TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Ernährungsphysiologie

Human plasma and peripheral blood mononuclear cell (PBMC) proteome databases for biomarker discovery studies *in vivo*

Carolin Heim

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. D. Haller

Prüfer der Dissertation:

1. Univ.-Prof. Dr. H. Daniel
2. Univ.-Prof. Dr. J. J. Hauner

Die Dissertation wurde am 04.05.2010 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 16.08.2010 angenommen.

Table of Contents

Zusammenfassung 1

Summary 2

1. Introduction 3
   1.1. Biomarker Discovery in Nutritional Research 3
   1.2. Impact of Dietary Protein Intake on Human Metabolism 4
   1.3. Use of Proteomics Applications to Human Plasma and Blood Cells in Nutritional Intervention Studies 7
   1.4. The Plasma Proteome 9
   1.5. Proteome Analysis of Plasma and PBMC 10
      1.5.1. Sample Preparation 11
      1.5.2. Plasma Sample Processing 13
      1.5.3. Protein Separation Techniques 16
      1.5.4. Protein Identification 19
      1.5.5. Search Algorithms 21
      1.5.6. Plasma Protein Databases 21
      1.5.7. The Sub-Proteomes of Human Plasma 22
   1.6. Identification of the Plasma Proteome 23

2. Aim and Outline of the Thesis 25

3. Materials and Methods 26
   3.1. Chemicals and Consumables 26
   3.2. Instruments 26
   3.3. The High-Protein Intervention Study 26
      3.3.1. Volunteer Recruiting 26
      3.3.2. Study Design 26
      3.3.3. Sample Collection 27
      3.3.4. SDS Electrophoresis of the Protein Powder 27
   3.4. Sample Preparation for Proteomics Analysis 28
      3.4.1. PBMC Preparation 28
      3.4.2. Plasma Pre-Fractionation 28
      3.4.3. Protein Measurement 29
   3.5. Electrophoretic Analysis of Plasma and PBMC 29

3.5.1. Protein Separation Using 2D Gel Electrophoresis 29
3.5.2. 1st Dimension 30
3.5.3. 2nd Dimension 31
3.5.4. Staining of Gels 31
3.5.5. Image Analysis 32
3.6. Protein Identification 32
3.6.1. Sample Preparation for MS Analysis 32
3.6.2. MALDI Mass Spectrometry 33
3.6.3. Spectra Processing and Database Search 33
3.7. Analysis of Amino Acids and Clinical Parameters 33
3.7.1. Measurement of Amino Acids in Plasma and Urine 33
3.7.2. Clinical Parameters in Plasma and Urine 33
3.8. Statistical Analysis 34

4. Results and Discussion 35
4.1. Method Development 35
4.1.1. Sample Pre-Fractionation in Plasma 35
4.1.2. Optimizing Electrophoresis for Plasma and PBMC 36
4.2. 2DE Separation for Determining Within- and Between-Laboratories Variability 40
4.3. In-House Proteome Databases for Human Plasma and PBMC Samples 41
4.3.1. The Plasma Database 41
4.3.2. The PBMC Database 45
4.4. A High-Protein Diet Human Intervention Study 49
4.4.1. Content of the Protein Powder 49
4.4.2. Clinical Chemistry Parameters 51
4.4.3. Amino Acid Profiles during the Study Course 55
4.4.4. Analysis of the Plasma Proteome after Protein Supplementation 62
4.4.5. Analysis of the PBMC Proteome after Protein Supplementation 69

5. General Remarks and Conclusions 74
5.1. Proteomics Applications in Human Intervention Studies 74
5.2. The “Stability” of the Plasma and PBMC Proteomes 75
5.3. Application of 2D Maps of Plasma and PBMC in Biomarker Discovery 76
5.4. High-Protein Supplementation and Its Impact 77
5.5. Future Perspectives 78

6. Appendix 80
6.1. Abbreviations 80
6.2. Tables 83

7. References 95

Erklärung 104

Lebenslauf 106

Publikationen 107

Danksagung 111

List of Figures

Figure 1-1 Profiling techniques in nutritional research 3
Figure 1-2 Nitrogen balance in adults (modified from [7]) 4
Figure 1-3 Reference intervals for 70 protein analytes in plasma (from [24]) 9
Figure 1-4 Sample analysis of plasma and PBMC using 2DE and MALDI-TOF-MS 11
Figure 1-5 Categorization of 1427 high-confidence proteins (from [39]) 24
Figure 3-1 Outline of the high-protein intervention study 27
Figure 4-1 Separation of plasma spots before and after antibody depletion 35
Figure 4-2 Different protein amounts on PBMC gels 36
Figure 4-3 Comparison of different rehydration methods for PBMC samples 37
Figure 4-4 Comparison of continuous and discontinuous buffer system and gel composition in the 2nd dimension 38
Figure 4-5 Different staining techniques after 2D electrophoresis 39
Figure 4-6 Variation of spots from plasma, platelets and PBMC between laboratories 41
Figure 4-7 Proteome map with identified proteins as derived from depleted plasma samples 42
Figure 4-8 Biological functions of all proteins included into the plasma database according to SwissProt 44
Figure 4-9 Proteome map derived from PBMC 46
Figure 4-10 Biological functions of all proteins included into the PBMC database according to SwissProt 47
Figure 4-11 Clinical chemistry parameter levels in plasma and urine 51
Figure 4-12 BUN/creatinine-ratio and urea/creatinine-ratio during the study course 54
Figure 4-13 Changes in amino acid concentrations in plasma and urine 56
Figure 4-14 Amino acid pathways altered through protein supplementation 60
Figure 4-15 Amino acid correlation amongst plasma and urine samples 62
Figure 4-16 Gels of baseline plasma and PBMC samples from the intervention study 63
Figure 4-17 Expression profiles of all significant spots in plasma 65
Figure 4-18 Altered spots in plasma during the trial 68
Figure 4-19 Expression profiles of all significant spots in PBMC 70
Figure 4-20 Altered spots in PBMC during the trial 73

List of Tables

Table 4-1 Identified proteins of the plasma database sorted by their biological processes as stated in SwissProt ... 43
Table 4-2 Identified proteins of the PBMC database sorted by their biological processes as stated in SwissProt ... 47
Table 4-3 Nutrition information Protein90Plus as given by the manufacturer ... 50
Table 4-4 Content of the Protein90Plus supplement ... 50
Table 4-5 Clinical parameters in plasma during the trial ... 52
Table 4-6 Clinical parameters in urine during the trail ... 53
Table 4-7 Interpretation of BUN/creatinine-ratio and urea/creatinine ... 54
Table 4-8 Amino acid content of Protein90Plus as declared by the manufacturer ... 55
Table 4-9 Regulated 2D spots in the plasma baseline of the trial ... 64
Table 4-10 Regulated protein spots in the plasma high-protein and wash-out phase of the trial ... 66
Table 4-11 Regulated 2D spots in the PBMC baseline samples of the trial ... 69
Table 4-12 Regulated 2D spots in the PBMC high-protein and the wash-out phase of the trial ... 71
Table 6-1 Proteins included into the plasma and PBMC databases ... 83
Table 6-2 Identified proteins in the plasma database ... 86
Table 6-3 Identified proteins in the PBMC database ... 88
Table 6-4 Characteristics of the 11 volunteers included into the trial ... 90
Table 6-5 Regulated spots in plasma during the baseline of the trial ... 90
Table 6-6 Regulated spots in plasma during the high-protein intake phase of the trial ... 90
Table 6-7 Regulated spots in plasma during the wash-out phase of the trial ... 91
Table 6-8 Regulated spots in PBMC during the baseline period of the trial ... 91
Table 6-9 Regulated spots in PBMC during the high-protein intake phase of the trial ... 91
Table 6-10 Regulated spots in PBMC during the wash-out phase of the trial ... 92
Table 6-11 Identification of regulated protein spots in PBMC not listed in the database ... 92
Table 6-12 Plasma amino acids in the trial ... 93
Table 6-13 Urinary amino acids in the trial ... 94

Zusammenfassung

Die vorliegende Arbeit hatte zum Ziel, Datenbanken für das Proteom von Blutplasma und mononukleären Blutzellen (PBMC) des Menschen zu erstellen und anhand einer humanen Interventionsstudie mit einer hohen Proteinzufuhr zu prüfen, inwieweit Markerproteine einer metabolischen Adaptation identifiziert werden können.

Humanstudien erlauben vielfach nur eine minimal invasive Probennahme. Entsprechend sind Blut mit seinen PBMC oder Urinproben als Probenmatrix die am häufigsten benutzten Quellen. Plasma besitzt nach heutigem Erkenntnisstand das umfassendste Proteom, da es neben den klassischen Plasmaproteinen auch solche Proteine beinhaltet, die aus Gewebsverletzungen und Entzündungen hervorgehen. Der enorme Konzentrationsbereich von Plasmaproteinen stellt für die Analytik jedoch eine große Herausforderung dar. Proteine, die als Biomarker fungieren könnten, aber in geringer Konzentration vorliegen, werden häufig von den abundanten Proteinen verdeckt. Grundlage der Proteomanalyse in dieser Arbeit war die klassische 2D Elektrophorese kombiniert mit der Matrix-Assisted Laser Desorption Ionization (MALDI) Massenspektrometrie zur Proteinidentifizierung. Aus den Plasma- und PBMC-Proben von Probanden wurden Proteomkarten erstellt und mit Datenbanken vernetzt, die Informationen über die dargestellten Proteine beinhalten. Mit Hilfe dieser Datenbanken kann die Identifizierung von Proteinen in künftigen Interventionsstudien deutlich beschleunigt werden und ermöglicht eine schnelle und geeignete Ermittlung von veränderten Proteinen und ihren Spiegeln. Schwankungen biologischer oder analytischer Art wurden ohne Intervention im Proteom von Plasma und PBMC untersucht. Dabei zeigten sich beträchtliche inter-individuelle Unterschiede im Proteom der Probanden.

In einer humanen Interventionsstudie wurde die Anwendung der entwickelten *in-house* Datenbanken geprüft. Dabei wurde der Einfluss eines Proteinsupplements in Probanden auf das Proteom von Plasma- und PBMC-Proben untersucht. Die Aminosäureprofile im Plasma und Urin sowie klinische Parameter lieferten zusätzliche Informationen über die Stoffwechselantworten. Die Probanden reagierten auf die hohe Proteinzufuhr mit einem deutlichen Anstieg der Harnstoffkonzentration in Plasma und Urin. Es konnten jedoch keine signifikant erhöhten Konzentrationen von Proteinen und Aminosäuren aus dem Harnstoffzyklus als Indikatoren der metabolischen Adaptation gefunden werden. Die Plasmaanalyse ergab Hinweise auf einen Anstieg der Proteinsynthese in der Leber, während in den PBMC vermeintlich vermehrt Strukturproteine als Reaktion auf die hohe Proteindosis gebildet wurden.

Summary

The project presented in this thesis aimed at the generation of databases for the proteome of human plasma and peripheral mononuclear blood cells (PBMC) with a proof of principle by application in a human intervention study with a high-protein supplementation over 5 days in human volunteers.

In many cases, human studies only allow minimally-invasive sampling. Therefore, blood and PBMC derived from blood samples as well as urine samples are mostly used as sources. Plasma is reported to possess the most complex proteome that covers proteins derived from tissue leakage and inflammation markers besides the classical plasma proteins mainly secreted by liver and immune cells. However, the huge concentration range of plasma proteins poses a major analytical challenge. Low-abundant proteins which may represent biomarkers of diseases are frequently masked by highly-abundant plasma proteins. To overcome these difficulties we established and standardized analytical methods with depletion of the most-abundant plasma proteins by prefractionation over antibody columns. Classical two-dimensional electrophoresis and Matrix-Assisted Laser Desorption Ionization (MALDI) mass spectrometry for protein identification were applied to plasma and PBMC samples. From the 2D gels corresponding proteome maps were created and linked to databases containing information of the displayed proteins with biomarkers of interest included. These databases may reduce the burden of protein identification processes in future human intervention studies and may allow a quick and convenient detection of proteins with altered levels. The biological and/or analytical variability of plasma and PBMC proteomes was assessed in volunteer samples with a remarkable inter-individual variation.

A human volunteer study was performed to search for changes in plasma and PBMC proteome markers representing a signature for a high-protein dietary intervention. Compliance was tested by assessing plasma amino acid and urinary excretion levels before, during, and after the intervention with the expected increase in urea production. However, no significantly elevated concentrations of urea cycle amino acids and proteins could be identified that would indicate a metabolic adaptation. Plasma analysis resulted in an increase in selected hepatic proteins that may be taken as a measure of increased synthesis, whereas in PBMC some structural proteins changed in response to the intervention.

1. Introduction

1.1. Biomarker Discovery in Nutritional Research

Current nutrition and food research intends to characterize the impact of diet and food components on human metabolism at the cellular and molecular levels. The goal is to identify biomarkers that can serve as measures of the health status of the human body and where nutrition can be applied to improve health or even prevent disease. Especially diabetes and cardiovascular disorders are caused and promoted in genesis by an unhealthy lifestyle and diet [1]. Typically, nutrition-related effects on the human metabolism are very small, within the normal health state of the body and extremely time-dependent [2]. Therefore, the identification of alterations in human homeostasis after food intake and dietary interventions remains as a real challenge [3, 4].

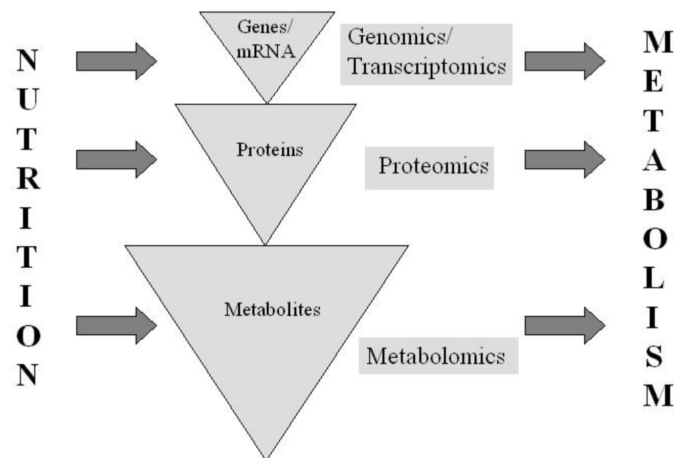

Figure 1-1 Profiling techniques in nutritional research

Biomarkers are considered to indicate disease risks and are mostly derived from large epidemiological studies [3]. Assessing the effects of diet on human metabolism by employing the profiling techniques of transcriptomics, proteomics and metabolomics aims at the detection of such biomarkers that can be altered to prevent disease at an early state by dietary interventions and optimized advice [5]. For this approach new phenotypical biomarkers have to be identified with sensitive technologies. Ideally, these techniques screen for a large number of parameters, as nutrients interact with many targets at once and can influence human metabolism through the inter-connected biological pathways [2].

Genomic, transcriptomic, proteomic and metabolomic approaches aim at the identification of genes, transcripts, proteins and metabolites in a particular sample at a certain time point. Assessing changes in expression levels of transcripts, proteins and metabolites by the platform technologies upon dietary treatments allows comprehensive coverage of biological adaptation [3]. Therefore, the omics-technologies provide promising platforms to identify or even predict disorders that can be caused or prevented by nutrition [4].

1.2. Impact of Dietary Protein Intake on Human Metabolism

Amino acids from dietary proteins fulfill numerous functions in the human body. The proteinogenic amino acids provide building blocks and nitrogen for the synthesis of proteins, nucleotides and other nitrogen-containing molecules. They can influence signal transmission through hormones and act as neurotransmitters. As a carbon source, amino acids take part in the energy homeostasis through formation of ATP and creatine. Glucogenic amino acids serve as precursors for *de novo* glucose synthesis and glucose homeostasis. Glutamine and alanine serve as nitrogen carriers in the blood and in inter-organ metabolisms of nitrogen. Sulfur-containing amino acids provide organic sulfur for synthesis of other sulfated biomolecules. Glutathione, containing glycine, glutamate and cystein, is the most important water soluble antioxidant and taurine serves as an osmolyte. Ammonia derived from amino acid degradation is part of the acid-base balance in the liver and kidneys. The adult human contains approximately 10 kg protein with some 2.5 kg in collagenes. As a result from a high protein turnover, a daily amount of 250-300 g of proteins is degraded or newly synthesized. The main part of this, up to 100 g per day, in muscles and up to 50 g per day in the liver. Nitrogen homeostasis is a highly controlled mechanism that regulates protein and amino acid degradation and synthesis according to the individual demand. Processes that are involved in nitrogen homeostasis are the protein turnover, amino acid oxidation, urea production and nitrogen excretion (Fig. 1-2). Nitrogen losses occur in the form of urea (about 80-85%), ammonia and creatinine but also through the faeces (10-15%) and several other losses (sweat <5%). The amount of excreted urea reflects the protein uptake. Dietary protein intake is therefore necessary to maintain the nitrogen balance of the human body [6, 7].

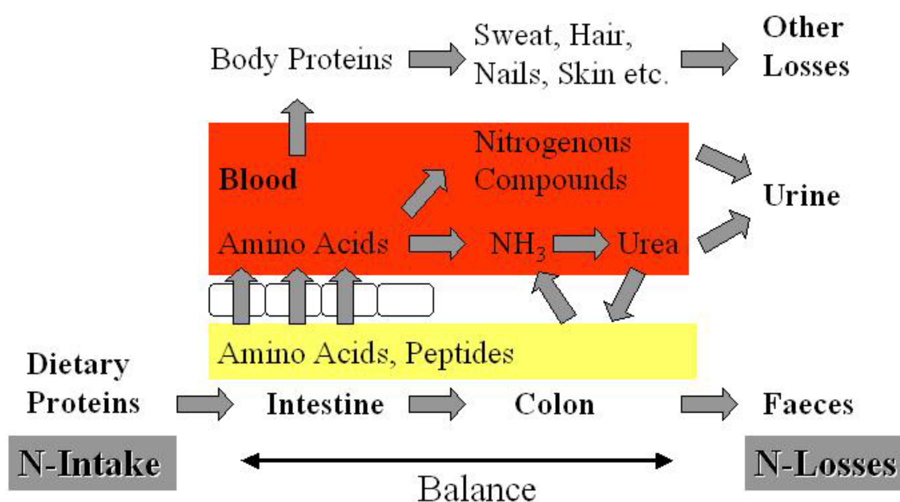

Figure 1-2 Nitrogen balance in adults (modified from [7])

Ingested proteins are absorbed in the form of free amino acids and as di- and tripeptides. After a protein-rich meal the liver is the central organ for plasma amino acid metabolism. Ammonia is eliminated in the form of urea and approx. 1/3 of the amino acids are used for liver needs and plasma protein synthesis. An increase in postprandial plasma amino acid levels is mainly due to branched-chain amino acids that are taken up and metabolized by the liver to only a minor extent. Those are trans-aminated in muscles, kidneys and the brain. In muscle they inhibit the uptake of glutamine and therefore stimulate protein biosynthesis in muscle cells in the presence of insulin [6-9].

Bioavailability of amino acids from a given protein source also has to be considered. The quality of a protein source is classified through the Protein Digestibility Corrected Amino Acid Source (PDCAAS) established by the Food and Agriculture Organization (FAO 1991). It describes the utilization of amino acids in a test protein compared to a reference amino acid pattern. This utilization is then corrected for differences in protein digestibility. Milk-derived whey protein followed by casein show the highest PDCAAS values due to a high content of essential and branched-chain amino acids. Casein intake induces a moderate, long-lasting effect of high plasma amino acid levels up to 7 hours after ingestion [10]. The recommended daily allowance (RDA) level for proteins recommended for body maintenance of healthy adults is reported as 0.8 g/kg BW and day. This level does not consider an elevated need of energy intake during exercise to overcome protein and amino acid oxidation and repair processes after muscle damage. Recommendations for athletes vary between absolute intake ranges of 1.0 to 1.6 g/kg BW and day for endurance athletes and up to 2 g/kg BW and day during strength and power exercise. Heavy protein consumption exceeding 5 g/kg BW and day is considered as a risk factor [10, 11].
However, a high dietary protein intake has become popular especially for power and endurance athletes. The aim of a protein supplementation in the first instance is building up muscle mass during exercise periods. Several beneficial effects of protein rich diets have been discussed also for non-athletes mainly based on increased satiety effects and benefits during weight loss programs. Although the mechanisms of actions of the proteins are not yet fully elucidated it is also important to point out that a chronic high protein intake may be negative [12, 13] especially when additionally an impaired renal function is given.

