) and exercise high fat diet group (exeHFD)

	Bodyweight in gram after 8 months					
Group	N	Mean	SD	Min	Max	% weight gain
coND	10	405	35	350	515	62
exeND	10	382	28	330	450	53
coHFD	10	517	63	410	710	107
exeHFD	10	463	67	380	660	85

5.2.2 Irisin

After eight months of training irisin concentrations were compared before and directly after an exercise session. In both diet groups no significant difference could be shown. In detail, neither in the normal diet group nor in the high fat diet group a significant difference in irisin levels could be found before and after exercise. Interestingly, there were also no differences between the diet groups.

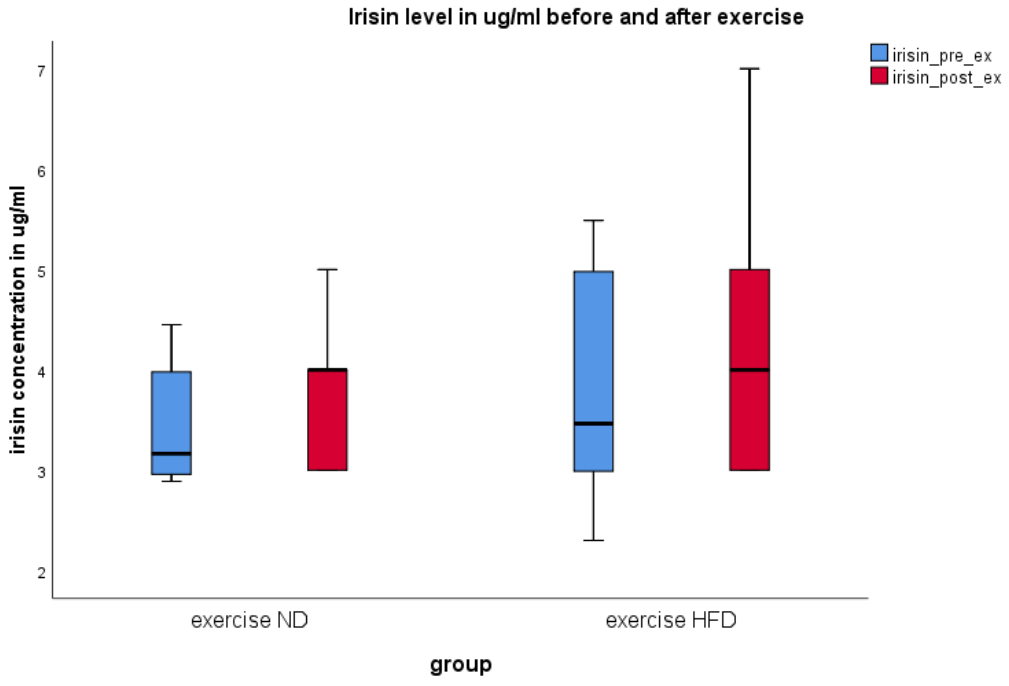

Figure 24: irisin levels before (blue boxplots) and after (red boxplots) exercise in normal and high fat diet rats

The mean irisin levels with their standard deviation, minima and maxima are listed in the following table.

Table 12: mean irisin levels (µg/ml) of the normal diet group (ND) and the high fat diet group (HFD) pre and post exercise

	Irisin levels (µg/ml)				
Group	N	Mean	SD	Min	Max
exeNDpre	10	3,41	0,54	3	4
exeNDpost	10	3,90	0,73	3	5
exeHFDpre	10	3,81	1,13	2	5
exeHFDpost	10	4,40	1,34	3	7

5.2.3 Sclerostin

Before exercising, baseline sclerostin levels were significantly lower in the high fat diet group compared to the normal diet group. The significant decrease (p = 0.002) in baseline sclerostin levels in high fat animals was no longer present post-exercise (p = 0.108). Also, exercise did not show any effect in the normal diet group.

