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Impact of an High Protein Diet in Healthy Young Men

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Verfasserin / Verfasser: Rita Leonore Babeluk

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Betreuerinnen: Ao. Univ.-Prof. Dipl.-Ing. Dr. Marcela Hermann
Dipl. – Ing. Dr. Maria Zellner

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1. Abstract

Backround: Meat and animal products are one of the most favourite foods in the western world. There are some high protein (HP) studies that show a positive effect on on sarcopenia, glucose homeostasis, bone health, weight management and satiety. But there are only a few studies that characterise the physiological effects of a HP diet. Further there is no study done yet that investigate the impact of a HP diet on certain proteomes.

Objective: In this study the impact of an HP diet on platelet proteome was explored, whereas the platelets were used as a model for muscle cells since they have several similarities like the contractile protein system. Further there are several studies concerning changed protein diet and the homocysteine pathway. However, their results are very contradictorily. In this study we want to adduce the final evidence about the correlation of homocysteine, folate and vitamin B_{12} and a HP diet.

Methods: In this randomised intervention study 23 young men took part. In the one week run in period all individuals consumed a normal protein (NP) diet (1.5 g protein/kg body weight). In the intervention period they were separated into two groups: a HP group (3.0 g protein/kg body weight) and a NP group. In both diets the protein derived from an animal source. Intervention time lasted for 3 weeks. Blood was taken and cognitive tests were performed at the beginning and at the end of the intervention time. Platelets were isolated and analysed with 2 D DIGE analysis.

Results: The protein that showed the strongest effect with HP diet in platelets was Monoamine oxidase B (Mao-B) wich is a dopamine degrading enzyme. It was significantly decreased with HP diet. Vitamin B_{12} concentration was significantly increased with HP diet in plasma and correlated inversely with the Mao-B concentration in platelets. Further the improvement of a reaction time test correlated with the decrease of Mao-B in plateles.

Discussion: The inverse correlation between vitamin B_{12} and Mao-B could indicate that vitamin B_{12} is directly regulating Mao-B expression. Further the improved reaction time indicates enhanced cognitive functions when consuming an animal HP diet. These improved cognitive functions could be explained with the correlation of the reaction time test, Mao-B and components of the methylation cycle like vitamin B_{12}.

2. Zusammenfassung

Hintergrund: Fleisch und tierische Produkte sind eines der beliebtesten Nahrungsmittel in der westlichen Welt. Es gibt einige Studien über erhöhten Protein Konsum die einen positiven Effekt in Bezug auf Sarkopenie, Glukose Homeostase, Knochengesundheit, Gewichtsmanagement und Sättigungsgefühl zeigen. Es gibt jedoch nur wenige Studien, die die physiologischen Effekte einer Ernährung mit erhöhtem Proteinanteil zeigen. Weiters wurde bis jetzt noch keine Studie durchgeführt, die sich auf den Einfluss einer Diät mit erhöhtem Proteinanteil auf Proteome konzentriert.

Zielsetzung: In dieser Studie wurde der Einfluss einer Diät mit erhöhtem Proteinanteil auf das Thrombozytenproteoem untersucht, wobei Thrombozyten als Modell für Muskelzellen verwendet wurden, da diese verschiedene Ähnlichkeiten besitzen, wie zum Beispiel das kontraktile System. Weiters gibt es einige Studien die den Zusammenhang von Diäten mit verschiedenen Proteinmengen mit dem Homocysteinzyklus beschreiben. Diese sind jedoch sehr widersprüchlich. In dieser Studie wollen wir den eindeutigen Beweis für einen Zusammenhang von Homozystein, Folat und Vitamin B_{12} mit einer Diät mit erhöhtem Proteinanteil beweisen.

Methoden: An dieser randomisierten Studie nahmen 23 junge Männer teil. In einer einwöchigen Einlaufphase konsumierten alle Teilnehmer eine Diät mit einer normalen Menge an Protein (1,5 g Protein/kg Körpergewicht). In der Interventionszeit wurden sie in zwei Gruppen aufgeteilt: eine Gruppe mit erhöhtem Proteinkonsum (3,0 g Protein/kg Körpergewicht) und eine Gruppe mit normalem Proteinkonsum. Bei beiden Diäten war die Proteinquelle eine tierische. Die Interventionszeit dauerte 3 Wochen. Zu Beginn und am Ende der Interventionszeit wurde den Probanden Blut abgenommen und kognitive Tests wurden durchgeführt. Die Thrombozyten wurden isoliert und mittels 2 D DIGE Analyse analysiert.

Ergebnisse: Das Protein das den stärkesten Effekt nach einer Diät mit erhöhtem Proteinanteil zeigte ist Monoaminoxidase B (Mao-B), welches ein Dopamin abbauendes Enzym ist. Es war signifikant vermindert nach einer erhöhten Protein Diät. Die Vitamin B_{12} Konzentration im Plasma war signifikant erhöht nach einer erhöhten Proteindiät und korrelierte invers mit der Mao-B Konzentration in den

Thrombozyten. Weiters korrelierten die Ergebnisse des Reaktionstests mit dem Abfall von Mao-B in Thrombozyten.

Diskussion: Die inverse Korrelation von Vitamin B_{12} mit Mao-B könnte daruf hindeuten, dass Vitamin B_{12} Mao-B direkt reguliert. Weiters zeigt die verbesserte Reaktionszeit verbesserte kognitive Funktionen bei einer erhöhten Protein Diät auf tierischer Basis. Diese verbesserten kognitiven Funktionen könnten durch die Korrelationen von Reaktionstest, Mao-B und den Komponenten des Methylierungszyklus, wie z.B.: Vitamin B_{12}, erklärt werden.

3. Introduction

Meat intake is an important contributor to dietary protein and therefore has a potential impact on a range of nutritional and health outcomes. In western countries meat is an important part of the daily menu. Especially in Austria meat is one of the most favourite foods. (Grüner Bericht 2004)

Nitrogen balance studies have led to the calculation of the RDA (recommended dietary allowance) for protein for young healthy adults.(Campbell 2002; Castaneda 1995) However, only a few studies have addressed the question about the biochemical consequences of changed protein amount intake in humans. Also most of the studies that were done concern low protein intake. Castenada et al. showed for example that a low protein diet in elderly women (0.45 g protein / kg / day compared to 0.92 g protein / kg / day) leads to a significant loss of muscle function, immune response and lean tissue mass, though they all had a neutral nitrogen balance, after a 9 weeks study period. (Castaneda 1995)

There are some high protein (HP) studies showing a positive effect on sarcopenia, glucose homeostasis, bone health, weight management and satiety (Rodriguez 2008) but only a few studies that address the physiological effects. Mikkelsen et al. showed for example that a HP diet increases the 24 h energy expenditure with 3% compared to a low protein diet. Further they showed that animal protein induced 2 % higher increase in 24 h energy expenditure than vegetable protein did. (Mikkelsen 2000)

Although an increased meat diet is frowned upon, relatively little evidence has been gathered regarding the effect of protein on the development of chronic diseases. While there was initial suspicion that HP diets increased the risk for renal failure, studies have shown that kidney problems occur only in people with pre-existing kidney disease. It has also been shown that there is no correlation between HP, animal HP diet and gout. Gout is the most common form of inflammatory arthritis. (Choi 2004) It is arising by the release of monosodium urate cristals into synovial spaces, causing an inflammatory response and thereby the recurrent attact of acute arthritis. (Williams 2008) Since uric acid is the end product of the purine metabolism, and purine derives mainly from diet, gout is significanty influenced by nutrition. Purine rich foods and beverages are meat (especially offal) beer and some vegetables like spinach, peas, beans, mushrooms and asparagus. Low purine foods are for examle

dairy products or fruits. (Fam 2002) Choi et al. showed a coherence between increased meat consumption and gout, but as said before there was no coherence found regarding to an animal HP diet, since this includes dairy product which decrease the risk. (Choi 2004)

In recent years, health concerns have been raised about the consumption of meat increasing the risk of cancer. Taylor et al. found a correlation between meat consumption and the risk for breast cancer in pre- and postmenopausal women. (Taylor 2007) Another study showed that increased red and processed meat consumption can increas the risk for pancreatic cancer. (Nothlings 2005)

Only a few studies have concentrated on the physiological effects of a HP diet that derives from animal source. In order to understand the possible effect of animal HP intake on physiological functions, it is necessary to know the underlying cause of change in the cell (Proteomics), and the relation between these changes and biochemical regulators of the processes.

In this study probable changes in plasma proteome, plasma amino acids and in platelets proteome were examined. Platelets were used as a peripheral model for muscle cells.

3.1. Digestion and absorption of proteins and amino acids – protein and amino acid metabolism

In order to follow the effects of a HP diet in blood it is essential to track all pathways and start thereby in the mechanisms of digestion and absorption of alimentary proteins and amino acids.

After food intake the gastric acid starts to cleave proteins partially and hydrolyses them partially to peptides. Additionally, the gastric acid catalyses the formation of pepsin from pepsinogen. Pepsin can cleave all proteins (except keratin and myosin) to polypeptides. Supplementary trypsin and chymotrypsin, derived from pancreatic juice, hydrolyse proteins to polypeptides in the small intestine.

In the small intestine there are several ezymes – like enterokinase, elastase, dipeptidase, aminopeptidase or prolidase – that cleave polypeptides and peptones to dipeptides and amino acids.

Amino acids are absorbed rapidly out of the gut lumen, whereas small peptides are hydrolyzed to amino acids when entering the mucosa cells. Only free amino acids can make their way to the liver. There are several transport mechanisms that vary in

specificity and velocity but all of them need a free amino group in α-position of the amino acid.

Vegetable protein is harder to absorb for the human body than animal protein because of the cell wall. 90 % of alimentary protein is absorbed and processed by the body.

After the transport to the liver, the free amino acids get processed. Liver and kidneys are the most important organs for amino acid metabolism. One part of the amino acids is catabolised in different procedures to store the carbon scaffold as glycogen or fat. The amino group is used for the urea cycle. Some of the degradation products are intermediates of the citric acid cycle. Another part of the amino acids is used for the formation of tissue proteins. Most of the amino acids used for this purpose are amino acids from catabolised tissue proteins that were transported back to liver or kidney for recycling. The third main application for free amino acids is the formation of nitrogen containing products like purine bases, creatine or adrenalin. Additionally the non-essential amino acids are required for the transformation into essential amino acids.

Alimentary proteins are important for several reasons: synthesis of body mass (especially in children and pregnant women), novel synthesis of proteins that were catabolised in metabolism, they can act as enzymes, hormones, transporters, plasma proteins for maintenance of osmotic proportion, antibodies and clotting factors, contractile proteins in muscle tissue and as an important part of cells and tissues. (Elmadfa 1998)

3.2. Amino acids

Since dietry protein gets catabolized into amino acids during digestion, an analysis of the plasma amino acids in an HP study is very important. It has been shown in rat skeletal muscle that the amino acid concentration plays a crucial role in translational regulation. With amino acid supplementation the protein synthesis rate could be increased twofold. (Vary 1999) Further it was shown that both intravenous (Volpi 1998) and oral amino acid supplementation increased skeletal muscle protein synthesis in elderly. (Paddon-Jones 2004)

Amino acids can be separated into proteinogenic amino acids and nonproteinogenic amino acids. The function of proteinogenic amino acids is to be building block of proteins and the one of nonproteinogenic amino acids is to attend metabolism.

Amino acids are chiral (except Glycin). There are 20 proteinogenic amino acids, which are all L-α-amino acids.

More than 100 nonproteinogenic amino acids are known so far. Nearly all of them are derivatives of proteinogenic amino acids. They function as intermediates of proteinogen amino acids in metabolism, precursors of low-molecular compounds and they are important in biosynthesis of urea. (Löffler 2002a)

Amino acids can be devided into essential, semi-essential and non-essential. Essential amino acids are amino acids that are necessary but cannot be synthesized by the human body. They have to be supplied through food. There are eight amino acids that are essential for humans: isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine.

Semi-essential amino acids can be synthesized by the body but in case of injury or during growth they have to be conducted with food. Tyrosine and cysteine are semi-essential amino acids.

Non-essential amino acids can be synthesized from carbon compounds and ammonia by the human body. They don't have to be delivered to the body by food necessarily. Non-essential amino acids are: apartate, asparagine, glutamate, glutamine, glycine, alanine, serine, proline and arginine. (Löffler 2002b)

3.3. Muscle cells

As described above an amino acid supplementation can increase skeletal muscle protein synthesis in elderly and thereby could decrease the risk of sarcopenia. (Paddon-Jones 2004; Volpi 1998) Sarcopenia is the degenerative loss of skeletal muscle mass and strength and is strongly associated with aging. One of the main mechanisms contributing to sarcopenia is the declining muscle protein synthesis in ageing. (Short 2004) So with a HP diet there may also be an effect on the skeletal muscle protein synthesis.

3.3.1. Skeletal muscle cells - Physiology

Muscle cell is an umbrella term for several kinds of cell types which all have a contractile system involving actin and myosin. In mammals four main classes of muscle cells are present: skeletal muscle, heart (cardiac) muscle, smooth muscle cells and myoepithelial cells. (Alberts 2002b)

Skeletal muscles account for 40 % of the body weight and are responsible for almost all intended movements. Skeletal muscle cells, also called muscle fibres, consist of syncytia i.e. multinucleated cells. In adult persons muscle cells can have a diameter around 15-200 µm and a length up to 15 cm. Muscle fibres are condensed to a muscle bundle which are condensed to the muscle itself. The cytoplasm membrane contains a collagen layer which ends in sinew at the end of the muscle. In the cytoplasm the sarcomeres - contractile units that consist of actin and myosin – form the myofibril. Further the sarkoplasmatic reticulum, several nuclei and a large number of mitochondria are located in the cytoplasm. (Speckmann 2008)

3.3.2. Skeletal muscle cells - Contraction

Myofibrils, that are nearly as long as the muscle fibre itself, consist of many contractile subunits called sarcomeres. They down date its striated appearance and consist of overlapping thick and thin filaments. The thin filaments consist of actin and are fixed at the Z disc with the plus end at the end of each sarcomere. The thick filaments consist of bipolar myosin II structures. Several accessory proteins are responsible for the uniformity in filament organisation, like the already mentioned Z-disc which consists of CapZ and α-actin, nebulin or Titin which functions a “feather” against overexpansion of the muscle (see figure 1).

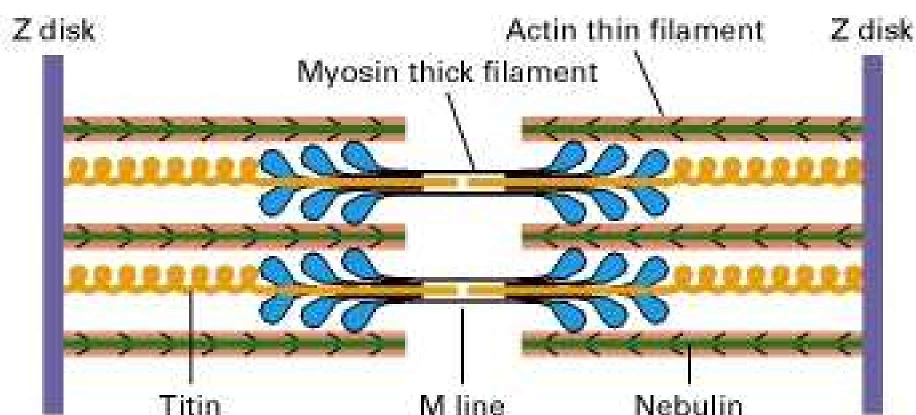

Figure 1: Filament organisation of sceletal muscle cells. Actin thin filaments are attached to the Z disk. Nebulin is attached at the thin filaments and the Z disk. Titin connects the myosin thick filament with the Z disk. Mayosin thick filament is attchaed to the actin thin filament. (Lodish 2000)

At the start of contraction the myosin head is attached to the actin filament. One molecule of ATP binds which implicates a slight conformation change in the inner of the protein which results in a movement for 5nm which leads to a reduction of affinity to the actin filament. ATP gets hydrolysed to ADP and Pi and affinity increases again. The myosin head can bind to the actin filament 5 nm farther. This mechanism - performed with a high number of motor domains of the myosin II filaments at the same time - enables the sarcomere to shorten for 10% in less than $^{1}/_{50}$ second. For proper muscle contraction rapidly large amounts of ATP molecules are needed. (Alberts 2002a)

Muscle contraction is induced by increased calcium concentration in the cytosol implied by an arriving action potential at the neuromuscular end-plate. When the action potential reaches the sarcolemma, calcium ions can invade the muscle fibres through voltage dependent calcium channels. The muscle fibres get activated and the muscle tenses. The calcium channels stay open as long as the action potential sustains. (Löffler 2002c)

3.4. Blood platelets

3.4.1. Blood platelets as a peripheral model for muscle cells

Since muscle biopsies in many clinical study settings are too elaborate and costly and also not suitable for routine diagnostic we decided to study possible biochemical mechanisms of a HP diet on muscle cells, in blood platelets which are described to reflect several structural and biochemical features of muscle cells.

Like muscle cells platelets have a predominantly aerobic glycolytic metabolism and considerable glycogen stores. Also the energy metabolism is analogical in both cell types. Blood platelets and muscle cells both perform glycolysis actively and for ATP generation they are using large amounts of glycogen. Platelets have to be able to deliver large amounts of energy very fast.This energy is needed for aggregation, granule release and clot retraction. (Gnatenko 2003; Karpatkin 1970)

Like muscle cells, platelets are contractile and motile. Also in proteome composition they have several similarities. Both cell types retain large quantities of actin, myosin and regulatory proteins like gelsolin and actin-binding protein. Further the Ca^{2+}-transport system in platelets has several similarities to the one in muscle cells. The Ca^{2+} storage organelle in platelets is called dense tubular system and is analogous to

the sarcoplasmatic reticulum of muscle. Further the surface-connected canalicular system can be compared to the T-tubule system in muscle cells. (Fischer 1989)

3.4.2. General abilities of platelets

Blood platelets are the smallest cells that circulate in blood. They have a diameter of 1 – 4 µm. The concentration of platelets in blood is 170 – 400 * 10^3 cells per µL. After their pinch-off of megacaryocytes in bone marrow they circulate in blood for 7 – 10 days until they get catabolized in spleen and lungs. (Silbernagl 2007) Though they have no nucleus, therefore contain no nuclear DNA, platelets retain cytoplasmatic, megakaryocyte-derived mRNA which enables them to perform protein translation. (Gnatenko 2003)

However, their main function is to perform haemostasis in case of injury. Thereby platelets get activated, aggregate and form the primary haemostatic thrombus (white thrombus). This leads to the activation of the coagulation cascade which further leads to formation and activation of thrombin that degrades fibrinogen to fibrin. Fibrin attaches to the primary haemostatic thrombus and forms the much more stable secondary haemostatic thrombus (red thrombus) together with the aggregated platelets. When the wound is healing, the red thrombus is slowly degraded by fibrinolysis. (Gawaz 2001)

They also have an important immunological function because they can also act as cells of the innate immune system by activating leukocytes. (Weyrich 2004)

Platelets have several inversions of the outer membrane that form the open canalicular system. This system increases the platelet surface by several folds. The canalicular system gives the platelets a sponge-like structure and proteins or ions are sponged into the canals. The increased surface area is important for a fast Ca^{2+} absorption to release the granula and activate the platelets. (Beck 2002)

3.5. Blood plasma

The main function of blood plasma is to transport nutrients. Plasma contains large amounts of metabolites, nutriments, electrolytes, vitamines and hormones dissolved in the aqueous solution (plasma) or attached to transporter proteins.

In a HP diet changes in the plasma proteome, plasma amino acid concentrations and other plasma parameters like cholesterol, triglycerides, iron, zinc, vitamin B12 are

from great interest as the can be an indication for risk factors for cardiovascular diseases, osteoporosis or deficiency symtoms.

3.5.1. Cholesterol and triglycerides

The cholesterol status in a healthy individual is highly associated to its nutrition habits. Further the cholesterol status is an important parameter for the risk of cardiovascular diseases and therefore the measurement of these values is very important in an HP study.

Cholesterol is a very important component of plasma membranes. It can be synthesized by the human body or supplemented by food. In blood cholesterol and triglycerides are transported by lipoproteins. Lipoproteins are differentiated between VLDL, IDL, LDL, HDL and Chylomicrons. They are classified by their physical and functional attitudes. All lipoproteins consist of lipids and proteins, so called Apolipoproteins that are transport proteins.

LDL transports cholesterol into the tissue where it is needed as a component of cell membranes or for example as a precursor of steroid hormones. If there is too much cholesterol in the blood the LDL can attach to blood vessel walls and accumulate there, which increases the cardiovascular disease risk. Therefore LDL transports the so called “bad cholesterol”. HDL transports the “good cholesterol” because it transports excess cholesterol out of the tissue for degradation in the liver and excreted by the gall. For measurement of the cholesterol parameters in medicine not only the single LDL or HDL values are important, only the LDL/HDL cholesterol quotient gives meaningful information about the cholesterol status of the person since these lipoproteins should be in equilibrium.

Triglycerides adapt very fast when nutrition habits are changed. The adaption of HDL and LDL to nutrition changes on the other hand is quite slow. They need about 6 weeks to even out when nutrition is changed into a constant and healthy way. Papakonstantinou et al. for example showed in a 4 weeks HP study in type 2 diabetes, obese individuals a significant decrease of triglycerides with HP diet. The total and LDL cholesterol on the other hand also decreased but there was no significant difference between the HP and the NP diet found. (Papakonstantinou 2010b) Further it was shown in hypercolesterolemic rabbits that a consumption of soy yoghurt – which is plant protein – decreased the total cholesterol levels and

increases the HDL levels and thereby decreases the risk of cardiovascular diseases. (Cavallini 2009)

3.5.2. Homocysteine, Folate and Vitamin B_{12}

Since the meat, fish milk products and eggs can derive the only sufficient vitamin B_{12} source the measurement of this parameter is quite important in an HP diet study, where the protein derived form animal products. Folate and homocysteine are part of the same pathway like vitamin B_{12}. (see figure 22, page70) To be able to find links all these parameters were also measured.

Vitamin B_{12} is an enzyme that methylizes homocysteine to methionine that further is necessary for the methylation of lipids and DNA.

High homocysteine levels are highly associated with cardiovascular diseases. (Omenn 1998) It was shown that an increased folate intake could decrease the homocysteine levels and thereby decrease the risk for cardiovascular diseases. (Boushey 1995)

Referring to HP diet it was shown that an HP diet composed mainly by plants has a positive (decreasing) effect on the homocysteine levels whereas an HP diet deriving from animal sources has a negative effect. Further the homocysteine values correlated significantly with the folate but not with the vitamin B_{12} values. (Yakub 2010)

Further high homocysteine levels are associated with lower bone density. Krivosíková et al., showed that vegetarians have higher homocysteine levels and lower vitamin B_{12} levels that people who eat meat. Additionally they showed by correlations with bone markers, that vegetarians have a higher risk for osteoporosis correlating to hyperhomocystemia. (Krivosikova 2010) Clifton et al., on the other hand could not show a significant change of homocysteine or folate with a HP diet. Only the vitamin B_{12} levels increased with HP diet. (Clifton 2008)

When reading the publications about the relationship between HP diet or rather diet in general and homocysteine, vitamin B_{12} and folate, the controversity of the results is surprising. It seems as if the regulation of these three factors by diet is multifactorial.

4. Aim of the study

There is an increasing number of observational studies suggesting a positive role for protein in promoting optimal health at intakes beyond the Dietary Reference Intakes (DRI), such as weight management and satiety, sarcopenia, glucose homeostasis, and bone health.

Many studies describe that a change in the protein diet influences muscle physiology as well as the composition of the muscle cells.

The impact of an HP diet on certain proteomes in the body has so far not been analyzed. In this study we want to research the impact of an HP diet on muscle cells and therefore we are using platelets as a model. Human blood platelets are contractile and motile cells which share many features with muscle cells. Similarities between the contractile protein system of platelets and muscle have made the platelets a popular model for muscle disease. Platelet is a "minimuscle" with a protein contractile system (actin and myosin) similar to that of skeletal muscle.

Furthermore there are several studies concerning changed protein diet and the homocysteine pathway. However, their results are very contradictorily. In this study we want to adduce the final evidence about the correlation of homocysteine, folate and vitamin B_{12} and a HP diet.

5. Study design

5.1. Subject selection

24 young healthy men were chosen for the study. (One of them dropped out because of flu) They had to be between 20 and 30 years old, non-smoking, had to have stable weights (i.e. ± 2 kg within the previous 3 months) and a body mass index between 18.5 and 25. Exclusion criteria were the use of any medication on a daily basis, heavy physical exercise,smoking, chronic diseases or an illness in shoulder, arm or hand. The individuals had to guarantee to only consume foods and beverages provided by the Department of Human Nutrition, Faculty of Life Sciences, University of Copenhagen except for water and salt.

5.2. Experimental design

Before the study started there was a 3 days registration period where the individuals had to perform a weighted food diary and a physical activity diary. Afterwards a one-week run-in period, where all individuals received a diet with average protein content of 1.5 g/kg (15 E %), was performed. Then the individuals were separated into two groups: a NP (normal protein) group and an HP (high protein) group. The NP group, containing 12 individuals, continued with the average protein content diet of 1.5 g/kg (15 E %) whereas the HP group, containing 11 individuals, received a high protein content diet with the double amount of the recommended protein content in daily nutrition of 3 g/kg (30 E %). This period lasted for three weeks. The protein primarily derived from meat and fish.