Studies with athletes uncovered several beneficial effects of protein supplementation during exercise periods. The most obvious are weight loss and increased muscle glycogen synthesis [9, 14-18]. After protein rich meals, plasma insulin levels were found to be increased as well as fasting plasma glucagon. High insulin concentrations in plasma lead to an increased glucose uptake into skeletal muscle and a higher glycogen synthase activity especially after an increase in plasma amino acid concentrations. Elevated glucagon levels stimulate gluconeogenesis. Both hormones have an impact on satiety as well [14, 16, 19]. The branched-chain amino acids (BCAAs) leucine, isoleucine and

valine make up to about one third of the skeletal muscle protein. Leucine appears to play a role in many metabolic processes, including stimulation of protein synthesis via mTOR and modulation of the insulin signaling pathway. Supplementation of BCAAs was shown to reduce protein degradation rates and muscle glycogen depletion during aerobic exercise, and proven to upregulate muscle protein synthesis in catabolic conditions. Some researchers have investigated whether leucine plays a key role in controlling metabolic processes of dietary amino acids ingested in doses above the RDA level [9, 10]. Due to the effect of proteins on glucose metabolism it is further speculated whether patients suffering from type 2 diabetes could improve their glycaemic control through a dietary supplementation [19].

Several studies have demonstrated a satiety effect of a protein rich diet and ghrelin levels are considered to be part of this effect. Ghrelin is a hormone produced in the endocrine cells of the stomach and is referred to as the only orexigenic hormone stimulating food intake. Screening for changes in the transcriptome of circulating leukocytes indicated also that ghrelin level changes in blood after high-protein meals may mediate the effects. It is however not yet clear whether insulin is responsible for this ghrelin suppression. Furthermore, genes related to energy homeostasis were down-regulated, whereas satiety-related genes showed higher expression levels after consumption of high-protein meals [20]. Some studies suggest that the satiety effect of protein is even higher than the effects of fat and carbohydrates [17] and, therefore, a high-protein intake could have an impact in weight loss in obesity prevention. Several studies could also show a reduction of fasting plasma homocysteine concentrations by high-protein diets suggesting that such diets could be preventive for cardiovascular diseases. However, supplementation with protein powder instead of elevating the protein content of the normal diet increased postprandial homocysteine concentrations but did not affect the fasting state levels [21].

Formation of renal stones, osteoporosis, cardiovascular disease and even cancer are often discussed in the context of high-protein diets although a clear link between high-protein intake and these diseases could not be shown [10]. Renal diseases due to the elevated glomerular filtration rates could also not be detected [10, 22]. Supplementation with high protein doses above 5 g/kg BW and day could exceed the liver's capacity to convert nitrogen into urea and therefore cause hyperaminoacidemia, hyperammonemia, hyperinsulinemia and can cause nausea, diarrhea and even death [11]. Thus it is recommended to stay within the safe intake range in order to avoid toxicity. Daily protein doses below 2.8 g/kg BW and day did not have an impact on renal functions in athletes with regular protein consumption [23]. High-protein intake is also widely believed to lead to a wash-out of calcium from bones and cause osteoporosis. Due to the high phosphate content of a protein rich diet this effect is even reversed during protein supplementation. Furthermore, most protein supplements are enriched with calcium. The main source of high calcium levels found in urine after supplementation with

protein powders in volunteers therefore is of dietary origin and does not come from bone degradation [10]. A high dietary intake of the sulfur-containg amino acids methionine and cysteine may lead to acidosis after their oxidation to SO_4^{2-}, which is then eliminated by the kidneys after neutralization. This indeed is the case as shown in studies with moderate protein content and the absence of alkalinizing salts in the diet [10].

Taken together, a high-protein intake via the diet is well tolerated by healthy humans when not exceeding the capacity for hepatic elimination of nitrogen by urea synthesis and sufficient water for renal elimination and excretion of urea. Whilst for rats it has been shown that a high-protein diet causes adaptive changes in protein synthesis for hepatic genes, mainly of the urea cycle, it is not known whether the same is true for humans. Since PBMC express selected genes of the urea cycle and circulate in the blood, reaching various sites in the body, we used proteome profiling techniques to analyze whether changes in PBMC and plasma proteins can be identified under a high-protein diet.

1.3. Use of Proteomics Applications to Human Plasma and Blood Cells in Nutritional Intervention Studies

In contrast to cell or animal experiments, human intervention studies require easily accessible sample sources. All body fluids such as blood, urine and saliva therefore represent sample targets, especially if they can be obtained in sufficient amounts. The most interesting of these body fluids seems to be blood, including plasma and blood cells, which are the only cell types that can be obtained in sufficient amounts using minimal-invasive sampling methods. Blood plasma, the fluid compartment of blood, carries inter-organ metabolites and signaling molecules and therefore mediates the communication between cells and tissues directly or indirectly. It thus reflects also the conditions of organs and physiological processes associated with them. Moreover, proteins in the blood actively participate in many life-sustaining body functions such as the transport of nutrients and metabolites, inflammation, hormone signaling, and many more [24].

Blood is drawn in sufficient amounts for analysis of several clinical parameters in most clinical and nutritional studies. In contrast to tissue samples, it is relatively stable against proteolytic degradation [25]. Among blood cells, peripheral blood mononuclear cells (PBMC) seem to be the most valuable ones, because they are the most easily accessible. PBMC can be isolated directly from whole blood by centrifugation and therefore represent an easily accessible source for cells in the human body. Target molecules (as proteins for example) can be quickly obtained from the cellular fraction, and excess plasma can be directly stored for later use. PBMC consist of 80-85% lymphocytes that are known to be responsible for inflammatory responses in the human immune system and in the repair processes after tissue injury. In the nearby environment they form cytokines like interleukin-6, TNF-α, interferon-γ and others as an acute answer to inflammation [6, 26, 27]. 5% of white blood cells are monocytes that become activated in inflammatory conditions. They produce biologically active molecules such as cytokines, chemokines, adhesion molecules and enzymes for signal transduction in

many disease states [28]. As they circulate through the body it is likely that PBMC can serve as a "monitor" of tissue and organ responses. Both plasma and PBMC therefore serve as an easily accessible sample source, and may contain valuable information on the current health status of the organism.

In contrast to transcriptomics, proteomics detects all expressed proteins including their isoforms, interactions and modifications. Proteomics is the only platform that provides targets to interfere with, as proteins can be directly influenced by extrinsic factors. Thus, proteomics is ideally used to screen on a large scale for changes in response to external factors [29, 30]. Despite its high potential, proteomics science has to deal with several analytical difficulties. Anderson and Anderson remarked in 1998: "Considered objectively, there is every reason to expect that proteomics will ultimately exceed genomics in total effort, though this effort will sorely be limited by the availability of scientists able to deal with protein's nonideal properties." [31]. Since the early 1980s proteomics technologies have developed significantly and the different techniques are applied as powerful diagnostic tools today. They allow measurement and also identification of many proteins and therefore can provide an overall image of the proteome of a cell or body fluid under a certain condition [24, 31]. The pharmaceutical industry has established this technology for the development of novel drugs and it is clear that proteomics will become a strong tool in nutritional science as well [32, 33].

Proteome profiles of plasma and PBMC react to external signals which make these blood components a valuable sample source and may provide a better understanding of effects of nutrients in human metabolism. So far, several studies have uncovered alterations linked to nutritional factors in the PBMC genes [34], whereas studies on the protein level are still sparse. However, changes in the proteome of human PBMC in response to dietary interventions can be shown as well [35, 36]. Therefore, plasma and blood cell proteomes are potential candidates for the discovery of biomarkers that indicate early stages of disorders, respond to dietary ingredients, and may uncover the effects of nutrition on human health.

As the proteome of PBMC underlies quick changes due to external factors, the method of sampling and the sample processing prior to proteomic analysis is very important and has to be optimized and standardized. Protein profiles are expected to vary between subjects anyway, whereas it was shown before that gene expression profiles within the same person appear to be relatively constant [20]. The most crucial issue is platelet contamination of the PBMC fraction in the isolation process. Various isolation methods can result in enormous differences in platelet content within the PBMC sample and therefore in a varying amount of platelet-specific proteins in the analysis [37].

1.4. The Plasma Proteome

Plasma is believed to contain the most complex human-derived proteome. It includes far more than just the classical "plasma proteins" that carry out their functions in circulation, but also secretion products and proteins derived from leakage from cells (Fig. 1-3).

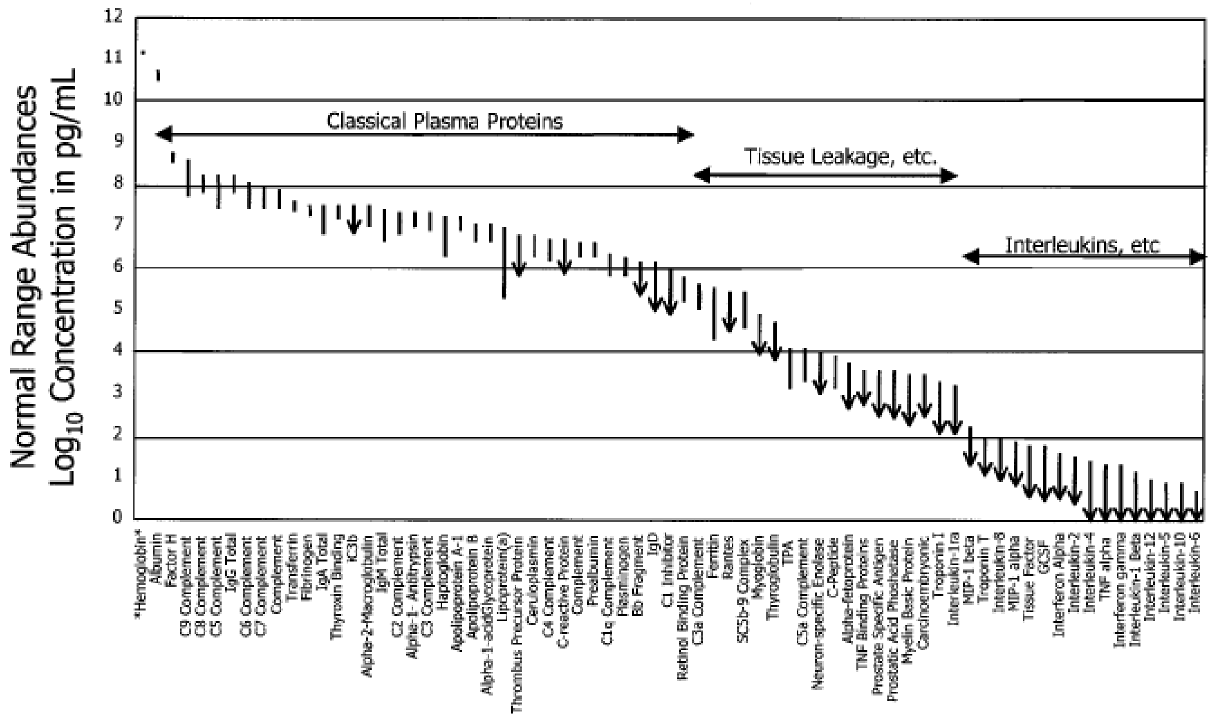

Figure 1-3 Reference intervals for 70 protein analytes in plasma (from [24])

The total number of proteins in human plasma can still not be quantified and is expected to exeed 100,000 [38] covering a huge quantitative dynamic span of at least 10 orders in magnitude. This makes analysis a difficult task. Albumin is the most-abundant plasma protein making up to 55% of all plasma proteins with concentrations of about 35-50 mg/ml. Low-abundant proteins are for example cytokines, such as interleukin-6, appearing in concentrations of pg/ml.

The classical plasma proteins are mainly secreted by the liver and the intestine. Immunoglobulins also constitute a high-abundant protein fraction in plasma. About 10 million different sequences of antibodies are believed to be present in human plasma. Tissue leakage products or other secretion products may be present only transiently by release from cells or tissue by damage in disease states, and may represent diagnostic biomarkers. Receptor ligands, such as peptide and protein hormones and cytokines, mostly have a relatively short residence time in plasma, as their molecular weight is typically below the kidney filtration cut-off of about 45 kDa [24].

First *in silico* methods have been applied to explore the origin of the various proteins in plasma – as a surprisingly high number of "unexpected" proteins was identified. For example, a large number of intracellular proteins and even proteins of nuclear origin have been found in plasma. Whether they are indicators of normal tissue renewal processes or measures of tissue (organ-specific) damage is currently under investigation. In this context, the isoform-specific proteins (in case of selected enzymes well established clinical markers) in plasma are interesting for further exploration.

1.5. Proteome Analysis of Plasma and PBMC

The huge dynamic range of protein abundancy in plasma makes analysis of this sample a difficult task. To overcome this problem either very sensitive technologies are needed to allow detection and identification of less-abundant proteins, or the proteins of interest must be enriched in the sample itself [39]. Recently, various efforts have been put into the development of sensitive methodologies that improve plasma as well as blood cell proteome analysis. Proteome analysis of blood proteins depends on many variables, most importantly the method of sampling, sample processing and sample storage. It is obvious that the optimization and standardization of these experimental parameters is needed in order to reduce analytical variation [40].

The Human Proteome Organization (HUPO) Plasma Proteome Project, in which worldwide 38 laboratories contributed to the goal of providing a first map of the human plasma proteome, has made major contributions to, and provided important recommendations for, sample analysis. In a pilot phase sample treatment procedures were standardized. Different technologies were used to test their advantages and limitations in plasma proteome analysis. A comparison of reference specimens of human plasma and serum was used to test these technology platforms by comparison based on protein numbers. The long-term goals are the complete identification of the human plasma and serum proteome, the identification of variation of plasma constituents within individuals and across individuals [41]. A wealth of biological information is already available from the Human Plasma Proteome Project (HPPP), and essentially all is in the public domain. The HPPP has defined a variety of critical parameters determining the quality of analysis of the plasma proteome, and gives recommendations on the most reliable methods for sample collection, storage, treatment and analysis [42]. In the following section the state-of-the-art of plasma proteome analysis methods will be discussed in order to provide an overview of current technologies and their advantages and disadvantages.

The present study is based on the HUPO recommendations for plasma proteome analysis. Two-dimensional gel electrophoresis (2DE) followed by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) was used for plasma and PBMC proteomics, and Liquid Chromatography coupled to Tandem Mass Spectrometry (LC-MS/MS) for amino acid analysis. Sample complexity in plasma was beforehand reduced through antibody-based depletion of

the proteins with highest abundance from the crude sample. Results obtained from the applied technologies and possible alternatives are discussed later (chapter 5.1.). Figure 1-4 shows the different steps in sample analysis for plasma as used in the present study (Fig. 1-4).

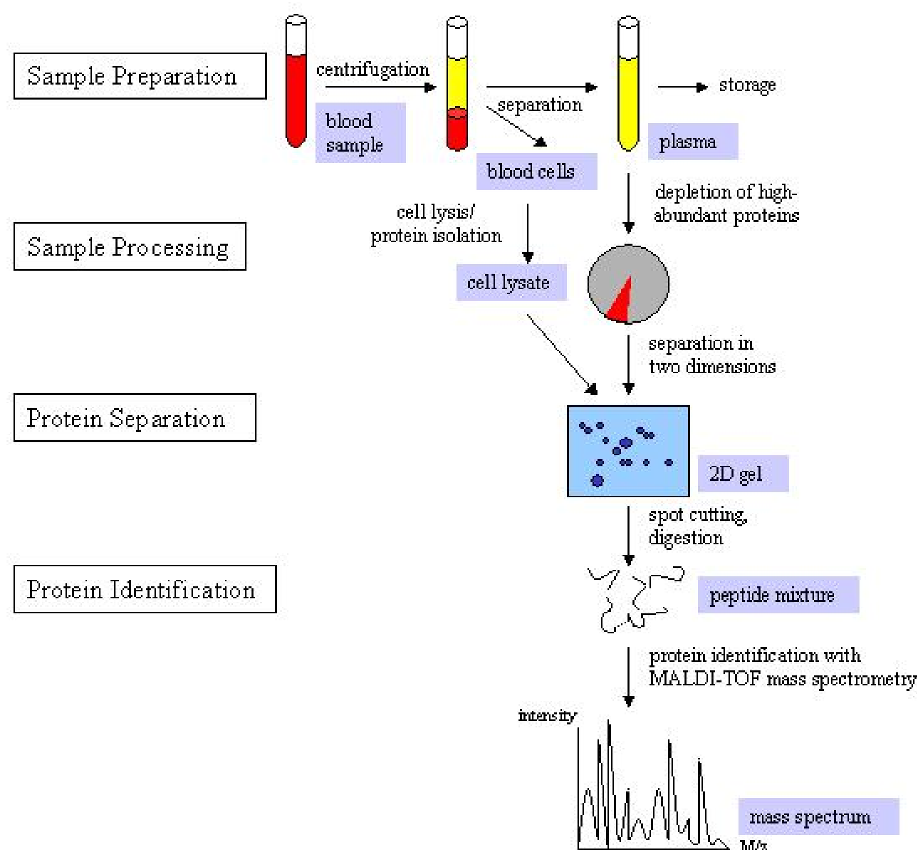

Figure 1-4 Sample analysis of plasma and PBMC using 2DE and MALDI-TOF-MS

1.5.1. Sample Preparation

Method of Sampling

In most experiments venous blood is collected in 10 ml vacutainer tubes. Without any special additives the blood will clot and coagulate. In this case after centrifugation the supernatant is referred to as ‘serum’. Alternatively, tubes may contain certain additives to prevent the blood from coagulating. After centrifugation for cell removal, this type of sample is called ‘plasma’. Common additives are EDTA, heparin and citrate. It is obvious that serum and plasma will differ with regards to the composition of the proteome. For instance, serum will not contain large quantities of blood-clotting factors, whereas plasma does.

There are also important qualitative differences. It has been observed that the proteins in serum suffer more from *ex vivo* degradation. During the clotting process natural proteases are released from the blood cells into serum, which in turn influences protein stability. This seems especially problematic

when the analysis is performed at the level of small peptides, since breakdown products appear then in the normal peptidome (as the sum of all low-molecular mass peptides). An extra complication of clotting is observed when analytical techniques like Surface-Enhanced Laser Desorption Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF/MS) are used.
It appears that the clotting process is extremely difficult to standardize and is highly influenced by factors such as temperature and processing time. This can affect the reproducibility of SELDI experiments when serum is used as the bio-sample. Plasma is therefore preferred to serum. Nevertheless, the generation of standardized proteomics procedures to analyze serum is advocated because in many studies performed in the past, including those on the effects of nutrition, samples have already been stored as serum.

The preparation of plasma samples demands closer attention with respect to sample quality. Since clotting is omitted platelets will remain intact, and because of their small mass the centrifugation step to spin down blood cells is not able to remove most of these. Moreover, during sample centrifugation, processing and storage, components may leak from the platelets contaminating the plasma proteome and peptidome. It seems therefore advisable to put effort in the removal of platelets. This can be achieved by a second centrifugation step at higher speed. Alternatively, one can filter the plasma over a 0.2 µm membrane that should have low protein-binding characteristics. Platelets can be stabilized by the addition of citrate and even more so by EDTA as anti-clotting agents for generating plasma samples. These treatments have the added advantage that their chelating activity stabilizes the platelets and at the same time inactivates many proteases that need metal ions as a cofactor.
One issue that needs to be addressed is that the addition of a concentrated citrate solution as anti-clotting agent results in some degree of dilution of the plasma samples. Generally, 15% lower protein concentrations are achieved compared to heparin or EDTA plasma. This of course can have an effect on the number of detectable and identifiable proteins, although no direct comparisons are available [43].

From the data obtained in the HPPP it seems that a nutrigenomic experiment, aiming to detect dynamic changes in the plasma proteome or peptidome, may be best performed with EDTA-treated and platelet-depleted plasma samples. Of course, for a nutrigenomic experiment the requirements for standardization go beyond the use of the best sampling method. Besides nutrients many other factors influence the blood fluid proteome, such as the moment of sampling, the amount of physical activity of the volunteer before sampling and others. All these potentially interfering parameters need to be controlled by standardizing the sampling procedure. In some human studies blood sampling may also be part of more complex sampling protocols, and this of course requires extra attention on standardization of sample preparation for proteome analysis.