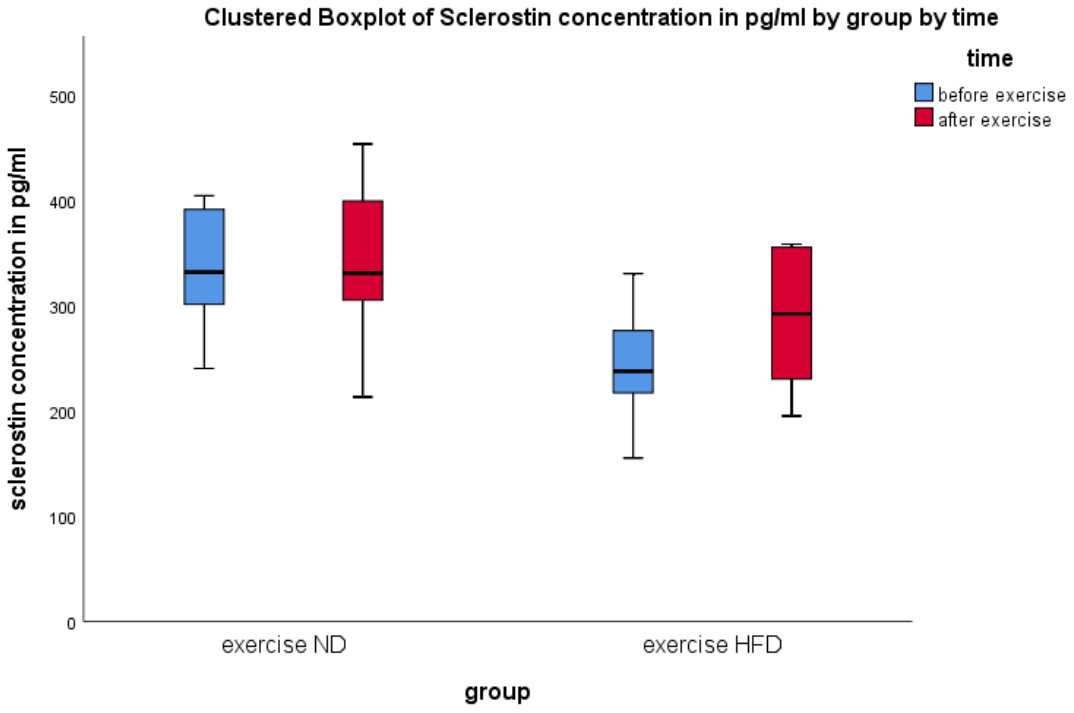

Figure 25: sclerostin concentration before (blue boxplots) and after (red boxplots) exercise in normal and high fat diet rats

*Table 13: mean sclerostin levels (pg/ml) of the normal diet group (ND) and the high fat diet group (HFD) pre and post exercise; * baseline levels of sclerostin were significantly lower in the high fat diet group compared to the normal diet group (p=0,002), post exercise there is no significant difference*

	Sclerostin levels (pg/ml)				
Group	N	Mean	SD	Min	Max
exeNDpre	10	333,5*	56	240	404
exeNDpost	10	338,5	70	213	453
exeHFDpre	10	244,7*	52	155	330
exeHFDpost	10	289,5	59	195	358

5.2.4 Correlation of body weight, irisin and sclerostin

Body weight, irisin and sclerostin measurements were analysed if any correlation could be found. As shown in the diagrams below and the corresponding coefficients of determination, no statistically significant correlation was detectable between the body weight and irisin, the body weight and sclerostin or irisin and sclerostin. P-values of those correlations are over 0,05 (body weight and irisin p = 0,16, R=0,327, body weight and sclerostin p = 0,09, R=0,386, sclerostin and irisin p = 0,62, R=0,115).