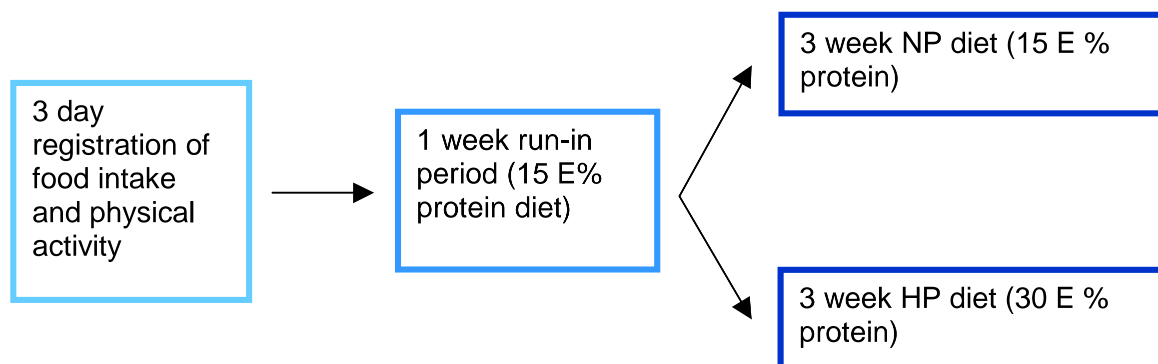

Figure 2: Timetable of study period and protein amount expressed as a percentage in every step of the experiment

During the study period the individuals received all foods and drinks from Department of Human Nutrition in Copenhagen. As mentioned before, the individuals were not allowed to consume any other food or beverages than they received from the department. The individuals had to consume lunch at the department under superstition on weekdays and the rest of the meals (dinner, snack, breakfast and drinks) were provided daily for takeaway and consumption at home. On Fridays the food for the weekend was provided as a package for taking home. The leftovers had to be brought back to the department for registration.

5.3. Diets

As mentioned before, there are two different diets applied in this study. In the run-in period, respectively the NP group a diet with 15 E % from protein per day was performed by the individuals. The HP group got twice the protein amount of the NP group, thus 30 E % protein per day. The diets varied in the ratio of protein to carbohydrate and had different compositions. The diets were matched for n-3 PUFA and dietary fiber content during the study period.

Macronutrient composition of diets	***NP group***	***HP group***
Protein	15 E %	30 E %
Fat	30 E %	30 E %
Carbohydrate	55 E %	40 E %

Table 1: Diet composition of the NP group and the HP group expressed as a percentage.

5.4. Measurements

During the study period there were two time points where blood was taken from the individuals. The first time point (baseline) was at the end of the run-in period. The second time point (after treatment) was after the 3 weeks of intervention period, respectively at the end of the study period. After blood was taken, plasma and platelets were isolated and sent to Vienna.

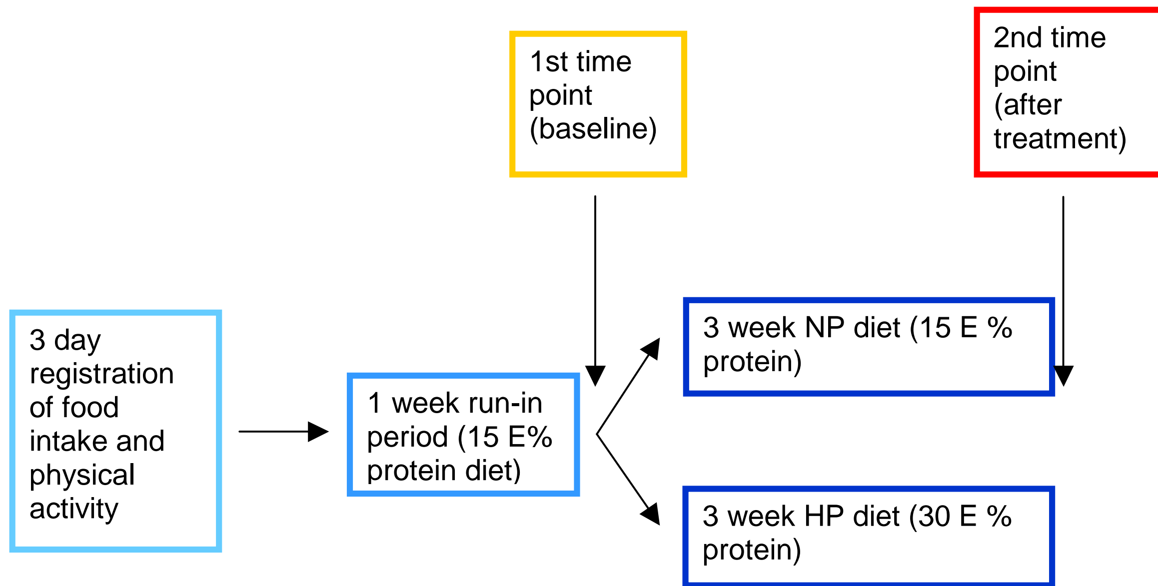

Figure 3: timetable of study period and protein amount expressed as a percentage in every step of the experiment and time points that show the time point where blood was taken from the individuals.

In Vienna platelet and plasma proteome was examined with 2D DIGE technique, plasma amino acids were measured with HPLC and plasma parameters (haptoglobin, vitamin B_{12}, folate, cholesterol and triglycerides, albumin and homocysteine) were measured.

6. Material and Methods

6.1. Equipment and Material

6.1.1. Blood collection

- Hematology analyzer, MicroDiff 10, Beckman Coulter, USA and SYSMEX KX-21N, Sysmex Digitana, CH
- Vacutainer tubes, Greiner, Austria
  - Vacuette EDTA K3 tubes
  - Vacuette trisodium Citrate 3,8%

6.1.2. Gel filtration

- Refrigerated centrifuge, Universal 30 RF, Hettich
- Columns for gelfiltration – Econo-Pac 15mm diameter, Bio Rad, USA
- Sepharose 2B, Sigma-Aldrich, USA
- D-PBS, Gibco, USA: 2.67 mM KCl, 1.47 mM KH_2PO_4, 137.93 mM NaCl, 8.1 mM Na_2HPO_4
- Urea buffer: 7 M urea, 2 M Thiourea, dissolve in ddH_2O, add 1% Amberlite IRN-150L and stir for 1hour, filtrate and add 4% CHAPS, pH 8.5 at 4℃, aliquote and store at 4℃

6.1.3. Affinity chromatography – ProteomeLab IgY-12 LC2

- Affinity column: Proteome Lab IgY-12 LC2, Beckman Coulter, USA
- Dilution buffer: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl
- Stripping buffer: 0.1 M Glycine-HCl, pH 2.5
- Neutralization buffer (10 x): 1M Tris-HCl, pH 8.0

6.1.4. Trichloacetic acid precipitation

- Sample shaker, Labinco L46
- TCA solution: 6.1 N TCA solution, 80 mM DTT
- Acetone solution : acetone, 20 mM DTT

6.1.5. Bradford measurement

- ELISA reader, Wallac Victor^2, Perkin Elmer, USA
- Bradford protein assay: Coomassie Plus, Pierce, USA

6.1.6. CyDye sample labelling

- CyDye DIGE Fluors (minimal dyes), 25-8010-65, GE Healthcare, USA: N, N-dimethylformamide, anhydrous, 99.8 % (DMF), Sigma-Aldrich, Germany; CyDye stock solution: dissolve 5nmol of cyDye powder in 5µL of DMF
- CyDye working solution: 0.45 µL CyDye stock solution, 2.0 µL DMF
- Lysine stock solution: 10 mM Lysinmonohydrochloride

6.1.7. Isoelectric Focussing

- IPGphor, GE Healthcare, USA
- IPG-Strips: Immpbiline Dry Strip Gels, GE Healthcare, USA
- Rehydration buffer: 7 M urea, 2 M thiourea, dissolve in dH_2O, add 1 % Amberlite IRN-150L and stir for 1 hour, filtrate and add 4 % CHAPS and some crumbs bromine phenolblue, aliquote and store at 4°C, before use add 1% Ampholyte and 70mM DTT

6.1.8. Second Dimension Ettan DALT six – SDS PAGE

- Ettan DALTsix Electrophoresis Unit, GE Healthcare, USA
- Typhoon TRIO – Variable Mode Imager, GE Healthcare, USA
- Lower Tris Buffer (LTB) (4 x): 1.5 M Tris, dissolve in dH_2O, pH 8.88, store at 4°C
- Acrylamide (30 %): 29.2 % acrylamide, 0.8 % N-N´ methylenbisacrylamide, dissolve in dH_2O
- N,N,N´,N´-tetramethylene-diamine (TEMED) 99 %, Sigma-Aldrich, Germany
- Ammonium peroxidisulfate (APS), Merck, Germany
- Running buffer (10 x): 250 mM Tris, 190 mM Glycin, 1 % SDS (add fresh before every experiment)
- Molecular weight standard: Precision Plus Protein unstained standards, Bio Rad 161-0363, USA
- Agarose sealing solution: 0.5 % Agarose, 0.1 % SDS, dissolve in 371 mM LTB, heat shortly in the microwave, add some crumbs bromine phenolblue, aliquote and store at –20°C
- Equilibrationbuffer: 50 mM Tris, 6 M urea, 30 % Glycerol, 2 % SDS, dissolve in dH_2O, pH 8.88, aliquote and store at –20°C, before use add either 10 mg DTT per mL equilibrationbuffer or 25 mg iodacetamide per mL equilibrationbuffer

6.1.9. Silver staining – compatible for mass spectrometry

- Scales - Sartorius, 4.8kg , Laboratory LC 4800 P
- Analytical balance - Sartorius , Analytic AC 210 S
- Fixing solution: 50% EtOH, 5% acetic acid
- Washing solution: 50% EtOH
- Sensitizing solution: 0,025% $Na_2S_2O_3$
- Silver solution: 0,15% silver nitrate
- Developing solution: 0,04% Formaldehyde (37%), 2% Na_2CO_3
- Stop solution: 5% acetic acid
- Storage solution: 1% acetic acid

6.1.10. Preparation for mass spectrometry

- Speedvac
- Trypsin: Roche 1418025

6.1.11. High pressure liquid chromatography (HPLC)

- HPLC-Glasspitzvials 1,1 cm³, Stölzle 58025 12 32 05
- HPLC-Standardvials 2,0 cm³, Stölzle 58012 12 32 00
- Bördelkappen zu HPLC-Vials, Stölzle 89540 11 00 04
- MilliQ-plus Millipore water; Waters Millipore, Austria
- O-phtaldehyde reagent
- Buffer A
- Buffer B
- Acetonitrile: Merck 100 030, LiChrosolv or FLUKA 00687
- Tetrahydrofurane: Merck 108 101, LiChrosolv
- Methanol: Merck 106 009, LiChrosolv
- 2-Mercaptoethanol: MERCK 15 433 or SIGMA M-6250
- Sodium acetate: Merck 6264, Suprapur
- 0,1M acetic acid: MERCK 100 063
- Fluropa: SIGMA D-6518
- 1M Boratbuffer (Fluoraldehyd): PIERCE 27 035
- 5-Sulfosalicylsäure-Dihydrat: MERCK 100 691

6.2. Methods

6.2.1. Preparation of plasma and platelet proteome samples

6.2.1.1. Blood collection

Peripheral venous blood samples were taken from antecubital vein. The first 7 mL were taken into EDTA vacutainer tubes with stasis. This blood sample was taken to compile a haemogram and to isolate plasma. Plasma isolation was performed by two centrifugation steps. First the whole blood was centrifuged at 2095 g at 4℃ for 15 minutes. Then 1 mL of the supernatant was centrifuged at 15000 g at 4℃ for 10 minutes. After the second centrifugation step the supernatant is purified plasma, which was aliquoted and stored at –80℃. The plasma was later used for amino acid analysis, 2D DIGE gels and blood parameter analysis.

Afterwards blood was taken without stasis into 4 citrate vacutainer tubes (9 mL). To separate thrombocytes from erythrocytes and leukocytes, the citrated whole blood was centrifuged for 20 minutes at 80 g at room temperature. On the bottom of the tube there is the erythrocyte layer. On top of the erythrocytes there are the leukocytes, and on the top of the tube there are the thrombocytes and plasma proteins. This yellow supernatant is called platelet rich plasma (PRP). PRP has to be removed very carefully to ensure, that none of the leukocytes are taken with it. The thrombocyte concentration was counted on the Blood Analyzer.

6.2.1.2. Gel filtration

To separate thrombocytes from plasma proteins, size exclusion chromatography was performed.

Chromatography is a technique for separating molecules in a solution from each other. It is possible to disperse the analytes towards different parameters. In size exclusion chromatography for example molecules are separated by their size. In affinity chromatography certain proteins for example can be isolated because of antibodies that are fixed in the chromatography column. Other examples for chromatography are ion exchange chromatography, thin layer chromatography, high pressure liquid chromatography (HPLC) or gas-liquid chromatography. Though, the principle of all these methods is similar: There is always a stationary phase and a mobile phase. The stationary phase is a substance that is fixed and interacts with the substance that is supposed to be separated. The mobile phase is mixed with the analyte and is in motion. Every molecule has its substance specific retention time.

Retention time is the time a substance needs to go through a column. It depends on the affinity between the analyte and the stationary phase, the solubility in the mobile phase and its diffusion properties.
In this study size exclusion chromatograph was performed to separate platelets from plasma proteins, since thrombocytes are considerable bigger and thereby eluted faster than plasma proteins, which invade in pores of sepharose.
Sepharose-2B that was stored in ethanol, PBS and frits were degassed in a vacuum desiccator for 45 minutes. Columns were filled with 19 mL of Sepharose-2B. When the sepharose was condensed to the 11 mL mark of the column, the frit was placed at the top of the sepharose. The column was washed twice with PBS to remove the ethanol left in the sepharose. The frit avoids the column running dry and assures even application of PRP.
1 mL of PRP was applied onto column. When it was infiltrated totally, 2.5 mL PBS were applied. Thrombocytes were eluted with 1.5 mL PBS and collected. The eluate, that is highly cloudy because of the thrombocytes, is called gel-filtered platelets (GFP). The concentration of thrombocytes in GFP was counted on a Blood Analyzer.

6.2.1.3. Plasma sample pools

For EDTA plasma proteome analysis sample pools were used. 4 (once 3) Individuals were pooled in one group. The pooled individuals had to be in the same experimental group and in the same clinical investigation day.

	Baseline		After treatment	
	sample (individuals)	pooled samle	sample (individuals)	pooled samle
15 E% meat	02-K101-01 02-K101-03 02-K101-07 02-K101-08	SV1	03-K101-01 03-K101-03 03-K101-07 03-K101-08	SN1
	02-K101-10 02-K101-11 02-K101-13 02-K101-14	SV2	03-K101-10 03-K101-11 03-K101-13 03-K101-14	SN2
	02-K101-18 02-K101-21 02-K101-24 02-K101-25	SV3	03-K101-18 03-K101-21 03-K101-24 03-K101-25	SN3
30 E% meat	02-K101-02 02-K101-04 02-K101-06 02-K101-12	SV11	03-K101-02 03-K101-04 03-K101-06 03-K101-12	SN11
	02-K101-15 02-K101-17 02-K101-19 02-K101-20	SV22	03-K101-15 03-K101-17 03-K101-19 03-K101-20	SN22
	02-K101-22 02-K101-23 02-K101-26	SV33	03-K101-22 03-K101-23 03-K101-26	SN33

Table 2: List of individual samples that were pooled for plasma proteome analysis.

6.2.1.4. Affinity chromatography – ProteomeLab IgY-12 LC2

For pasma 2D DIGE gels it is important to remove the high abundant proteins from the plasma. Otherwise the high abundant proteins would cover the low abundant proteins on the gel with their stron signal and a proteome ananlysis would not be possiple. Therefore we used affinity chromatography after pooling the EDTA plasma samples.

In affinity chromatography receptors, antibodies or enzymes are fixed in the stationary phase. When a protein alloy is applied on the column, some proteins can bind specifically and the rest is flushed out of the column. With an eluate the affinity between stationary phase and protein can be reduced and these specific proteins can also be removed from the column. This is a possibility to filter one protein of interest out of a mixed sample.

In this study the ProteomeLab IgY-12 LC2 column is used. It can remove simultaneously twelve highly abundant plasma proteins, namely human serum

albumin, IgG, α1-antitrypsin, IgA, IgM, transferring, haptoglobin, α1-acid glycoprotein (orosomucoid), α2-macroglobulin, HDL (apolipoproteins A-I and A-II) and fibrinogen. As ligands affinity purified avian (IgY) antibodies are used. The column has a capacity of about 1875 µg of proteins. The respective yields of depleted proteins according to the manufacturer are 200 µg. Thus 11 % of the total amount of applied proteins should be recovered in the depleted protein fraction.

As a pre-treatment before every run the column has to be equilibrated for 20 minutes with 1x dilution buffer at a flow rate of 1 mL/min. In the meantime the sample can be prepared. 25 µL of plasma sample are mixed with 100 µL 1x dilution buffer and centrifuged in filter tubes for 1 minute at 10.000 rpm to remove any particles from the sample. Then the 125 µL diluted and pooled (see chapter 4.2.1.3) plasma sample can be loaded on the column at a flow rate of 0.1 mL/min. 600 µL of 1x dilution buffer are applied on the sample at a flow rate of 0.1 mL/min. The 600 µL are the volume that is needed to get the sample right in front of the entrance of the column. While still soaking up dilution buffer with a flow rate of 0.1 mL/min the 1.5 mL flow-trough are discarded. The next 1.5 mL of the flow-trough contain the sample and are collected in an Eppendorf tube with a flow rate of 0.2 mL/min. The column in washed with 6 mL of dilution buffer at a flow rate of 1 mL/min and afterwards the bound proteins are eluted with 35 mL stripping buffer. To neutralize the pH after elution the column is treated with 6 mL neutralization buffer. If there is another sample to run the column is equilibrated with dilution buffer for 20 minutes again, otherwise the column is washed with 6 mL dilution buffer (incl. 0.02 % NaN_3) and stored at 4℃. Under proper conditions the IgY micro beads should be reusable for 100 times.

6.2.1.5. Trichloacetic acid (TCA) precipitation

Both the gel-filtrated platelets (GFP) and the plasma samples after affinity chromatography are TCA pecipitated.

Therefore 500 mL of ice-cold TCA solution were mixed with 1.5 mL of GFP respectively plasma. The mixture was stored 60 minutes at 4° C. Cells were lysed and proteins precipitated. After one hour the precipitate was centrifuged for 10 minutes at 10.000 g at 4℃. A white protein pellet was formed. The pelled was washed four times with ice-cold acetone solution. It is possible to store the samples at –80° C at this step of preparation and go on wit h the procedure any other day. The pellet was dried by air evacuation and resolubilized in denaturizing urea buffer by

shaking over night at 4° C. The next day the soluti on was centrifuged for 10 minutes at 10.000 g at 4°C, aliquoted and stored at –80°C .

6.2.1.6. Bradford measurement

Bradford method was used for protein concentration measurement after TCA precipitation. A BSA (bovine serum albumin) dilution series in PBS with seven different concentrations (0 – 0.3 mg/mL) was used as standard (see table 1).

	conc. [mg/mL]	µL BSA [1mg/mL]	µL PBS
S1	0	0	950
S2	0.05	50	900
S3	0.10	100	850
S4	0.15	150	800
S5	0.20	200	750
S6	0.25	250	700
S7	0.30	300	650

Table 3: BSA standard dilutions, aliquots to 190 µL and stored at –20℃

190µL of each standard were mixed with 10µL of the used sample buffer. Thereby interfering influences of the sample buffer should be compensated. The sample was diluted 1: 20 in PBS. 20 µL of every standard dilution and sample were applied three times on a 96-well microtiter plate. 200 µL of Bradford Reagens were applied on standards and sample as fast as possible. After 5 minutes the absorption was measured in an ELISA-reader at a wavelength of λ = 595 nm.

The mean value of all three same samples and standards was calculated and multiplied with the dilution factor. A standard curve was created where the values of the sample were inserted to calculate the protein concentration.

6.2.1.7. Assembling of an internal standard

After concentration measurement two internal standards are prepared: one internal standard for the ETDA plasma DIGE analysis and one for the platelet proteome

ananlysis. The internal standard is of prime importance to minimize the technical variance. It consists of the same amount of protein of each sample in the study.
The internal standard of the platelets samples was made of 60 µg of each sample. Since the platelet internal standard was not only used for this study but also for another study, the internal standard consisted of the high protein diet study samples as well as of 105 samples from another study.
The internal standard for the plasma proteome analysis was made of 15 µg of each sample.
After the pooling of the samples to prepare an internal standard, Bradford was performed again, to know the exact protein concentration of the internal standard.

6.2.2. 2D – differential gel electrophoresis (DIGE)

Both the platelets and the plasma proteome were analysed with 2D DIGE. Due to CyDye labelling of the samples it is possible to compare two smples with each other on one DIGE gel. Because of the internal standard that is applied on every gel it is also possible to compare samples that are on different gels.
The first step in 2D DIGE is the sample labelling. Afterwards the first dimension is run followed by the second dimension.

6.2.2.1. CyDye sample labelling

Samples were labelled immediately before IEF was performed. One sample was labelled with Cy 5, the comparing sample with Cy 3 and the internal standard with Cy 2 and focussed together on one IEF strip. The dyes bind covalent by amid bond between their NHS group and the ε-amino group of the lysines of the proteins in the sample. This technique is described to be a minimal labelling technique, because the dye constitutes the limiting factor. Only 1 – 2 % of all the lysines are labelled. The CyDyes cause no change of the isoelectric point of the proteins and only increase molecular weight by about 500 Da. The labelling reaction is stopped by an overspill of lysine added after 40 minutes.
Samples were centrifuged for 10 minutes at 10 000 g at 4° C before use to remove possible precipitates. 17 µg sample were mixed with 0.35 µL CyDye working solution and incubated on ice for 40 minutes in the dark. 0.5 µL lysine [10 mM] were added to stop labelling reaction and incubated on ice for 10 minutes in the dark. Cy 5 and Cy 3 samples and Cy 2 internal standard were pooled.

6.2.2.2. Isoelectric Focussing (IEF) pH 4 – 7

Isoelectric focussing serves for separating proteins with the isoelectric point by applying voltage. Thereby IPG-strips, a gel matrix with immobilized pH gradient on a plastic carrier, are used. The strips have to be expanded before use, because they are dried. Proteins that are supposed to be separated are stored in urea buffer.

The sample is infiltrated into the strip by passive rehydration. The pooled CyDye samples were mixed with 70 mM DTT and 1% ampholyte pH 4 – 7 and filled up to 450 µL with rehydration buffer. The rehydration mix was applicated to IPG strip holders, a special ceramic container with electrodes on each end, and IPG-strip (24 cm) was applied with the gel layer downwards. The sample has to be arranged even. To prevent running dry, it was overlaid with 2 mL oil. The strips were reswelled for 12 hours.

Immediately before IEF was started, small filter pads soaked in rehydration buffer with 70 mM DTT and 1 % ampholyte pH 4 – 7 were put between electrodes and IEF strip. Then IEF was started. (see table 4)

Step		Voltage [V]	Time [h]
1	Step-n-Hold	200	0:01
2	Step-n-Hold	10	0:01
3	Gradient	200	2:00
4	Step-n-Hold	200	2:00
5	Step-n-Hold	300	2:15
6	Gradient	5000	7:30
7	Step-n-Hold	5000	2:20
8	Step-n-Hold	500	3:00

Table 4: IEF parameters for 24 cm IEF strips pH 4 - 7; 50 µA/strip at 20℃

Step 8 has only been a safety step, for preventing the strip being without voltage when the IEF was over. When step 7 was finished the strips were stored at –80℃ until second dimension was performed.

6.2.2.3. Isoelectric Focussing (IEF) pH 6 – 9

Separating proteins from pH 6 – 9 works by applying the sample by “cup-loading” on the strip. First the strip has to reswell for 10 hours in rehydration buffer with 150 mM DTT and 2 % ampholyte pH 6 – 9. Therefore the strip is laid in a reswelling tray with the gel layer downwards and overlaid with 2 mL oil. For applying the sample after rehydration, the strip is put into strip holder specially constructed for cup loading with the gel layer upwards. On both electrodes at the ends of the strip holders, filter pads are placed exactly at the end of the gel layer of the strip. The filter pad at the anode is dipped into rehydration buffer before applying, whereas the one at the cathode is soaked with 175 µL rehydration buffer with 3.5 % DTT and 2 % ampholyte. During isoelectric focussing, the DTT migrates from cathode to anode. To avoid the cathode end of the strip being without DTT, the filter pad is soaked in this special mixture.
Electrodes are put on the filter pads and the “cup” is placed right in front of the anode. For not drying-out the strip is overlaid with 4.5 mL oil. The dyed and pooled samples are filled up with rehydration buffer to 55 µL, 150 mM DTT and 2% ampholyte pH 6 – 9 are added. The sample mix is transferred into the “cup” and isoelectric focussing is started. (see table 5)

Step		Voltage [V]	Time [h]
1	Step-n-Hold	300	0:01
2	Step-n-Hold	300	3:00
3	Gradient	1400	6:00
4	Step-n-Hold	1400	8:00
5	Gradient	3500	3:00
6	Step-n-Hold	3500	2:20
7	Step-n-Hold	500	3:00

Table 5: IEF parameters for 24 cm IEF strips pH 6 - 9; 50 µA/strip at 20℃

Step 8 has only been a safety step, for preventing the strip being without voltage when the IEF was over. When step 7 was finished the strips were stored at –80℃ until second dimension was performed. (Zellner 2005)

6.2.2.4. Second Dimension Ettan DALT six – SDS PAGE

After separating the proteins by their pI with isoelectric focussing the proteins have to be separated by their molecular weight. This is done in a polyacrylamide matrix in a 24 cm SDS (Natriumdodecylsulfate) gel.

During sample preparation SDS was added which results in the proteins being negatively charged. Further DTT was added to the sample which causes destruction of the secondary and tertiary structures of the proteins. By applying voltage the proteins migrate depending on their molecular weight respectively on the length of the polypeptide chain through the gel towards the anode with different velocity. Bigger proteins need more time to cover the same distance than smaller proteins would need.