Storage

The storage of serum/plasma to preserve the proteome should follow the general rules for the storage of biological materials. The storage temperature should be kept as low as –80ºC. Lower temperatures like that of liquid nitrogen may be preferred, but the improvement of conservation in going from –80ºC to –180ºC seems to be relatively limited. Samples should be stored in minimal aliquots sufficient for analysis so as to minimize repeated freeze/thawing. This may induce denaturing of some proteins, give proteases more chance to exert their degrading activity and might lead to disruption of platelets.

The addition of protease inhibitors should be a matter of careful consideration in the light of the methods of proteome analysis that later will be applied on the samples. Some protease inhibitors are peptides or proteins and their addition to the sample may interfere with the subsequent analysis. The same holds true for inhibitors that bind to the proteins. Often these chemical inhibitors are not very specific and will attach not only to proteases but also to other proteins.
It should be mentioned that the effect of prolonged storage of blood fluids (i.e. over a period of years) on the conservation of the proteome has not been studied. Such a study would provide essential information for future experiments [43].

1.5.2. Plasma Sample Processing

The dynamic range of blood proteins is at least 10^{10} and, as expected, the proteins appearing in higher concentrations are identified more easily. A complicating factor is that these proteins and their breakdown products interfere with the intermediate or lower-abundance proteins. This leads to serious problems in the visualization and identification of protein present at lower concentration levels in case of most of the analytical techniques. To deal with this problem, sample processing prior to protein separation and analysis is necessary. Various so-called 'clean-up' methods have been designed to either remove the most-abundant proteins from the sample or to reduce complexity of the sample through pre-fractionation steps prior to proteomic analysis. Therefore, either antibody-based or dye-based depletion methods are used, or membrane filtration for separation of low-molecular-weight proteins, including many biomarkers from the high-molecular-weight fraction, is applied [44, 45]. Within all these methods immunodepletion is the technique with the highest resolution and detection rate of low-abundance proteins [46] and is further discussed below.

Antibody-Based Depletion of Highly-Abundant Proteins

Surprisingly, when samples depleted of the top six abundant proteins are analyzed by two-dimensional gel electrophoresis (2DE), the number of newly identified proteins does not dramatically increase, but rather so the number of isoforms of the same set of proteins. Apparently, the depletion leads somehow to the unmasking of protein isoforms, which do already belong to the group of next-to-high-abundant proteins. From a scientific point of view, this broadening of the scope of protein isoforms is very

interesting, because it allows studying the effect of nutrition on the body's protein isoform management.

A variety of these methods, usually based on affinity-extraction with antibodies, have already been taken into commercially available kits. Some of those remove only albumin and immunoglobulins, while the more advanced systems remove up to twenty proteins. Although the available kits seem to work reasonably well, all of them result more or less in the loss of some medium- and low-abundant proteins due to non-specific binding to the extraction material or to the extracted proteins. Antibody-based columns showed the lowest rate for unspecific binding [47-49].

In addition, albumin is a natural carrier protein in blood, and many compounds including proteins, peptides, hormones, fatty acids and xenobiotics are bound to it. To a varying degree - depending on the applied materials and conditions - all albumin-removing methods lead to some loss of the target proteins. Of course, this may introduce an unknown degree of experimental variation in the quantity of less-abundant plasma proteins. Therefore, there is a need for the improvement of the clean-up methods [50, 51].

From the currently available methods, the MARS (Multiple Affinity Removal System) of Agilent Technologies was reported to be the best after comparison with other antibody-based methods [51]. The system is based on a High-Performance Liquid Chromatography (HPLC) column filled with a carrier resin to which polyclonal antibodies have been attached for selective removal of albumin, transferrin, alpha-1-antitrypsin, haptoglobin, IgG and IgA. The use of polyclonal instead of monoclonal antibodies ensures the removal of different structural forms of the proteins. Moreover, the columns can be used in tandem to increase the purification efficiency [25, 51]. The MARS method still has some disadvantages. It requires relatively expensive HPLC equipment, which is used at room temperature. Repeated use of the column carries the risk of cross-contamination. As a consequence, dye-based affinity depletion methods are still considered useful for certain experiments. The material is relatively cheap and is supplied as spin columns, allowing a high throughput of samples that can be rapidly processed in the cold. The major problem with the dye-based methods is the relatively high degree of (non-specific) retention of target proteins, in particular via binding to extracted albumin. This approach of affinity removal and treatment of samples might be considered useful and handy for standardized protein fractionation, but is far from ideal as a means of perfect removal of interfering proteins [52].

The depletion of the six most-abundant proteins seems to be only a first step towards the ultimate aim of reducing sample complexity. Approximately 99% of the serum protein consist of about 20 proteins including apolipoproteins and complement factors. An attractive aspect of antibody-based affinity purification is the fact that, in principle, the number of extracted proteins can be extended without any limits. Recently, spin columns with polyclonal antibodies against the 12 human blood proteins with

the highest concentrations in plasma have been put into the market (Genway Biotech, Amersham, Beckman). Another column (Sigma) claims to deplete even 20 proteins from the sample [53].

Pre-Fractionation/Pre-Separation of Samples

Sample pre-fractionation is used to further reduce the complexity of blood plasma and, therefore, to simplify the sample prior to final separation and mass analysis. Important issues concerning the quality of the analysis itself are reduction of separation speed, higher protein recovery and lower cost. A comparison of several pre-fractionation methods showed that each approach resulted in the identification of unique proteins that could not be detected with any other method. Therefore, it is recommended to combine the available methods to get the highest number of protein identifications in plasma. Widely used strategies include liquid chromatography, solution phase electrophoresis, free-flow electrophoresis and flat-bed electrophoresis [54].

Various methods for pre-fractionation of plasma samples have been tested for validity and practical applications (time, cost, efficiency) and are summarized briefly here.

Electrophoretic Methods

Proteins are separated based on their molecular mass on polyacrylamide gels. After electrophoresis in the first dimension and staining with common methods, bands can be excised from the gel and either digested directly ahead of mass spectrometric analysis, or further fractionated in multi-dimensional chromatographic steps.

The Gradiflow BF 400 allows pre-fractionation of complex samples in a membrane-based electrophoresis system. Proteins are separated due to their charge and size on uncharged membranes, resulting in four fractions containing proteins with an isoelectric point (p*I*) p*I*>albumin, p*I*<5.25 combined with the molecular weight (MW) MW>125 kDa, p*I*<5.25, together with MW<albumin and an albumin fraction containing over 80 % of the total albumin amount present in the sample. With this strategy especially the detection of proteins of extreme p*I* or mass range is improved. Limitations of this technique lie in a limited resolving power, sampling efficiency, accuracy and therefore confidence. Further fractionation with cation-exchange chromatography leads to an increase in the number of proteins that can be identified [46, 55].

Sample pre-fractionation on Sephadex gels provides a cheap, simple and quick method based on isoelectric focusing (IEF) in granulated gels. Proteins are focused according to their p*I* before fractions are scraped from the gel and transferred directly to a narrow pH range Immobilized pH Gradient Gel (IPG) strip for further gel electrophoresis. Therefore, the sample is not diluted and no proteins are lost through precipitation. Using this method, the number of proteins detected on 2D gels is much higher than running narrow-range IPG strips without pre-fractionation [56].

Chromatography

Chromatofocussing is a pre-fractionation method utilizing differences in protein p*I* to separate proteins. By application of a positively charged anion exchange column the system is set to a pH of 8.5 in the beginning with a decreasing pH gradient down to pH 4.0 during chromatography. Proteins with a p*I* below that of the mobile phase are negatively charged and stay on the column, whereas the ones with a higher p*I* are washed out. Proteins move down the column with the mobile phase when their p*I* is identical to that of the solvent. Fractions are collected automatically and can be further analyzed with common techniques, decreasing the detection limit down to ng/ml levels.

In order to overcome disadvantages in classical electrophoresis, a common alternative is the shotgun or Multi-Dimension Protein Identification Technology (MudPIT) where complex protein mixtures are digested prior to multi-dimensional separation, mostly with Strong Cation Exchange Chromatography (SCX) and Reversed Phase Liquid Chromatography (RP-HPLC). With MudPIT strategies a higher throughput and the identification of an increased number of proteins can be achieved. The techniques are improving constantly and identifications should improve as well.

A highly sensitive MudPIT method, covering a concentration range of up to nine orders of magnitude, is the protein array pixelation. After depletion of several proteins, the sample is further fractionated by Microscale Solution Isoelectric Focusing (MicroSol-IEF) followed by SDS-PAGE electrophoresis. As described by Tang *et al.*, the plasma sample is applied to a special device with seven chambers separated by immobilized gel membranes at pH values of 3.0, 4.4, 4.9, 5.4, 5.9, 6.4, 8.1 and 10.0. Albumin, therefore, can be reduced to a single fraction. For the second dimension, short SDS minigels are used where each protein fraction is loaded to a separate gel lane. As a result, low-resolution 2D arrays are obtained where protein clusters can easily be located due to their p*I* and mass range after visualization with common staining techniques. Subsequently, these protein gels are cut into equal pieces and digested with trypsin, the derived peptides can be further analyzed by liquid chromatography coupled to Electrospray Ionization-Tandem MS (LC-ESI-MS/MS) as described below. With this 4D method 3104 non-redundant proteins could be detected with high-confidence criteria. The detection limit could be reduced down to pg/ml levels [57, 58].

For Magnetic Bead Separation magnetic particles (size >1 µm) with a pore size of 40 nm are coated with hydrophobic surfaces containing C3, C8 and C18 residues. Protein samples, therefore, can be fractionated due to hydrophobic interactions with the different beads. Beads are separated from the sample solution, washed, and proteins are released from the bead surface with a water/acetonitrile mixture. Subsequently, the different fractions can be analyzed in a mass spectrometer. As an example, the detection limit for angiotensin II in serum using this method is 2 ng/ml. Thus, it serves as a very sensitive and reproducible pre-fractionation technique for complex samples [59].

1.5.3. *Protein Separation Techniques*

Protein separation is necessary prior to MS analysis due to the enormous complexity of the proteome of human plasma and PBMC. Separation techniques are either based on electrophoresis or on

chromatographic approaches. Further reduction of the complexity can be obtained by sample pre-fractionation as described above.

Electrophoresis Approaches

Two-dimensional gel electrophoresis is still the only method that allows separation of complex protein mixtures and a visual comparison of proteins from at least two gels. The method was first developed in 1974 [60] and is used up to now. A major advantage of this method is its simplicity and the direct display of the separated proteins that makes the results directly accessible [30]. Proteins are separated according to their p*I* on immobilized pH gradient gels in the first dimension, and then transferred onto a polyacrylamide gel for separation according to their MW in the second dimension. After this separation, spots can be excised from the gel and used for in-gel digestion before applying the sample to mass spectrometry. Care should be taken though when extracting peptides from the gel due to low extraction efficiency. A major disadvantage of 2D electrophoresis is the intense masking of lower-abundant proteins by those present at high concentrations. This effect cannot be reduced completely by depletion steps. However, after pre-fractionation many protein isoforms can be identified with 2D electrophoresis, providing detailed structure analysis of the sample. After spot normalization information about the relative abundance of these protein isoforms can be gained from this method [61-63].

Variation between different gels even within the same 1st and 2nd dimension run cannot be avoided. It results from changes in temperature or in slightly different buffer or gel compositions. The reproducibility of 2DE was improved enormously by implementation of ready-made IPG strips, polyacrylamide gels and buffers. The resolution and the detection limit can further be advanced, especially for proteins and peptides with extreme mass and p*I,* by the introduction of narrow-range IPG strips, covering a small and defined pH region. Pre-fractionation prior to analysis with narrow-range pH is recommended. As 2D electrophoresis cannot be completely automated, the method is not suitable for high-throughput strategies. Sensitivity of protein separation on gels is dependent on the staining technique. So far, proteins in the range of 10-60 µg/ml can be resolved without any problems, using classical staining techniques [64, 65]. New fluorescence stains could further improve the dynamic range of detected proteins.

To overcome the limits of low sensitivity and reproducibility in 2D electrophoresis, fluorescent labeling, as done in the Differential Gel Electrophoresis technology (DIGE), is introduced. Currently, three commercially available cyanine dyes, Cy2, Cy3 and Cy5, are used to label the ε-amino groups of lysine residues. By adding Cy-dyes, the charge and molecular weight of the proteins changes only slightly and mass analysis of labeled proteins is still possible. Up to three samples with different Cy-dyes can be mixed and run on the same gel, making it possible to identify reliably changes in protein expression levels and reducing the number of gels needed, thus saving time and labor. By the use of a labeled internal standard, gels can even be standardized. The detection range of the DIGE technique

covers up to four orders of magnitude in comparison to classical 2D gels with Coomassie staining that covers only about one order of magnitude in protein abundance [66-69].
Another sensitive and high-throughput method for protein separation under native or denatured conditions is Free Flow Electrophoresis (FFE). FFE coupled to rapid RP-HPLC in off-line mode results in a high-resolution separation, detecting protein concentrations down to low ng/ml levels under non-denaturing conditions. Compared to gel-based methods, FFE provides the opportunity of automated sample loading with a continuous sample feeding. In addition, as no gel media is used, sample recovery can be improved, thereby increasing the amount of proteins detected with this method by 2-fold compared to 2DE techniques [70-72].

Chromatographic Approaches

Several chromatographic approaches are utilized as fractionation methods in proteomics, most useful in the form of orthogonal multi-dimensional techniques. Separation is based on characteristic properties like p*I*, mass, charge, hydrophobicity and affinity for ligands. Anion-exchange chromatography is based on a Weak Anion Exchange (WAX) column and a buffer gradient, increasing the amount of sodium perchlorate. The limited number of fractions suggests its use as pre-fractionation step in combination with multi-dimensional separation techniques. Cation-exchange chromatography is performed with SCX capillary columns and a multi-step salt gradient of ammonium chloride adjusted to pH 3 with formic acid. Collected fractions can be further separated either with other chromatographic techniques or 2D electrophoresis followed by MS analysis online or offline. By offline combination of SCX capillaries and mass spectrometry a linear salt gradient instead of steps is applied, improving fractionation for particular proteins that are only co-eluted when using the multi-step gradient. Better sharpening and separation of the peaks can be achieved by the use of acetonitrile in the mobile phase. The number of fractions can be expanded, as the length of the columns and the flow rate is increased compared to the online strategy. Therefore, more proteins are identified with the offline method than directly connected to a mass spectrometer. However, automation is less possible compared with the online method. Shen *et al.* were able to cover about eight orders of magnitude in protein abundance by using ultra high-efficiency strong cation exchange chromatography in a 2D LC-MS/MS strategy.
RP-HPLC techniques make use of silica columns with a buffer gradient, increasing the amount of acetonitrile in aqueous solution and separation based on different protein hydrophobicity. This method is very useful for proteins, although the most hydrophobic fractions show co-elution due to the high affinity of proteins to the column material, resulting in lower resolution. Most of this co-elution can be suppressed by using a WAX column ahead of RP-HPLC. Another disadvantage lies in the fact that this method is more time-consuming than other approaches, leading to further loss of low-abundant proteins. On the other hand greater amounts of sample can be handled with RP-HPLC [73].

Shotgun Techniques

The idea of shotgun approaches is to use direct tryptic digestion of proteins, in order to obtain shorter peptides that can be applied directly to MS or MS/MS analysis. In this way, a labor- and time-saving method can be applied together with high efficiency. No pre-fractionation of complex protein mixtures is necessary and even proteins with extreme MW and p*I* can be detected in a high-throughput method. The detection limit covers concentration ranges down to the ng/ml level. Shortcomings are that protein isoforms can barely be resolved, and the reproducibility rate is relatively poor (45.7%). Compared to 2D electrophoresis, this technique is less useful to quantify differential protein expression levels [74].

The Accurate Mass and Time Tag Technology (AMT) consists of a high-resolution liquid chromatography system coupled to mass spectrometry in a shotgun approach. Highly accurate data from mass measurement and elution times obtained from complementary RP capillary chromatography are stored in a database and serve as mass and time tags for subsequent biomarker analysis. Therefore, a quick identification and improved quantification of peptides of interest can be achieved with this method. For database setup, mass analysis is performed with an Ion Trap Mass Spectrometer (IT-MS) with the MS/MS strategy being replaced by a Fourier Transform Ion Resonance Cyclotron Mass Spectrometer system (FT-ICR-MS) in the subsequent routine analysis. MS/MS/MS could further improve confidence in peptide identifications, as shotgun approaches deliver less confident results due to partial digestion, missed cleavage sites and cleavage that results from the normal biological process. Compared to other shotgun approaches, this technique allows an increased throughput as well as the analysis of comparative samples. Apart from database setup that has to be performed only once, measurement is very fast with this method, and replicate results can be compared easily. However, peptides must be identified prior to the analysis itself. AMT should be combined with protein depletion or enrichment techniques in order to improve sensitivity [75].

1.5.4. *Protein Identification*

Mass spectrometry has become the method of choice for analyzing complex protein mixtures in combination with complex protein databases. The discovery and development of protein ionization techniques was awarded with the 2002 Nobel Prize in chemistry, and, after that, further attracted interest for protein applications [76].

As no single analysis technique can be used to identify the whole plasma proteome, proteins are mass-analyzed and identified with mass spectrometry after separation. With the obtained mass spectra proteins can be identified in databases like IPI, NCBI or Swiss-Prot by application of different search algorithms. Several highly sensitive systems are currently used, with ESI and MALDI as the most common ones [76].

In Matrix Assisted Laser Desorption Ionization (MALDI) proteins or peptides are included in a matrix crystal with absorption properties at the laser wavelength. Irridiation by pulsed laser beams leads to “soft” ionization, making analysis of complex protein or peptide mixtures possible without any fragmentation. Usually, MALDI is coupled to a Time-of-Flight (TOF) analyzer, either working in

linear or in reflector mode. The reflector mode also allows the analysis of fragments occurring from induced protein decay, to gain sequence information through tandem mass spectrometry (MS/MS). MALDI-TOF can be utilized to obtain the mass fingerprint that is unique for each protein. In this method the peptide mass fingerprint obtained from MS is matched with the calculated peptide masses of each entry in the database. Usually, MALDI is combined with 2D PAGE. This proteomics approach can also be automated for high-throughput analysis, although gel-based separations limit the throughput. Mass analysis by MALDI rapidly gives confident results with high sensitivity [30, 76, 77].
Surface Enhanced Laser Desorption Ionization (SELDI) is used to bind subsets of proteins and peptides selectively onto the surface of a SELDI-chip. Chips coated with certain (bio-) chemical entities like hydrophobic hydrocarbons or specific metal ions are incubated with plasma or serum, in order to bind and concentrate the reactive proteins. Multiple surfaces can be used in one single experiment to increase separation power. Fractionation of proteins with SELDI is still limited but generally useful for increasing the number of proteins identified. As with many analytical approaches, also the SELDI method is hampered by non-specific binding of the most-abundant proteins. Depletion of these proteins from the sample before the use of the affinity chip is therefore advised. The peak intensity provides information about the relative protein abundance instead of absolute abundance, as intensities are influenced by sample preparation protocols, instrument settings and data normalization. Additional information about different expression levels can be obtained by use of antibodies in a Western blot. Comparisons of data even from multiple sites show a great consistency in relative peak intensity and therefore a good reproducibility. This is supported by standard quality control chips and standardized protocols [78, 79].
Electro Spray Ionization Mass Spectrometry (ESI) is preferably used in combination with capillary electrophoresis due to the very low flow rates improving the ionization efficiency. The sensitivity of ESI is inversely related to the flow rate and the diameter of the LC column, respectively. The optimum flow rate would be in the range of few nanolitres per minute. To obtain online analysis, chromatographic columns are directly connected to the ionization chamber of the ESI instrument with an integrated spray tip, reducing the post-column dead volume. Despite extensive system optimization, the reproducibility between different runs is not very satisfying [77, 80].

In contrast to all described analytical methods, the use of antibody-mediated technology to (semi-) quantify proteins in serum or plasma is not as much influenced by the presence of the abundant proteins. In addition, use of protease inhibitors does usually not interfere with the analysis. On the other hand, this way of proteome analysis is still limited by the availability of suitable antibodies. However, a multiplex assay for up to 100 blood proteins has been developed and is on the market (Rules Based Medicine, Inc). It is likely that in the near future antibody assays will be set up for many more (maybe even all) plasma/serum proteins after their identification by other methods. Research

groups, having already stored serum samples from previous nutrition studies, might want to save their material until the 'complete blood proteome antibody test' has become available.