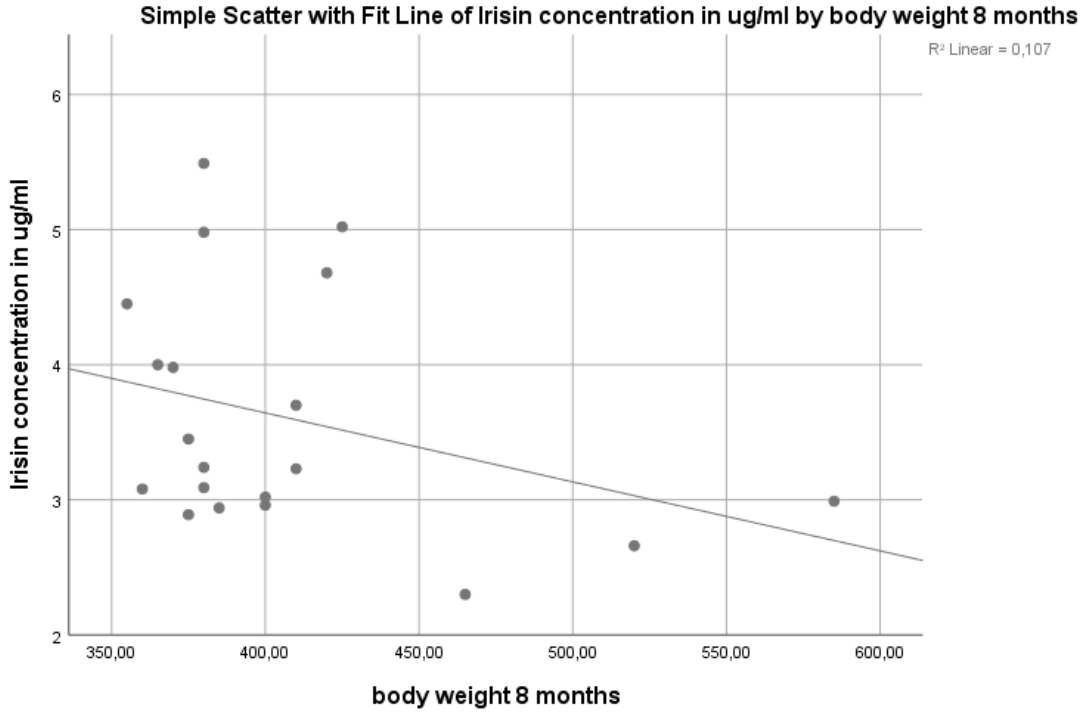

Figure 26: scatter plot of the body weight in grams and irisin concentration in µg/ml