With Ettan DALT six system it is possible to prepare and run six gels at the same time. For gel casting low fluorescence glass plates are used. At first the glass plates are cleaned with double distilled water and high pressure oxygen to minimize contamination of the sample and to get rid of dust particles that could have fluorescent abilities and complicate further analysis. To minimize variations in the gel preparation the acryl amide solution was always prepared freshly and filtered before usage. After degassing the gel solution (see table 6), APS and TEMED were added under gentle mixing and the gel solution was filled into the gel casting system till about 5 mm underneath the glass plates. In 2D electrophoreses instead of using stacking gel the IPG strip is directly applied onto the resolving gel and therefore the area to keep free for the strip can be quite small. Afterwards 2 mL of water-saturated butanol are applied on each gel and the gel mixture is let polymerise for 1 hour. For the platelet proteome analysis a 11.5 % polyacrylamide gel was used and for the EDTA plasma analysis a 9 % polyacrylamid gel was used.

Resolving gel (26 cm x 20 cm, 1 mm thick)		
Solution	**11.5 %**	**9 %**
LTB (4x) pH 8.88	125 mL	125 mL
Acrylamide (30 %)	193 mL	150 mL
SDS	0.5 g	5 mL (10% SDS)
Aqua dest.	178 mL	220 mL
Degas for 1 hour		
APS (10 %)	3.75 mL	3.75 mL
TEMED	165 µL	165 µL

Table 6: Preparation of 26 cm resolving gels for 2D-DIGE containing 11.5% or 9% acryl amide

During polymerisation the running buffer is prepared and cooled down to 10℃ in the electrophoresis chamber. The polymerised gels and glass plates are taken out of the pouring chamber, polyacrylamide remains on the outside of the glass plates are removed with distilled water and the top of the gel is washed twice with running buffer to get rid of the butanol. To prevent the gels from running dry they are overlaid with running buffer until the IEF strips are ready to be applied.

Before the IEF strips are applied, two equilibration steps must be performed. At first the strips are gently shaked in equilibration buffer containing DTT for 20 minutes. DTT reduces disulfide bonds and unfolds the protein totally. Afterwards the strips are shaked in equilibration buffer containing iodacetamide for 15 minutes. Iodacetamide removes excess DTT and alcylates the sulfhydryl groups.

After equilibration the strips are applied on the gels whereas the lower pH rage is supposed to be on the left side. A small filter pad soaked with 3 µL of molecular weight standard is placed at the end of the strip at the right side. Strip and filter pad were coated with agarose sealing solution and remaining air bubbles between the strip and the gel are removed. The gels are put into the Ettan DALT running chamber and the Upper Buffer Chamber, which was filled with the remaining cool running buffer, was applied on top of them. For the electrophoretic separation an overnight protocol was used (see table 7).

Voltage [V]	Time [hours]
30	1,5
50	3
110	16

Table 7: Setting for gel electrophoresis of 26 cm polyacrylamide gels. The run was stopped when the tracking dye bromphenol blue had just run out of all gels.

On the next day, the gels were scanned in the Typhoon TRIO scanner. After a prescan laser intensity (PMI) was adapted to the gels and then the scan was started. The gels were scanned inside the glass plates to prevent warpage. Therefore DIGE glass plates are made from non-fluorescent glass. After scanning the 2D DIGE gels can be analyzed with the DeCyder software.

6.2.3. 2D DIGE gel analysis – DeCyder 2D Software, version 6.5

DeCyder 2D™ software allows detection, quantification and statistical analysis of Ettan DIGE system gels. Before statistical analysis is possible, several steps are performed including spot detection, background subtraction, in-gel normalization and gel-to-gel matching. After preparing the gels in the DIA (Differential In-gel Analysis) tool 300 to 400 spots were matched manually in the BVA (Biological Variation Analysis between gels) tool. Automated spot matching was performed and afterwards corrected manually in some gel areas. Statistical analysis was also performed in the BVA tool.

For gel to gel matching one image has to be assigned as master image to which all other spot maps will be matched sequentially. In DeCyder one gel image is referred to one spot map, which can either be a Cy 2, Cy 3 or Cy 5 image. To determine the master gel, all gel images were looked at to find the one with the best labeling and separation quality.

The optimum number of spots for spot detection was also determined in the DIA tool. Starting from these calculations the estimated number of spots was assessed to be 2500 for spot detection in pH 4 -7 gels. In pH range 6 -9 the number of spots for spot detection was 2800.

In the DIA module spots are detected and quantified. Therefore, a cumulative image of the three individual CyDye gel images is used to ensure that all spots are included. After spot detection the DIA algorithms calculate spot protein abundances for each

image, which are expressed as ratios to compare the expression levels of corresponding spots. Therefore the spot volumes have to be calculated first. The spot volume (V) is defined as the sum of pixel intensity within the spot boundary and is always given with background subtracted (V_g). Background subtraction is performed by excluding the lowest tenth percentile pixel value on the spot boundary from all other pixel values within the spot boundary. The spot volume then corresponds to the sum of these corrected values.

Spot ratios are calculated as follows, volume of sample image spot (Vg(S)) over volume of internal standard image spot (Vg(IS)). See formula 1.

$$Spot\ ratio\ (Ri) = \frac{V_g(S)i}{V_g(IS)i} \qquad (1)$$

Ri ... spot ratio; indicates change in spot value between two images
i ... refers to all spots that are included in analysis

Then all ratio values are logarithmised and combined to create a histogram. Since the majority of protein spots should have an unchanged expression, the main peak of the histogram should be centered on the value zero. Though, usually the main peak of the raw log volume ratios is not centered on zero and therefore normalization is necessary.

Intra-gel normalization is done by fitting a Gaussian curve to the main peak (C) of the raw log volume ratio histogram to calculate the normalization factor. Any values below 10 % of the main peak height are excluded from the model curve fitting procedure. The normalization factor is represented by the centre of the resulting model curve (C’). The volume values in the standard spot maps (V_g(IS)i) are then multiplied by 10^C’. See formula 2.

$$V_g(IS)i_{norm} = V_g(IS)i * 10\text{^}C' \qquad (2)$$

For automated matching in the BVA – after matching 300 to 400 spots manually – a pattern recognition algorithm, that matches one single spot in the one gel to a single spot in another gel based on its neighboring spots, was used.
The normalization procedure between gels in the BVA module is based on the integration of an internal standard sample on each gel. This standard image is then used for inter-gel normalization. The intra-gel normalized volume ($V_g(IS)i_{norm}$) from the DIA workspace is divided by its corresponding procedure in BVA is generally the same as in the DIA module, but as primary image the standard image (IS) is used in the calculations. The normalized volume ratios are also referred to as standardized abundance (SA). See formula 3.

$$SA = \frac{V_g(S)\,i}{V_g(IS)\,i_{norm}} \qquad (3)$$

As a result of this analysis a table, called appearance table, is created in the BVA module, in which the SA-values of each protein spot on each spot map are contained. The logarithm of the normalized volume ratios also referred to as standardized log abundance (log SA) is used for further statistical analysis. (2010; Michlmayr A. 2007) Statistical analysis was also performed in BVA. Paired and unpaired Student t-test were calculated for all spots in all possible combinations of the experimental groups and time points.

6.2.4. Silver staining – compatible for mass spectrometry

In the DeCyder software a statistical analysis of all spots on the gels can be performed. To identfy spots of interest the gels must be dyed and the interesting spot has to be analyzed with mass spectrometry. Therefore after scanning the gels, the gels were taken out of the glass plates and shaked in fixing solution for 20 minutes. Without fixing solution the proteins would diffuse in the gel. Then the gels are washed with washing solution for 10 minutes to get rid of the acetic acid that would have a bad influence on the silver stain. After washing with water for 10 minutes the gels are incubated with water overnight.

At the next day the gels were incubated with sensitizing solution for 3 minutes exactly and after washing twice with water for 1 minute the gels were shaked in silver solution for at least 45 minutes. The next two washing steps (1 minute in water) minimize the background of the silver stain. The gels are incubated in developing solution until the intensity of the stain is as desired. The developing is stopped with stop solution 5 minutes 3 times. The gels can be shrik-wrapped and stored until preparation for mass spectrometry is done after incubating the gels in storage solution over night.

6.2.5. Preparation for mass spectrometry

After silverstaining the gels, the spots of interest are cut out of the gel with a scalpel. 4 to 5 spots, differing in molecular weight and isoelectric point in a great amount, were pooled in one reaction tube. After washing the spots with 1 % acetic acid solution for about half an hour, 500µl 15 mM $K_3Fe(CN)_6$ / 50 mM $Na_2S_2O_3$ is added to the spots and destained for 2-3 minutes. The supernatant is discarded and spots are shaked with 500µl 50% Methanol / 40% H_2O / 10% CH_3COOH for 4 times for five minutes to wash out the destaining reagent. For adjusting the right pH, the spots are washed in 500 µL 50 mM NH_4HCO_3 (pH > 7.5) for 5 minutes. Disulfide bonds are reduced during an incubation of 50 mM NH_4HCO_3 (pH > 7.5) and 10 mM DTT for 30 minutes at 56°C, followed by a washing step in 500 µL 50 mM NH_4HCO_3 (pH > 7.5) for 5 minutes. For alkylation of the disulfide bonds the spots are incubated in 50 mM NH_4HCO_3 (pH > 7.5) and 50 mM Iodacetamide for 20 minutes at room temperature in the dark. After another washing step with 50 mM NH_4HCO_3 (pH > 7.5) the supernatant is discarded and spots are incubated with 100 µL Acetonitril until the spots have an homogen white color. Supernatant is discarded and the pieces of gel are dried in the speedvac for 20 minutes. Per spot 5 µL trypsin solution is applied and incubated for 20 minutes on ice. Per spot 10 µL 50 mM NH_4HCO_3 (pH > 7.5) is applied and incubated at 37℃ over night.

At the next day the tubes are centrifuged for 1 minute at 1000 rpm and then 20 µL 50 mM NH_4HCO_3 (pH > 7.5) are applied and incubated for 30 minutes. After 15 minutes in the ultrasound bath the supernatant is transferred into a silicon covered reaction tube and 20 µL of 5 % formic acid 50 % acetonitrile is applied to the gels. After 10 minutes in the ultrasound bath the supernatant is transferred into the silicon covered reaction tube and 20 µL of 5 % formic acid 50 % acetonitrile is applied to the gels.

After 10 minutes in the ultrasound bath the supernatant is transferred into the silicon covered reaction tube and the volume of the supernatant is reduced to 20 µL in the speedvac. Samples where either stored at -80 ℃ or brought to the mass spectrometry directly.

6.2.6. High pressure liquid chromatography (HPLC)

For the EDTA plasma amino acid analysis HPLC was performed. HPLC is another type of chromatography and works in principle in analogous manner like all other chromatographies described before. There is a stationary phase fixed in the column and a mobile phase mixed with the analytes. Though with this method it is not only possible to separate substances it is also a technique for quantification and identification of analytes with the help of standards.

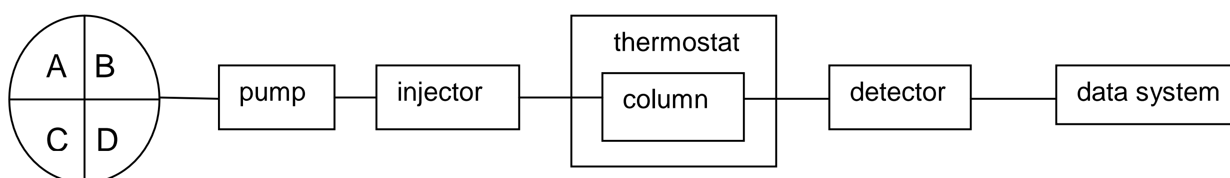

Figure 4: Diagrammatic assembly of a simple HPLC basic system. A, B, C, D are the reservoirs filled with mobile phase or analytes.

Figure 4 shows a diagrammatic assembly of a simple HPLC system. At the beginning there are reservoirs filled with the mobile phase, the eluate or the analyte. With the injector a defined amount of analyte is added to the mobile phase flow. The column is packed with anorganic porose microparticles and warmed up to a specific temperature with the thermostat. The thermostat is important to achieve good reproducibility of retention times, since retention factors are dependent on temperature. The pump forwards the mobile phase with certain velocity through the chromatography column. In the detector the different fractions are analyzed. There are different kinds of detectors, for example UV detectors, fluorescence detectors, electrochemical detectors or mass spectrometer with ESI ionisation. All these detectors are concentration dependent. The data system registers the signals, converts them from analogue to digital and saves them on the computer.

In this experiment reversed-phase chromatography was used which means that the stationary phase is nonpolar and elution force decreases with increasing polarity.

SiO_2 Particles were used as stationary phase that were derivatized with octadecylsilan (ODS) to decrease retention times that would have been much too long otherwise. Further the mobile phase was a polarity gradient in this experiment to achieve better separation. Separation was done at 22° C and signals were detected with a fluorescence detector. Fluorescence detectors irradiate analyte molecules with light, the molecules get activated and emit light and after going through several filters and fences the molecules can be identified and quantified because of the wavelength and the intensity of the emission.

20 µL of sulfosalicylic acid (30 %) containing 1 mM β-(2-thienyl)-DL-alanine, used as internal standard, were added to 200 µL EDTA plasma for deproteinisation. After incubating the samples for 30 minutes at -20℃ they were brought to room temperature again and centrifuged for 5 minutes at 15000 g. The supernatant was used for analysis.

The column was pre – derivated with o-phtalehyde reagent (OPA) for one minute. 7 µL of the plasma supernatant were mixed with 800 µL destilled and deionisized water and 7 µL of this dilution were injected onto the column by a Spark Triathlon autosampler. The particle column, packed with 3 µm big SiO_2 – ODS C18, had a size of 150 mm x 3 mm and the chromguard column sized 10 mm x 3 mm. Separation was performed at a temperature of 22° C using a ste p gradient of buffer A and buffer B at a flowrate of 0.5 mL/min. Detection was obtained with a fluorescence detector with an excitation wavelength of 330 nm and an emission wavelength of 440 nm. Data acquisition and evaluation were carried out by Beckman System Gold software.

6.2.7. Go/NoGo test

The Go/NoGo test is a complex reaction time test. Subjects see geometric shapes in different colors in increasing frequency and have to press or not press a button with the left or the right hand, depending on the shape they see. The frequency stresses the subjects. The wrong or missing reactions are measured and are a dimension for the reaction time of the subjects. (Zimmerman 2007)

7. Results

7.1. Influence of High Protein diet on platelet proteome

To get an overview if a change in the dietry protein amount intake has an influence on the basic molecular composition of platelets a proteome analysis was done by 2D DIGE in the pH ranges 4-7 and 6-9.

Significant protein expression changes between the platelet proteomes of the NP and the HP groups were calculated by student t-test. A HP dependent diet effect was evaluted with three different calculation steps: The HP protein profile was mached within the study group by comparing the expression levels of baseline and after 3 weeks intervention time (1), the HP and the NP proteomes of the two study groups were compared after 3 weeks intervention time (2) and the HP proteome after 3 weeks and the NP proteome at the baseline (3) were compared.

Additionally the NP group was compared at the baseline and after 3 weeks intervention time (4), the HP and the NP groups were at the baseline (5) and the NP group at the after treatment time point and the HP group at the baseline (6) were compared. However the change in the diet of the NP group may have an influence on platelet protein composition.

Moreover also a substantial number of false positives may accumulate at the 0.01 confidence level because 1% of sample differences will be significant by change even if no changes in protein expression exist between the two samples

Therefore proteins that happened to be significant in the t-tests of 4-6 are supposed to be not HP related protein expression changes.

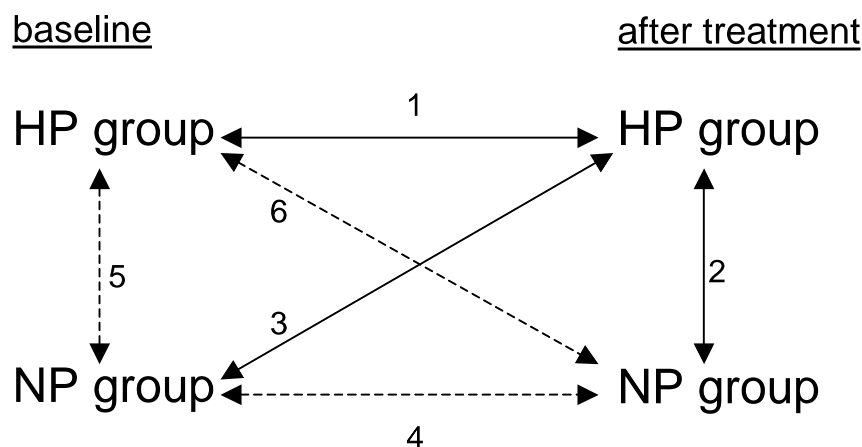

Figure 5: Diagram of calculation groups for statistical analysis to find HP diet associated changes (1-3) or false positive changes (4-6).

If the same individuals were compared within the same treatment group, paired student t-test was made. Unpaired t-test had to be calculated for comparison across the treatment groups (of the HP and NP group). Additionally an unpaired t-test was calculated within a treatment group to have an adequate comparison of the significant changes also between the two different treatment groups. The significant range was defined $p < 0.05$ and the number of significant events was counted. Not all the significant events derived from proteins. Some significant spots derived from artefacts, too low abundant or badly separated protein. These events were also counted. To have also an overview of another reason for false positive events the percentage of well defined protein spots of all significant events was calculated.
Significantly changed spots, that were associated with HP and NP diet, were analysed with mass spectrometry.

7.1.1. High protein diet related platelet protein changes in 2D DIGE gels – pH 4 - 7 analysis

In average 2075 ± 163 spots are counted across all individual acidic 2D DIGE gels and the average number of matched spots to the master gel is 1412 ± 85.

	1 paired t-test	**1** unpaired t-test	**2** unpaired t-test	**3** unpaired t-test	**4** paired t-test	**4** unpaired t-test	**5** unpaired t-test	**6** unpaired t-test
significant events	248	248	53	302	338	335	50	420
protein spots	203	202	30	224	265	264	31	336
artefacts	45	46	23	78	37	71	19	84
% protein	82	81	57	74	78	79	62	80

Table 8: Number of significant events, significant spots and artefacts for all possible combinations of groups and time points. Percentage of significant protein spots of all significant events. Numbers (1-6) describe the calculation groups in statistical analysis (see figure 5)

The number of significant spots is not exactly the same when calculated with paired or unpaired t-test in both, the HP and the NP diet group. Though, the significant spots are always the same except one change in the HP group and two changes in the NP group. The percentages of clearly defined protein spots from all significant events across the calculation groups are between 74 % and 82% apart from two exceptions. When comparing the two after treatment groups, the percentage of significant protein is 57 % and when comparing the two groups at the baseline the percentage of

significant protein is 62%. At figure 6 the total significant events, the number of artefacts and the number of significant proteins are shown across all possible combinations of comparison between the two study groups.

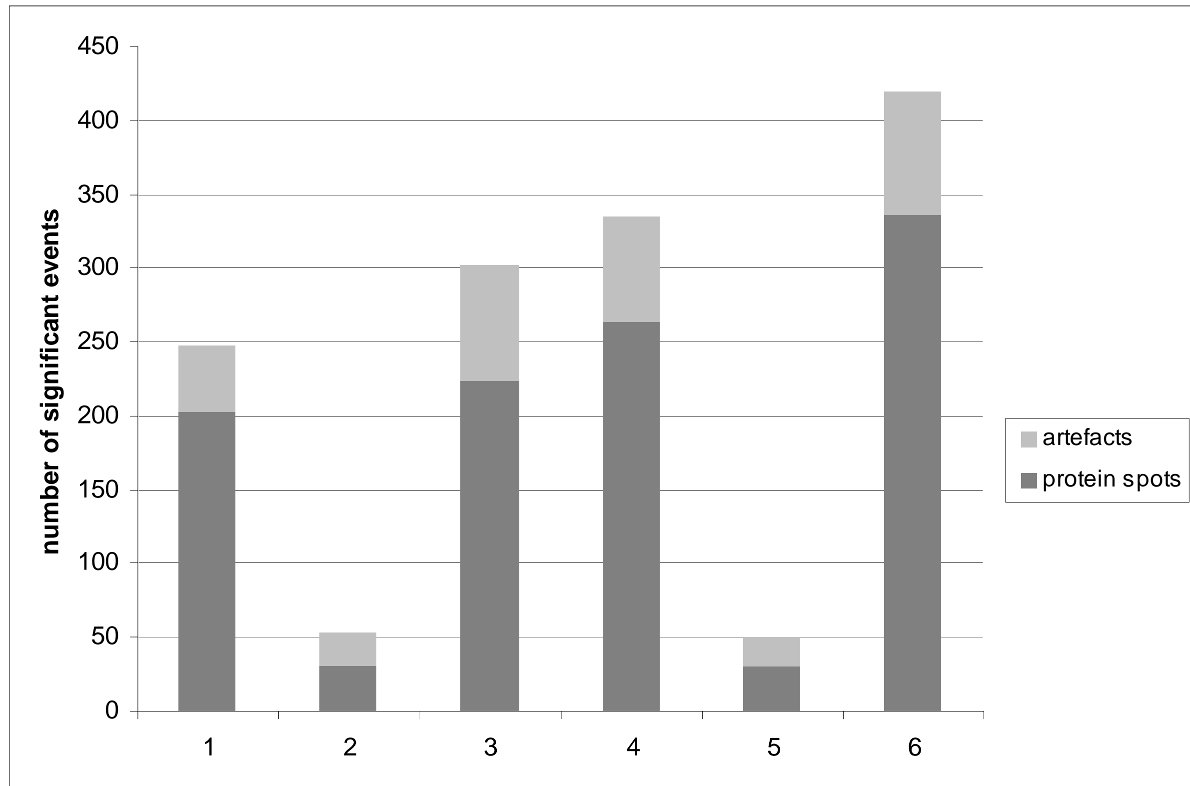

Figure 6: Number of HP and NP-related protein spot changes in pH range 4-7: All possible combinations of groups and time points were calculated to get an overview of possible interference of the standardized nutrition and grouped individuals. Numbers (1-6) describe the calculation groups in statistical analysis (see figure 5)

7.1.2. High protein diet related platelet protein changes in 2D DIGE gels – pH 6 -9 analysis

In average 2075 ± 163 spots are counted across all individual alkaline 2D DIGE gels and the average number of matched spots to the master gel in pH range 6-9 is 1072.39 ± 65.40.

	1 paired t-test	1 unpaired t-test	2 unpaired t-test	3 unpaired t-test	4 paired t-test	4 unpaired t-test	5 unpaired t-test	6 unpaired t-test
significant events	104	104	44	265	195	197	43	132
Protein spots	89	89	22	223	167	169	31	95
artefacts	15	15	22	42	28	28	12	37
% protein	86%	86%	50%	84%	86%	86%	72%	72%

Table 9: Number of significant events, significant spots and artefacts for all possible combinations of groups and time points. Percentage of significant protein spots of all significant events. Numbers (1-6) describe the calculation groups in statistical analysis (see figure 5)

The number of significant spots is not exactly the same when calculated with paired or unpaired t-test NP diet group. Though, the significant spots are always the same except three changes. The percentages of significant proteins from all significant events across the calculation groups are between 72 % and 86% apart from one exceptions. When comparing the two after treatment groups, the percentage of significant protein is 50. At figure 7 the total significant events, the number of atrefacts and the number of significant proteins are shown.

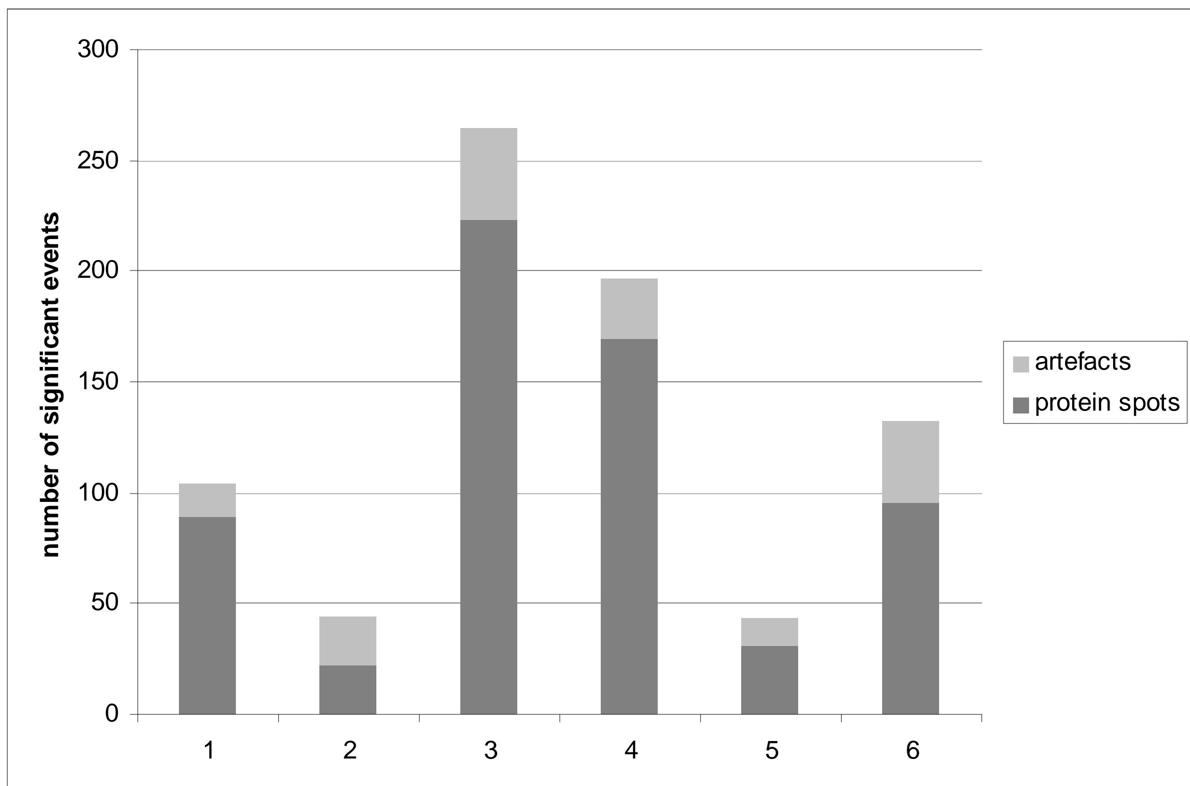

Figure 7: Number of HP and NP-related protein spot changes in pH range 6-9: All possible combinations of groups and time points were calculated to get an overview of possible interference of the standardized nutrition and grouped individuals. Numbers (1-6) describe the calculation groups in statistical analysis (see figure 5)

7.1.3. Corrected Platelet proteome analysis – elimination of supposed plasma protein contamination in in platelet proteome analysis

After the identification of protein identities by mass spectrometry of significantly changed proteins related to HP and NP diet it was conspicuous that mainly plasma proteins were significantly increased in the platelet proteome in the HP as well as in the NP study group.