1.5.5. Search Algorithms

In proteomic experiments, a significant number of protein matches is incorrect due to a high rate of false positives. One reason for that is based on the fact that a peptide sequence or peptide mass usually does not uniquely define a protein. Results from search engines should therefore be interpreted carefully in order to understand strengths and weaknesses of different search algorithms. Scoring functions of these engines are based on two different algorithms.

Heuristic algorithms as used for SEQUEST, Spectrum Mill, X!Tandem or Sonar correlate experimental MS/MS spectra with theoretical spectra and count the number of peaks common to two spectra ("shared peak count").

The second approach is the probabilistic algorithm, as used in search engines like MASCOT or Peptide Prophet that provide information about the probability, that a peptide sequence produced a spectrum by chance. The MASCOT algorithm itself is based on the probability, that a match is a random event. By adding the size of the database, an objective score is created, demonstrating the significance of a given hit. MASCOT, Sonar and X!Tandem give the most sensitive hits, and SEQUEST combined with Peptide Prophet reduce the number of false positives [81].

In general, it can be said that the combination of a high-sensitivity algorithm with a high-specificity algorithm should increase the confidence in protein identification.

1.5.6. Plasma Protein Databases

Usually, proteomics data sets are publicly available in the form of long lists and tables, containing enormous amounts of data. In order to prevent unstructured data storage, several publicly available and accessible databases have been developed in the HUPO Plasma Proteome Project Pilot Phase.

The HPPP Database (http://www.plasmaproteomedatabase.org) was set up to provide information about all protein isoforms found in the literature and assembled due to genetic origin. Additional information considering SNPs (single nucleotide polymorphisms), PTMs (post-translational modifications), tissue expression and sub-cellular localization information is provided for each isoform. In total, 4932 protein entries derived from 2446 genes and based on the 3020 proteins, identified by laboratories participating in the pilot phase of the HPPP, is included in the dataset. In addition, information on 2672 PTMs, 11381 SNPs and 248 cleavage sites can be drawn from the database [82, 83].

Martens *et al.* developed the Proteomics Identification Database (PRIDE) as a publicly available and easily accessible database. It serves as a central storage tool with a web-based interface for data submission. The database can be accessed under http://www.ebi.ac.uk/pride [84].

PeptideAtlas is a genome-mapped database derived from LC-MS/MS proteomics data, leading to databases for human and *Drosophila melanogaster*. A unique database is built from data derived from

proteomics experiments using human plasma and serum, the Human Plasma PeptideAtlas. The database is publicly available in the internet under http://www.peptideatlas.org [85].

1.5.7. The Sub-Proteomes of Human Plasma

An attractive way to study an increased number of blood proteins is by generating sub-proteomes enriched for proteins carrying a defined protein modification. Antibodies against such a modification can be used for this purpose, as done for example by precipitation of phosphorylated proteins with anti-phosphoserine or –phosphotyrosine antibodies. Until now, most of these applications have not received sufficient attention to culminate in standardized protocols. An analytical method based on (bio-) chemical fractionation is the SELDI approach.

Based on different selection methods, a sub-proteome of glycosylated proteins has also been defined. Using hydrazine chemistry, glycosylated proteins are coupled to a resin via their sugar chains. After trypsin digestion and washing, the N-glycosylated peptides are released from the carrier by digestion with the enzyme PNGase F. The mixture of peptides is then collected, whereas the sugar moieties remain attached to the carrier. Peptides can be separated and sequenced by LC-MS/MS, whereas quantitative comparison of peptides between different mixtures can be done by the isotope-coded affinity tags (for instance with the ICAT approach) labeling procedure in advance of LC-MS/MS. Although not yet reported, the procedure may be extended with the enzymatic release of O-linked glycosylated peptides. Another way of establishing a sub-proteome is by affinity purification of the glycosylated proteins via their binding to lectins. This can be done with both, serum and plasma, resulting in mixtures of comparable protein composition. Of course, glycoproteins involved in the blood coagulation, like fibrinogen, are missing from serum. For both approaches the advantage is, that albumin, being a non-glycosylated protein, is not a constituent of the glycoprotein sub-proteome, although minor amounts of albumin cannot be excluded because of non-specific binding to the resin or the trapped glycosylated proteins. Although about 150 glycoproteins have been identified in this way, their range of biological functions seems confined, since one third was contributed by keratins (5), apolipoproteins (6), complement factors (13), immunoglobulin heavy or light chains (23) [82, 86, 87].

The plasma peptidome includes the low-molecular-weight proteome with molecular masses of peptides <15 kDa. Plasma peptides are of interest because of their specific functions in biological processes. They can occur as hormones, growth factors, cytokines or through proteolytic cleavage of high-molecular-weight proteins. Analysis of the peptidome with a differential peptide display (DPD) includes a RP-HPLC separation with subsequent MALDI-TOF analysis of 96 chromatography fractions. By combination of all 96 mass spectra, an *in silico* 2D display of peptides in the range of 750-15 000 Da shows the m/z ratio for all peptides on the x-axis and their retention times on the chromatographic column on the y-axis. Peptide fragmentation and sequence analysis is done by MALDI-TOF/TOF-MS or by combining ESI with a quadrupole and a time-of-flight mass analyzer (ESI-qTOF-MS/MS), resulting in peptide identification from a database search. With this method it is possible to analyze peptides down to the picomolar concentration range [88, 89].

Other subproteomes categorize proteins according to their origin or their body functions. Based on literature data, “functional subproteomes” from the identified proteins of HPPP have been defined. These include for example about 110 proteins related to inflammation, including cytokines, adhesion molecules and chemokines, 362 proteins of liver origin, 345 proteins with cardiovascular function, DNA-binding proteins, and many others have been separated out from the HPPP database [82].

1.6. Identification of the Plasma Proteome

In 1977, first approaches resulted in the display of 20 proteins from human plasma using 2D electrophoresis [90]. Because of the huge dynamic range and the lack of sufficiently sensitive analysis technologies, this number was only increased up to 55 in 1991 [91]. In 2002, a list of 289 proteins found in plasma or serum could be retrieved from literature [24].

As up to the present several methods for protein separation and identification have been established, all these technologies result in quite different results in protein analysis. Anderson *et al.* merged four different methodologies in order to describe the plasma proteome. These included a literature search, multi-dimensional analysis of either proteins or tryptic peptides, using chromatography followed by electrophoresis and mass spectrometry, and multi-dimensional chromatographic analysis of low-molecular-weight peptides followed by mass spectrometry. 195 identified proteins and peptides of all four methods appeared in more than one dataset, and far less, only 46, were redundant in all four datasets [92].

Li *et al.* identified 560 unique serum proteins using five different approaches. Of these, 2-dimensional electrophoresis combined with MALDI-TOF-MS/MS after anion-exchange chromatography yielded 78 proteins, whereas an offline shotgun-nanospray strategy identified 330 unique proteins. The 2DE method, however, included more information about p*I* isoforms and the relative abundance of serum proteins. Of all proteins, 37 were identical in all five approaches [93]. In 2005, a list of 1427 high-confidence plasma proteins from literature identified with various methods was published (Fig. 1-5), and the proteins were categorized due to their cellular origin and biological function. The dynamic range of these proteins was estimated to be about 10^8, still not displaying the very low-abundant proteins from plasma [39].

Shen *et al.* could expand the number of identified proteins in plasma up to 2392 through affinity depletion of IgG and albumin and separation with ultrahigh-efficiency capillary liquid chromatography. Proteins were mass-analyzed using an ion-trap mass spectrometer. Some of the proteins are in correlation with diseases and can be considered as biomarkers of risk [94].

When plasma is fractionated with anion-exchange chromatography followed by a second reversed-phase fractionation, 2254 different proteins could be identified by LC-MS/MS analysis. It could be clearly shown that extensive fractionation results in an increased number of detected proteins [95]. However, this also increases complexity of protein analysis. The Pilot Phase of the HUPO Plasma Proteome Project yielded a total number of 3020 proteins based on two or more peptides from

different analysis techniques. This is the starting point for the next phase in that project that will include further determination of subproteomes in plasma, quantitative data and bioinformatics [96].

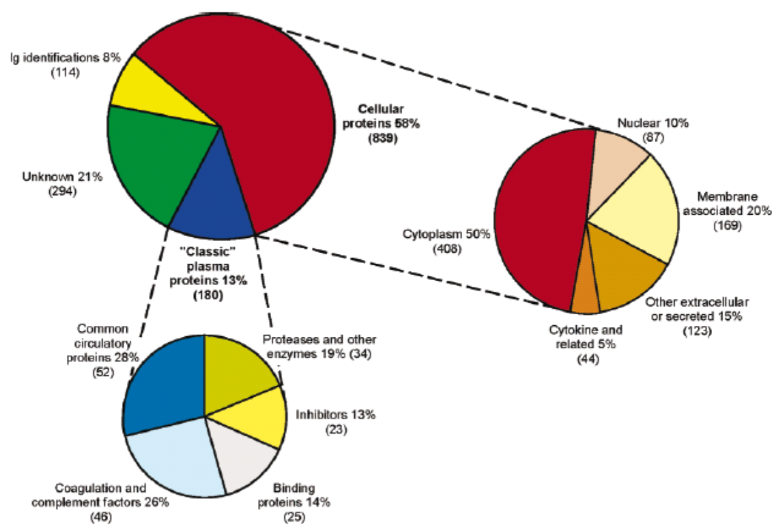

Figure 1-5 Categorization of 1427 high-confidence proteins (from [39])

2. Aim and Outline of the Thesis

Nutrition-related biomarker discovery recently has become very popular. However, effects of nutrients on human metabolism often are very small and complex. As for humans, sample sources to analyze these interactions are limited to easily accessible body fluids. The plasma and peripheral mononuclear blood cells (PBMC) are reported to reflect the health status of the human body. As easily accessible sample sources they are ideal targets for biomarker discovery in human studies. Assessing the plasma and PBMC proteome therefore provides the possibility to uncover adaptive changes in human metabolism.

The present work used proteome analysis of plasma and PBMC samples to assess changes in response to a dietary intervention in human volunteers. First, sample preparation and separation using 2D electrophoresis was optimized and standardized based on analytical recommendations from the HUPO PPP. Resolution of low-abundant proteins in 2D gels was improved through removal of the 12 most-abundant proteins with antibody-based depletion. The variability and "stability" of the human plasma and PBMC proteome was investigated in collaboration with NuGO partners, including four different proteomics centres, to assess basal variation.

Proteome 2D maps for plasma and PBMC were created in order to accelerate the identification process for altered proteins. Mass analysis of all visible protein spots on the gels was undertaken with MALDI-TOF-based mass spectrometry. All identified spots were included in an in-house proteome database. Based on the established proteome maps the plasma and PBMC proteomes of 10 volunteers were analyzed after a high-protein supplementation, as frequently used by athletes. Volunteers received 2 g/kg BW and day of a commercially available protein supplement on top of a controlled in-house diet over a time period of five days. Baseline variation was investigated during repeated measurements after overnight fasting as well as the wash-out of the protein supplement. In addition to plasma and PBMC proteome data, amino acid levels in plasma and urine were determined with LC-MS/MS as well as clinical chemistry parameters.

3. Materials and Methods

3.1. Chemicals and Consumables

Unless otherwise described all chemicals and consumables were purchased from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) or Sigma (Taufkirchen, Germany) and of *pro analysis* quality. All ready-made IPG buffers and IPG strips came from GE Healthcare (München, Germany).

3.2. Instruments

Special laboratory equipment which was used in addition to common centrifuges, rotators etc is indicated below.

3.3. The High-Protein Intervention Study

3.3.1. Volunteer Recruiting

11 male volunteers were recruited from the (ZIEL) at the Technical University of Munich. One volunteer was excluded due to an illness, 10 healthy men participated in the study. The average age of participants was 29.73 ± 6.76 years, body-mass index (BMI) values were reported with 23.95 ± 3.73 kg/cm^2 (mean ± SD). All participants were healthy and did not take any medication (for details see Table 6-4).

3.3.2. Study Design

The study period consisted of a one-week pre-phase, a five day intervention phase with a standardized diet and protein supplementation and a wash-out phase of 10 days. Participants should proceed with their normal diet during the pre-phase and the wash-out phase. During the intervention all volunteers were given a standardized diet supplemented by 2 g of the commercially available protein powder Protein90Plus (Power System) per kg body weight. The protein powder was taken during the day after mixing it with fruit juice. The volunteers were not allowed to eat or drink anything additional except tap water, coffee and tea.

Seven urine and blood samples were taken in the morning after an overnight fasting period from each volunteer. BL1, BL2 and BL3 were taken on days 0, 5 and 7 in the pre-phase in order to assess the baseline levels. HP1 and HP2 were taken during the intervention period on days 9 and 11. Finally, WO1 and WO2 were taken on days 15 and 21 after finishing the supplementation to assess the wash-out phase (Fig. 3-1). Blood samples were used for proteome analysis, clinical chemistry and determination of amino acid concentrations. Clinical chemistry parameters and amino acid concentrations were also detected in urine samples at each time point.

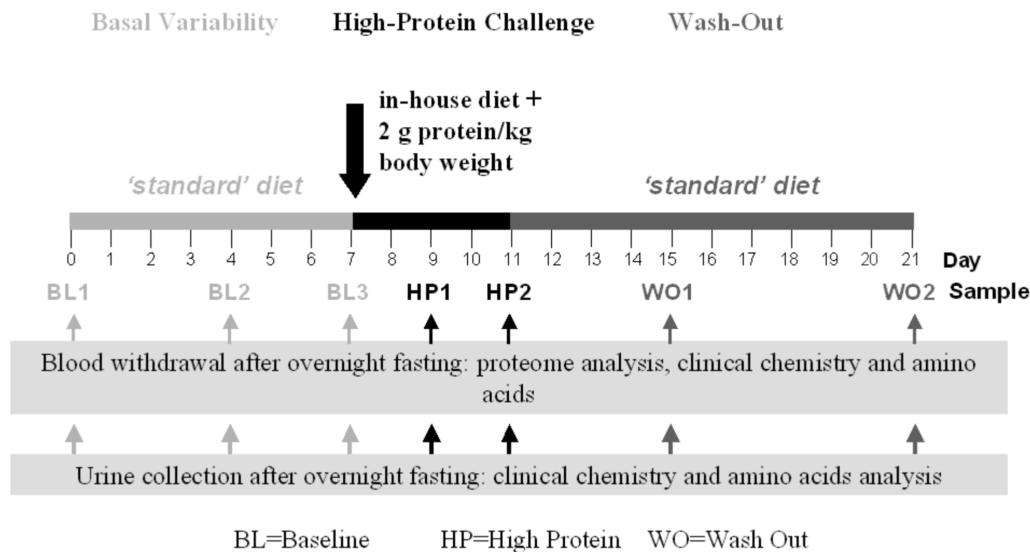

Figure 3-1 Outline of the high-protein intervention study

Ethical permission for the study was obtained from the Medical Ethical Committee at the Technical University of Munich.

3.3.3. Sample Collection

Blood was drawn from the arm by venipuncture using the Vacutainer CPT tubes (BD, Heidelberg, Germany) for PBMC isolation containing sodium heparin as anticoagulant. Tubes were inverted 8-10 times and centrifuged at room temperature and 1500-1800 x g for 20 minutes. The buffy coat above the gel barrier containing the PBMC (approximately 2 ml from each vacutainer) was carefully removed with a glass pipette into an empty 15 ml falcon tube. The plasma layer on top of the tube was removed as well and aliquoted into 1.5 ml Eppendorf tubes. Plasma was stored at -80°C. Morning urine samples were collected in 1 mM sodium azide. Cell debris and particles were removed with 1000 x g centrifugation at 4°C for 8 minutes. Urine samples were stored at -80°C.

3.3.4. SDS Electrophoresis of the Protein Powder

Analysis of the protein content in Protein90Plus was caried out with SDS electrophoresis on EXEL® 8-18 SDS gels (Pharmacia, Erlangen, Germany). The protein powder was dissolved by 30 minutes incubation in a reducing buffer containing 5 mM TRIS acetate, 6 M urea, 1% SDS, 8 mM tris-(2-carboxyethyl-) phosphine (TCEP, Calbiochem, Darmstadt, Germany) and bromophenol blue. 1 ml of the solution was incubated for another 5 minutes at 100°C and then mixed with 10 µl 20% TCEP solution. 3 µl of the sample was pipetted onto cellulose acetate paper (Schleicher & Schüll, Kassel, Germany) and used for electrophoresis. Electrophoresis was carried out at 12°C, 600 V and 35 W until the bromophenol blue front reached the anode. Staining was carried out at 50°C for 75 minutes using 1 tablet Coomassie R-350 (PhastBlue R, Pharmacia) in 2 liters 10% acedic acid before destaining in

10% acedic acid at room temperature. Finally, the gel was incubated for another 15 minutes in 15% glycerine solution and dried overnight. The gel was scanned using the image scanner Umax Astra 1120 (GE Healthcare) and analyzed with the software Total Lab 2008 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Quantitative measurement of protein fractions was carried out through comparison with the following calibrants: casein (300, 600, 900, 1200, 1500 µg/ml), β-lactoglobuline (60, 120, 180, 240, 300 µg/ml), α-lactalbumine (30, 60, 90, 120, 150 µg/ml), as well as BSA, lactoferrine und IgG (each 20, 40, 80 µg/ml).

3.4. Sample Preparation for Proteomics Analysis

3.4.1. PBMC Preparation

PBMC were washed with at least 3 volumes of freshly prepared balanced salt solution containing 1 volume salt solution (5.5 mM D-glucose, 5 mM $CaCl_2$*2 H_2O, 0.98 mM $MgCl_2$*6H_2O, 5.4 mM KCl, 0.145 M TRIS, pH adjusted to 7.6) and 9 volumes of 0.14 M NaCl solution. The PBMC solution was centrifuged at 200 x g and 4°C for 10 minutes. After carefully removing the supernatant, cells were re-suspended in 6-10 ml balanced salt solution and centrifuged again as described above. 200 µl lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, 0.04% pharmalytes pH 3-10 (GE Healthcare), 0.06% proteinase inhibitor cocktail (made of 1 tablet Roche Complete Mini Proteinase Inhibitor Cocktail (Roche, Mannheim, Germany) dissolved in 300 µl H_2O)) were added to the cell pellet per CPT tube. Cells were kept on ice for 30 minutes and sonicated afterwards.
PBMC protein solutions were desalted using 800 µl of ice-cold acetone per 200 µl of lysed protein solution. The mixture was kept at –20°C overnight for precipitation. Afterwards, proteins were centrifuged at 4°C and 10 000 x g for 30 minutes, the supernatant was removed and the pellet allowed to air-dry. The pellet was resuspended using 200 µl lysis buffer without pharmalytes. The sample was stored at –80°C after protein measurement.

3.4.2. Plasma Pre-Fractionation

In order to reduce complexity of plasma samples sample pre-fractionation using antibody-based protein depletion was tested. The 12 most-abundant proteins (albumin, IgG, fibrinogen, transferrin, IgA, IgM, HDL apoAI, HDL apoAII, haptoglobin, α1-antitrypsin, α1-acid glycoprotein and α2-marcroglobulin) were depleted from crude plasma through an IgY-12 high capacity proteome partitioning kit according to the manufacturer's protocol (Beckman Coulter, Krefeld, Germany). The flow-through (500 µl per aliquot) of two runs was pooled.
Concentration of flow-through was carried out at each of the 4 investigating centres using the Millipore Ultrafree-0.5 Centrifugal spin columns using a cut-off of 5000 Dalton (Millipore, Schwalbach, Germany), according to the manufacturer's instructions. Samples were centrifuged to the lower mark (= 25 µl), which took about 5-7 minutes. Concentrated depleted plasma (25 µl) was removed with a fine-tip pipette into an Eppendorf tube, and 210 µl of rehydration buffer was added to

the Millipore Ultrafree-0.5 Centrifugal spin columns. The solution was aspirated up and down twice and removed into an Eppendorf containing the concentrated depleted plasma.