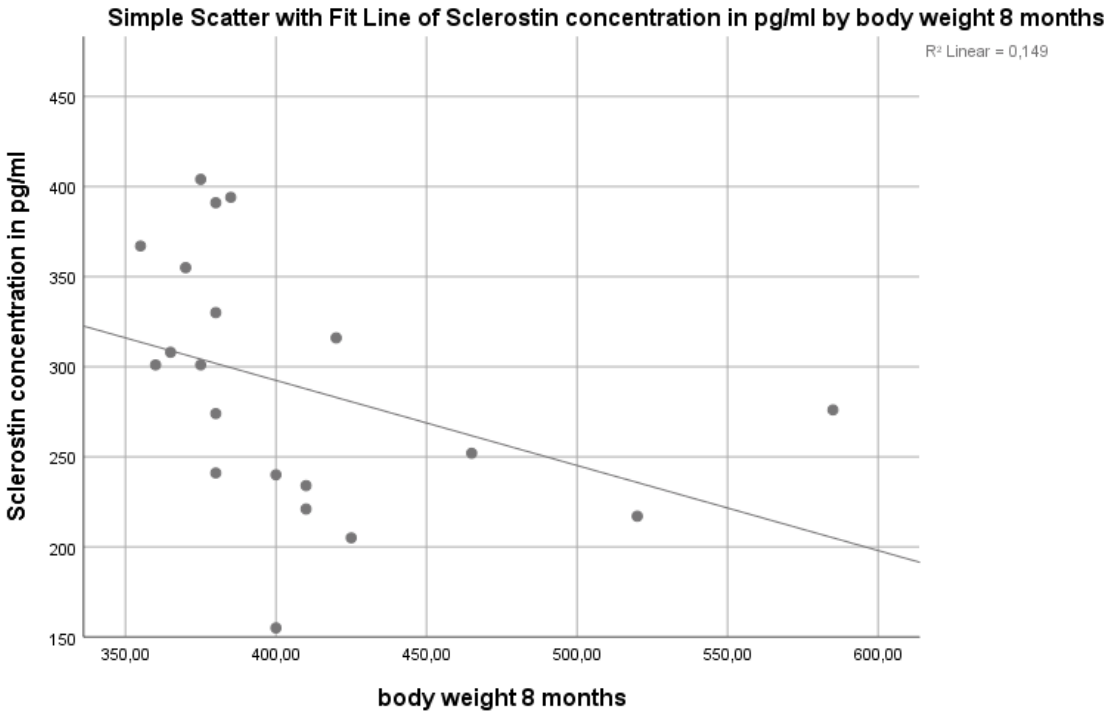

Figure 27: scatter plot of the body weight in grams and sclerostin concentration in pg/ml

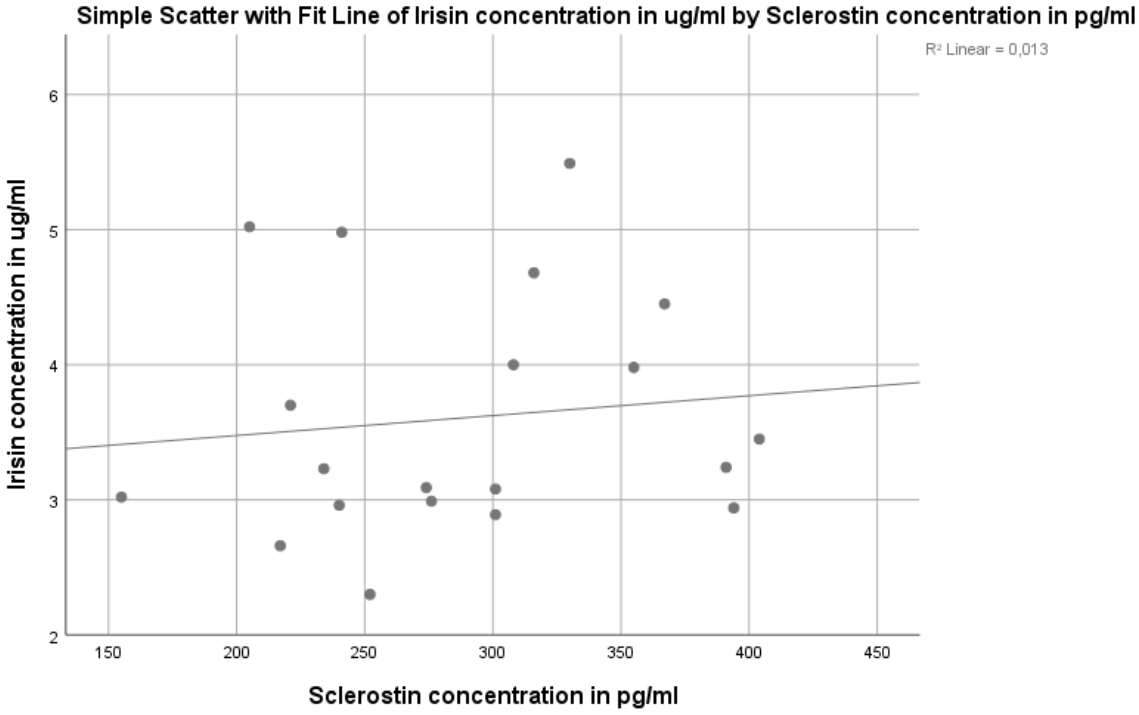

Figure 28: scatter plot of sclerostin concentration in pg/ml and irisin concentration in µg/ml