Further it was conspicuous that several proteins that were previously observed as platelet proteins with very low biological variance (Zellner 2009) were decreased in platelet samples of HP as well as NP in the after treatment measurements compared to the baseline (table 10a and 10b).

pI range 4-7

Protein Name	SwissProt Acc.No.	CV	% change HP diet	paired t-test HP diet
14-3-3 protein gamma	P61981	0,084	-6	0,019
Peroxiredoxin-6	P30041	0,086	-1	0,65
Growth factor receptor-bound protein 2	P62993	0,088	-4	0,31
F-actin capping protein beta subunit (Cap Z beta)	P47756	0,088	-4	0,15
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	P62136	0,089	-8	0,12
Myosin light polypeptide 6	P60660	0,092	-4	0,29
Microtubule-associated protein RP/EB family member 2	Q15555	0,092	-6	0,12
Rab GDP dissociation inhibitor beta (Rab GDI beta)	P50395	0,093	-8	0,046
Programmed cell death 6-interacting protein (PDCD6-interacting protein)	Q8WUM4	0,095	3	0,45
Alpha-soluble NSF attachment protein (SNAP-alpha)	P54920	0,095	-5	0,017
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta 1	P62873	0,095	-4	0,22
14-3-3 protein theat	P27348	0,099	-4	0,38
14-3-3 protein zeta/delta	P63104	0,099	-4	0,38
GRP75 Mortalin	P38646	0,104	1	0,81
Protein disulfide-isomerase A6	Q15084	0,112	-1	0,68
Integrin α-IIb	P08514	0,143	-8	0,55
Nucl. assembly prot 1	P55209	0,177	-4	0,39

Table 10a: Platelet proteins with low biological variance (pH 4-7) decreased with HP diet. Ratio and paired t-test values were calculated from the HP group at the two time points. CV is the coefficient of variance of the proteins. Ratios of the NP group are also all decreasing with time (data not shown).

pI range 6-9

Protein Name	SwissProt Acc.No.	CV	% change HP diet	paired t-test HP diet
Profilin-1	P07737	0,074	1	0,79
Cyclophilin A	P62937	0,082	-2	0,57
Cyclophilin A	P62937	0,092	-2	0,64
Triosephospahate-Isomerase	P60174	0,103	-2	0,68
Mitogen-activated protein kinase 1 (ERK2)	P28482	0,103	-6	0,16
Voltage-dependent anion-selective channel protein 3	Q9Y277	0,112	-7	0,11
Fructose-bisphosphate aldolase A	P04075	0,115	-2	0,63
Calponin-2 (Calponin H2; smooth muscle) (Neutral calponin)	Q99439	0,115	-9	0,083
Tyrosyl-tRNA synthetase; cytoplasmic	P54577	0,12	-1	0,87
Dual specificity protein phosphatase 3	P51452	0,121	-1	0,77
Actin-related protein 2/3 complex subunit 2	O15144	0,125	-6	0,19
Isocitrate Dehydrogenase	P48735	0,128	-8	0,067
Protein-L-isoaspartate(D-aspartate) O-methyltransferase	P22061	0,128	4	0,36
Glyceraldehyde-3-phosphate dehydrogenase	P04406	0,129	-2	0,77
Proteasome subunit alpha type 2	P25787	0,129	-2	0,72
Proteasome subunit alpha type 4	P25789	0,137	-3	0,63
Proteasome subunit alpha type 7	O14818	0,147	-5	0,54
Glyceraldehyde-3-phosphate dehydrogenase	P04406	0,155	-4	0,63

Table 11b: Platelet proteins with low biological variance (pH 6-9) decreased with HP diet. Ratio and paired t-test values were calculated from the HP group at the two time points. CV is the coefficient of variance of the proteins. Ratios of the NP group are also all decreasing with time (data not shown).

Further many different plasma proteins like albumin and haptoglobin were found to be highly significantly increased in the platelet proteome analysis in all after treatment measurements of the HP and NP study group (see table 15). Haptoglobin was increased by 69 % (p = 0.0113) after 3 weeks in the HP diet group and by 268 % (p = 0.0059) after 3 weeks in the NP diet group. Albumin was increased by 233 % (p = 0.0038) after 3 weeks in the HP group and by 323 % ($p = 8.9 * 10^{6}$) after 3 weeks in the NP group. Other plasma proteins that were significanty increased after 3 weeks intervention time in both groups were Fibrinogen, Apolipoproteins C-III precursor protein, cytoplsamatic antiproteinase, apolipoprotein A-I precursor protein or C4b-binding protein alpha.

Normally small amounts of plasma proteins like haptoglobin and albumin can be detected in platelets which are believed to take place in their open canalicular

system. This pore system soaks them up and thereby plasma proteins can appear in proteome analysis.

There are two possible reasons for this event. The first reason is that there is a severe plasma contamination in all after treatment samples and therefore these highly abundant plasma proteins were increased in the platelet analysis. And the second reason is that albumin and haptoglobin (which has the highest increase after 3 weeks treatment) are that highly increased in plasma, so that – since the open canalicular system soaks proteins up relatively to their concentration in plasma – the effect of this high increase is visible even in platelet analysis. Therefore haptoglobin and albumin in plasma were measured (see 4.7.3). Since no significant changes could be found in plasma after 3 weeks treatment in HP and NP group the significant increase of the huge amount of different plasma proteins in the platelet proteome are supposed to be a contamination during separation of plasma proteins from platelets by gel filtration. Probably the plasma contamination happened because of too short columns during gel filtration at the second timepoint after 3 weeks tratment. When a collumn is too short in size exclusion chromatography the eultion profile is too small and the fractions that are supposed to be separated are contaminated (see 4.2.1.2.). Thereby the platelet fraction was contaminated with plasma proteins.

53 % of all significantly changed spots derived from plasma contamination. If the contamination would be smaller than 5 % there would be no interference to the normalisation of the DeCyder software. But since one of the normalisation steps of the DeCyder program normalises every spot on a gel by a median of all spot intensities of the gel, the results were distorted. Because the median of all spot intensities of the plasma contaminated samples differed a lot from the non-contaminated samples. Therefore the normalization step of the DeCyder software even worsened the results.

To get rid of the interference in the normalisation by the plasma contamination in the Decyder Programm normalisation had to be done manually in a different way. Therefore the median of every single gel of 4 proteins with low biological variance at the pH 4 – 7 and 5 proteins of pH 6 – 9 were calculated and every spot was normalized with the median of its gel. (see tables 11 and 12)

pI range 4-7

Spot id	Protein Name	SwissProt Acc. no.	CV-all
2041	14-3-3 protein gamma	P61981	0.084
2062	Peroxiredoxin-6	P30041	0.086
2094	Growth factor receptor-bound protein 2	P62993	0.088
1954	F-actin capping protein beta subunit (Cap Z beta)	P47756	0.088

Table 12: Platelet proteins with low biological variance were taken for normalization for pI range 4 – 7.

pI range 6-9

Spot id	Protein Name	SwissProt Acc. no.	CV-all
1724	Profilin-1	P07737	0.074
1671	Cyclophilin A	P62937	0.082
1672	Cyclophilin A	P62937	0.092
1488	Triosephospahate-Isomerase	P60174	0.103
1115	Mitogen-activated protein kinase 1 (ERK2)	P28482	0.103

Table 13: Platelet proteins with low biological variance were taken for normalization for pI range 6 – 9.

Afterwards the spots had to be standardised (as usual standardised abundance in the DeCyder software) with the analogous spot in the internal standard on the same gel to further decrease technical variance of 2D electrophoresis. These corrections in the normalisation to overcome the interference of plasma contamination were done for both, the values of the first and the second time point.

After these two normalisation steps the adjusted standardised abundances could be drawn on as basis for further calculations. Natural logarithm, common logarithm and ^{2}log were calculated and t-test and ratios for every spot in every possible combination of group and time point (see above) were assessed.

After this approach to focuss the normalisation exclusivly on platelet proteins to exclude the interference of the plasma contamination now all the remaining platelet proteins with low biological variance (that were not chosen for normalisation) did not change significantly and had ratios very close to 1. (see table 13a and 13b)

pI range 4-7

Spot ID	Protein Name	SwissProt Acc. No.	% change HP diet	paired t-test HP diet
2041	**14-3-3 protein gamma**	**P61981**	**-1**	**0,221**
2062	**Peroxiredoxin-6**	**P30041**	**3**	**0,324**
2094	**Growth factor receptor-bound protein 2**	**P62993**	**1**	**0,647**
1954	**F-actin capping protein beta subunit (Cap Z beta)**	**P47756**	**1**	**0,324**
1810	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	P62136	-3	0,515
2192	Myosin light polypeptide 6	P60660	1	0,651
1910	Microtubule-associated protein RP/EB family member 2	Q15555	-1	0,427
1546	Rab GDP dissociation inhibitor beta (Rab GDI beta)	P50395	-3	0,14
772	Programmed cell death 6-interacting protein (PDCD6-interacting protein)	Q8WUM4	9	0,094
1918	Alpha-soluble NSF attachment protein (SNAP-alpha)	P54920	0	0,898
1884	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta 1	P62873	1	0,77
2047	14-3-3 protein theat	P27348	2	0,788
2047	14-3-3 protein zeta/delta	P63104	2	0,788
1062	GRP75 Mortalin	P38646	6	0,096
1518	Protein disulfide-isomerase A6	Q15084	3	0,59
568	Integrin α-IIb	P08514	-2	0,602
1367	Nucl. assembly prot 1	P55209	1	0,757

Table 14a: Platelet proteins with low biological variance after correction. (pH 4-7) Ratio and paired t-test values were calculated from the HP group at the two time points. Median of proteins that are marked ´**fat**´ (bold) was used for normalization.

pI range 6-9

Spot ID	Protein Name	SwissProt Acc. No.	% change HP diet	paired t-test HP diet
1724	**Profilin-1**	**P07737**	**0**	**0,886**
1671	**Cyclophilin A**	**P62937**	**1**	**0,713**
1672	**Cyclophilin A**	**P62937**	**1**	**0,577**
1488	**Triosephospahate-Isomerase**	**P60174**	**-1**	**0,683**
1115	**Mitogen-activated protein kinase 1 (ERK2)**	**P28482**	**-5**	**0,04**
1395	Voltage-dependent anion-selective channel protein 3	Q9Y277	-6	0,013
1096	Fructose-bisphosphate aldolase A	P04075	-1	0,714
1248	Calponin-2 (Calponin H2; smooth muscle) (Neutral calponin)	Q99439	-8	0,022
700	Tyrosyl-tRNA synthetase; cytoplasmic	P54577	0	0,88
1581	Dual specificity protein phosphatase 3	P51452	-2	0,151
1351	Actin-related protein 2/3 complex subunit 2	O15144	-5	0,022
1013	Isocitrate Dehydrogenase	P48735	-7	0,028
1510	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	P22061	6	0,107
1177	Glyceraldehyde-3-phosphate dehydrogenase	P04406	0	0,839
1530	Proteasome subunit alpha type 2	P25787	0	0,559
1432	Proteasome subunit alpha type 4	P25789	-2	0,499
1061	Proteasome subunit alpha type 7	O14818	-4	0,403
1200	Glyceraldehyde-3-phosphate dehydrogenase	P04406	-3	0,659

Table 15b: Platelet proteins with low biological variance after correction. (pH 6-9) Ratio and paired t-test values were calculated from the HP group at the two time points. Median of proteins that are marked ´**fat**´ (bold) was used for normalization.

After the two normalization steps ratios and paired and unpaired t-test of all possible combinations of the two groups at the two time points were calculated. A paired t-test could not always be accomplished, since in some of the combinations of the groups there were not the same individuals that were compared.

7.1.4. Influence of HP diet on platelet 2D DIGE pH 4-7 analysis- corrected to get rid of plasma protein contamination

After correcting the plasma contamination, the number of significantly changed protein spots increased dramatically when the baseline values and the after 3 weeks treatment values were compared. There was nearly no change in the number of significant protein spots when the two groups were compared at the baseline or after 3 weeks respectively.

	1 paired t-test	1 unpaired t-test	2 unpaired t-test	3 unpaired t-test	4 paired t-test	4 unpaired t-test	5 unpaired t-test	6 unpaired t-test
significant events	392	297	42	285	389	295	41	381
protein spots	320	254	24	219	317	246	23	311
artefacts	72	43	18	66	72	49	18	70
% protein	82%	86%	57%	77%	81%	83%	56%	82%

Table 16: Number of significant events, significant spots and artefacts for all possible combinations of groups and time points. Percentage of clearly defined protein spots from all significant events. Numbers (1-6) describe the calculation groups in statistical analysis (see figure 5 page 36)

The percentages of significant proteins from all significant events across the calculation groups are between 77 % and 86% apart from two exceptions. When comparing the two after treatment groups, the percentage of significant protein is 57 and when comparing the two groups at the baseline the percentage of significant proteins is 56%. At figure 8 the total significant events, the number of artefacts and the number of significant proteins are shown.

In comparison to the number of significant protein spots, the proportion of significant protein spots and artefacts was similar before and after correction of the plasma contaminaton.

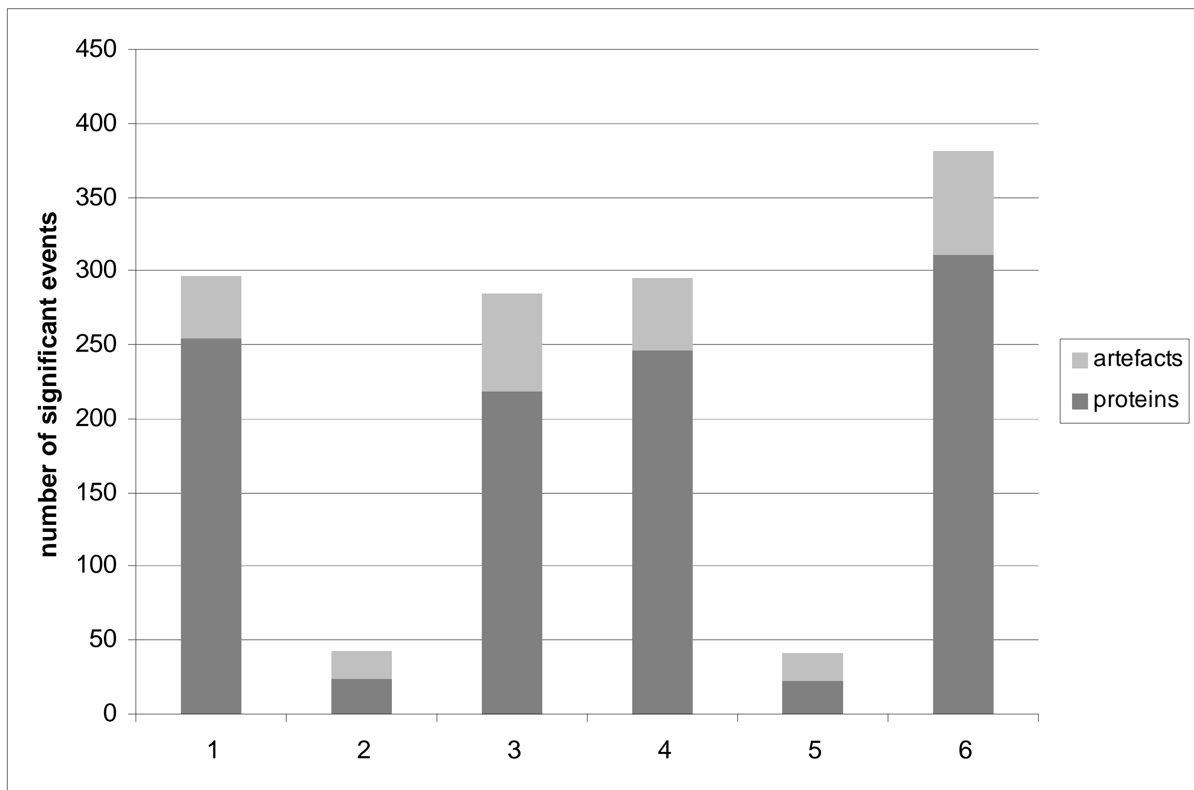

Figure 8: Number of HP and NP-related protein spot changes in pH range 4-7: All possible combinations of groups and time points were calculated to get an overview of possible interference of the standardized nutrition and grouped individuals. Numbers (1-6) describe the calculation groups in statistical analysis (see figure 5 page 36)

Significantly changed spots were identified with mass spectrometry. On table 15 all identified significant proteins are listed. The percentage of change between the groups and t-test are named.

1 (3 weeks HP / baseline HP)				
spot number	acessnr	protein name	% change	t-test (unpaired)
S980	**Q86UX7**	Unc-112-related protein 2	55	0,0005
S1733	**P00738**	Haptoglobin?	69	0,0006
S1453	**P02679**	Fibrinogen gamma	221	0,0006
S1487	**P02679**	Fibrinogen gamma chain	35	0,0007
S1013	**P04003**	C4b-binding protein alpha	247	0,0008
S1486	**P02679**	Fibrinogen gamma chain A	29	0,0020
S1714	**P00738**	Haptoglobin	207	0,0025
S1098	**P02679**	Albumin	245	0,0031
S1330	**P02675**	Fibrinogen beta chain [Precursor]	36	0,0032
S2118	**P02647**	Apolipoprotein A-I precusor	354	0,0035
S1750	**P00738**	Haptoglobin?	256	0,0040
S1004	**P04003**	C4b-binding protein alpha ?	214	0,0046
S1343	**P02675**	Fibrinogen beta chain	33	0,0060
S1693	**P00738**	Haptoglobin	50	0,0071
S1401	**P02675**	Fibrinogen beta chain	51	0,0071
S682	**P02787**	Serotransferrin precursor ?	17	0,0091
S966	**Q05682**	Caldesmon	21	0,0094
S516	**P01023**	alpha-2-macroglobulin	246	0,0113
S1499	**P02679**	Fibrinogen gamma chain	12	0,0122
S1451	**P02679**	Fibrinogen gamma chain precursor	28	0,0142
S1326	**P02675**	Fibrinogen beta chain	32	0,0147
S1489	**P02679**	Fibrinogen gamma	24	0,0173
S1389	**P02675**	Fibrinogen beta chain	26	0,0189
S1433	**Q14141**	Septin-6	11	0,0190
S2155	**P19105**	Myosin regulatory light chain 2; nonsarcomeric	25	0,0202
S2221	**P02656**	Apolipoprotein C-III precursor	209	0,0216
S991	**Q05682**	Caldesmon	24	0,0226
S938	**Q15942**	Zyxin	29	0,0265
S2152	**P24844**	Myosin regulatory light chain 2; smoothmuscle isoform	22	0,0274
S1885	**O43396**	Thioredoxin-like protein 1	8	0,0284
S2142	**P32119**	Peroxiredoxin 2 ?	89	0,0319
S947	**Q15942**	Zyxin	39	0,0458
S463	**Q9Y4L1**	150 kDa oxygen-regulated protein [Precursor] ?	18	0,0477

2 (3 weeks HP / 3 weeks NP)				
spot number	acessnr	protein name	% change	t-test (unpaired)
S2051	**P60900**	Proteasome subunit alpha type-6	15	0,0096
S752	**P12814**	alpha-actinin 1	91	0,0183

3 (3 weeks HP / baseline NP)				
spot number	acessnr	protein name	% change	t-test (unpaired)
S980	**Q86UX7**	Unc-112-related protein 2	79	0,0000
S1453	**P02679**	fibrinogen gamma	234	0,0002
S1487	**P02679**	Fibrinogen gamma chain	41	0,0003
S2118	**P02647**	Apolipoprotein A-I precusor	488	0,0004
S1733	**P00738**	Haptoglobin?	70	0,0006
S1013	**P04003**	C4b-binding protein alpha	46	0,0006
S1486	**P02679**	Fibrinogen gamma chain A	30	0,0012
S1098	**P02679**	Albumin	265	0,0013
S1330	**P02675**	Fibrinogen beta chain [Precursor]	41	0,0014
S1750	**P00738**	Haptoglobin?	284	0,0017
S1343	**P02675**	Fibrinogen beta chain	39	0,0019
S1004	**P04003**	C4b-binding protein alpha ?	213	0,0029
S1489	**P02679**	Fibrinogen gamma	26	0,0067
S1401	**P02675**	Fibrinogen beta chain	48	0,0076
S1326	**P02675**	Fibrinogen beta chain	36	0,0088
S516	**P01023**	alpha-2-macroglobulin	263	0,0088
S1501	**P10644**	cAMP-dependent protein kinase type I-alpha regulatory subunit	12	0,0097
S1714	**P00738**	Haptoglobin	81	0,0101
S2161	**P00738**	Haptoglobin	16	0,0138
S2142	**P32119**	Peroxiredoxin 2	73	0,0264
S1499	**P02679**	Fibrinogen gamma chain	11	0,0280
S1791	**P04899**	Guanine nucleotide-bindingproteinG(i), alpha-2 subunit	-10	0,0291
S1451	**P02679**	Fibrinogen gamma chain precursor	24	0,0308
S1299	**P10809**	60kDa heat shock protein, mitochondrial	11	0,0309
S1693	**P00738**	Haptoglobin	37	0,0335

4 (3 weeks NP / baseline NP)				
spot number	acessnr	protein name	% change	t-test (unpaired)
S1013	**P04003**	C4b-binding protein alpha	336	0,0000
S2118	**P02647**	Apolipoprotein A-I precusor	647	0,0000
S1750	**P00738**	Haptoglobin?	386	0,0000
S1004	**P04003**	C4b-binding protein alpha ?	275	0,0000
S1453	**P02679**	fibrinogen gamma	236	0,0000
S1098	**P02679**	Albumin	331	0,0000
S1714	**P00738**	Haptoglobin	268	0,0000
S1733	**P00738**	Haptoglobin?	204	0,0001
S980	**Q86UX7**	Unc-112-related protein 2	81	0,0001
S1343	**P02675**	Fibrinogen beta chain	49	0,0001
S516	**P01023**	alpha-2-macroglobulin	343	0,0003
S1330	**P02675**	Fibrinogen beta chain [Precursor]	43	0,0004
S1489	**P02679**	Fibrinogen gamma	35	0,0006
S1486	**P02679**	Fibrinogen gamma chain A	33	0,0007
S1693	**P00738**	Haptoglobin	90	0,0011
S1487	**P02679**	Fibrinogen gamma chain	34	0,0017
S1326	**P02675**	Fibrinogen beta chain	38	0,0029
S1451	**P02679**	Fibrinogen gamma chain precursor	39	0,0055
S1142	**Q9BR76**	Coronin-1B	52	0,0060
S1401	**P02675**	Fibrinogen beta chain	42	0,0161
S2161	**P00738**	Haptoglobin	377	0,0178
S2221	**P02656**	Apolipoprotein C-III precursor	223	0,0242
S1299	**P10809**	60kDa heat shock protein, mitochondrial	10	0,0323
S991	**Q05682**	Caldesmon	40	0,0387
S463	**Q9Y4L1**	150 kDa oxygen-regulated protein [Precursor] ?	17	0,0463

5 (baseline HP / baseline NP)				
spot number	acessnr	protein name	% change	t-test (unpaired)
S2216	**O75947**	ATP synthase subunit d, mitochondrial ?	-13	0,0265

6 (3 weeks NP / baseline HP)				
spot number	acessnr	protein name	% change	t-test (unpaired)
S1013	**P04003**	C4b-binding protein alpha	338	0,0000
S1714	**P00738**	Haptoglobin	307	0,0000
S1750	**P00738**	Haptoglobin?	347	0,0000
S2118	**P02647**	Apolipoprotein A-I precusor	470	0,0000
S1098	**P02679**	Albumin	306	0,0000
S1004	**P04003**	C4b-binding protein alpha ?	276	0,0000
S1453	**P02679**	fibrinogen gamma	223	0,0001
S1733	**P00738**	Haptoglobin?	203	0,0001

S1693	**P00738**	Haptoglobin	207	0,0003
S1343	**P02675**	Fibrinogen beta chain	43	0,0005
S516	**P01023**	alpha-2-macroglobulin	321	0,0008
S2221	**P02656**	Apolipoprotein C-III precursor	379	0,0009
S1330	**P02675**	Fibrinogen beta chain [Precursor]	37	0,0010
S1486	**P02679**	Fibrinogen gamma chain A	31	0,0011
S1142	**Q9BR76**	Coronin-1B	55	0,0014
S980	**Q86UX7**	Unc-112-related protein 2	56	0,0018
S1489	**P02679**	Fibrinogen gamma	33	0,0024
S1451	**P02679**	Fibrinogen gamma chain precursor	43	0,0027
S1389	**P02675**	Fibrinogen beta chain	38	0,0028
S1487	**P02679**	Fibrinogen gamma chain	29	0,0045
S1326	**P02675**	Fibrinogen beta chain	34	0,0049
S1942	**P02649**	Apolipoprotein E3 precursor	93	0,0064
S463	**Q9Y4L1**	150 kDa oxygen-regulated protein [Precursor] ?	22	0,0080
S966	**Q05682**	Caldesmon ?	60	0,0105
S772	**Q8WUM4**	Programmed cell death 6-interacting protein (PDCD6-interacting protein) ?	13	0,0132
S1401	**P02675**	Fibrinogen beta chain	45	0,0152
S1078	**P43304**	Glycerol-3-Phosphat-DH	28	0,0164
S991	**Q05682**	Caldesmon	47	0,0186
S2152	**P24844**	Myosin regulatory light chain 2; smoothmuscle isoform	21	0,0195
S2216	**O75947**	ATP synthase subunit d, mitochondrial ?	11	0,0259
S1499	**P02679**	Fibrinogen gamma chain	11	0,0302
S1083	**P43304**	Glycerol-3-phosphate dehydrogenase; mitochondrial [Precursor]	22	0,0318
S1318	**P31146**	Coronin-1A(Coronin-like rotein p57)	16	0,0331
S682	**P02787**	Serotransferrin precursor ?	11	0,0416
S1312	**P31146**	Coronin-1A ?	14	0,0441
S1671	**P30740**	leukocyte elastase inhibitor	-9	0,0466

probable plasma contamination

probable interference of control diet

Table 17: List of identified significant spots of pH 4 – 7 gels. Proteins that probably derive from plasma contamination are marked with grey. Proteins that probably derive from interference of control diet are marked with light blue. Ratios and unpaired t-test results are named. Numbers (1-6) describe the calculation groups in statistical analysis (see figure 5 page 36)

As visible on table 15 after the normalization with low variabel platelet proteins the significant increase of the plasma proteins in the after treatment values of the HP and NP group persists. Some of the proteins – like Unc-112-related protein 2, Caldesmon or 60kDa heat shock protein, mitochondrial – that are significantly changed with HP diet are also significantly changed in the NP diet and therefore seems not to be related mainly to the HP diet.