3.4.3. Protein Measurement

Protein concentration was measured using the Bradford assay [97]. 800 µl bidest. H_2O was pipetted into a cuvette, 200 µl of protein reagent solution (BioRad, München, Germany) and 1 µl of sample were added. Samples should not contain any ampholytes. Absorption was measured in a photometer at 595 nm against BioRad/water solution as a blank. Protein concentrations can be calculated from a standard curve using BSA solutions in concentrations of 0.5, 1, 5, 7.5, 10, 15 and 20 µg/µl.
After protein measurement pharmalytes according to the concentration in the lysis buffer were added to the protein solution.

3.5. Electrophoretic Analysis of Plasma and PBMC

3.5.1. Protein Separation Using 2D Gel Electrophoresis

The methods used for protein separation with 2D electrophoresis were adapted based on the references given by Görg *et al.* [62]. IPG strips (GE Healthcare, Freiburg, Germany) were rehydrated to a final thickness of 0.5 cm before using them in the first dimension. There are three possibilities to rehydrate IPG strips. For active and passive rehydration the sample is already added to the rehydration buffer, whereas rehydration for cup loading is working without adding the sample solution.

Passive Rehydration

For passive rehydration of 18 cm strips 350 µl of rehydration solution in total was added into each lane of the reswelling tray (for 24 cm gels 460 µl rehydration solution, respectively). The rehydration solution consisted of the sample volume according to the protein amount used, 15 µl of freshly prepared 30% DTT solution (20 µl for 24 cm gels) and filled up to the final volume of 350 µl (460 µl for 24 cm gels) with rehydration buffer (7 M urea, 2 M thio urea, 4% CHAPS, 2% pharmalytes pH 3-10 (GE Healthcare), 0.5% IPG (GE Healthcare)). The solutions were mixed in a separate tube. Linear 18 cm IPG strips of pH 3-10, 6-11, 6.2-7.5 and pH 4-7 were used as well as 24 cm strips pH 4-7. The protective cover was removed from each IPG strip and the strip placed in the reswelling tray (GE Healthcare) with the gel side down making sure that no air bubbles were tracked underneath the strip. 1 ml of silicone oil was added onto each IPG strip in order to protect the gel from drying out. Rehydration was done at room temperature over night.

Active Rehydration

Active rehydration was carried out according to the method described above. Instead of a reswelling tray, the same amount of rehydration solution was pipetted into separate ceramic trays with electrode

inlays, and the IPG strip was placed upon this solution. Rehydration was undertaken overnight at 50 V on the cooling plate of the Ettan IPGphor II (GE Healthcare).

Rehydration before Cup Loading

Rehydration when using cup loading is done according to the protocol for passive rehydration without adding the sample to the rehydration buffer. Therefore, the total amount of rehydration solution containing only rehydration buffer and DTT was reduced to 340 µl, for 24 cm gels to 430 µl, respectively. IPG strips were kept in the reswelling tray overnight.

3.5.2. 1st Dimension

The manifold was placed on the Ettan IPGphor II system and 108 ml of silicone oil was spread evenly. Then the rehydrated IPG strips were taken from the rehydration trays, placed onto tissue paper with the gel side up in order to remove excess oil and rehydration solution. Afterwards they were placed in the manifold with the positive end near the anode and the negative end of the strip reaching the bottom of the manifold. All strips should be at the same level to avoid different separation distances. Paper wicks were wetted with 150 µl of desalted water and placed near the anodic end of the IPG strips, at the cathodic end paper wicks were wetted with 150 µl of 3.5% DTT solution in desalted water and placed onto the end of the strip. The electrodes were fixed onto the paper wicks with the side screws. When using cup loading the cups were placed close to the anode, and sample solution was added according to the protein amount loaded onto each strip. A maximum of 150 µl could be filled into each cup. The program was started. After approximately 1 hour the paper wicks were changed once and the run continued until the protein separation was completed.

Electrophoresis program:

Voltage	Step	Duration
1. 500 V	Gradient	0:01 h
2. 4000 V	Gradient	1:30 h
3. 8000 V	Step-n-hold	60,000 Vh (24 cm strips)
		25,000 Vh (18 cm, pH 3-10)
		30,000 Vh (17+18 cm, pH 4-7)
		30,000 Vh (18 cm, pH 6-11)
		30,000 Vh (18 cm, pH 6.2-7.5)
4. 500 V	Step-n-hold	until removed

After the electrophoresis IPG strips were removed from the manifold and sealed in plastic foil. Strips were kept at -20°C when they were used for the 2nd dimension on the same day or stored at -80°C up to several weeks.

3.5.3. 2nd Dimension

SDS gel electrophoresis in the 2nd dimension was carried out with isocratic (12.5%) acrylamide gels of 1 mm thickness in an Ettan Dalt II system (GE Healthcare). Acrylamide gels were cast according to the method of Laemmli [98]. For test reasons also Duracryl gels were used. 30% Duracryl gels (30T 2.2C Proteomic Solutions, Saint Marcel France) with a bis-acrylamide crosslinking grade of 0.65% were cast according to the IFR protocol (IFR protocol for casting 2nd dimension SDS-PAGE gels, personal correspondence). To run the 2nd dimension gels, IPG strips were incubated at room temperature for 15 minutes in equilibration buffer (1.5 M TRIS-HCl, pH 8.8, 6 M urea, 26% glycerol, 2% SDS) containing 1% DTT before transfer to a second equilibration step with equilibration buffer containing 4% iodoacetamide and incubation for 15 minutes at room temperature. The strips were then rinsed in water and SDS-buffer, applied ono the top of the gel cassette and fixed in position by overlaying with molten 0.5% agarose containing bromophenol blue to visualize the electrophoresis front. After cooling of the agarose solution, 3 µl of a stained low-molecular-weight calibration marker were applied to a small paper wick and inserted in a well formed on the right of the cassette by insertion of a 1 mm spacer. The IPG strips were then overlaid with the agarose solution. The 2nd dimension run was performed overnight. After 1 hour with 4 mA of current *per* gel, 12 mA were applied to each gel until the bromophenol blue had reached the bottom of the gel in the Ettan Dalt II System. Up to 12 gels could be loaded in the same run.
Two different buffer systems were used for the 2nd dimension electrophoresis:

Continuous Buffer System:
5 mM TRIS, 0.19 mM glycin, 0.1% SDS in bi-distilled water.

Tricine Discontinuous Buffer System:
Upper running buffer: 200 mM TRIS acetate buffer (Sigma), 200 mM tricine (Sigma) and 14 mM SDS in a total volume of 3 liters.
Tank buffer: 25 mM TRIS acetate buffer (Sigma), pH 8.3 in a total volume of 0.5 liters.
After the electrophoresis was completed gels were removed from the glass plates and placed in a fixing solution containing 40% ethanol and 10% acetic acid (up to 6 gels per tray) for at least 2 hours on the shaker.

3.5.4. Staining of Gels

Gels were stained either with Flamingo™ fluorescent stain, Krypton™ fluorescent stain or Commassie Brilliant Blue solution.

Flamingo ™ Staining

Flamingo™ staining was performed according to the manufacturer's instructions. In brief, 10 x Flamingo™ Fluorescent Gel Stain solution was diluted and at least 250 ml was used per gel. Gels were incubated in the dark overnight and destained afterwards for 30 minutes. Flamingo-stained gels were scanned using the Typhoon TRIO+ Variable Mode Imager (GE Healthcare) with the 532 nm laser and the 610 nm bandpass emission filter (settings for SYPRO®Ruby fluorescence stain). The resolution was 100 µm and the photomultiplier was set to 450 V. The gel file was then converted to a 16 bit tif-image for further comparison. After scanning they were sealed in plastic bags and stored in the dark at 4°C.

Krypton ™ Staining

The staining was undertaken according to the instructions given in the manual. Krypton™ Fluorescent Protein Stain (Pierce, Rockford, IL, USA) was diluted 10-fold and the gels were stained in the dark overnight. After destaining in 5% (v/v) acedic acid solution for 5 minutes gels were scanned using the Typhoon scanner with the 532 nm laser and the 580 nm emission filter.

Coomassie Staining

Coomassie staining was carried out for four days using Coomassie Brilliant Blue solution (10% $(NH_4)_2SO_4$, 2% phosphoric acid, 25% methanol and 0.625% CBB G250). Gels were destained in bidistilled water until complete removal of background staining. Scanning was done using the Umax ImageScanner (GE Healthcare).

3.5.5. Image Analysis

Spots were identified and analyzed using the ProteomWeaver 3.1 software (BioRad) or with the Delta2D 3.6 software (Decodon, Greifswald, Germany). Background subtraction and normalization were automatically carried out by the software program. Spots were considered as regulated when the intensities differed significantly ($p<0.05$) and by at least 2-fold intensity. Each analysis group consisted of at least 4 gels *per* treatment group or time point.

3.6. Protein Identification

3.6.1. Sample Preparation for MS Analysis

Spots were prepared manually for MS analysis. Spots on the fluorescent SDS gels were stained with Coomassie Brilliant Blue in order to visualize them.

The selected spots were manually picked with a sterile skin picker and transferred into solvent resistant tubes. After washing with 50 mM NH_4HCO_3 and acetonitrile/ 50 mM NH_4HCO_3 1:1 (v/v) for removal of Coomassie following a shrinking step with pure acetonitrile spots were dried in a SpeedVac. Dried spots were rehydrated for 1 h at 4°C and trypsinized overnight at 37°C using 5 µl of

0.002 µg/µl sequencing grade-modified trypsin (Promega, Mannheim, Germany). Excess trypsin was removed and peptides were extracted from the gel plugs with 1% TFA for 10 minutes with sonication.

3.6.2. MALDI Mass Spectrometry

Supernatants from the peptide extraction were used for MALDI-TOF analysis and co-crystallized onto a Bruker AnchorChip™ target (Bruker Daltonics, Bremen, Germany) with HCCA as the matrix using the double-layer method from Bruker Daltonics. A peptide calibration standard (#206195, Bruker Daltonics) was applied for external calibration. Proteins were analyzed with the Autoflex mass spectrometer (Bruker Daltonics) in positive ion reflector mode. The software for spectrometer control was FlexControl (Bruker Daltonics).

3.6.3. Spectra Processing and Database Search

Spectra were processed with the FlexAnalysis 2.0 software (Bruker Daltonics) and sent into the MSDB database automatically through the BioTools 3.0 software (Bruker Daltonics) and the MASCOT search engine (version 1.9.00, www.matrixscience.com). Matrix peaks with an m/z value below 900 were not included in the search. The following identification parameters were set: allowance of 0 or 1 missed cleavages, peptide mass tolerance of 50-150 ppm, trypsin as digestion enzyme, carabamidomethyl modification of cysteine; methionine oxidation as variable modification; and charged state as MH^+. Results with probability based MOWSE scores greater than 54 were identified as significant in the human database ($p<0.05$).

3.7. Analysis of Amino Acids and Clinical Parameters

3.7.1. Measurement of Amino Acids in Plasma and Urine

45 amino acids and derivatives were analyzed in plasma and urine using the LC-MS/MS and the iTRAQ-labeling method. 40 µl of sample were treated according to the AA45/32 Kit Phys Reag (Applied Biosystems, Darmstadt, Germany). The samples were then automatically (autosampler type HTC PAL, CTC Analytics AG, Switzerland) injected into the column (AAa C18 column 4.6x 150 mm, Applied Biosystems) of the LC system 1200 series (Agilent Technologies, Waldbronn, Germany) and mass-analyzed with the 3200 Qtrap (Applied Biosytems). Spectra were processed using the Analyst 1.4 software (Applied Biosystems).

3.7.2. Clinical Parameters in Plasma and Urine

Classical Measurements

Ammonia in plasma and urine was measured enzymatically using the Ammonia UV Test by BioChemica (Ralf Greiner BioChemica GmbH, Flacht, Germany). Urea concentrations in plasma and urine were determined with the Urea Liquicolor Test #10505 (Human Gesellschaft für Biochemica und Diagnostica, Wiesbaden, Germany) and urinary creatinine levels were analyzed with the Sigma

Diagnostics Kit #555 (Sigma Diagnostics, St. Luis, MO, USA), according to the manufacturers' instructions. Osmolarity in urine was determined with a semi-micro osmometer (Knauer, Berlin, Germany) and glucose in urine was measured with the Glucose Hexokinase FS-kit (DiaSys International, Holzheim, Germany) according to the instructions.

Clinical Chemistry in Plasma

Several clinical chemistry parameters in plasma were analyzed with the Piccolo™ system (HITADO Diagnostic Systems GmbH, Möhnesee, Germany). The used Comprehensive Metabolics panel (HITADO) included measurement of alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, albumin, total protein, total bilirubin, urea nitrogen, creatinine, calcium, glucose, chloride, potassium, sodium and total carbon dioxide. For the analysis 100 µl of plasma was directly injected into the panel. Measurement was carried out automatically.

3.8. Statistical Analysis

Results are displayed as the mean value ± SD or ± relative standard deviation RSD. Values with a p-value <0.05 were considered to be statistically significant. Statistical significance was analyzed using ANOVA or paired t-test. For graphical display of bar charts the software GraphPad Prism 4 (GraphPad Software Inc. La Jolla, CA, USA) was used. Statistical analysis of proteome data was conducted within the Delta2D software package. Software-based data analysis of amino acid and clinical chemistry data was carried out using the freely available program R (http://www.r-project.org/). An analysis of variance for each variable with "subject" and "sample" as factors was conducted. If a statistical significance was found (p.adjusted <0.05) Tukey analysis was performed to identify the significantly changed samples. To adjust p-values for multiple testing, the p.adjust-function was used within the R-library limma, using the Benjamini-Hochberg method [99]. Pearson correlations were determined using the rcorr function within the Hmisc package [100]. The packages lattice, latticeExtra, plyr, RColorBrewer and reshape were used for data transformation and creating plots and graphs [101, 102].

4. Results and Discussion

4.1. Method Development

As a first step sample preparation and 2D electrophoresis for plasma and PBMC was established in view of standard protocols and with regard to the cost factor. For PBMC, an existing protocol was optimized, whereas the plasma preparation procedure was not based on preliminary work.

4.1.1. Sample Pre-Fractionation in Plasma

To overcome the problem of high-abundant plasma proteins covering other relevant proteins, a pre-fractionation was performed prior to 2D gel analysis. A Beckman Column that depletes 12 proteins from crude plasma samples was used and demonstrated to improve resolution of lower-abundant protein spots on 2D gels as shown in Figure 4-1. The dominating albumin smear as well as the IgG chains and transferrin could successfully be removed and distinct spots hidden underneath appeared after depletion. The crude plasma sample containing 0.35 µg/µl protein was concentrated to a level of 0.26 µg/µl protein. Therefore, a large proportion of the total protein content was removed by the proteome partitioning kit.

It is often discussed whether removal of high-abundant proteins from plasma samples can also remove lower-abundant proteins and peptides either through the technology itself or due to carrier protein interactions. Especially albumin binds low-molecular-weight molecules, resulting in a loss of these in the following analyses. When detecting low-molecular-weight proteins and proteins at very low concentration levels, e.g. like cytokines, this issue has to be considered [52, 103].

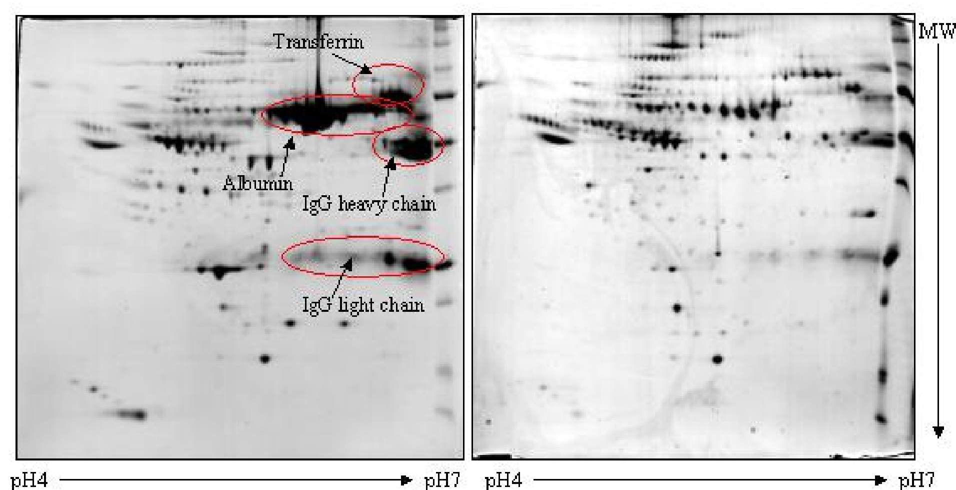

Figure 4-1 Separation of plasma spots before and after antibody depletion

2D gels of crude plasma (left) and plasma after depletion of the 12 most-abundant proteins with the Beckman column (right). 240 µg of protein was loaded onto 17 cm gels pH 4-7 using active rehydration. The second dimension was carried out with the discontinuous buffer system on 12.5% SDS-polyacrylamide gels. Gels were stained with Coomassie.

4.1.2. Optimizing Electrophoresis for Plasma and PBMC

pH Splitting

On the one hand, 2D gels with a broad pH range separate most of the proteins that are present in a sample. On the other hand, resolution can be quite poor on these gels, and narrow pH gradient gels could help to improve the spot resolution and visualization of low-abundant proteins. A comparison between plasma and PBMC samples, separated on gels with ph 3-10 and narrow ranges of 4-7 and 6-11, clearly favored the narrow-range gels, as more spots are visible (data not shown). The majority of spots appeared in the acidic pH range of 4-7, whereas only few spots can be found in the basic range, with poorer resolution at the very basic end. Basic proteins are difficult to resolve on two-dimensional gels as they tend to precipitate. Therefore, a range of 4-7 was used in all further experiments.

Amount of Protein

The amount of protein loaded onto the gels differs from sample to sample, depending on the appearance of prominent spots in comparison to rather faint spots. Thus, protein loading was optimized for plasma and PBMC. Figure 4-2 shows the procedure for PBMC samples. Increasing the amount of protein not necessarily increases the number of appearing spots, but prominent spots more and more dominate the spot pattern. The best resolution was yielded using 200 or 300 µg of PBMC sample. Decreasing this amount down to 100 µg resulted in less spots on the gels. For plasma, the same procedure showed that protein loads between 100 and 200 µg per gel gave the best spot pattern. Loading 500 µg of protein onto plasma gels resulted in an overload of the prominent albumin spots and less other spots on the gels (data not shown). As sample pre-fractionation with the Beckman column is laborious and cost-intensive, it was therefore decided to load 100 µg of plasma onto each gel.

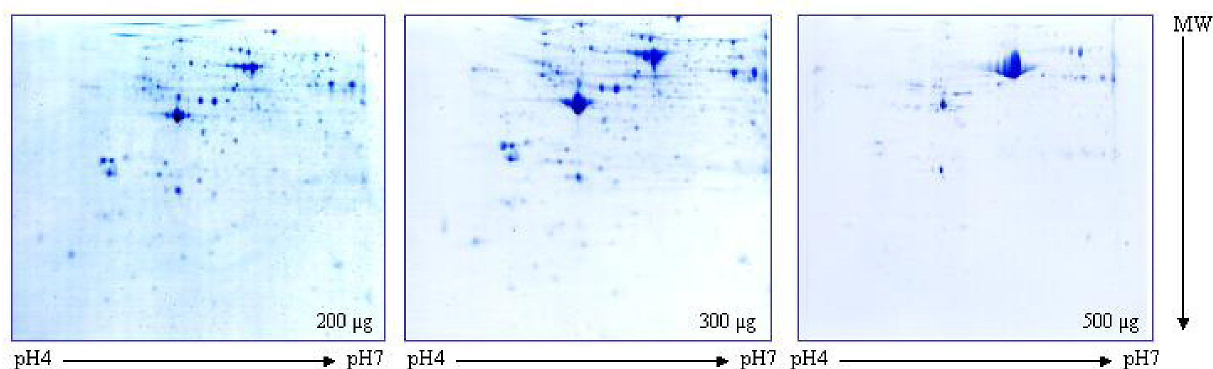

Figure 4-2 Different protein amounts on PBMC gels

2D gels of PBMC lysates with different protein amounts. 200 (left), 300 (middle) and 500 µg (right) of protein was loaded onto 18 cm gels, pH 4-7 using passive rehydration. The second dimension was carried out with the continuous buffer system on 12.5% SDS-polyacrylamide gels. Gels were stained with Coomassie.

Strip Length

To achieve an optimum resolution, IPG strips with different lengths were tested. Results of 17 cm and 18 cm strips did not differ from each other. Spot patterns that appeared on 24 cm strips were slightly pulled apart, but there was no clear improvement of resolution or spot number (data not shown).