6 Discussion

6.1 Human pilot study

The aim of the human pilot study was to determine possible individual effects of physical activity on irisin and sclerostin expression. The suspected hypothesis, that expression of irisin is induced by physical activity, was examined, but could not be verified. In the conducted pilot study, the irisin baseline parameters ranged from 2,13 µg/ml to 3,82 µg/ml. This matches the information of the manufacturer, where an irisin range from 0,2 to 5 µg/ml is given. Hwang et al. (27), Duran et al. (45) and Colaianni et al. (33) reported similar ranges of irisin results, whereas for example Huh et al. (10), Cai et al. (46) and Fukushima et al. (47) announced levels about 1 to 150 ng/ml. To this date, mass spectrometry is viewed as the gold standard. Using this technique, the usual concentration of irisin found in human blood is 3-5 ng/ml (21). Overall, it seems as if there are more studies with value ranges in the nanogram than in the microgram area, as this study does. For a quick overview, a few studies and their value ranges can be found in the annexe. Due to the fact that different studies had different settings and that there is no standardisation of irisin range, we used the first measurements as our reference values.

Performing linear regression on irisin, sclerostin and other laboratory parameters a moderate negative correlation between irisin and cholesterol could be shown. Here we have to mention the slightly higher cholesterol levels of one of our probands. Nevertheless Oelmann et al. also ascertained significant inverse associations between irisin and cholesterol levels in a population-based study in Pomerania (48) as well as Benedini et al. (49). In contrast a moderate positive correlation between irisin and BMI appeared in our study. One could assume that this mismatches the correlation above, but actually BMI does not correlate with cholesterol levels. This is also shown by Hussaid et al., at least in diabetes patients in 2019 (50). Regarding the influence of fasting onto irisin levels, no significant correlation could be found. Bearing in mind that all of our probands were between 50 and 60 years old, sclerostin and age of probands showed a strong positive correlation. When comparing the individuals´ irisin levels, it seems as if every person has their characteristic range. The individual irisin measurements were significantly different from the measurements of the other participants ($p< 0,05$). This phenomenon was also recognised previously, e. g. plasma sodium clustered around a patient-specific

point (51). As mentioned above linear regression on irisin and BMI could show a moderate positive correlation. This might indicate that the different constitution of each individual proband is one possible reason for the characteristic range of irisin levels. Additional to personal specifics of laboratory parameters, another potential explanation of the different irisin levels could also be the difference in age of probands. In this study the age gap was not substantial, but differences of irisin levels depending on the age have been shown beforehand (52).

The suggested different expression of irisin depending on the intensity of the physical activity (53) could not be reproduced. Although the probands underwent three different intensities of ergometric exercise no statistically significant difference appeared. Admittedly, the intervals between exercising and blood drawing were much closer in the study of Tsuchiya et al. (53) than in this pilot study. This might influence the different outcome. Newer studies show contrary results: irisin levels even decrease directly after physical exercise (26, 54).

We have to clearly point out that the results of the pilot study are not suitable to generate an overall hypothesis regarding physical activity and myokines, e.g. irisin and sclerostin because of the small number of probands and time points of blood collection. When summarizing the conclusions it should also be kept in mind that the methods and training protocols vary from study to study and therefore might lead to different results (12).

6.2 Animal study

The results of the animal study are in accordance with those of the human pilot study where no statistically significant differences in irisin or sclerostin expression before and after physical activity could be shown. The study design is in line with previous studies (55), therefore we can refer to our results in this setting as robust. One clear confirmation of this study is the lower gain in body weight due to regular physical exercise, regardless of the dietary habits. Both diet groups had a gain in body weight, but in the normal diet group this was due to the natural growth of the initially juvenile rats. The rats of the normal diet group gained less body weight than the high fat diet group rats, and above all the exercising rats gained significantly less than the control group within the high fat diet group.