The following table show only significantly changed proteins that are supposed to be changed only because of the HP diet.

2 (3 weeks HP / 3 weeks NP)				
spot number	acessnr	protein name	% change	t-test (unpaired)
S2051	**P60900**	Proteasome subunit alpha type-6	15	0,0096
S752	**P12814**	alpha-actinin 1	-9	0,0183

1 (3 weeks HP / baseline HP)				
spot number	acessnr	protein name	% change	t-test (unpaired)
S1433	**Q14141**	Septin-6	11	0,0190
S2155	**P19105**	Myosin regulatory light chain 2; nonsarcomeric	25	0,0202
S938	**Q15942**	Zyxin	29	0,0265
S1885	**O43396**	Thioredoxin-like protein 1	18	0,0284
S2142	**P32119**	Peroxiredoxin 2 ?	-11	0,0319
S947	**Q15942**	Zyxin	39	0,0458

3 (3 weeks HP / baseline NP)				
spot number	acessnr	protein name	% change	t-test (unpaired)
S1501	**P10644**	cAMP-dependent protein kinase type I-alpha regulatory subunit	12	0,0097
S2142	**P32119**	Peroxiredoxin 2	-37	0,0264
S1791	**P04899**	Guanine nucleotide-bindingproteinG(i), alpha-2 subunit	-10	0,0291

Table 18: Identified significant spots of pH 4 – 7 gels associated with HP diet. Numbers (1-3) describe the calculation groups in statistical analysis (see figure 5 page 36)

7.1.5. Influence of HP diet on platelet proteome 2D DIGE pH 6-9 analysis-corrected to get rid of plasma protein contamination

The number of singnificantly changed spots after correction of the plasma contamination differed a lot from before. But in contrast to pH 4 – 7 they didn't increase. All except one decreased. The comparison of the HP diet at the two different timepoints increased after the correction of the plasma contamination.

	1 paired t-test	**1** unpaired t-test	**2** unpaired t-test	**3** unpaired t-test	**4** paired t-test	**4** unpaired t-test	**5** unpaired t-test	**6** unpaired t-test
significant events	212	80	56	200	198	161	35	96
protein spots	158	66	30	171	147	138	24	71
artefacts	54	14	26	29	51	23	11	25
% protein	75%	83%	54%	86%	74%	86%	69%	74%

Table 19: Number of significant events, significant spots and artefacts for all possible combinations of groups and time points. Percentage of clearly defined protein spots from all significant events. Numbers (1-6) describe the calculation groups in statistical analysis (see figure 5 page 36)

The number of significant spots differs a lot when calculated with paired or unpaired t-test in the HP and the NP diet group. In the HP group there are 212 events found when calculated with paired t-test, but only 80 when calculated with unpaired t-test. Though, all of the 80 significant events are also found in the paired t-test calculation. In the NP group there are 37 significant events that are different, depending on the t-test that is performed. Further it is worth to mention that at comparison 1 (3 weeks HP / baseline HP) there are much less significant protein spots than in comparison 4 (3 weeks NP / baseline NP) which is actually used only as control. The percentages of significant proteins from all significant events across the calculation groups are between 69 % and 86% apart from one exception. When comparing the two after treatment groups, the percentage of significant protein is 54%. At figure 9 the total significant events, the number of artefacts and the number of significant proteins are shown.

In comparison to the number of significant protein spots, the proportion of significant protein spots and artefacts was similar before and after correction of the plasma contaminaton.

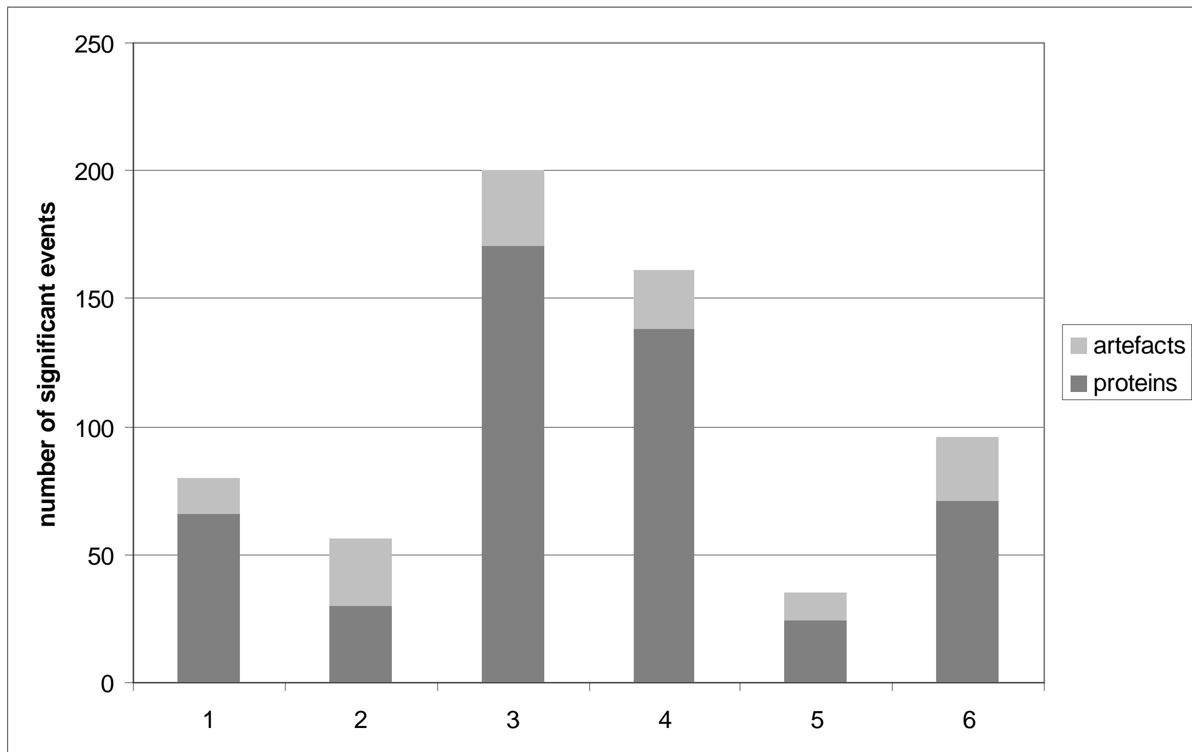

Figure 9: Number of HP and NP-related protein spot changes in pH range 6-9: All possible combinations of groups and time points were calculated to get an overview of possible interference of the standardized nutrition and grouped individuals. Numbers (1-6) describe the calculation groups in statistical analysis (see figure 5 page 36)

Significant changed spots were identified with mass spectrometry. On table 18 all identified significant proteins are listed. The percentage of change between the groups and t-test are named.

1 (3 weeks HP / baseline HP)				
spot number	**acessnr**	**protein name**	**% change**	**t-test (unpaired)**
B1386	**Q9Y277**	Voltage dependent anion selective channel protein 3	-8	0,0055
B645	**P27338**	Monoamine Oxidase B	-31	0,0087
B206	**P19367**	Hexokinase-1	-12	0,032
B202	**Q7KZF4**	Staphylococcal nuclease domain-containing protein	-15	0,0111
B760	**P02675**	Fibrinogen beta chain	22	0,0278
B448	**P43405**	Tyrosine-protein kinase SYK	-10	0,0284
B726	**P02675**	Fibrinogen beta chain	14	0,0364

2 (3 weeks HP / 3 weeks NP)				
spot number	acessnr	protein name	% change	t-test (unpaired)
B1460	**P18669**	Phosphoglycerate mutase 1	-8	0,0203

3 (3 weeks HP / baseline NP)				
spot number	acessnr	protein name	% change	t-test (unpaired)
B871	**Q13418**	Integrin linked kinase	-66	0,0000
B518	**P02671**	Fibrinogen alpha chain	41	0,0050
B726	**P02675**	Fibrinogen beta chain	21	0,0050
B528	**P02671**	Fibrinogen alpha chain	46	0,0051
B760	**P02675**	Fibrinogen beta chain	33	0,0056
B541	**P02671**	Fibrinogen alpha-chain	52	0,0089
B759	**P02675**	Fibrinogen beta chain	29	0,0098
B539	**AAA52426**	Fibrinogen alpha chain	33	0,0114
B527	**P02671**	Fibrinogen alpha chain	30	0,0115
B1460	**P18669**	Phosphoglycerate mutase 1	-8	0,0146
B154	**P53396**	ATP-citrate synthase	-30	0,0154
B1431	**P00915**	Carbonic Anhydrase 1	-203	0,0172
B202	**Q7KZF4**	Staphylococcal nuclease domain-containing protein	-16	0,0176
B206	**P19367**	Hexokinase-1	-13	0,0187
B863	**Q13418**	Integrin linked protein kinase 1	-6	0,0238
B614	**P02675**	Fibrinogen beta chain ?	22	0,0272
B1714	**P68871**	Haemoglobin subunit beta	-289	0,0274
B1395	**Q9Y277**	Voltage-dependent anion-selective channel protein 3	-8	0,0275
B1128	**Q15942**	Zyxin ?	-12	0,0482

4 (3 weeks NP / baseline NP)				
spot number	acessnr	protein name	% change	t-test (unpaired)
B750	**P02671**	Fibrinogen alpha chain	61	0,0095
B538	**P02671**	Fibrinogen alpha chain	43	0,0097
B1408	**P02671**	Fibrinogen alpha-chain	28	0,0128
B517	**P02675**	Fibrinogen beta chain	31	0,0295
B264	**Q13418**	Integrin linked kinase	-34	0,0475

5 (baseline HP / baseline NP)				
spot number	acessnr	protein name	% change	t-test (unpaired)
B871	**Q13418**	Integrin linked kinase	-35	0,0174

6 (3 weeks NP / baseline HP)				
spot number	**acessnr**	**protein name**	**% change**	**t-test (unpaired)**
B1510	**P22061**	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	13	0,0236

probable plasma contamination

probable interference of control diet

Table 20: List of identified significant spots of pH 6 – 9 gels. Proteins that probably derive from plasma contamination are marked with grey. Proteins that probably derive from interference of control diet are marked with light blue. Ratios and unpaired t-test results are named. Numbers (1-6) describe the calculation groups in statistical analysis (see figure 5 page 36)

On table 18 after the normalization with the low variable platelet protein of the spot volumes there are still some significantly increased standardized abundances of spots in all after treatment values left that probably derive from plasma contamination. All of these spots are fibrinogen. Some of the proteins that are significantly changed with HP diet are also significantly changed with NP diet, when there was no obvious difference in food composition. These proteins were excluded of the results.

Table 19 show significantly changed platelet proteins that are supposed to be only influenced by the HP diet.

1 (3 weeks HP / baseline HP)				
spot number	**acessnr**	**protein name**	**% change**	**t-test (unpaired)**
B1386	**Q9Y277**	Voltage-dependent anion-selective channel protein 3	-8	0,0055
B645	**P27338**	Amine Oxidase	-31	0,0087
B206	**P19367**	Hexokinase-1	-12	0,032
B202	**Q7KZF4**	Staphylococcal nuclease domain-containing protein	-15	0,0111
B448	**P43405**	Tyrosine-protein kinase SYK	-10	0,0284

2 (3 weeks HP / 3 weeks NP)				
spot number	**acessnr**	**protein name**	**% change**	**t-test (unpaired)**
B1460	**P18669**	Phosphoglycerate mutase 1	-8	0,0203

3 (3 weeks HP / baseline NP)				
spot number	**acessnr**	**protein name**	**% change**	**t-test (unpaired)**
B1460	**P18669**	Phosphoglycerate mutase 1	-8	0,0146
B154	**P53396**	ATP-citrate synthase	-30	0,0154
B1431	**P00915**	Carbonic Anhydrase 1	-203	0,0172
B202	**Q7KZF4**	Staphylococcal nuclease domain-containing protein	-16	0,0176
B206	**P19367**	Hexokinase-1	-13	0,0187
B1714	**P68871**	Haemoglobin subunit beta	-289	0,0274
B1395	**Q9Y277**	Voltage-dependent anion-selective channel protein 3	-8	0,0275
B1128	**Q15942**	Zyxin ?	-12	0,0482

Table 21: Identified significant spots of pH 6 – 9 gels associated with HP diet. Numbers (1-3) describe the calculation groups in statistical analysis (see figure 5 page 36)

After the normalization by the low variable platelet proteins in the pH range 6 - 9 to eliminate the influence of plasma contamination on the first normalization step, the results from pH 6 – 9 did not considerably change or some of the significantly changed proteins lost or worsened their significance. Monoamine oxidase B for example differed in the ratio by 2 % and significance decreased by 0.0033. Further proteins, like voltage-dependent anion-selective channel protein 3 that are supposed to be proteins with low biological variation suddenly were significantly changed with HP diet.

Since most plasma proteins occur in 2D separation of pH range 4 – 7 and hardly none of the plasma proteins has a pI larger than 7, the primary analysis of the results of the gels from pH 6 – 9 seemed to be the proper one:

1 (3 weeks HP / baseline HP)				
spot number	**acessnr**	**protein name**	**% change**	**t-test (unpaired)**
645	**P27338**	Amine Oxidase	-33	0,0054
206	**P19367**	Hexokinase-1	-12	0,037
1431	**P00915**	Carbonic Anhydrase 1	-29	0,042

3 (3 weeks HP / baseline NP)				
spot number	**acessnr**	**protein name**	**% change**	**t-test (unpaired)**
154	**P53396**	ATP-citrate synthase	-31	0,0051
206	**P19367**	Hexokinase-1	-16	0,0094
1431	**P00915**	Carbonic Anhydrase 1	-214	0,014
1013	**P48735**	Isocitrate Dehydrogenase	-12	0,017
1460	**P18669**	Phosphoglycerate mutase 1	-11	0,018
1714	**P68871**	Haemoglobin subunit beta	-313	0,019
448	**P43405**	Tyrosine-protein kinase SYK	-14	0,021
6	**P51659**	Peroxisomal multifunctional?	10	0,023
1227	**Q15942**	Zyxin ?	-10	0,031
201	**P19367**	Hexokinase-1	-29	0,034
515	**P43304**	Glycerol-3-phosphate dehydrogenase;mitochondrial precursor	-19	0,04

Table 22: Identified significant spots of pH 6 – 9 gels associated with HP diet. Numbers (1,3) describe the calculation group in statistical analysis (see figure 5 page 36)

7.1.6. HP related decrease of Monoamine Oxidase B expression in platelets

Mao-B expression in platelets significantly decreased by 33% with HP diet ($P < 0.0054$) (paired t-test). Mao-B has with this p value the highest significance from all HP diet related proteins. A slight decrease of Mao-B expression was also observed in the NP group but becomes not significant. However therefore no significant decrease of Mao-B expression could be found by comparing the after treatment values of the NP and HP group (see figure 10).

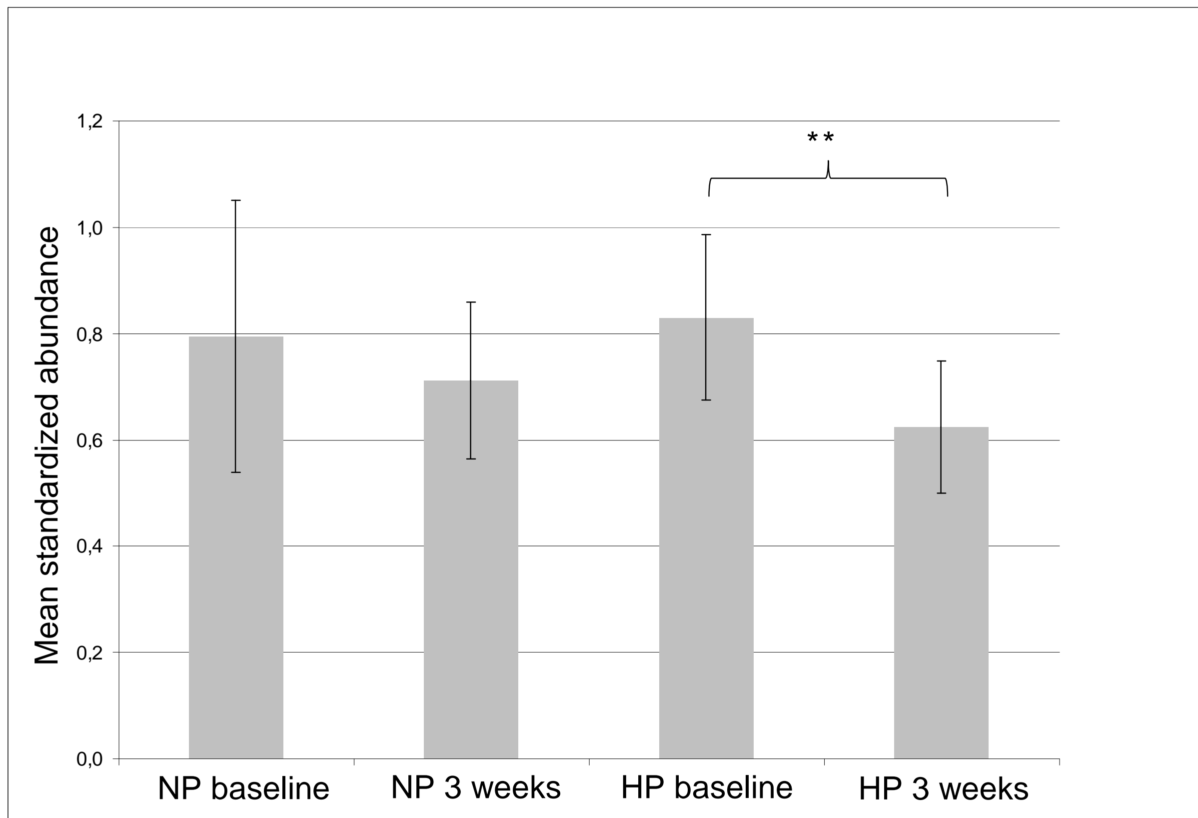

Figure 10: Platelet Mao-B expression of the study individuals in the NP (n =12) and HP group (n = 12) at the baseline and after 3 weeks treatment measured by 2D DIGE. Results are presented as means ± standard deviations. Significances are calculated with paired student t-test **P < 0.0054

Figure 10 shows the mean standardised abundance of Mao-B expression across the study groups and Figure 11 and 12 show Mao-B expression in each individual of the NP and the HP group and thereby illustrate that the protein expression changes of Mao-B are very uniform in the HP group in comparison to the NP group.

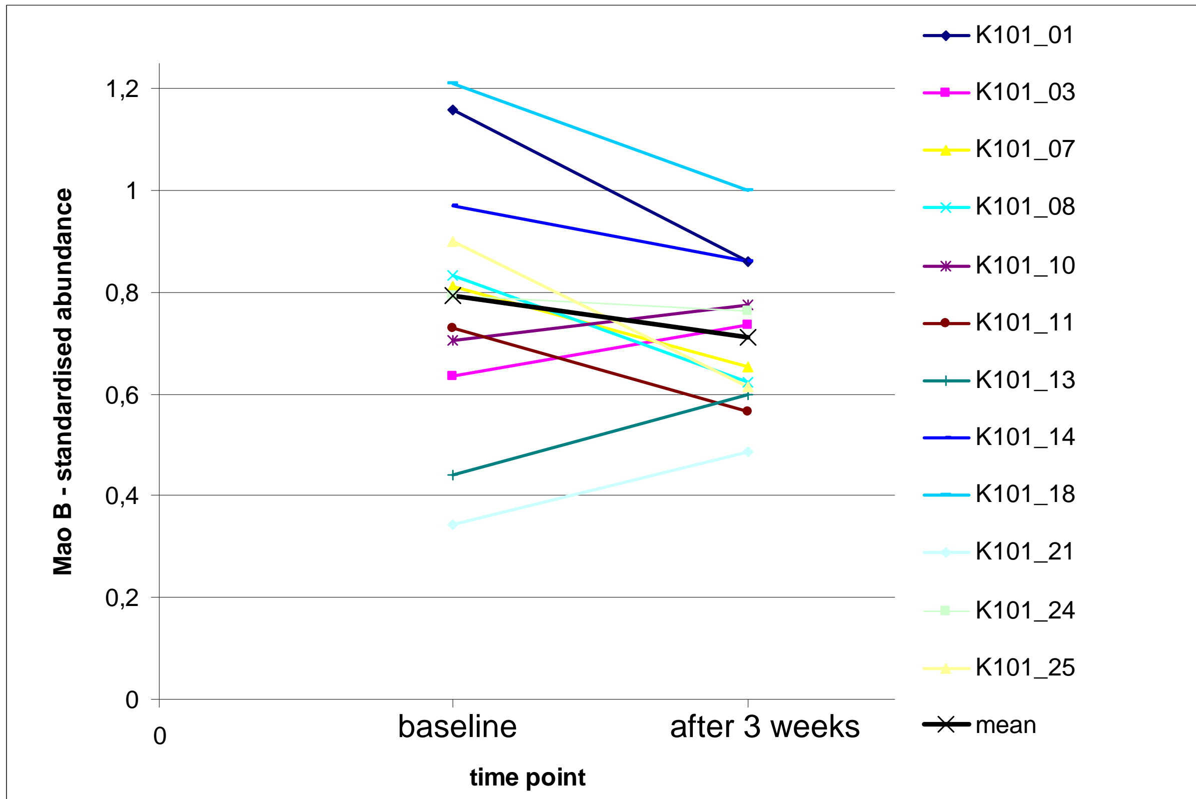

Figure 11: Standardized abundance of Mao-B expression from each individual of the NP group.

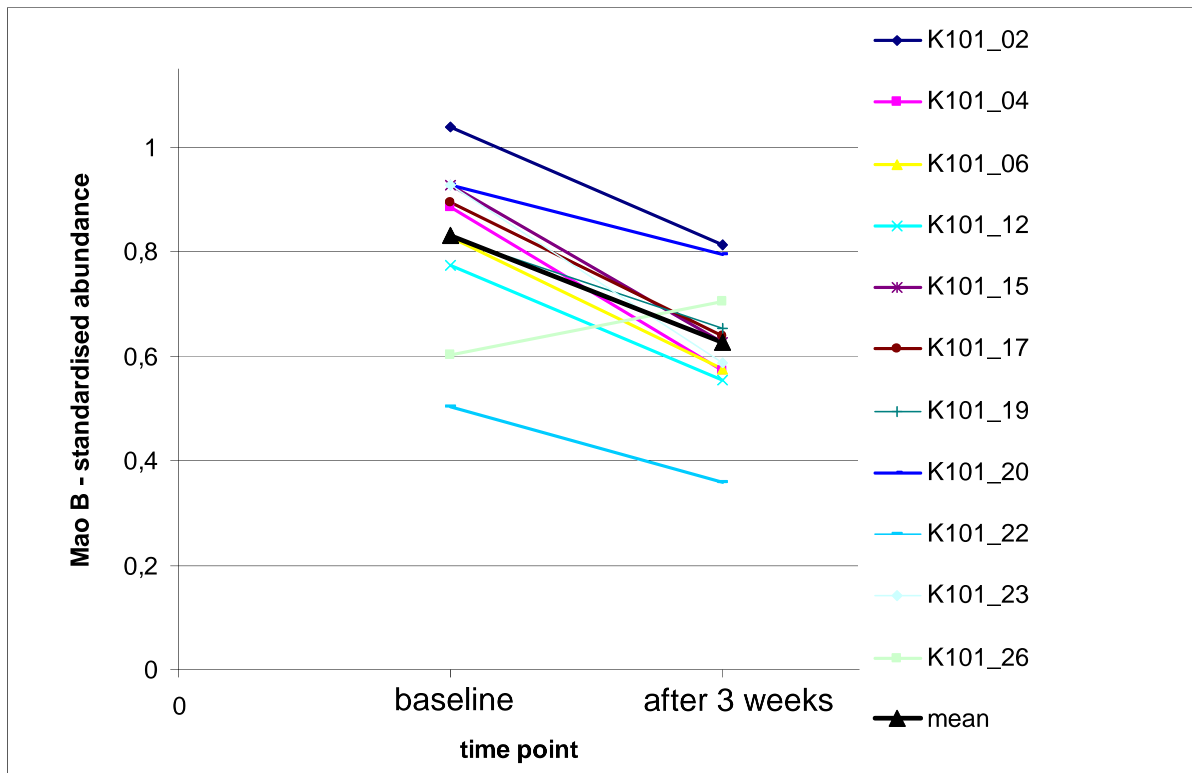

Figure 12: Standardized abundance of Mao-B of each individual of the HP group

7.1.7. Platelet Monoamine oxidase B expression and reaction time correlates significantly

To evaluate the influence of a high protein diet on cognitive functions of the study individuals a Go/NoGo test was performed.