Rehydration Methods

Through the rehydration step in 2D electrophoresis, the IPG strips are reswollen up to the final thickness. The sample can either be applied into the rehydration buffer by in-gel rehydration, or the protein solution is applied just before starting the first dimension through cup loading. In-gel rehydration can be undertaken passively on the bench or actively by applying a low current on the cooling plate of the electrophoresis unit. The temperature during rehydration also plays an important role as protein carbamylation can occur at higher temperatures (usually >37°C). Temperatures <10°C carry the risk of urea cristallization. The optimum method depends on the protein nature in each sample source and has to be determined individually [61].

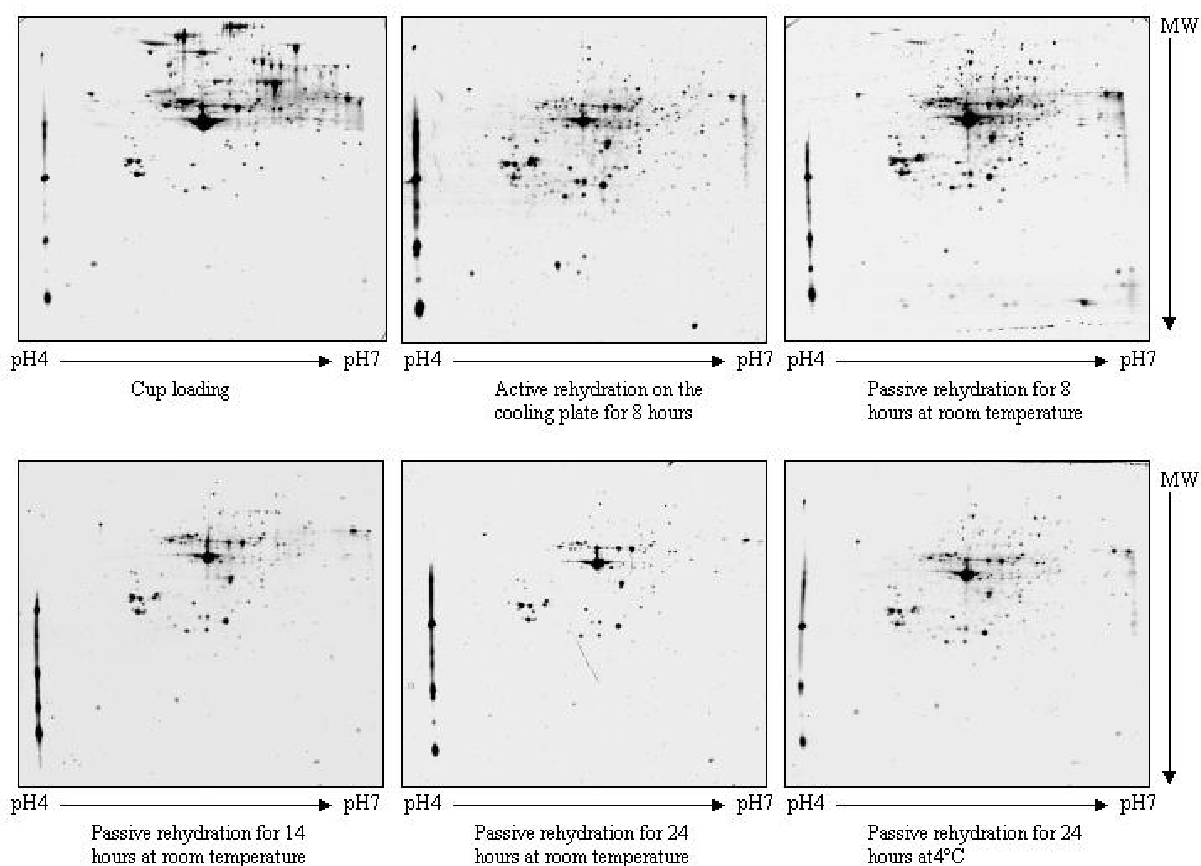

Figure 4-3 Comparison of different rehydration methods for PBMC samples

Rehydration and protein loading was carried out either by cup loading, active rehydration or passive rehydration, either at room temperature or at 4°C. Passive rehydration times were either 8 hours, 14 hours or 24 hours. 2D gels of PBMC lysates with 200 µg of protein separated on 18 cm IPG strips in the first dimension at pH 4-7. The second dimension was carried out with the continuous buffer system on 12.5% SDS-polyacrylamide gels. Gels were stained with Coomassie.

As shown in Figure 4-3, several different rehydration methods were tested for PBMC samples in order to optimize this step. The gels were analyzed with the ProteomWeaver software, and visible spots were counted. Cup loading resulted in the highest spot number with 774 detected spots. However, the spots were sometimes not clearly separated from each other. Passive rehydration at room temperature for 8 hours yielded 601 nicely separated spots, whereas the spot number after active rehydration was only 530. Prolonging the rehydration time in passive rehydration resulted in lower spot numbers (339 spots for 14 hours and 152 spots for 24 hours) compared to 8 hours 601 spots). In contrast, carrying out passive rehydration at 4°C could enhance the number of detected spots up to 523. As many proteins in plasma and PBMC samples are identical, they are also expected to get absorbed into the gel with the same rehydration method. Indeed, also for plasma, passive rehydration resulted in the highest number of nicely separated spots (data not shown). Based on these findings, we decided to carry out passive rehydration for 8 hours at room temperature in all plasma and PBMC proteomics experiments.

2nd Dimension Buffer

In the second dimension, either a continuous tank buffer or a discontinuous buffer system can be used. A higher resolution of proteins with a high-molecular-weight especially in plasma can be observed using the discontinuous buffer system [37]. This could be due to the use of tricine in the upper tank buffer. In our laboratory, plasma samples were run on both buffer systems in order to find out whether a discontinuous buffer would imrove results (Fig. 4-4). For plasma, it could clearly be observed that the discontinuous buffer improves resolution in the high-molecular-weight part of the gel, whereas the lower-molecular-weight spots remain unaffected. For PBMC samples this effect could not be observed after changing the buffer system. As proteome analysis of plasma samples could thereby be further improved, the discontinuous buffer system was used for all plasma proteomics, whereas PBMC gel electrophoresis was done using the continuous system.

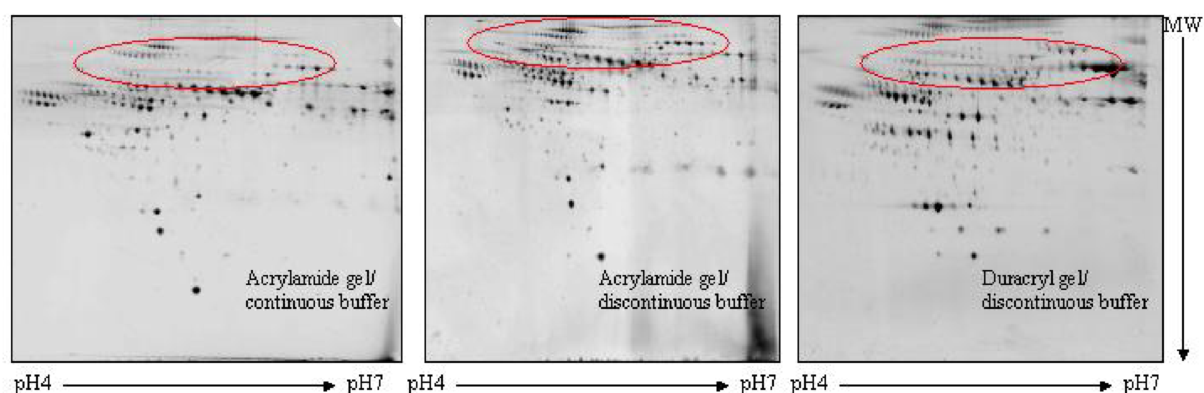

Figure 4-4 Comparison of continuous and discontinuous buffer system and gel composition in the 2nd dimension

100 µg of depleted plasma sample was applied to 18 cm IPG strips pH 4-7 and run either on 21.5% acrylamide (left and middle) or 12.5% duracryl gels (right) in either the continous (left) or the discontinuous (middle and right) buffer system. Gels were stained with Flamingo stain.

Gel Matrix

Acrylamide gels are rather complicated to handle in the laboratory due to their thickness of 1 mm. Gel ruptures during the staining or scanning process are quite common. An alternative option is the use of 30% duracryl gels which are similar in their resolution, but do not tear that easily. In Figure 5, a comparison between acrylamide and duracryl gels is shown. Some spots appear clearer in the acrylamide gels, whereas others turn out to be more prominent in the duracryl gels. Testing plasma samples on duracryl gels yielded in an elongated spot shape compared to the round spots on acrylamide gels. In addition to that, duracryl gels are very cost-intensive. Despite the difficult handling, we decided to keep acrylamide gels as the standard gels for the 2nd dimension electrophoresis.

Staining Techniques

The maximum number of spots identified on the gels depends mainly on the staining technique applied. Coomassie Brilliant Blue is still widely used due its easy handling and good quantitative linearity with low background staining [37]. Fluorescent stains like Flamingo can detect 0.25-5 ng of protein, covering a linear quantitative range of three to four orders in magnitude, according to the manufacturer's information. The detection limit of Coomassie is around 50-100 ng of protein. As high-resolution fluorescent stains are very expensive, decisions on cost *versus* resolution needed to be made. All staining techniques discussed below are compatible with mass spectrometry analysis.

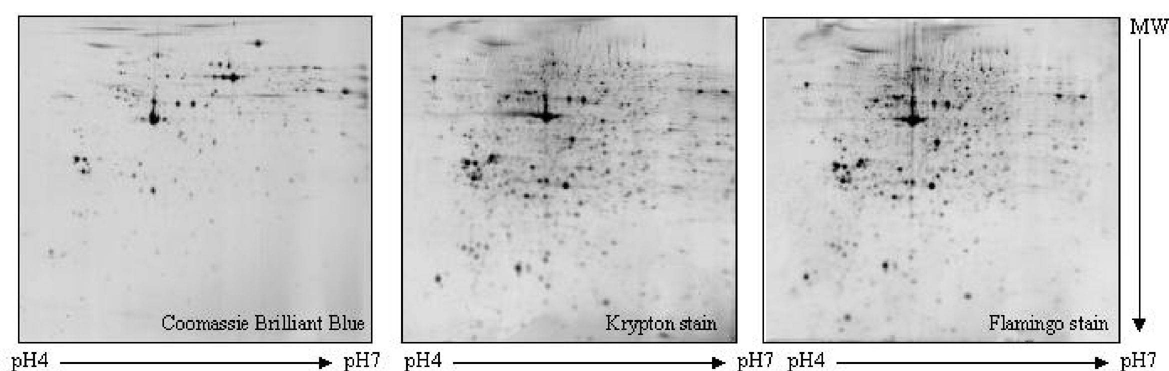

Figure 4-5 Different staining techniques after 2D electrophoresis

PBMC samples separated on 18 cm IPG strips pH 4-7 in the first dimension 300 µg of protein was loaded onto each gel. The second dimension was carried out on 12.5% acrylamide gels with the discontinuous buffer system. Colloidal Coomassie staining (left) and the fluorescent stains Krypton™ (middle) and Flamingo™ (right) were used for spot visualization.

A comparison of different gel staining techniques is shown in Figure 4-5. After Coomassie staining, 398 spots could be detected with the ProteomWeaver software. Staining with Krypton yielded 889 spots and Flamingo staining yielded 1110 spots. All staining was done on gels from the same sample. Within a method comparison exercise during the NuGO project (see chapter 4.2.) another fluorescent

stain, Sypro Ruby (BioRad), was compared with Flamingo stain and Coomassie. Sypro Ruby yielded on average 886 spots, whereas Flamingo gave on average 1172 spots per gel [37]. These findings demonstrate that the Flamingo stain is superior and the most sensitive dye that can be applied. Despite its poor sensitivity and a long-lasting staining procedure, Coomassie has the advantage of an end-point staining combined with low cost. Spots that can be seen on a gel by eye can normally also be detected in the Bruker Autoflex mass spectrometer used here for the protein analysis. To take advantage of the enormous sensitivity of the fluorescent stains, fluorescence-based spot pickers and a more sensitive mass spectrometry would be needed.

4.2. 2DE Separation for Determining Within- and Between-Laboratories Variability

The variation of 2D electrophoresis approaches applied to the same samples was tested within a project of the European Nutrigenomics Organisation (NuGO). All participating partners analyzed the same samples from depleted human plasma, platelets and PBMC, using their own technology in order to optimize standard procedures in proteome analysis. The coefficient of variation was calculated for each set of 5 gels *per* sample, using either software-based automatic matching of spots or manual matching. Manual matching improved the overall CV, as incorrect overlays could be eliminated before analysis. Between-laboratory variation was calculated from 10 selected spots per match set. These spots were also mass-analyzed and identified for protein identity with different mass spectrometry methods.

For PBMC analysis the CVs per match set based on all visible spots varied between 22% and 38% after automatic matching and 1-12% in 3 out of 4 laboratories after manual matching. The average between-laboratory variance was analyzed based on 10 selected spots per match set and was 34% (range 18-70%) for PBMC proteomics. 9 out of these 10 spots showed significant variation. The CV for depleted plasma was between 10% and 69% with automatic matching and could be improved by 1-16% through manual matching using all spots on the gels. The average variation between laboratories was 23% (range 4-54%). The test of variance was significant for 6 out of the 10 selected spots. Variation on platelet gels was 21-55% within laboratories and could be improved by only 3-5% after manual matching in three out of four laboratories. Between laboratories, the CV was 22% (range 5-60%). Based on the fact that not all selected spots could be clearly identified by all four laboratories, the unidentified spots were treated as “missing values”. Considering only those laboratories where the selected spots could be identified only slightly improved the between-laboratory variance (Fig. 4-6).

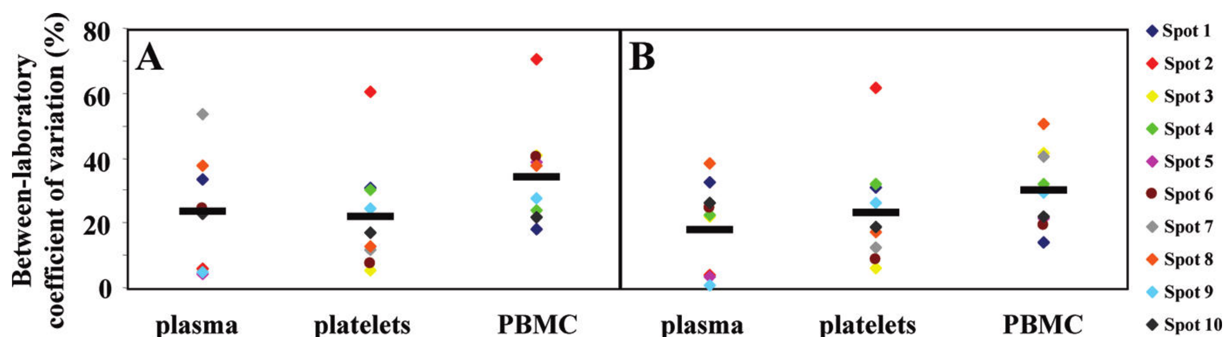

Figure 4-6 Variation of spots from plasma, platelets and PBMC between laboratories

Coefficient of variation in % (the bold line shows the mean value) between laboratories given for the 10 selected spots for each of the three datasets. All datasets were included in A, B: CV calculated only on those laboratories where the selected spots were identified.

The overall between-laboratory variance resulted from the use of different electrophoresis equipment and different protocols and procedures used at the different sites. The study could not distinguish between the technology-based effect or human-derived variation [37]. Within-laboratory variation may result primarily from slight variation in gel composition and/or running conditions even within the same electrophoresis run, based for example on temperature changes and modest buffer composition changes in first and second dimension separation. This emphasizes how crucial the standardization of all procedures is to minimize variability.

4.3. In-House Proteome Databases for Human Plasma and PBMC Samples

The generation of an in-house human plasma and PBMC reference database was for further use in human intervention trials, attempting to identify protein markers by directly comparing gels and altered protein spots without the need of further mass-spectrometry-based analysis. After 2-dimensional electrophoresis, according to adapted methods described above, gel images were used to build a clickable 2D map of plasma and PBMC proteins to be accessed via internet. These master gels can also be loaded directly into gel analysis programs like Delta2D and overlaid onto other gel images, as derived from plasma and PBMC samples during the software-based spot analysis. Protein identity for each spot was derived from the MALDI-TOF based peptide mass fingerprinting, followed by a MASCOT database search. Each spot of the proteome map was clearly identified at least three times with the same significant results. Therefore, each identified spot on the 2D map provides information about the protein identity of this spot, the score from the MASCOT database and the theoretical p*I* and MW data. Additional information is obtained from the SwissProt database, concerning the biological and molecular function of the identified protein (for details see Table 6-1).

4.3.1. The Plasma Database

119 clearly visible spots were excised from plasma gels and identified with mass spectrometry. For depleted plasma samples, a proteome map was generated, containing 103 spots that could be identified

with significant scores. This means an overall spot identification rate of 87%. Identified spots were marked on a representative 2D gel (Fig. 4-7) and information about the protein identity together with experimental details of each spot can be derived from Table 6-2. 33 different proteins could be allocated to those 103 spots.

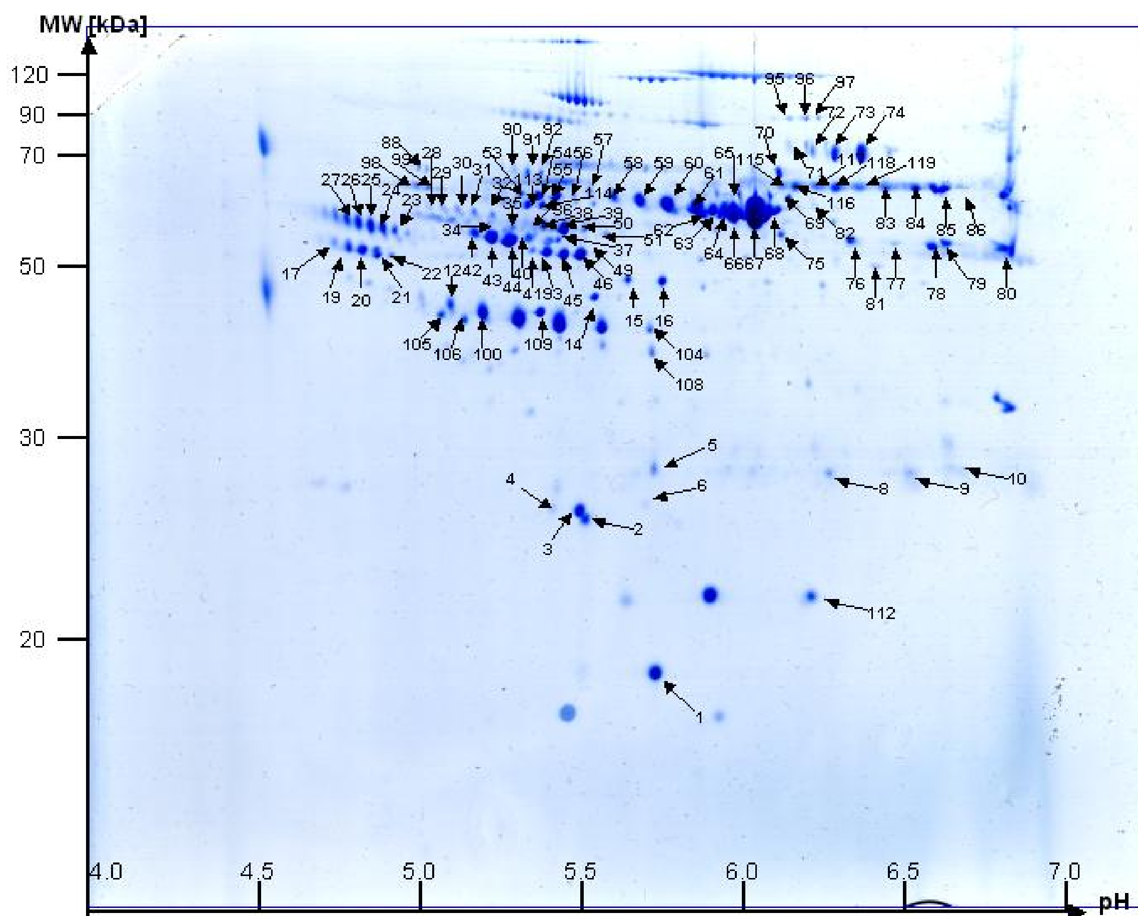

Figure 4-7 Proteome map with identified proteins as derived from depleted plasma samples

Proteome map derived from a 2D gel of depleted plasma. 100 µg of depleted plasma sample was applied to 18 cm IPG strips pH 4-7 and run on 21.5% acrylamide using the discontinuous buffer system in the second dimension. Staining was carried out with Coomassie-blue. Visible spots were dissected and mass-analyzed using MALDI-TOF MS. 103 spots were identified with significant scores in 3 or more individual gel analyses (numbered spots with arrows).