In our study sclerostin baseline levels were significantly lower in the high fat diet group compared to the normal diet group, but this fact was no longer present after physical exercise. In contrast Liao et al. (56) found exercise to be leading to a decrease of sclerostin levels. The study size was similar and they used the same breed of rats, but the animals were suffering from chronic kidney disease. The exercising animal group had significantly lower sclerostin levels than the group without exercise. (56) Here it has to be considered that a different study design was used, above all the examined animals suffered from chronic kidney disease. A chronic disease is likely to influence the expression of biomarkers and a direct comparison is therefore not reasonable.

Regarding the correlation between irisin, sclerostin and body weight, only a mild positive correlation between sclerostin and body weight, but no statistical significance could be shown. Potentially, there might be a statistically significant difference if the study was conducted on a larger number of animals.

6.3 Limitations and conclusion

Taken together, neither the human nor the animal study showed statistically significant different blood concentrations of irisin or sclerostin before and after physical exercise. Although we could not demonstrate a detectable effect in blood, one may speculate that physical exercise leads to an increased expression in muscle tissue, as Archundia-Herrera et al. investigated (16). In a cross-sectional study with thirty overweight adolescents they could find increased muscle irisin levels after high intensity-interval-trainings whereas the plasma irisin levels did not rise. We can conclude that in the rat model (and in other studies this is valid for humans too (57)) physical activity reduces the gain in body weight, compared to non-exercising individuals. There might be a connection between irisin levels and cholesterol values, irisin levels and BMI as well as sclerostin and age.

Due to the small number of probands and potentially the points of blood drawing, the human pilot study did not generate valid data to support the hypothesis that physical exercise induces the expression of irisin and sclerostin in blood. However, numerous references found a connection between those myokines and physical activity, but the exact pathways remain unknown. To comprehend the regulation of irisin and sclerostin, further research has to be done.

7 List of literature

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Annexe

Table 14: overview of irisin values from different studies

Study/ source	Irisin range	Method	Cohort
AdipoGen Life Sciences Irisin Competitive ELISA Kit MANUAL	0,2 to 5 µg/ml	Enzyme-linked immunoassay	
Jedrychowski et al. (21)	3 to 5 ng/ml	Tandem mass spectrometry	6 healthy males, 4 sedentary males as control
Choi et al. (58)	770 to 1140 ng/ml	Enzyme-linked immunoassay	401 men and women
Huh et al. (10)	50 to 166 ng/ml	Enzyme-linked immunoassay	117 middle-aged women
Cai et al. (46)	9.31 ± 2.45 ng/ml	Enzyme-linked immunoassay	575 primary schoolers
Hwang et al. (27)	1,2 to 2,6 µg/ml	Enzyme-linked immunoassay	424 healthy, pre-diabetic or diabetic men and women
Fukushima et al. (47)	1.20 ± 0.20 ng/ml	Enzyme-linked immunoassay	22 obese men and women
Duran et al. (45)	1: 3.16 ± 0.3 µg/ml 2: 2.86 ± 0.6 µg/ml 3: 2.83 ± 0.5 µg/ml	Enzyme-linked immunoassay	263 women 1: healthy, 2: impaired glucose tolerance or impaired fasting glucose, 3: diabetes type 2
Colaianni et al. (33)	2.59 ± 1.15 µg/ml	Enzyme-linked immunoassay	34 healthy children

Table 15: routine laboratory parameters

N=5	Mean	Min	Max	Sd
Fasting glucose (mg/dl)	90,6	77	100	8,38
TSH (µU/ml)	1,416	0,7	2,39	0,54
ALT (U/l)	20,4	15	26	4,54
AST (U/l)	22,6	17	25	2,87
Creatinine (mg/dl)	0,8	0,72	0,88	0,05
eGFR (ml/min/1,7)	85,392	72,81	97,54	8,06
Cholesterol (mg/dl)	207,6	167	244	26,08
LDL (mg/dl)	111,8	73	145	25,54
HDL (mg/dl)	79,8	66	87	7,98
CK (U/l)	104,8	60	168	39,68