The HP group had a significantly decreased mean reaction time (-25.5 +/- 11.6 msec) and a decrease in the number of errors in the Go/No-Go test. (Jakobsen 2010)

The main function of Mao-B is to degrade dopamine in the central nervous system which is an important neurotransmitter in the process of learning. Since platelets are also described to act as surrogate cells for neurons platelet Mao-B expression was correlated with their reaction time from the respective study individuals.

As shown on figure 13, there is a significant correlation (r = 0.477; p = 0.0213) between the reaction time and the Mao-B expression in platelets.

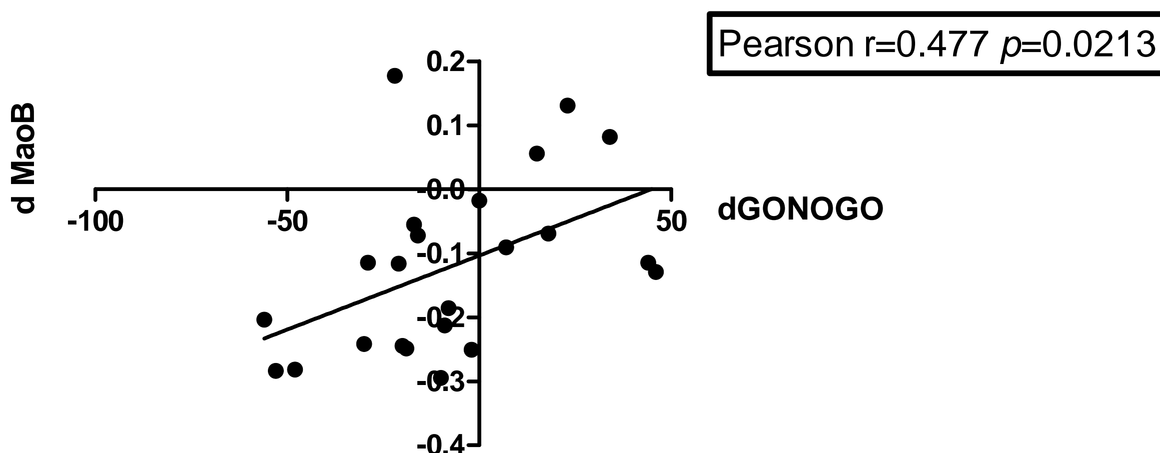

Figure 13: Correlation between reaction time measured with Go/NoGo test and Mao-B expression in platelets. Correlation was calculated with Pearson Test. d are the differences from baseline to the after treatment measurement. r is the correlation. p is the significance of the correlation

7.1.8. HP related increase of Myosin regulatory light chain 2 – MLC-2 expression in platelets

One study hypothesis was that the HP diet should increase muscle strength. In a previous proteomics study elderly subjects are characterised by a reduced phosphorylation of myosin regulatory chain isoform in the vastus lateralis muscle, which could contribute to the impairment of muscle performance (Gelfi, 2005). In our

study platelets should be a surrogate cell for muscle cells and therefore we were espcially interested in Myosin regulatory light chain.

Five isoforms of MLC-2 were identified on the 2D DIGE gels (pH 4-7) in this study. Two smooth muscle isoforms and three nonsarcomeric isoforms. Two of these 5 isoforms are significantly changed with HP diet in the platelet proteome, namely a MLC-2 nonsarcomeric isoform (spotnr. 2155) and a smooth muscle isoform (spotnr. 2152). (see figure 14)

The HP diet induced a significant increase of P19105 Myosin regulatory light chain 2; nonsarcomeric (25%; $P < 0.0202$; Spot number Spot number 2155) with the pI 4.61 but no change of the isoform with the pI 4.67 with the same MS identification P19105. In parallel the P24844 Myosin regulatory light chain 2; smooth muscle isoform (22% $P < 0.0274$ 2152) with the pI 4.72 increased in the HP group but change of the isoform with the pI 4.86 was observed. However P24844 with the pI 4.72 is also significantly decreased in the NP group and therefore cannot be clearly related to the HP-diet.

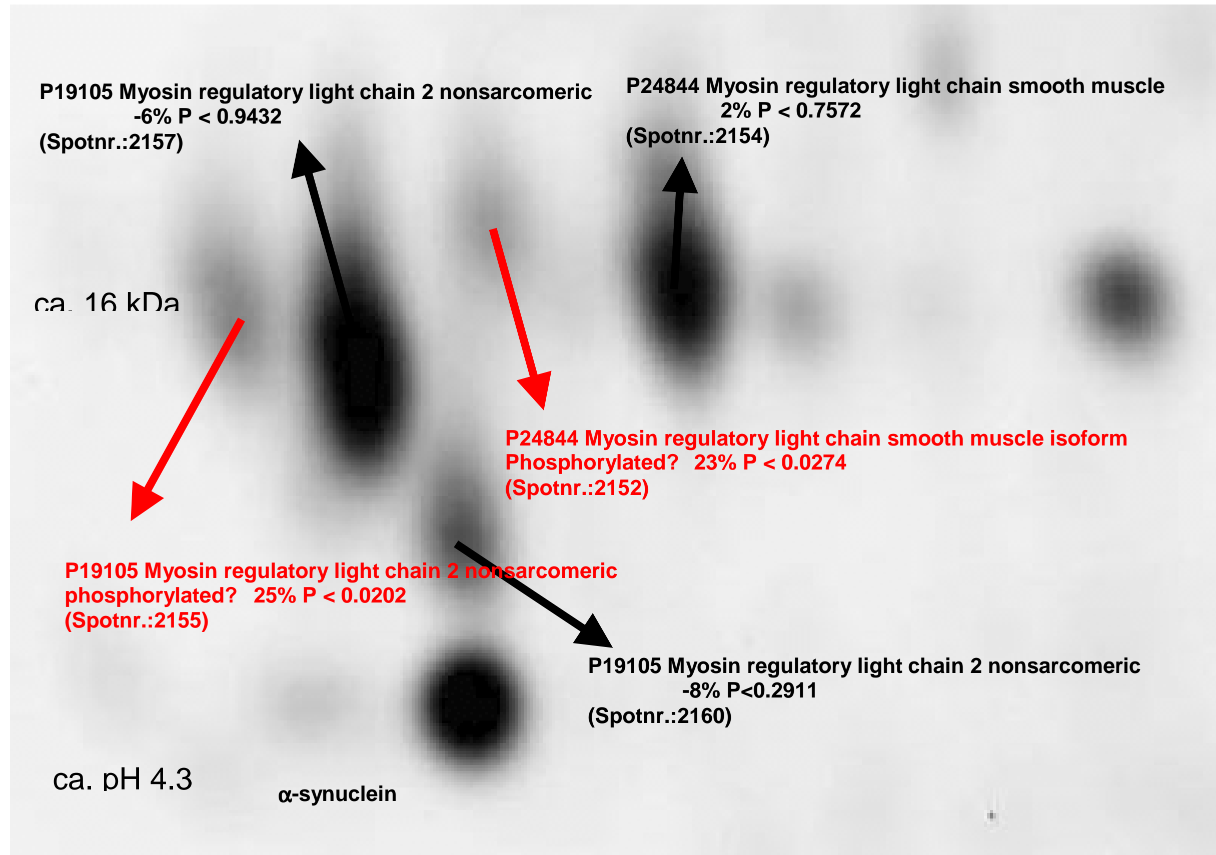

Figure 14: Partial view of 2D DIGE gel pH 4 – 7 showing the 5 isoforms of MLC-2. Percent changes and significances (unpaired student t-test) associated with HP diet are shown.

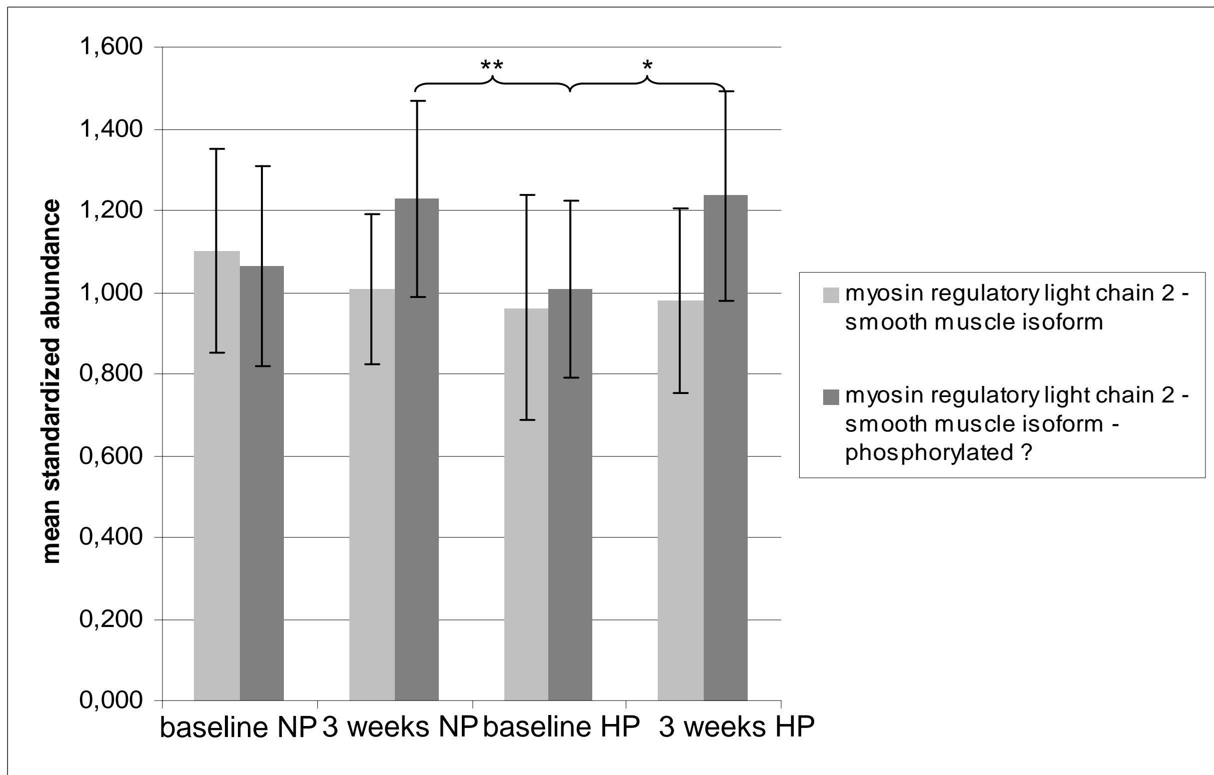

Figure 15: MLC-2 smooth muscle isoform expression of the study individuals in the NP (n = 12) and HP group (n = 12) at the baseline and after 3 weeks treatment measured by 2D DIGE. Results are presented as means ± standard deviations. Significances are calculated with paired student t-test **P < 0.0195; *P < 0.0274

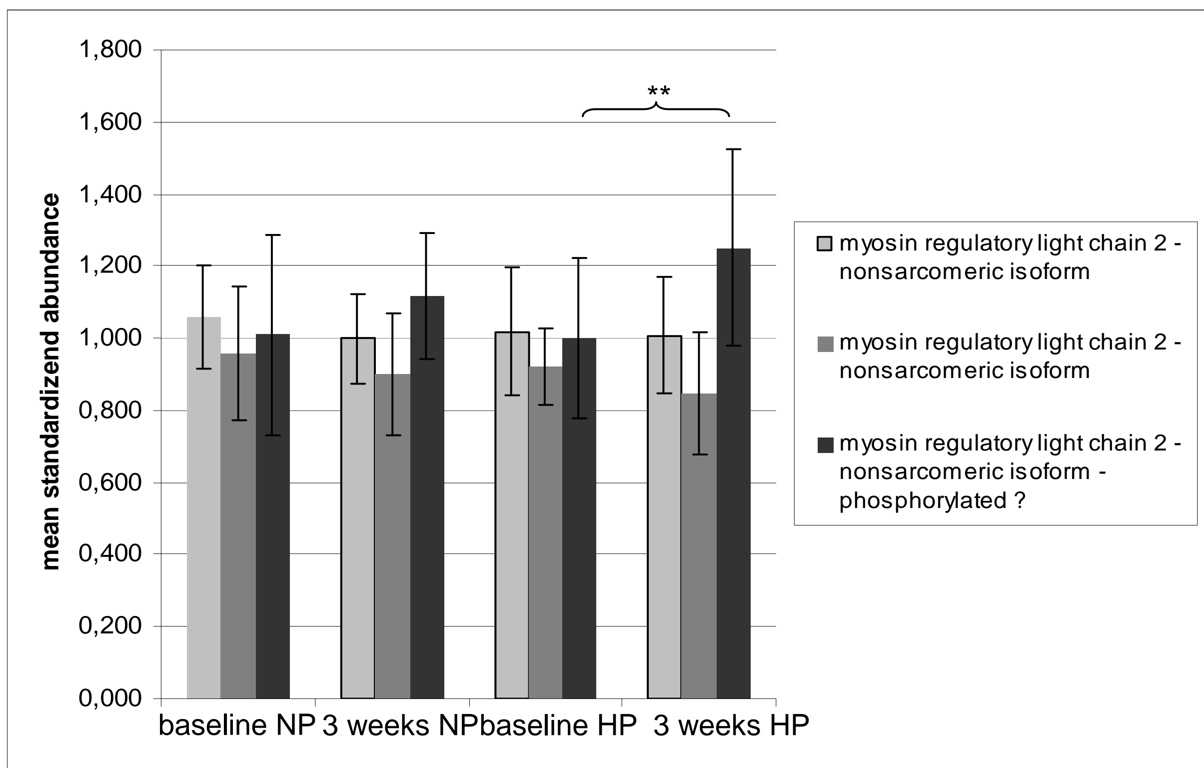

Figure 16: MLC-2 nonsarcomeric isoform expression of the study individuals in the NP (n = 12) and HP group (n = 12) at the baseline and after 3 weeks treatment measured by 2D DIGE. Results are presented as means ± standard deviations. Significances are calculated with paired student t-test **P < 0.0202

7.1.9. Myosin regulatory light chain 2 and muscle strength

A muscle strength test (HGE = Handgrip Endurance HGS = Handgrip Strength) was performed with the study individuals. There could be no significant changes monitored in muscle strength between the HP and the NP group after 3 weeks treatment. But HGE and HGS increased in both groups between baseline and after 3 weeks. This may be due to a training effect.

Since platelets were used as a surrogate cells for muscle cells, and myosin regulatory light chain 2 is an important factor for muscle contraction, the muscle strength results were correlated with the expression levels of all the myosin regulatory light chain 2 isoforms. With two of the isoforms (spotnr. 2154 and 2157) a significant correlation was found. (see figures 17 and 18) However, these two correlating spots were not significantly changed with HP diet.

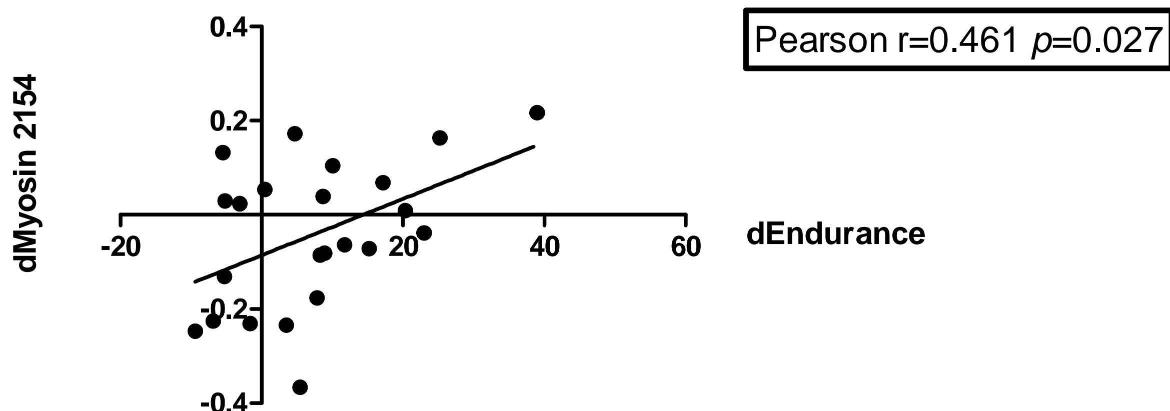

Figure 17: Correlation between MLC-2 isoform 2154 (=spotnr.) and endurance. Endurance: measured as handgrip enduance at 70 % of own max strength (sec). d: differences from first to final measurement. Correlation was calculated with Pearson Test. r is the correlation. p is the significance of the correlation

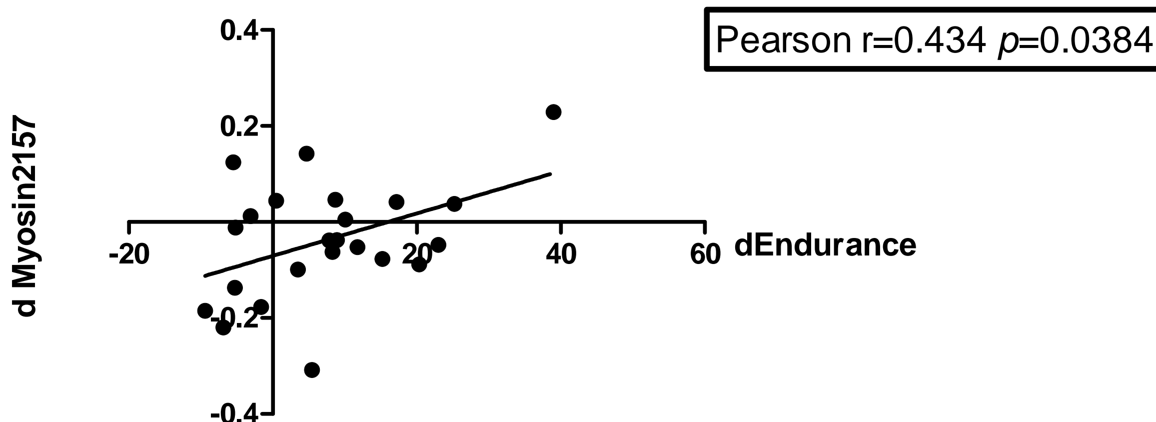

Figure 18: Correlation between MLC-2 isoform 2157 (=spotnr.) and endurance. Endurance: measured as handgrip enduance at 70 % of own max strength (sec). d: differences from first to final measurement. Correlation was calculated with Pearson Test. r is the correlation. p is the significance of the correlation

The isoforms that correlated with the muscle strength test were not significantly changed in the HP and NP diet group. There was no significant correlation between the muscle strength and MLC-2 nonsarcomeric isoform (spotnr. 2155) that was significantly changed with HP diet.

7.2. Influence of High Protein diet on plasma proteome

EDTA plasma was separated from blood cells by centrifugation and individuals were pooled to groups with 4 or 3 persons per group (see chapter 4.2.1.1 and 4.2.1.3). High abundant plasma proteins were filtered out by affinity chromatography. Plasma proteome was investigated with 2D DIGE gels in the pH range 4 – 7, since most of the plasma proteins have an acidic pI.

The total number of spots on the master gel is 2157. The average number of detected spots on the other gels is 2096 ± 151. The average number of matched spots to the master is 1221 ± 300.

Expression changes between the groups were estimated and their significance was calculated with student t-test. A HP diet effect was screened with 3 different calculations: The HP group was compared at the first time point and after 3 weeks intervention time (1), the HP and the NP groups were compared at the second time

point and the HP group at the second time point (2) and the NP group at the first time point (3) were compared.
Additionally the NP group was compared at the baseline and after 3 weeks intervention time (4), the HP and the NP groups were at the baseline (5) and the NP group at the after treatment time point and the HP group at the baseline (6) were compared.
However the change in the diet of the NP group may have an influence on plasma protein composition.
Moreover also a substantial number of false positives may accumulate at the 0.01 confidence level because 1% of sample differences will be significant by change even if no changes in protein expression exist between the two samples
Therefore proteins that happened to be significant in the t-tests of 4-6 are supposed to be not HP related protein expression changes.

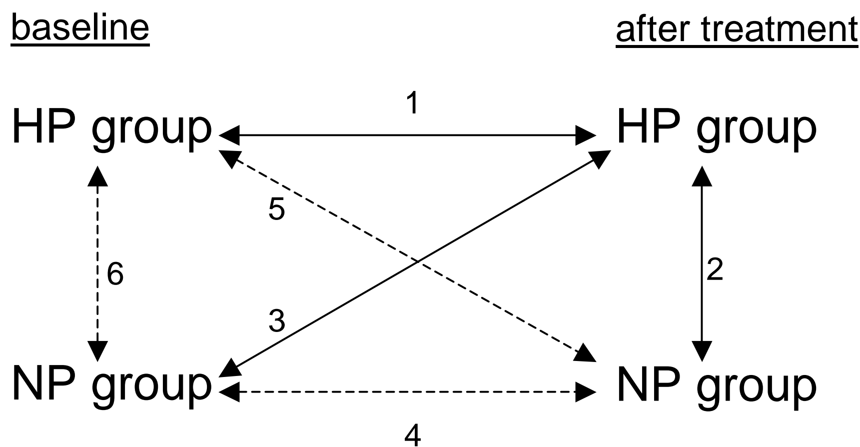

Figure 19: Diagram of calculations for statistical analysis to find HP diet associated changes (1-3) or false positive changes (4-5).

If the same individuals were compared within the same treatment group, paired student t-test was made. Unpaired t-test had to be calculated for comparison across the treatment groups (of the HP and NP group). Additionally an unpaired t-test was calculated within a treatment group to have an adequate comparison of the significant changes also between the two different treatment groups. The significant range was defined $p < 0.05$ and the number of significant events was counted. Not all the significant events derived from proteins. Some significant spots derived from

artefacts, too low abundant or badly separated protein. These events were also counted. To have also an overview of another reason for false positive events the percentage of well defined protein spots of all significant events was calculated (see table 21).

Significantly changed spots, that were associated with HP and NP diet, were analysed with mass spectrometry.

	1 paired t-test	**1** unpaired t-test	**2** unpaired t-test	**3** unpaired t-test	**4** paired t-test	**4** unpaired t-test	**5** unpaired t-test	**6** unpaired t-test
significant events	34	34	26	36	29	29	23	27
protein spots	25	25	20	26	16	16	17	19
artefacts	9	9	6	10	13	13	6	8
% protein	74%	74%	77%	72%	55%	55%	74%	70%

Table 23: Number of significant events, significant spots and artefacts for all possible combinations of groups and time points. Percentage of significant protein spots of all significant events. The numbers (1-6) describe the calculation groups in statistical analysis (see figure 19)

The number of significant protein spots with HP diet is similar and higher than with NP diet. The number of significant events is exactly the same when calculated with paired or unpaired t-test in the NP and the HP groups. The percentage of significant protein spots of all significant events is in a range between 55 % and 74%.

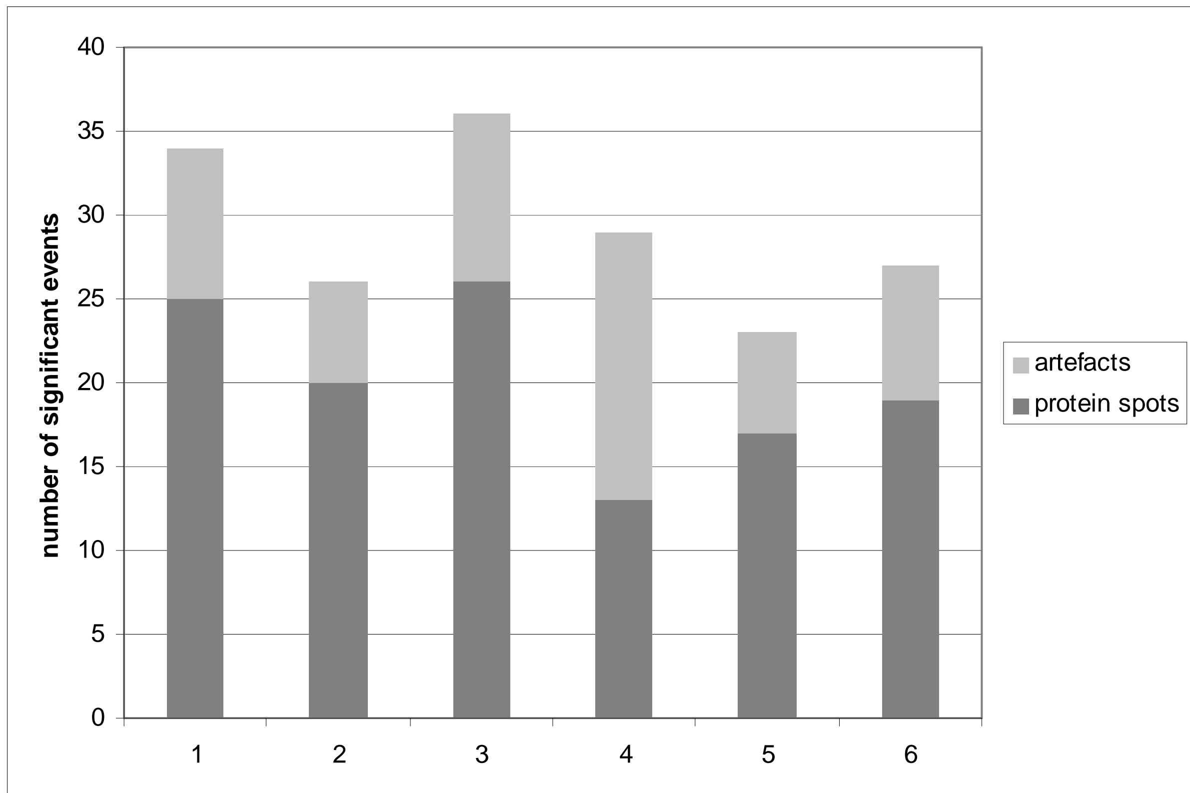

Figure 20: Number of HP and NP-related protein spot changes in pH range 4-7: All possible combinations of groups and time points were calculated to get an overview of possible interference of the standardized nutrition and grouped individuals. The numbers (1-6) describe the calculation groups in statistical analysis (see figure 19)

Significantly changed protein spots were identified with mass spectrometry. On table 22 all identified significant proteins are listed. The percentage of change between the groups and t-test are named.