The plasma proteins identified carry quite divergent biological functions (Fig. 4-8, Table 4-1) and could be classified into 7 different groups, according to their function as derived from the SwissProt database. Over-represented are immune response proteins with 25% of all identified entities, including the Ig kappa chain C region and the Ig mu chain C region (IgM) as typical immune globulins, but also complement component C6 and C9, which are considered as classical plasma proteins [24]. Antichymotrypsin, an acute phase protein which protects against proteolytic activity, can be found within the inflammation-related protein class. CD5 antigen like protein is associated with IgM and may play a role in inhibition of apoptosis [104]. The second largest group of proteins, according to

biological processes, is related to coagulation, including 21% of all identified proteins in plasma. Typical coagulation-related factors are fibrinogen, kininogen-1, antithrombin-III, beta-2-glycoprotein 1 and alpha-1-antitrypsin. All these proteins can be found with high abundance in blood plasma [92]. This is also the case for the 18% of represented transport proteins like prealbumin, albumin, vitamin D-binding protein, hemopexin and afamin. Vitamin D-binding protein is a multifunctional plasma protein that carries vitamin D and, linked to actin, prevents polymerization of actin. The main function of vitamin D-binding protein appears the buffering of free vitamin D concentrations in plasma. Plasma retinol-binding protein, which also belongs to that class, only occurs in plasma after tissue leakage and at lower concentrations. It does not belong to the classical plasma proteins, and therefore might represent an interesting biomarker. All other identified proteins relate to inflammatory processes (12%), lipid metabolism (9%) or represent structural proteins (12%) with a rather high abundance in plasma.

Table 4-1 Identified proteins of the plasma database sorted by their biological processes as stated in SwissProt

Biological Process	Identified Proteins
Transport	Transthyretin, plasma retinol-binding protein, vitamin D binding protein, hemopexin, serum albumin, serotransferrin, afamin
Lipid metabolism	Apolipoprotein A-I, apolipoprotein A-IV, zinc-alpha-2-glycoprotein
Immune response	Ig kappa chain C region, haptoglobulin, CD5 antigen-like, alpha-1-antichymotrypsin, complement factor B, Ig mu chain C region, complement component C6, complement component C9
Structural	Serum amyloid P-component, alpha-2HS-glycoprotein, zinc finger protein, gelsolin
Inflammation	C4b-binding protein alpha chain, complement C1s subcomponent, vitronectin, haptoglobulin
Coagulation	Fibrinogen gamma chain, kininogen-1, antithrombin-III, alpha-1-antitrypsin, beta-2-glycoprotein 1
Others	Angiotensinogen, alpha-1B-glycoprotein

In 2002, Anderson *et al.* published a first list of 289 identified plasma proteins detected with various methods. Not included in this list were two proteins from our plasma 2D map, including the C4b-binding protein alpha chain, also named proline-rich protein, that controls the classical pathway of complement activation. It is part of the innate immune response [105] and is secreted into circulation. Its presence in plasma was reported later [92]. Another protein not covered in the Anderson list is the zinc finger FYVE domain-containing protein, also named FYVE, rhoGEF and PH domain-containing protein 3. This protein promotes the formation of filopodia and therefore interacts with the actin cytoskeleton and cell shape. Its subcellular location is probably the cytoplasma and the cytoskeleton [106]. This protein has been found in plasma and may non-specifically bind to the ProteoPrep® depletion column that removes 20 high-abundance proteins from plasma [www.sigmaaldrich.com]. Although depletion with the Beckman column should remove up to 99% of these proteins, according to the manufacturer, albumin, fibrinogen, alpha-1B-glycoprotein, transferrin and IgM could still be detected in plasma samples. This is obviously based on the residual amount of 1-2% of these proteins not removed, or the possibility that the re-usable (up to 100 times) spin columns for plasma

fractionation leak after repeated use and remove less of the proteins. Using columns for single use could overcome the latter problem.

The relatively low number of identified proteins from the 103 spots probably also includes different isoforms of several proteins on the gels. These isoforms can occur after post-translational modification, alternative splicing or chemical modification during sample preparation. Such protein modifications lead to changes in the theoretical MW and p*I*. Glycosylation or phosphorylation, for example, results in a higher molecular weight and an alteration of the p*I* compared to the unmodified protein. Multiple modifications lead to spot chains that are typical, especially for glycosylated plasma proteins. These chains can clearly be visualized on the plasma gels (Fig. 4-7). With the used mass analysis technology it was however not possible to gain information about any protein modifications.

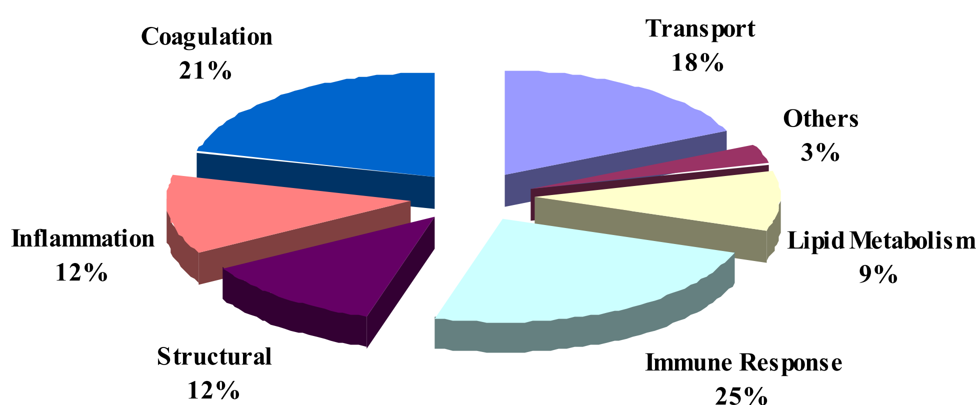

Figure 4-8 Biological functions of all proteins included into the plasma database according to SwissProt

Several of the identified proteins have been reported to serve as disease markers. Transthyretin, the apolipoproteins, haptoglobulin and alpha-1-antitrypsin all have been related to hepatitis B virus infections [107]. Apolipoprotein-I, albumin, prealbumin and zinc-alpha-glycoprotein decreased in sera of hepatitis C patients, whereas beta-2-glycoprotein 1 levels were found increased [108]. Fibrosis in hepatitis C patients furthermore led to an elevation in hemopexin and alpha-1B-glycoprotein and lowered the concentrations of haptoglobulin, alpha-1-antitrypsin and fetuin-A [109]. The fibrinogen gamma chain was found to be involved in cardiovascular disease development, and vitamin D-binding protein has been linked to cancer [94]. Alpha-1-antichymotrypsin, apolipoprotein A-I, hemopexin, transthyretin, alpha-1-glycoprotein, haptoglobulin, zinc alpha-2 glycoprotein and several proteins of the immunoglobulin family were found to be associated with Alzheimer's disease [94, 110, 111]. Lowered afamin levels were found in patients with ovarian cancer, as well as antithrombin 3, alpha-antitrypsin and serotransferrin, also linked to this disease [112]. Several glycoproteins, including alpha-1B-glycoprotein, complement factor C3, alpha-1-antitrypsin and serotransferrin, were identified

in search of breast cancer specific proteins [113], and alpha-1B glycoprotein was identified as a biomarker for pancreatic cancer [114]. Berhane *et al.* compiled a list of the proteins identified in the HUPO PPP Pilot Phase and their relation to cardiovascular diseases [115]. Several of those proteins are also found in our in-house plasma database.

A publicly available 2D map of human plasma proteins can be found at http://www.expasy.ch/swiss-2dpage. 626 identified spots represent 81 different proteins on a gel of pH 4-10. The plasma database constructed here based on in-house analysis can be compared with the Swiss 2DPAGE database and shows a large degree of confirmation. However, serum amyloid component, C4b-binding protein alpha chain and zinc finger FYVE domain-containing protein identified in our database are not yet included in the SWISS 2DPAGE of plasma. Meanwhile, some 325 unique proteins [115, 116] have been identified in plasma samples.

4.3.2. The PBMC Database

180 spots were excised from PBMC sample gels and analyzed by mass spectrometry. In total, 110 spots were identified with significant results in identification for 54 distinct proteins. The apparently low identification rate of 61% results from the limitation of MALDI analytics for very faint protein spots picked. Figure 4-9 shows the map derived for PBMC proteins that were included into the protein database (Table 6-3). In contrast to the plasma proteins identified, the majority (42%) of the PBMC proteins belong to the group of structural proteins (Fig. 4-10, Table 4-2). These proteins build the filament units of actin, and several actin-related proteins like vinculin, gelsolin and various actin subgroups could be identified. Vimentin, also found, has been related to the differentiation of monocytes into dendritic cells and therefore plays a role in the immune response of PBMC [117]. Around 7% of the identified proteins belong to the coagulation factor family found in plasma and may represent contaminations. During the PBMC isolation process platelet membranes may be destroyed, and platelet fragments together with bound fibrinogen for example may attach to the PBMC [26]. Pleckstrin, a platelet-specific protein, can also be found in PBMC samples due to platelet contamination, and such a contamination cannot be fully excluded during PBMC isolation and preparation [37].

Glutathione S-transferase P, heat shock 70 kDa protein 1 and the 78 kDa glucose-regulated protein precursor have been described in the context of stress response and as anti-apoptotic factors and may like other heat shock proteins play their prominent role in proper protein folding in PBMC. Protein disulfide-isomerase A6 is catalyzing the re-arrangement of disulfide bonds and therefore acts in redox homeostasis like the protein peroxiredoxin. The 14-3-3 protein family has numerous functions, and the isoforms zeta/delta have been found with highest expression levels in nerve terminals [116]. The intracellular chloride channel protein 1 is the only membrane protein that could be identified in the PBMC proteome samples. L-lactate dehydrogenase B chain, for example, was identified as a

biomarker for irreversible cell damage and liver diseases [116], and proteins such as alpha-1-antitrypsin, transthyretin and apolipoproteins are considered to serve as markers for virus infections [107], whereas fibrinogen beta and gamma are related to cardiovascular diseases [94]. Taken together, the PBMC proteome contains numerous protein entities that have the potential to be biomarkers of metabolic impairments and diseases.

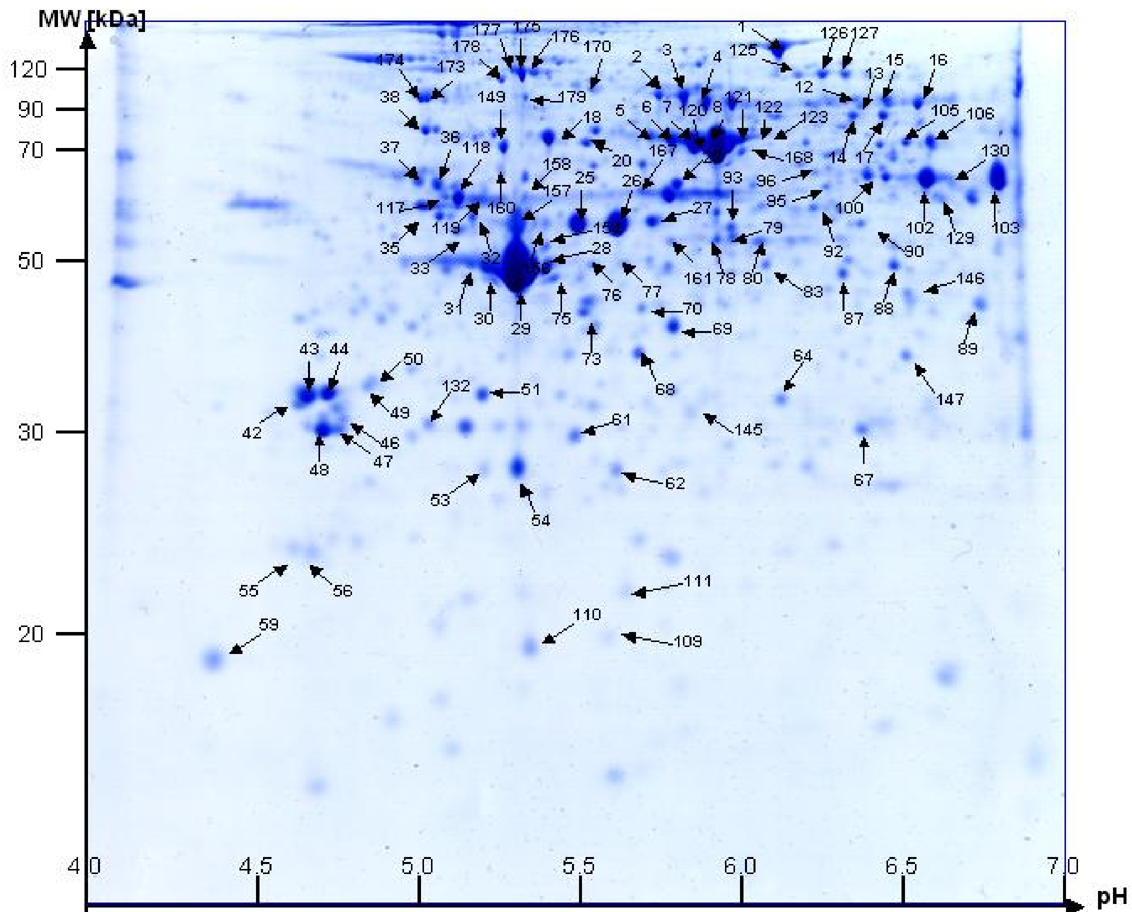

Figure 4-9 Proteome map derived from PBMC

Proteome map derived from a 2D gel of PBMC cell lysate. 300 µg of PBMC sample was applied to 18 cm IPG strips pH 4-7 and run on 21.5% acrylamide using the continuous buffer system in the second dimension. Staining was carried out with Coomassie Brilliant Blue. Visible spots were dissected and mass-analyzed using MALDI-TOF MS. 110 spots were identified with significant scores in 3 or more individual gel analyses (numbered spots with arrows).

In 2006, Ramirez-Boo *et al.* reported a porcine PBMC 2D map, using gels with a pH range of 5-8. From 100 analyzed spots, 76 distinct proteins could be identified. The majority, according to biological functions, also represented cytoskeletal proteins [26]. A Swiss 2D PAGE map of PBMC currently includes 77 spots derived from a first dimension separation of pH 4-8 with 62 protein entries. Taken together, the findings from human and porcine preoteome data in the public domain are highly comparable with the PBMC database entries defined here for the in-house analysis.

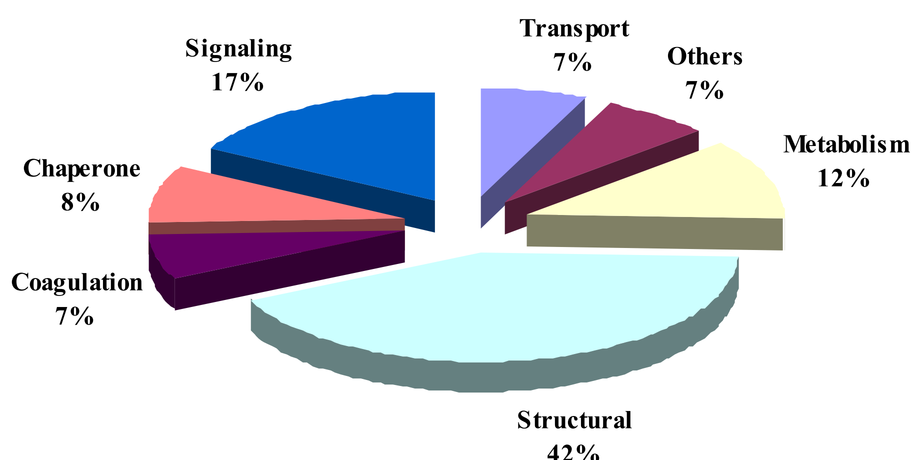

Figure 4-10 Biological functions of all proteins included into the PBMC database according to SwissProt

Table 4-2 Identified proteins of the PBMC database sorted by their biological processes as stated in SwissProt

Biological Process	Identified Proteins
Transport	Serum albumin, serotransferrin, chloride intracellular channel protein 1, transthyretin
Metabolism	Apolipoprotein A-IV, apolipoprotein A-I, glutathione S-transferase P, glutathione transferase omega-1, peroxiredoxin-6, L-lactate dehydrogenase B chain, glucose-6-phosphate 1-dehydrogenase
Structural	Vinculin, gelsolin, moesin, actin-like protein 2, actin-like protein 3, beta-actin, gamma-actin, tubulin beta chain, tropomyosin alpha-4 chain, tropomyosin alpha-3 chain, F-actin capping protein subunit beta, F-actin capping protein subunit alpha-1, F-actin capping protein subunit alpha-2, leukocyte elastase inhibitor, myosin-9, PDZ and LIM domain protein 1, coronin-1A, WD repeat protein 1, actin-related protein 2/3 complex subunit 5, vimentin, tubulin alpha-ubiquitous chain, fibronectin, keratin type I cytoskeletal 10, plastin-2, tubulin beta-1 chain, alpha-actinin-1
Coagulation	Coagulation factor XIII A chain, fibrinogen gamma chain, alpha-1-antitrypsin, fibrinogen beta chain
Chaperone	Heat shock 70 kDa protein, heat shock 70 kDa protein 1, T-complex protein 1 subunit beta, heat shock protein HSP 90-beta
Signaling	Protein disulfide-isomerase A3, protein disulfide-isomerase A6, 14-3-3 protein epsilon, 14-3-3 protein gamma, 14-3-3 protein zeta/delta, myosin regulatory light chain 2, myosin light polypeptide 6, pleckstrin, coronin-1C, rab GDP dissociation inhibitor beta, parvalbumin alpha, rho GDP-dissociation inhibitor 1
Others	78 kDa Glucose-regulated protein, elongation factor 1-gamma, purine nucleoside phosphorylase, eukaryotic initiation factor 4A-I

The in-house-built gel-based map with the identified proteins will enable the identification of regulated proteins in future studies, with ease and without the need for further mass analysis, by overlay of the experimental gels with the standard database gels. As the database also includes biological functions of the known proteins, data interpretation after protein identification can be shortened as well. In the future, overall sample analysis should take approximately only one week, including four days for staining and one night for destaining, when using the proteome databases and protein identification by matching gels. As MS analysis would need around three days, not considering

the time for literature search, the preset database has the potential to safe time in the identification process of altered spots.

Major drawbacks of 2D electrophoresis based proteomics approaches are the low reproducibility, a lack of separation of hydrophobic and membrane proteins and the limits in detecting low-molecular-weight proteins. However, the technique is able to display below 1 ng of protein per spot representing at least 3-4 orders in magnitude of dynamic range although not being able to cover the huge concentration range of more than 10 orders of the plasma proteome [61, 70, 118, 119]. The prime advantage is, that 2D electrophoresis is the only technique that can display many proteins at once and therefore can serve as a platform to create visible proteome maps [120]. Additional major advantages are the separation of intact proteins and the visualization of post-translational modifications, appearing frequently as spot chains on the gel. No other method exists that provides also information about the physical properties, such as molecular weight and p*I* of the analyte [61].

4.4. A High-Protein Diet Human Intervention Study

The application of the developed in-house databases for plasma and PBMC proteins was tested in a human intervention trial. In order to assess basal changes, all parameters were analyzed before the supplementation by repeated measurements. After this baseline phase, the effect of supplementation with 2 g/kg BW and day of a commercially available protein powder in male volunteers over a five-day period was investigated. To get an overall image of changes in human metabolism, plasma and PBMC proteins were measured together with amino acids and several clinical parameters in plasma and urine.