1 (3 weeks HP / baseline HP)				
spot number	**acessnr**	**protein name**	**ratio**	**t-test (unpaired)**
886	**P04217**	Alpha 1 beta Glycoprotein	-1,23	0,0088
691	**P09871**	Complement C1; s subcomponent	1,12	0,039
694	**P09871**	Complement C1; s subcomponent	1,11	0,041
892	**P04217**	Alpha 1 beta glycoprotein	-1,13	0,042

2 (3 weeks HP / 3 weeks NP)				
spot number	acessnr	protein name	ratio	t-test (unpaired)
1068	**Q96KN2**	Beta-ala-his-dipeptidase ?	1,43	0,012
1001	**P01042**	Kininogen ?	1,42	0,017
1328	**P0C0L5**	Complement C4	-1,26	0,032
1319	**P0C0L5**	Coplement C4	-1,27	0,037
931	**P01031**	Complement C5	1,13	0,04
1626	**P10909**	Clusterin precursor	1,2	0,043

3 (3 weeks HP / baseline NP)				
spot number	acessnr	protein name	ratio	t-test (unpaired)
1322	**P0C0L5**	Complement C4	-1,59	0,0048
724	**P01024**	Complement C3	-1,3	0,0075
696	**P01024**	Complement C3?	-1,25	0,011
1068	**Q96KN2**	Beta-ala-his-dipeptidase ?	1,37	0,013
1626	**P10909**	Clusterin precursor	1,25	0,021
460	**Q14624**	Inter-alpha-trypsin inhibitor heavy chain H4 precursor ?	-1,48	0,023
1965	**P02753**	Retinol binding protein	-1,22	0,024
1176	**P01019**	Angiotensinogen	-1,17	0,033
1299	**P0C0L5**	Complement C4	-1,26	0,034
1498	**P01024**	Complement C3	2,06	0,037
1001	**P01042**	Kininogen ?	1,35	0,038
1319	**P0C0L5**	Coplement C4	-1,48	0,04
717	**P01024**	Complement C3?	-1,23	0,041
562	**P01024**	Complement C3?	-1,32	0,042
1330	**P0C0L5**	Complement C4	-1,29	0,048
1486	**P0C0L5**	Complement C4	-1,27	0,049

4 (3 weeks NP / baseline NP)				
spot number	acessnr	protein name	ratio	t-test (unpaired)
1194	**P01008**	Antithrombin	-1,06	0,023
1470	**P01024**	Complement C3	1,8	0,027

5 (baseline HP / baseline NP)				
spot number	acessnr	protein name	ratio	t-test (unpaired)
1965	**P02753**	Retinol binding protein	-1,32	0,0069
1176	**P01019**	Angiotensinogen	-1,16	0,011
1322	**P0C0L5**	Complement C4	-1,57	0,045

6 (3 weeks NP / baseline HP)				
spot number	**acessnr**	**protein name**	**ratio**	**t-test (unpaired)**
645	**P00751**	Complement CB	1,15	0,011
1470	**P01024**	Complement C3	2,21	0,011
96	**P01024**	Complement C3	1,4	0,047

Table 24: List of identified significant spots of pH 4 – 7 gels. Ratios and unpaired t-test results are named. The numbers (1-6) describe the calculation groups in statistical analysis (see figure 19)

7.3. Concentration of plasma amino acids after HP diet

The effect of HP and NP diet on 19 different amino acids concetrations in plasma was also examined in this study from all study individuals at baseline and after 3 weeks treatment period. Mean ratios and deviation of the individuals in one group were calculated and significance was estimated with paired t-test.

In the plasma amino acid analysis several significant changes could be monitored in the HP diet as well as in the NP diet. Though the significant changes are not similar in the two experimental groups, the direction of the amino acid changes is almost always the same in both experimental groups.

amino acid	NP			HP		
	mean % change	deviation	paired t-test	mean % change	deviation	paired t-test
ASP	64	0,42	0,0010	42	0,66	0,2956
GLU	47	0,40	0,0055	21	0,30	0,0863
ASN	-2	0,07	0,2957	-4	0,18	0,5064
SER	6	0,09	0,0592	4	0,17	0,3851
GLN	0	0,05	0,9314	-8	0,08	0,0184
HIS	-9	0,11	0,0216	-9	0,14	0,0500
GLY	-8	0,22	0,2817	-23	0,09	0,0001
THR	-4	0,20	0,2740	-12	0,22	0,0762
ALA	-1	0,16	0,7131	-3	0,24	0,7035
ARG	1	0,11	0,9287	-8	0,22	0,1087
TAU	8	0,12	0,0698	-2	0,07	0,2891
TYR	3	0,10	0,4801	11	0,23	0,1532
A-A-BUT	-3	0,19	0,2378	-32	0,28	0,0213
VAL	1	0,14	0,9713	33	0,15	0,0000
MET	22	0,15	0,0005	15	0,27	0,1475
TRY	18	0,17	0,0046	20	0,14	0,0026
ILE	6	0,12	0,1555	16	0,22	0,0426
PHE	5	0,10	0,1168	17	0,18	0,0125
LEU	3	0,11	0,4365	10	0,21	0,2126
Total AA	0	0,09	0,8225	0	0,12	0,9850

Table 25: Mean ratios and deviation of the individuals in the NP and HP study groups and paired t-test comparing the individual concentrations at the two time points. The red marked amino acids are the ones that are significantly changed after 3 weeks in the HP group and the blue marked are significant changes in the NP group. Amino acids marked in green are significantly changed in both groups.

There was no change in total amino acis concentration between the groups.

Valine, methionine, tryptophan and phenylalanine belong to the group of essential amino acids. Valine significantly increased by 33 % in the HP diet group whereas Methionine is increased by 22 % in the NP diet group. Tryptophan increased significantly in both groups by about 20 %. Phenylalanine increased only in the HP diet group by 17 % after three weeks of changed nutrition.

Aspartate, glutamine, glutamate and glycine belong to the group of non-essential amino acids.

Aspartate significantly increased by 64 % in the NP diet group at the end of the study. Glutamine decreased by 47 % in the HP diet group. Glutamate significantly increased by 7 % in the NP diet group. Glycine decreased in the HP diet group by 23 % after three weeks of changed nutrition.

The significance of changes in amino acid concentration between the groups and time points was also calculated with unpaired t-test. Six different combinations of

groups and time points are possible: the comparison between the two time points of one group, the comparison between of the two groups at one time point and the comparison of the two different groups at two different time points. (see figure 19) On figure 21 the number of significant changed amino acids is charted. In the comparison of the amino acid concentrations at the two time points of the NP group, 5 amino acids showed significant changes in concentration levels. In the comparison of the amino acid concentrations at the two time points of the HP group 3 amino acids altered significantly. The evaluation of the two groups at the first time point showed no significant changes in amino acid concentrations. The concentration of 3 amino acids changed significantly between the two groups at the second time point. 4 significant amino acid concentration changes result from the comparison of the NP group at the first time point and the HP group at the second time point. Between the HP group at the first time point and the NP group at the second time point, significant concentration changes in 2 amino acids happened.

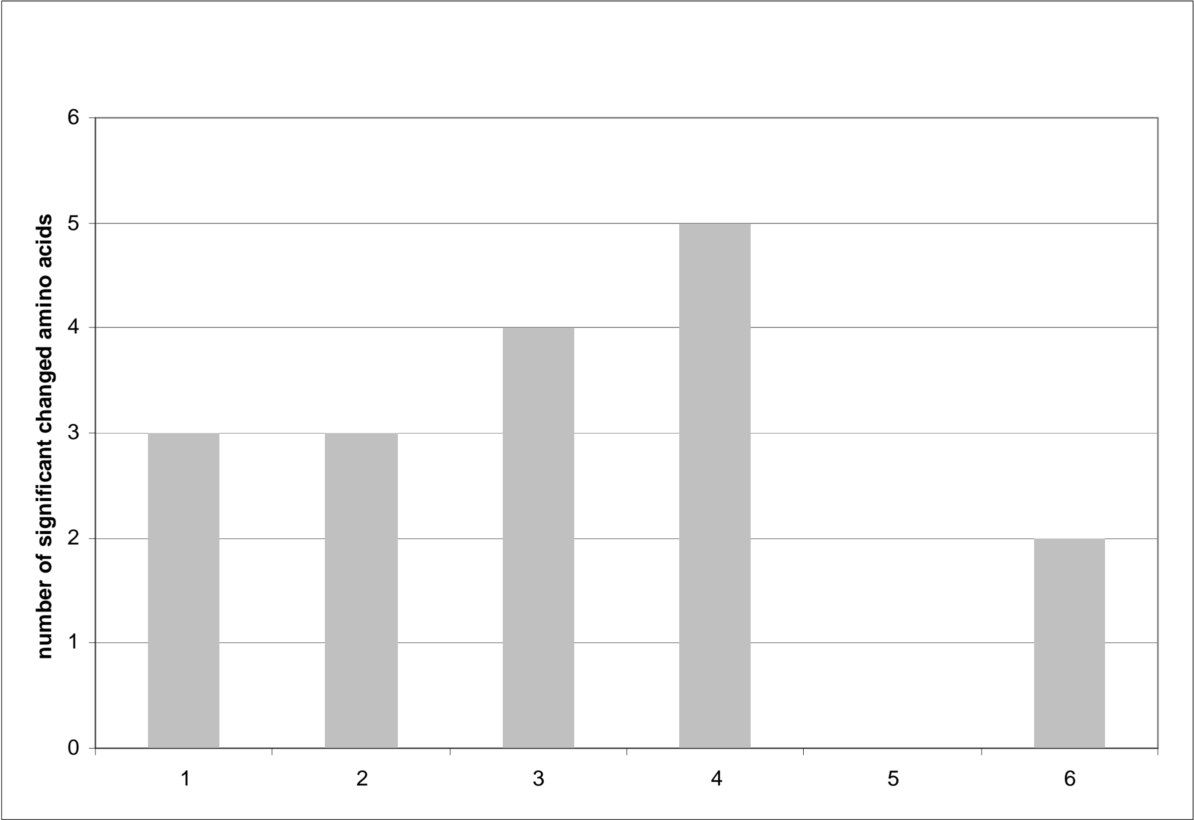

Figure 21: Number of HP and NP-related amino acid changes. All possible combinations of groups and time points were calculated to get an overview of possible interference of standardised nutrition and grouped individuals. The numbers (1-6) describe the calculation groups in statistical analysis (see figure 19)

7.4. Plasma parameter analysis

7.4.1. Cholesterols and triglycerides

Overall, fasting plasma total cholesterol at week 12 decreased by 7 % ($p < 0.002$) in the NP group. Fasting serum LDL cholesterol was by 9 % ($p < 0.009$) lower in the NP group after 3 weeks of the diet. Fasting plasma HDL cholesterol is significantly decreased by 7 % ($p < 0.012$) in the NP group and by 17 % ($p < 0.002$) in the HP group. The LDL/HDL cholesterol quotient is significantly decreased by 0.2 % ($p < 0.008$). In fasting serum triacylglycerol no significant changes could be found.

7.4.2. Homocysteine – Folate – Vitamin B_{12}

In this study a significant increase of vitamin B_{12} by 17 % ($p < 0.014$) and significant decrease of homocysteine by 18 % ($p < 0.013$) in the HP diet group were measured. A very strong and remarkable change is observed in the plasma folate concentration with a significant decrease by 48 % ($p < 0.002$) in the HP group and by 18 % ($p < 0.004$) in the NP group.

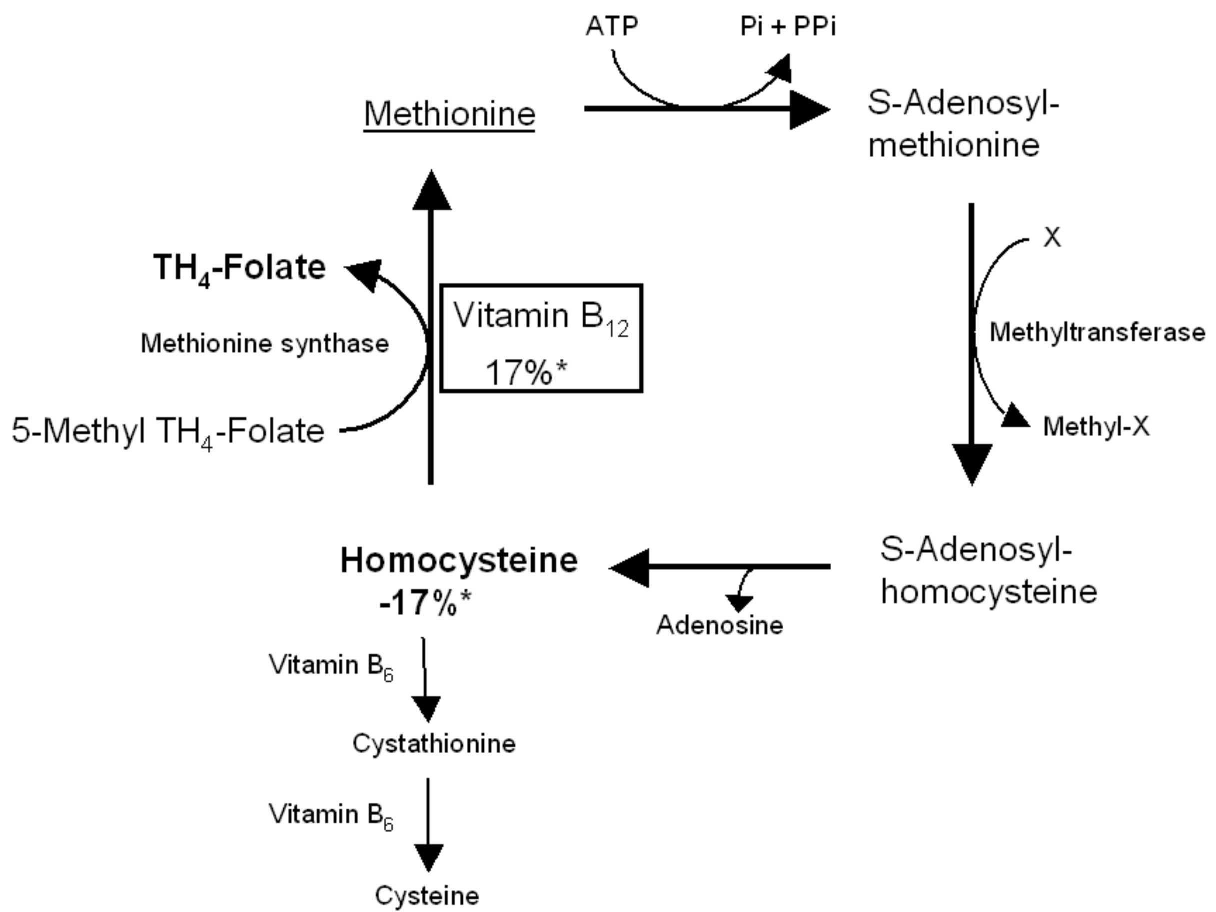

Figure 22: Homocysteine cycle. In presence of vitamin B12 and folate, homocysteine is metabolized to methionine. Thus, the concentration of homocysteine is inversely proportional to the concentration of vitamin B12. S-Adenosylmethionine provides the methyl group for DNA methylation. % change with HP diet of homocysteine and Vitamin B12 are shown.

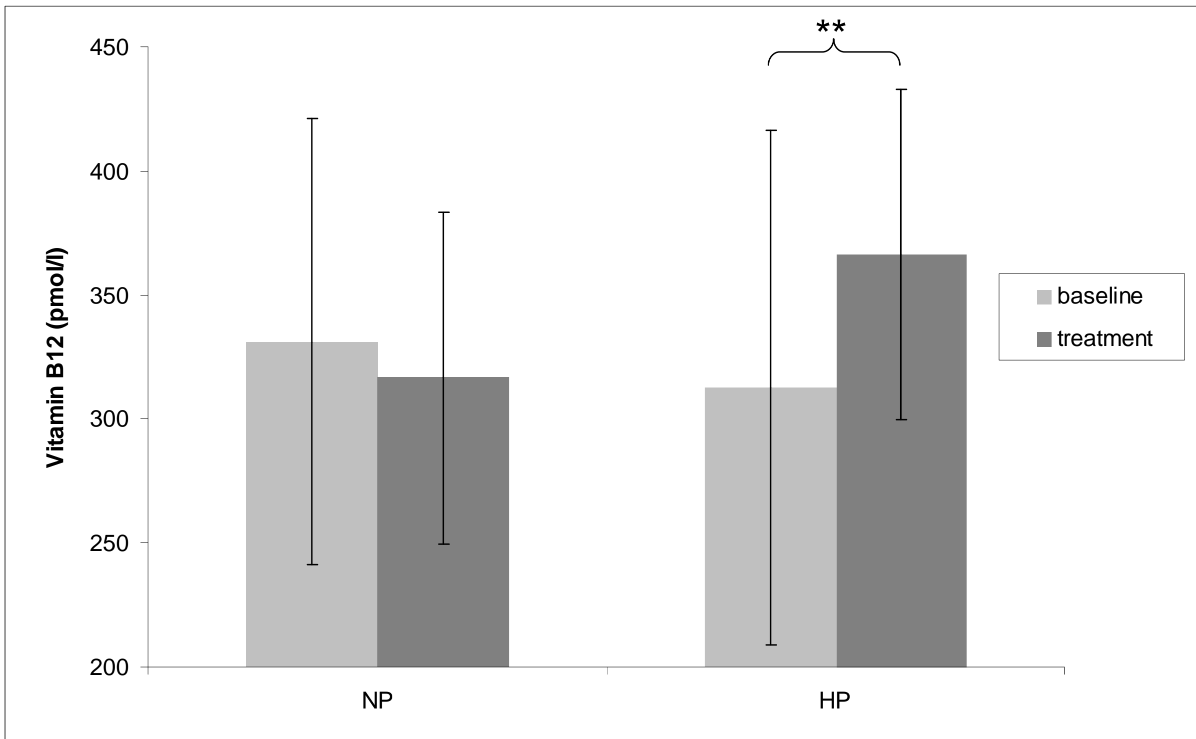

Figure 23: Vitamin B_{12} concentration in plasma of the NP and HP group at baseline and after treatment. Results are presented as means ± standard deviations. Significances are calculated with paired student t-test.

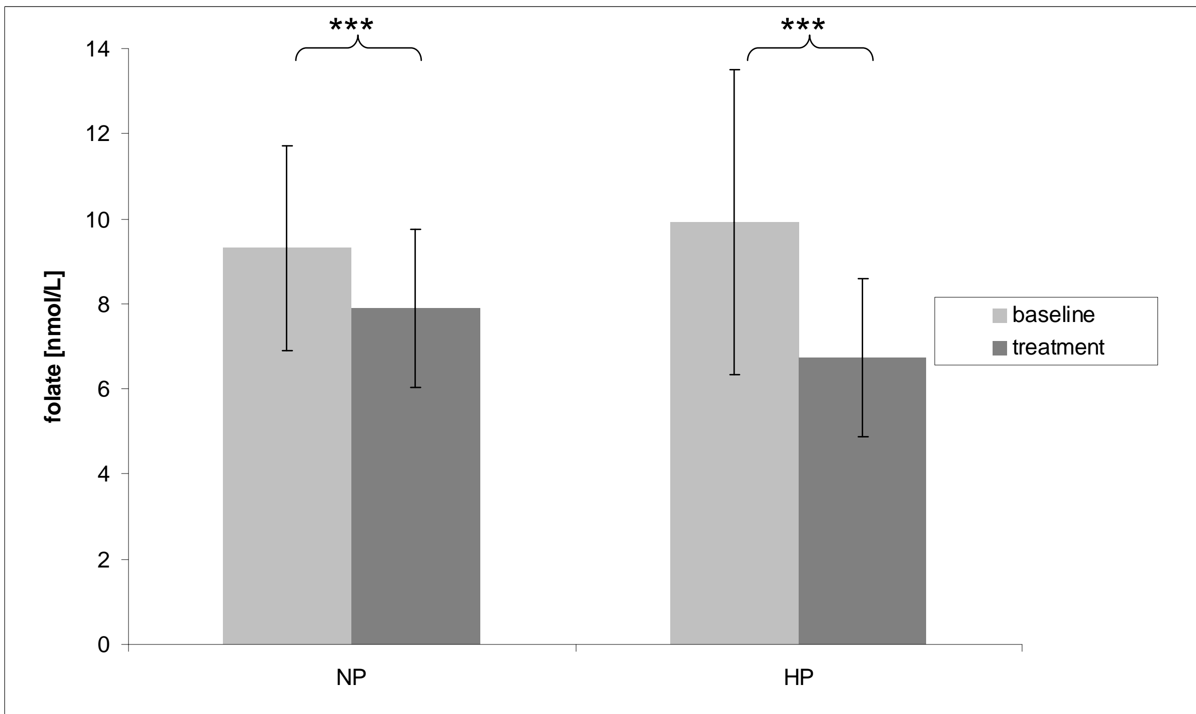

Figure 24: Folate concentration in plasma of the NP and HP group at baseline and after treatment. Results are presented as means ± standard deviations. Significances are calculated with paired student t-test.

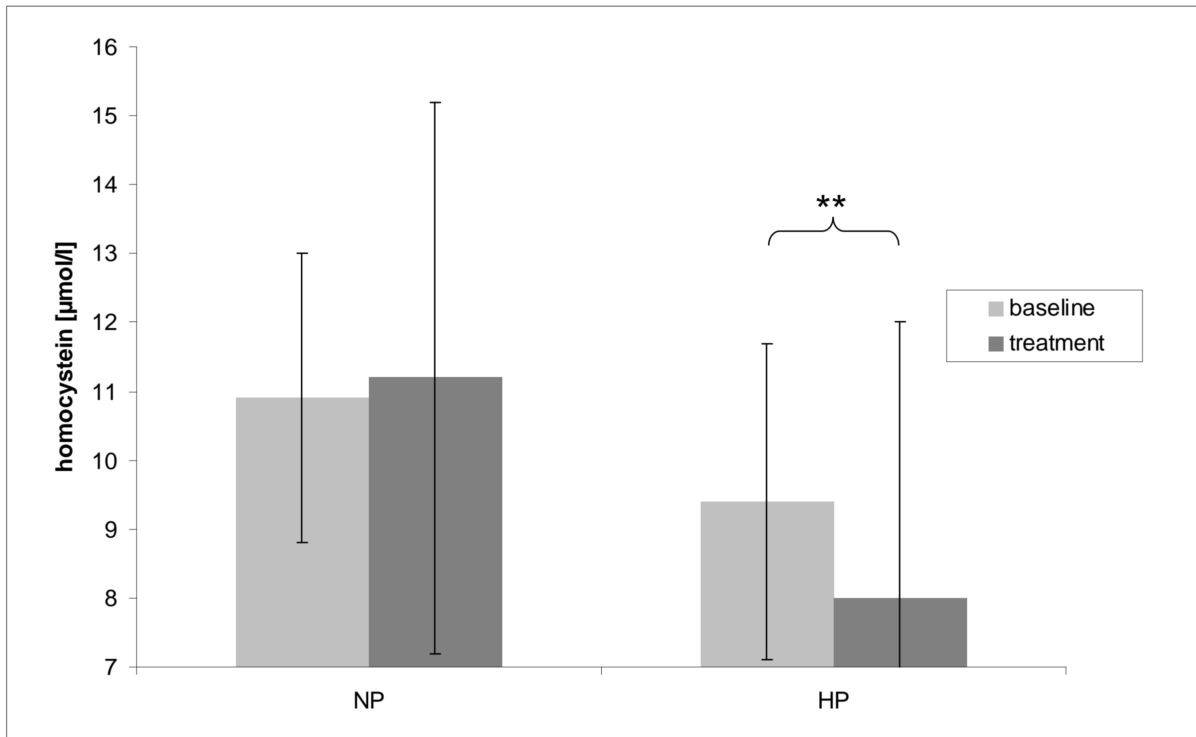

Figure 25: Homocysteine concentration in plasma of the NP and HP group at baseline and after treatment. Results are presented as means ± standard deviations. Significances are calculated with paired student t-test.

7.4.3. Platelet Monoamine oxidase B expression and plasma vitamin B12 concentration are inversely correlating

Mao-B activity in platelets is described to be inversely related to vitamin B_{12} concentration in plasma. (Regland 1991) Additionally it is described that MAO-B gene expression is selectively induced by reduced DNA methylation at specific CpC sites at the promoter. (Wong 2003)

In this study a negative correlation between MaoB expression in platelets and vitamin B_{12} concentration in plasma ($r = -0.46$, $p < 0.028$) was assessed.

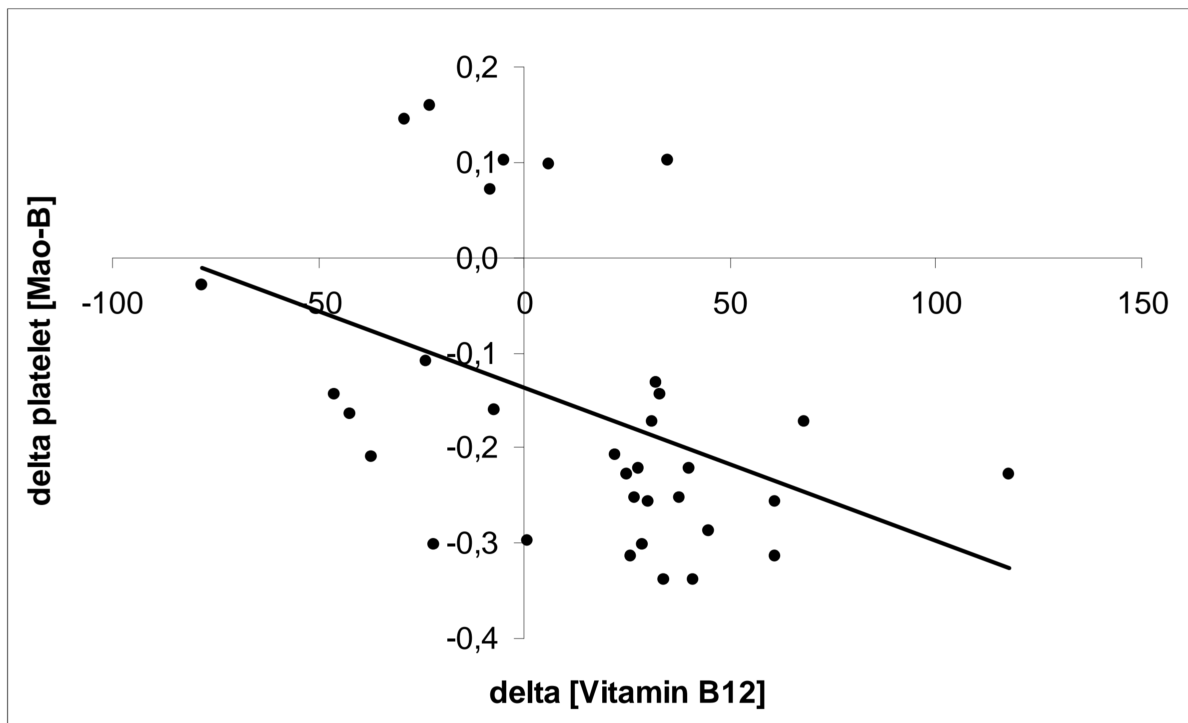

Figure 26: Correlation between maoB expression levels in platelets and vitamin B_{12} concentration in plasma. Delta: differences from first to second time point.

Since Mao-B expression is regulated by DNA methylation, vitamin B_{12} is part of the homocysteine cycle that leads to methylation and the correlation between Mao-B and vitamin B_{12} in this study, a connection between vitamin B_{12} and MAO-B promoter methylation can be suggested.

7.4.4. Albumin and Haptoglobin

The main function of haptoglobin is the transport of haemoglobin and it is an important marker for haemolysis and inflammation. Albumin is the most abundant plasma protein and belongs to the family of globular proteins. Its main function is to provide the maintenance of the colloid osmotic pressure.

These two parameters were measured to confirm the suspicion of having a plasma contamination in the platelet proteome samples (see chapter 4.1.3.). These two parameters could not be read out of the 2D DIGE plasma proteome analysis because during sample preparation all high abundant proteins were filtered out.

In plasma no significant changes were found in albumin and haptoglobin and therefore the suspicion of a plasma contamination was substantiated.

8. Discussion

In this study several significant changes in platelet proteome, plasma proteome and in plasma parameters like vitamin B_{12} or folate were characterised with an animal HP diet. In the following chapters the various results are put in context with each other and are compared to similar studies. This might give an insight on the consequences of an animal HP diet.

8.1. Influence of High Protein diet on platelet proteome

After analysing the influence of a HP diet in the platelet proteome with 2D DIGE gels in pH range 4 – 7 and 6 – 9, 25 significantly changed proteins could be identified. The strongest expression changes were detected with monoamine oxidase B and myosin regulatory light chain 2 nonsarcomeric isoform and zyxin.

Significant changes in the platelet proteome were not only visible with HP diet, but also with NP diet. This might be due to the plasma contamination of the gel-filtered platelets that occur in HP as well as NP after treatment values. An additional explanation other changes in HP and NP-related protein expression changes could be the rather short run-in phase of the study. The individuals could not adapt that fast – within a week – to the new way of nutrition and therefore the baseline values are still in an adaption phase.

8.1.1. HP related decrease of Monoamine Oxidase B expression in platelets

Monoamine Oxidase B (Mao-B) was the protein that showed the biggest effect with HP diet. Additionally it correlated with a shortened reaction time and inversely with the vitamin B_{12} levels in plasma.

Mao-B belongs to the family of mitochondrial monoamine oxidases. Monoamine oxidases catalyse oxidative deamination of monoamines. The primary substrate of Mao-B is dopamine. Dopamine is a neurotransmitter that controls the flow of information in the central nervous system and further modulates during learning process. (Nieoullon 2002) It is known, that during aging (Fowler 1980) , in depression and Alzheimer's disease (Adolfsson 1980; Gottfries 1985) increased Mao-B activity leads to increased dopamine degradation (La 1997) which results in both oxidative stress and decreased dopamine levels in the central nervous system. (Youdim 2006)

Since dopamine is an important modulator of learning processes, a decrease can participate in the appearance, development and progression of Alzheimer's disease.
In this study the correlation between the Mao-B expression and the reaction time test seems to confirm the hypothesis that a decreased Mao-B level improves cognitive abilities, in particular considering the dopamine lowering function of Mao-B. Further it showes that a HP diet can lead to decreaed Mao-B expression levels and therefore induce these better cognitive functions.

8.1.2. HP related increase of Myosin regulatory light chain 2 – MLC-2 expression in platelets

There are five isoforms of myosin regulatory light chain 2 found in this study which two of them were significantly increased with HP diet.
Myosin regulatory light chain 2 plays an important role in the regulation of smooth muscle and non-smooth muscle contractile activity. The phosphorylation of MLC-2 by MLC kinase in presence of calcium and calmodulin increases actin-activated myosin ATPase activity and thereby regulates the contractile activity.

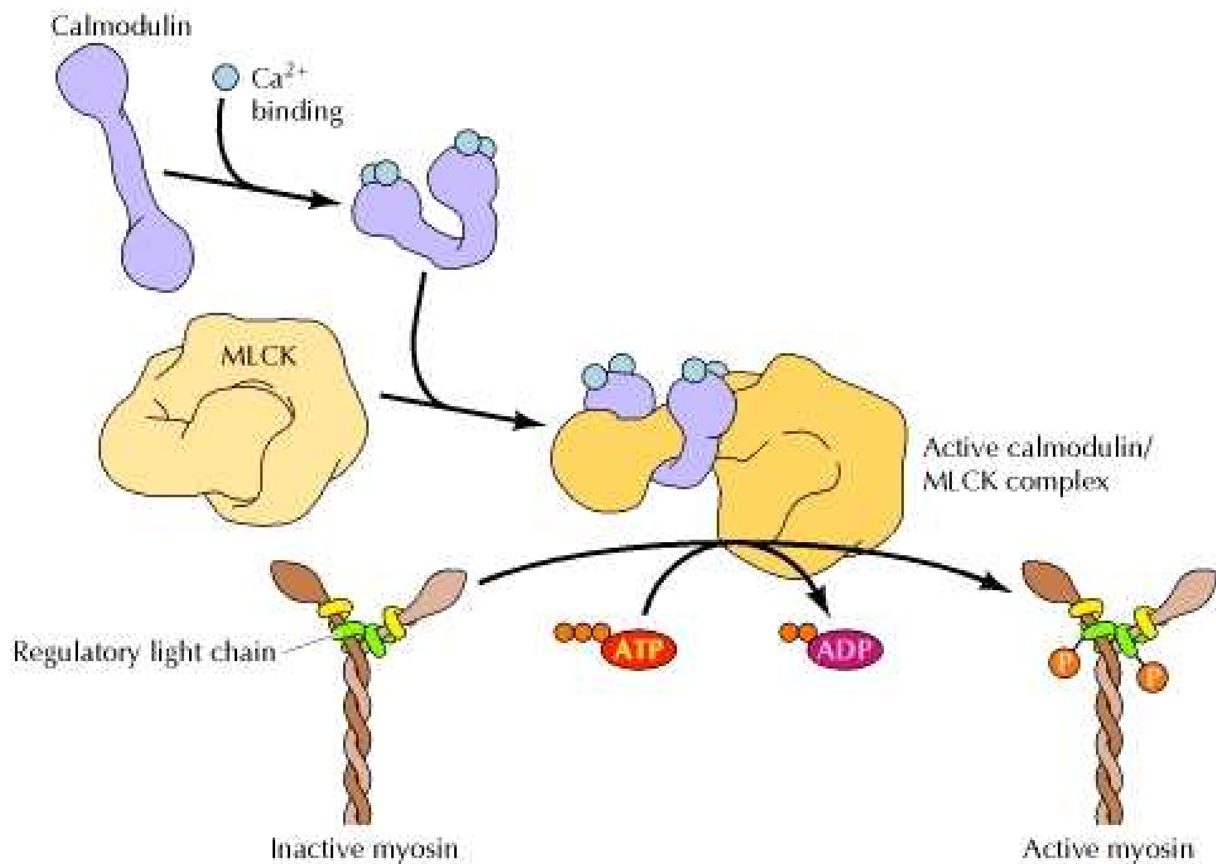

Figure 27: Activation of myosin by phosphorylation of myosin regulatory light chain 2. Calmodulin binds to Ca^{2+} and activates myosin light chain kinase (MLCK). The calmodulin – MLCK complex phosphorylates myosin regulatory light chain by cleavage of ATP and thereby activated myosin. (Cooper 2000)

The two isoformes that are significantly increased with HP diet in this study are supposed to be the phosphorylated ones. It has been shown by a 2D gel electrophoresis study that in aged skeletal muscle the phosphorylation of myosin regulatory light chain 2 is decreased and the pattern of their more acidic phosphorylated MLC-2 isoforms are quite similar to our HP related isoforms. (Gelfi 2006) Further He et al. say that the phosphorylation of MLC-2 are physiologically required for the contraction of the smooth muscle. (He 2008) Continuative it may be assumed that a HP diet may lead to increased muscle strength. However, hand grip measurements showed no increase in muscle strength after HP diet.

8.1.3. Myosin regulatory light chain 2 and muscle strength

Despite of the with HP diet increased assumed phosphorylated MLC-2 isoforms that might indicate increased muscle strength the muscle function test did not show any

improvements with HP diet. Both groups showed an increase in muscle strength but it is supposed to be a training effect.
Correlating the results of the muscle strength with the MLC-2 protein intensities of the DIGE gels showed that the two isoforms that are supposed to be the not phosphorylated ones are positively associated. This result contradicts our expectations and literature (Gelfi 2006; He 2008) that was found.

8.2. Influence of High Protein diet on plasma proteome

After analysing the plasma proteome with 2D DIGE in the pH range 4 –7, 12 proteins could be identified that were changed with HP diet. Actually there were much more proteins found but they had to be eliminated because they probably were a bias also affected by the control diet.
64 % of proteins changed by HP diet were also significanty changed during the control diet. This may be due to the fact that the run in phase of the study was only one week. This time was probably too short for the adaption to a new diet.
Most of the significant changed proteins in both groups are part of the complement system. The complement system is part of the inflammatory response and has never been implicated in HP diet or any other diets at all.

8.3. Concentration of plasma amino acids after HP diet

There was no change in total sum of amino acids after HP diet as well as after NP diet. But in the single amino acids several changes could be monitored in both, the NP group and the HP group. This could be also due to the fact that the nutrition habits of the individuals differed substantially from the food in this study and the short run in period.
In this study the branched chain amino acids, phenylalanin and tyrosin are increasing with HP diet. Harper et al. says that with a HP diet there is an adaptive increase of most amino acid degrading enzymes except for degrading enzymes for phenylalanine, tyrosine and branched chain amino acids (leucine, isoleucine and valine). (Harper 1984) That means that with HP diet branched chain amino acids, phenylalanin and tyrosin levels stay elevated while most of the other amino acids are declining. (Harper 1984; Harper 1989) Therefore it can be suggested that plasma phenylalanine, tyrosine and the branched chain amino acids are directly proportional to protein intake. (Harper 1989)

8.4. Plasma parameter analysis

8.4.1. Cholesterols and triglycerides

The significant decrease of LDL, HDL and total cholesterol in the NP group probably derives from the adaption to the new, constant way of nutrition – since there were no changes in alimentary composition with time in this group.

There was a significant decrease of HDL and total cholesterol with HP diet in this study.

Papakonstantinou et al. could also show in a 4 week intervention study that both a HP diet and a NP diet can improve LDL and total cholesterol levels in a comparable way. (Papakonstantinou 2010a) Further it was shown by Evangelista et al. that a HP diet improves LDL, HDL, total cholesterol and trigycerides levels. (Evangelista 2009)

The cholesterol values in this study probably decreased significantly because the individuals had a very unhealthy way of nutrition before the study started. With this controlled and regulated study diet a slow improvement of the cholesterol values was monitored after 3 weeks.

The decrease of HDL in the HP group was more distinct than in the NP group. Studying the results in more detail, the reason for this is obvious. Three of the individuals of the HP group had a very big decrease of their HDL values during the study, whereas in the NP group there was only one person with such strong individual changes.

8.4.2. Homocysteine – Folate – Vitamin B_{12}

Vitamin B_{12} was significantly increased and homocysteine was significanty decreased with HP diet in this study. Folate was significantly decreased with both HP and NP diet.

All three of them are take part in the methylation cycle. Methylation of DNA is a very important regulation mechanism of gene expression. The more a gene or a promoter of a gene is methylated the more tightly the DNA is packed and the harder the transcription of the gene is. For this methylation 5-methyl-TH_4-folate is required. Vitamin B_{12} takes the methyl group of methyl-TH_4-folate, attaches it to homocysteine and thereby TH_4-folate and methionine are generated.

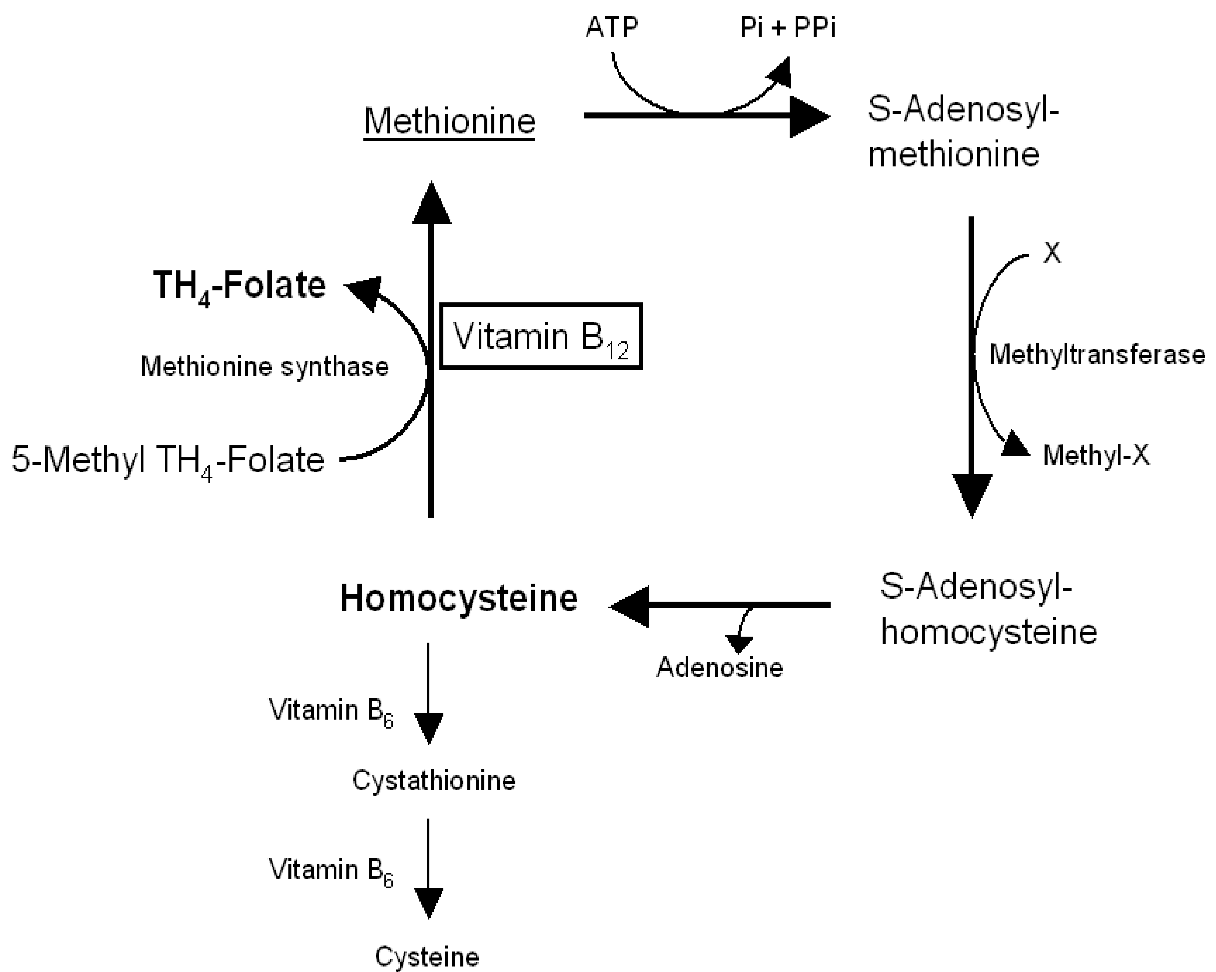

Figure 28: Homocysteine cycle. In presence of vitamin B12 and folate, homocysteine is metabolized to methionine. Thus, the concentration of homocysteine is inversely proportional to the concentration of vitamin B12. S-Adenosylmethionine provides the methyl group for DNA methylation.

Folate, homocysteine and vitamin B_{12} levels are highly governed by nutrition. Therefore significant effects on their levels were expected with a high protein diet. Since the main nutritional source for vitamin B_{12} is meat a significant increase of this parameter in the HP group was expected. Already Clifton et al showed before that with a HP diet the vitamin B_{12} levels are rising. (Clifton 2008)
Concerning the coherence between homocysteine and HP diet the literature is more contradictory. Jia et al showed an increase of homocysteine levels with HP diet in pigs for example. (Jia 2010) Haulrik et al. showed in contrast a decreased homocysteine level with HP diet. (Haulrik 2002) Noakes et al. could not observe any HP dependent changes in serum homocysteine at all. (Noakes 2005) In this study the extensive significant decrease of serum homocysteine with HP diet indicates a

positive effect of a HP diet from an animal source on the risk to sicken from cardiovascular diseases and osteoporosis.

Serum folate seems to response to any change in nutrition. (Clifton 2008; Noakes 2005) In this study both NP and HP diet caused an significant decrease in folate levels.

8.4.3. Platelet Monoamine oxidase B expression and plasma vitamin B12 concentration are inversely correlating

In this study the plasma vitamin B_{12} levels correlate significantly, inversingly with the Mao-B expression levels in platelets.

Regland et al. showed already a negative correlation between plasma vitamin B_{12} levels and Mao-B activity in plateles in megaloblastic anemia and dementia patients. Mao-B activity was significantly reduced (to apparently normal levels) after vitamin B_{12} supplementation. (Regland 1991) Our study shows that the decreased Mao-B activity that results from increased vitamin B_{12} levels are due to the decreased Mao-B expression. Increased vitamin B_{12} levels seem not only decrease Mao-B expression in platelets but also in all the other cells in the body like in the brain. This could explain the increased cognitive function with HP diet.

8.5. Future prospects

The main findings in this study are the increased cognitive abilities that can be explained due to the increased viamin B_{12} levels due to the HP diet from an animal source which results in a decreased Mao-B expression which is a key player in dopamin degradation. This might indicate a positive effect of an animal HP diet. To ensure this hypothesis futher studies are needed. It would be of special interest for example to compare HP diets that derive from an animal and a vegan source. Thereby effects can be excluded that originate from the HP diet and can be described to the source the protein comes from. Thus it would be possible to distinguish the effects of an animal HP diet.

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10. Appendix

10.1. Curriculum vitae – Rita Babeluk

Geboren am: 31. 08. 1986
Staatsangehörigkeit: Österreich
Familienstand: ledig

Ausbildung

seit 2004	Diplomstudium der Molekularen Biologie an der Universität Wien
18. 06. 2004	Maturaabschluss mit ausgezeichnetem Erfolg
1996-2004	Bundesgymnasium GRG23 Alterlaa
1992-1996	Öffentliche Volksschule Putzendoplergasse, 1230 Wien

Wissenschaftliche Arbeiten

1. 12. 2008 – 31. 10. 2010	wissenschaftliche Mitarbeit (auf 20 h Basis) im Institut für Physiologie an der Medizinischen Universität Wien bei Dr. Maria Zellner
1. 02. 2008 – 30. 11. 2008	wissenschaftliche Mitarbeit (auf 40 h Basis) im chirurgischen Forschungslabor des AKH Wien bei Dr. Maria Zellner
1. 02. 2005 – 31. 01. 2008	wissenschaftliche Mitarbeit (2 Tage pro Woche) im chirurgischen Forschungslabor des AKH Wien bei Dr. Maria Zellner

Publikationen

- ***Specific binding of hypochlorite-oxidized HDL to platelet CD36 triggers proinflammatory and procoagulant effects.***

  Assinger A, Koller F, Schmid W, Zellner M, **Babeluk R**, Koller E, Volf I.
  Atherosclerosis. 2010 Sep;212(1):153-60. Epub 2010 May 13.

- ***Biological variation of the platelet proteome in the elderly population and its implication for biomarker research***

  Winkler W, Zellner M, Diestinger M, **Babeluk R**, Marchetti M, Goll A, Zehetmayer S, Bauer P, Rappold E, Miller I, Roth E, Allmaier G, Oehler R
  Mol Cell Proteomics 2008; 7: 193-203

- ***Fluorescence-based western blotting for quantitation of protein biomarkers in clinical samples***

  Zellner M, **Babeluk R**, Diestinger M, Pirchegger P, Skeledzic S, Oehler R.
  Electrophoresis. 2008 Aug; 29(17): 3621-7.

Submitted

- ***A proteomics study reveals a predominant change in MaoB expression in platelets of healthy volunteers after high protein diet - Relationship to the methylation cycle***

  Zellner M, **Babeluk R**, Jakobsen LH, Gerner, Erich C, Roth E, Kondrup J.
  Journal of Neuronal Transmission

Abstracts

- **Impact of high protein diet on platelet proteome in humans**

  R. Babeluk, L.H. Jakobsen, J. Kondrup, Christopher Gerner, E. Roth, S. Baumann and M. Zellner

- **Implication of glutathione S-transferase omega 1 in Alzheimer's disease and its dependence on the presence of Apo E4**

  R. Babeluk, S. Kappel, E. Umlauf, M. Zellner

- **Different chemotherapeutic regime induces specific changes in the plasma proteome**

  S. Baumann, A. Michlmayr, **R.Babeluk**, T. Bachleitner, M.Gnant, U.Bartsch, T.Grünberger, M. Bergmann and R. Oehler

- **Comparative platelet proteome profinling of Alzheimer’s and Parkinson’s disease patients**

  M. Zellner, E. Umlauf, M. Diestinger, P. Pirchegger, E. Rappold, N. Kotzailias, C. Gerner, **R. Babeluk,** F. Garo, R. Oehler and J. Lamont

- **Comparative platelet proteome profiling from dementia and parkinson´s disease patients**

  M. Zellner, M. Diestinder, P. Pirchegger, E. Umlauf, E. Rappold, N. Kotzailias, C. Gerner, W. Winkler, **R. Babeluk**, F. Garo, R. Oehler

- **Platelet proteins with low biological variation for the use as normalisation tool**

  M. Zellner, R. Oehler, E. Umlauf, M. Ramberger, P. Pirchegger, **R. Babeluk**, J. Lamont, M. Veitinger

Berufsbezogene Fortbildungen und Posterpräsentationen

Sept 2006	AUPA Proteomics Symposium
Sept 2007	Kongress für Zivililsationserkrankungen und Alters-Assoziierte Erkrankungen – Prädiktion, Prävention und Therapiemonitoring
Sept 2007	AUPA Proteomics Symposium mit Kurs
Sept 2008	30th ESPEN Congress
Okt 2008	Phosphoproteomics Seminar with Practical Workshop
Juni 2009	5. PhD – Symposium 09
Aug 2009	31th ESPEN Congress
Okt 2009	3rd Central and Eastern European Proteomics Conference

Preise

- **Posterpreis:**

Implication of glutathione S-transferase omega 1 in Alzheimer’s disease and its dependence on the presence of Apo E4
R. Babeluk, S. Kappel, E. Umlauf, M. Zellner
AACC Annual Meeting; Chicago, Juli 2009

- **Student Travel Grant:**

Impact of high protein diet on platelet proteome in humans
R. Babeluk, L.H. Jakobsen, J. Kondrup, Christopher Gerner, E. Roth, S. Baumann and M. Zellner
3rd Central and Easter European Proteomics Conference; Budapest, Oktober 2009

weitere Jobs

9. 06. 2006 – 1. 02. 2008	Mitarbeiterin bei Patentanwalt Dipl.-Ing. Mag. Michael Babeluk
2005 – 2006	Servicekraft im Bereich Catering bei Großveranstaltungen
2001 – 2005	Nachhilfe für Unter- und Oberstufe Englisch und Mathematik

Sprachen

Deutsch – Muttersprache
Englisch – fließend in Wort und Schrift
Spanisch – Maturaniveau

Persönliche Interessen

Musik (Klavier, Cello), Lesen, Reisen

10.2. Abbrevations

AA = Amino Acids

ACE = Addenbrooke Cognitive Examination

BCAA = Branched Chain Amino Acids

BIA = Bioelectrical Impedance Analysis

DRI = Dietary Reference Intakes

E% = Energy Percent

HDL = High density lipoprotein

HGE = Handgrip Endurance

HGS = Handgrip Strength

HGW = Handgrip Work

HP = High Protein

HPLC = High pressure liquid chromatography

IDL = Intermediate density lipoprotein

LDL = Low density lipoprotein

Mao-B = Monoamine Oxidase B

MLC-2 = Myosin regulatory light chain 2

NP = Normal Protein

RDA = Recommended dietary allowance

TCA = Trichloacetic acid

VLDL = Very low density lipoprotein

2D DIGE = Two dimensional differential gel electrophoresis

10.3. Comment

Some data of this diploma thesis will be published. Chapter 5.3 and 6.3 are part of the manuscript: “Effect of a high protein meat diet on physiological functions: a randomised controlled dietary intervention trial in healthy men” that are submitted in Clinical Nutrition. Chaper 5.1.2, 5.1.5, 5.1.6, 5.4.2, 5.4.3, 6.1.1, 6.4.2 and 6.4.3 are part of the manuscript: “A proteomics study reveals a predominant change in MaoB expression in platelets of healthy volunteers after high protein diet - Relationship to the methylation cycle” that are in Journal of Neural Transmission.