For an 80 kg person the supplementation was 160 g protein in addition to a daily standard diet. The intake level that is recommended for body maintenance of non-athletic adults (RDA) is reported with 0.8 g/kg BW and day. However, an increase of this levels was suggested by Humayun *et al.* as a reduced antioxidant capacity and an increased exposure to oxidative stress were observed at this level compared to higher protein intake rates [121]. Athletes doing strength and power exercise should even elevate their protein supplementation to 2 g/kg BW and day. The maximum safe intake range for this person would be 285 to 365 g per day. Heavy consumption of protein can even reach ranges of 5 g/kg BW and day. This could be of danger, as the capacity of the liver for urea production for nitrogen elimination is exceeded [11]. The given protein amount in our study therefore corresponds to the regular intake in athletes and is not reported to be of any risk for the human body.

In order to eliminate diet-derived variation of any measured parameters in plasma, PBMC and urine, all meals during the intervention phase were provided at the study center, and the volunteers were asked to avoid ingestion of additional food. Drinks like coffee, tea and tap water were offered *ad libitum*. Eleven male volunteers participated in the study. As one volunteer was excluded due to illness, samples were analyzed from ten participants. For baseline measurements, samples were taken over a one-week pre-phase period without protein supplementation. Sample collection included blood withdrawal after overnight fasting, followed by isolation of plasma and PBMC, and morning urine samples. All parameters measured during the intervention were compared to the average baseline values and statistically analyzed in order to assess the effect of the protein supplementation. After the intervention phase two wash-out samples were taken to determine the adaptation back to the baseline level. Blood and urine samples were taken in the morning after overnight fasting of at least 12 hours.

4.4.1. Content of the Protein Powder

The given supplement was a commercially available protein powder that is recommended to promote muscles growth during exercise periods. According to the manufacturer, the protein supplement contains milk proteins, soy protein and egg white protein. Nutrition information, as given by the manufacturer, declared a protein content of 89 g/100 g with low supplementation rates of

carbohydrates (2.4 g/100 g) and vitamins (vitamin B6 2.9 mg/100 g and folate 290 µg/100 g). The composition is shown in Table 4-3.

Table 4-3 Nutrition information Protein90Plus as given by the manufacturer

Ingredient	per 100 g
Protein	89 g
Carbohydrates	2.4 g
Sugars	1.6 g
Fat	1.1 g
Saturated Fatty Acids	0.4 g
Dietary Fibers	<0.1 g
Sodium	0.4 g
Vitamin B6	2.9 mg
Folate	290 µg
Calcium	670 mg
Phosphorous	520 mg

The protein supplement was furthermore analyzed using SDS-PAGE to determine the origin of the proteins. Differences between the analyzed pure protein fraction (84.5%) and the raw protein content, declared with about 90%, are due to non-protein bound nitrogen, as well as systematic errors coming from a uniform Kjeldahl-factor for the analysis of the raw protein content. Protein values are declared as protein present in the raw protein fraction if not otherwise stated. As shown inTable 4-4, the protein powder consists of 79% casein with a whey protein fraction <5%.

Table 4-4 Content of the Protein90Plus supplement

Protein	Unit	A	B	Mean	SD	RSD
Lactoferrin	[%]	0.55	0.58	0.56	0.019	3.3
Serum Albumin (BSA)	[%]	2.76	2.72	2.74	0.028	1.0
Immunoglobulin	[%]	1.68	1.59	1.63	0.062	3.8
CAS2 (α-s2-Casein)	[%]	6.92	7.39	7.16	0.337	4.7
CAS1 (α-s1-Casein)	[%]	26.19	26.44	26.32	0.181	0.7
CASK + CASB (κ- + β-Casein)	[%]	39.97	40.42	40.19	0.318	0.8
γ-1-Casein	[%]	1.02	1.16	1.09	0.096	8.8
β-Lactoglobulin	[%]	3.03	2.96	3.00	0.049	1.6
α-Lactalbumin	[%]	0.71	0.81	0.76	0.065	8.6
γ-2,3-Casein	[%]	0.95	1.10	1.03	0.102	9.9
Pure Protein after Electrophoresis	[%]	83.78	85.16	84.47	0.978	1.2
Casein	[%]	78.08	79.47	78.77	0.984	1.2
Whey Protein	[%]	3.75	3.77	3.76	0.016	0.4
Whey Protein Fraction in Pure Protein	[%]	4.58	4.53	4.55	0.036	0.8

Analysis of the protein supplement (Lot No. 0717024, Exp. 07/2008) by SDS electrophoresis on EXEL® 8-18 SDS gels. Differences between the pure protein (84.5%) and the raw protein (90%) amount are due to non-protein bound nitrogen or can occur as systematic error due to a uniform Kjeldahl factor during analysis of the raw protein content.

Both fractions are major constituents of cow's milk. Caseins make up almost 80% of all milk proteins. They are carrying phosphate groups and form micelles that precipitate at pH 4.6. The whey protein fraction contains all proteins that are still soluble at pH 4.6, including β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins, lactoferrin and some minor proteins. The soluble fraction therefore is also rapidly taken up after ingestion, whereas caseins that are insoluble at gastric pH are metabolized with delay and are referred to as the "slow" proteins [8].

4.4.2. Clinical Chemistry Parameters

Several clinical parameters were assessed in order to gain information about the response of the volunteers after the high-protein intake. Urea and ammonia concentrations were both determined using enzymatic methods in plasma and urine. Other urine parameters included creatinine, glucose and osmolarity that were also analyzed with classical methods (Table 4-6). Additional clinical chemistry data were measured in plasma for BL3, HP1, HP2 and WO2 using the Piccolo™ Panel platform with the Comprehensive Metabolics panel. This included determination of alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, albumin, total protein, total bilirubin, urea nitrogen, creatinine, calcium, glucose, chloride, potassium, sodium and total carbon dioxide (Table 4-5).

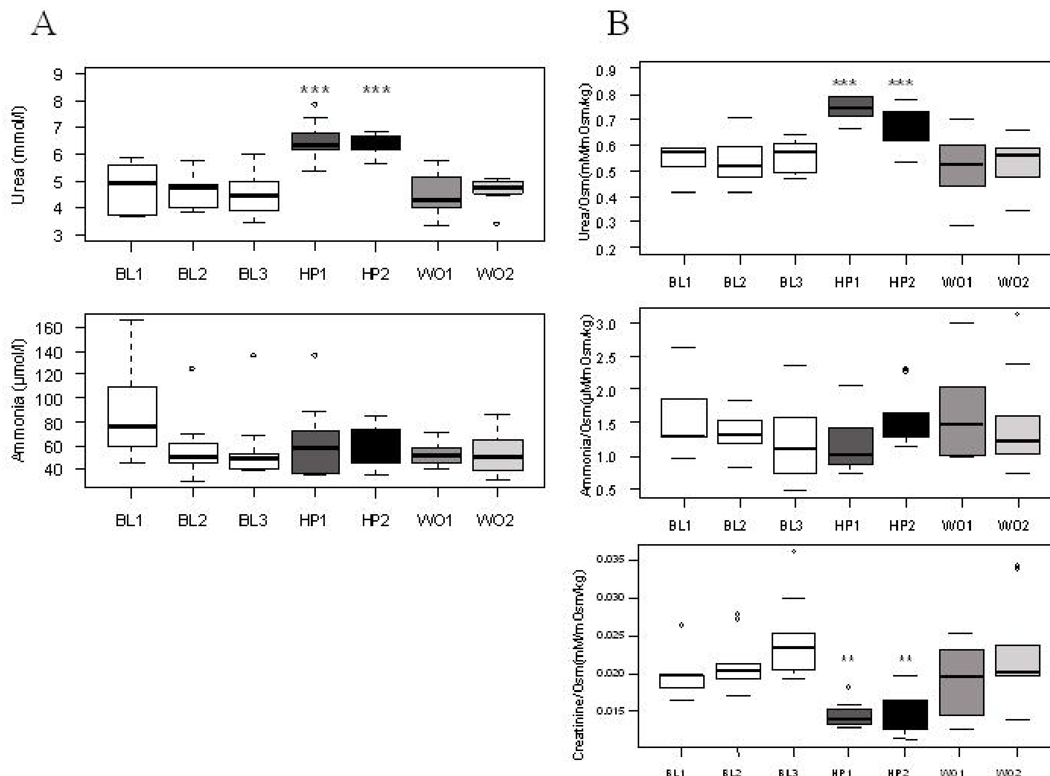

Figure 4-11 Clinical chemistry parameter levels in plasma and urine

Clinical chemistry parameters (urea, ammonia and creatinine) measured in plasma (A) and urine (B). The boxes represent the mean values from the 10 volunteers +/- SD, the lines below and above the extreme values. Values were tested for significance compared to the mean of all baseline values using ANOVA (** p<0.01. *** p<0.001).

The most important clinical chemistry parameters and their alterations in plasma and urine are shown in Figure 4-11. As creatinine levels in urine changed during the intervention, all urine values were normalized against osmolarity which remained constant.

Table 4-5 Clinical parameters in plasma during the trial

Parameter	Reference Range	BL1	BL2	BL3	HP1	HP2	WO1	WO2	HP *vs.* BL
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	p-Value
Ammonia (µmol/l)	<55	85.76 ± 36.04	56.65 ± 26.60	57.04 ± 29.02	63.06 ± 31.11	57.45 ± 17.38	53.62 ± 11.17	53.47 ± 18.86	0.084
Urea (mmol/l)	1.7-8.3	4.78 ± 0.91	4.61 ± 0.58	4.55 ± 0.85	6.48[c] ± 0.68	6.30[c] ± 0.42	4.52 ± 0.79	4.66 ± 0.52	<0.001
Alanine Amino-transferase (U/l)	10-47			19.90 ± 7.71	20.80 ± 7.36	22.80 ± 10.27		19.30 ± 10.17	0.470
Albumin (g/dl)	3.3-5.5			3.73 ± 0.21	3.69 ± 0.19	3.84 ± 0.28		3.92 ± 0.40	0.162
Alkaline Phosphatase (U/l)	53-128			49.40 ± 11.84	45.30 ± 12.92	47.80 ± 11.77		47.80 ± 13.20	0.235
Aspartate Amino-transferase (U/l)	11-38			25.10 ± 4.75	22.70 ± 2.91	24.80 ± 3.82		23.40 ± 4.22	0.363
Calcium (mg/dl)	8.0-10.3			7.84 ± 0.19	7.76 ± 0.28	7.78 ± 0.29		8.00 ± 0.71	0.518
Chloride (mmol/l)	98-108			97.70 ± 2.63	102.30 ± 2.16	99.70 ± 3.09		100.20 ± 5.98	0.222
Creatinine (mg/dl)	0.6-1.2			0.74 ± 0.14	0.57 ± 0.13	0.69 ± 0.20		0.76 ± 0.16	0.087
Glucose (mg/dl)	73-118			84.40 ± 7.47	82.90 ± 6.38	81.80 ± 5.20		87.00 ± 9.21	0.394
Potassium (mmol/l)	3.6-5.1			4.09 ± 0.26	4.25 ± 0.25	4.20 ± 0.22		4.27 ± 0.43	0.518
Sodium (mmol/l)	128-145			143.40 ± 4.01	140.90 ± 2.81	139.90 ± 4.09		143.80 ± 6.66	0.363
Total Bilirubin (mg/dl)	0.2-1.6			0.74 ± 0.14	0.66[a] ± 0.14	0.61 ± 0.09		0.85[a] ± 0.20	<0.001
Total Carbon Dioxide (mmol/l)	18-33			25.30 ± 1.89	25.50 ± 1.78	24.60 ± 1.96		24.80 ± 1.81	0.640
Total Protein (g/dl)	6.4-8.1			6.13 ± 0.47	6.10 ± 0.36	6.06 ± 0.35		6.26 ± 0.72	0.515
Urea Nitrogen (mg/dl)	7.0-22			11.90 ± 2.28	16.30[c] ± 1.64	15.90[c] ± 0.74		12.00 ± 1.76	<0.001

Clinical parameters in plasma measured enzymatically (urea, ammonia) and with the Piccolo™ system based Comprehensive Metabolics panel (only 4 time points). Mean values of the 10 volunteers and the standard deviation are given. The p-value is given for the high-protein time points compared to the baseline based on ANOVA analysis. Values with superscripts (a,b,c) indicate significant differences ([a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$) of the group compared to the baseline group (BL1+BL2+BL3).

Ammonia derived from amino acid metabolism in the liver is eliminated in the form of urea through kidney filtration of plasma. An elevated protein intake leads to an increased elimination of nitrogen, and therefore urea levels are expected to be increased due to higher protein catabolism. Plasma urea levels did show the expected response to protein supplementation, and urinary output increased as well. This effect disappeared in the wash-out phase and immediately reached the baseline level after the end of the intervention phase. Bound urea nitrogen (BUN) in plasma showed the same elevated levels for HP1 and HP2 samples (data not shown). Plasma free ammonia levels and urinary output did not reveal any effects of the supplement. It was reported before that the kidney is buffering acidic compounds by the formation of ammonia through conversion of glutamine into glutamate and its excretion into the urine [122]. Thus, bicarbonate is generated through metabolism of the substrates in the citric acid cycle and can help to buffer acid in the blood. Acidosis was observed in several studies after high-protein meals. It could be caused by metabolism of amino acids carrying sulfur-groups [15, 122, 123]. As ammonia levels in plasma and urine remained relatively constant during the whole study period, our findings do not provide evidence for states of acidosis caused by the high-protein intake.

Creatinine is a breakdown product of creatine phosphate in the skeletal muscle. Creatine is formed and metabolized at constant rates per muscle unit under normal conditions. Therefore, plasma creatinine levels are relatively stable and reflect the amount of muscle mass [124]. However, creatinine levels of the volunteers did not correspond to the amount of exercise as provided in the questionnaires (see Table 6-4). Creatinine levels in plasma were constant but urine levels responded with a significant decrease during the intervention period (Figure 4-11). After finishing the supplementation period, creatinine levels reached basal levels again at WO2. It is unclear what causes lowered creatinine levels during the protein supplementation. Creatinine uptake into the muscle could be one reason, but most probably there is a link between energy uptake and creatinine excretion in urine. As during the intake of the protein powder all volunteers reported a strong satiety effect, they ingested less during all offered meals compared to the baseline and the wash-out phase. This could also have an effect on creatinine levels in urine.

Table 4-6 Clinical parameters in urine during the trail

Parameter	BL1	BL2	BL3	HP1	HP2	WO1	WO2	HP *vs.* BL
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	p-Value
Ammonia (mmol/ (µOsm/kg)	1.55 ± 0.58	1.35 ± 0.33	1.22 ± 0.63	1.23 ± 0.50	1.57 ± 0.41	1.60 ± 0.66	1.46 ± 0.74	0.502
Creatinine (mmol/ (mOsm/kg)	0.020 ± 0.003	0.021 ± 0.004	0.014 ± 0.002	0.014 ± 0.002^{b}	0.015 ± 0.003^{b}	0.019 ± 0.005	0.023 ± 0.006	1.42E-06
Urea (mmol/ (mOsm/kg)	0.54 ± 0.07	0.53 ± 0.09	0.56 ± 0.06	0.74 ± 0.04c	0.68 ± 0.08c	0.53 ± 0.12	0.53 ± 0.09	8.14E-09
Osmolarity (mOsm/kg)	483 ± 245	565 ± 152	634 ± 227	595 ± 204	514 ± 120	593 ± 205	673 ± 236	0.386
Glucose	0.068 ± 0.055	0.097 ± 0.178	0.210 ± 0.208	0.045 ± 0.049	0.305 ± 0.179	0.192 ± 0.167	0.423 ± 0.218c	3.29E-05

Clinical parameters in urine measured enzymatically (ammonia, urea), with colorimetric analysis (glucose, creatinine), and using an osmometer (osmolarity). All parameters are normalized to the osmolarity. Mean values of the 10 volunteers and the standard deviation are given. The p-value is given for the high-protein time points compared to the baseline based on ANOVA analysis. Values with superscripts $^{(a,b,c)}$ indicate significant differences (^{a} p<0.05, ^{b} p<0.01, ^{c} p<0.001) of the group compared to the baseline group (BL1+BL2+BL3).

In the US, the plasma or serum BUN/creatinine-ratio is consulted as a marker for kidney function [124]. European countries and Canada use the urea/creatinine-ratio instead, which gives the same interpretation of the results. Critical values for these ratios and their interpretation for kidney disorders

are shown in Table 4-7. Under normal conditions, urea is re-absorbed during glomerular filtration, whereas creatinine is not. An increase of this ratio can be due to a decline in the blood volume and thus in renal blood flow, resulting in strongly elevated blood urea nitrogen levels compared to only slightly elevated creatinine. The normal range for BUN/creatinine is 10-20:1 and 0.1 for urea/creatinine. Both time points within the intervention phase show significantly increased ratios for these parameters (Fig. 4-12). During the wash-out period the ratios decreased again to baseline levels. The increase of the kidney function markers was more than 20 for the BUN/creatinine-ratio and more than 0.1 for the urea/creatinine-ratio. The decline of the BUN/CRE-ratio during the wash-out phase down to baseline values indicates that the kidney function is not sustainably affected by the high protein load. As plasma urea levels are clearly elevated due to the high-protein diet, this can therefore lead to the observed rise in the ratio without an increase in plasma creatinine levels. Several studies reported that daily protein doses below 2.8 g/kg BW did not have an impact on renal functions in athletes with regular protein consumption [23].

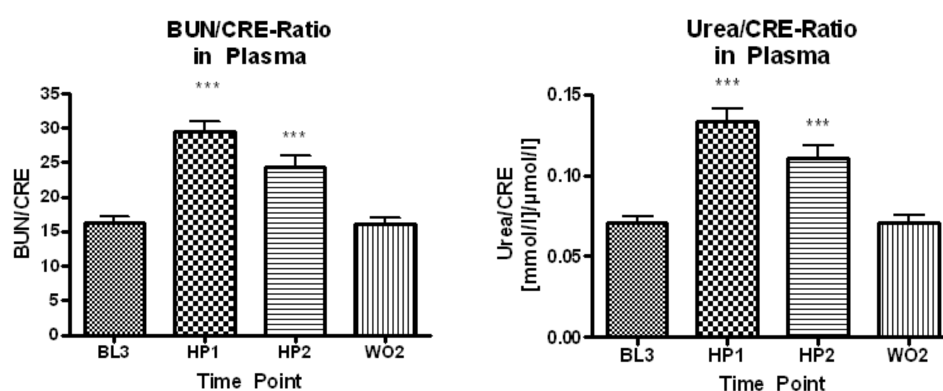

Figure 4-12 BUN/creatinine-ratio and urea/creatinine-ratio during the study course

BUN/CRE-ratio and urea/CRE-ratio measured in plasma. The bars represent the mean values from the 10 volunteers +/- SD. Values were tested for significance compared to the mean of BL3 values using t-test (*** p<0.001).

Table 4-7 Interpretation of BUN/creatinine-ratio and urea/creatinine

BUN/CRE	Urea/CRE	Location of Disorder	Mechanism
>20:1	>0.1	Pre-renal disease	Reduced flow causes high BUN relative to CRE
10-20:1	0.04-0.10	Normal or post-renal disease	Normal range. Post-renal: high BUN due to backflow, CRE not re-absorbed
<10:1	<0.04	Intrarenal disease	Renal damage causes low BUN re-absorption

Another clinical parameter that was altered due to the high-protein supplementation is bilirubin. Bilirubin is a degradation product of the heme group in hemoglobine. It is transported in plasma bound to albumin. In the liver, it is conjugated to glucuronic acid and thereby made water-soluble. The major part is excreted through the intestine, only small amounts are found in urine [6]. Total bilirubin levels decreased in plasma at HP1 during the protein supplementation, and were found to be increased

above basal levels after finishing the supplementation at WO2. The data suggest that there is a decrease in heme degradation during the protein intervention. All other clinical parameters were not significantly altered, and none of the clinical parameters was changed during the wash-out phase compared to the baseline period. The measured values also were within the reference range and did not give any indication of critical concentration changes and pathophysiological conditions.

4.4.3. Amino Acid Profiles during the Study Course

A major part of amino acids absorbed from the gut is metabolized in the liver. They can either be used for biosynthesis of plasma and liver proteins or converted to α-keto carbonic acids after elimination of ammonia. From these keto acids glucose is formed and oxidized to carbon dioxide and water for ATP synthesis. The liver is the organ of urea cycle activity for elimination of the nitrogenous compounds of amino acids, and urea excretion is mainly via the kidneys. Urine of healthy individuals can contain 1-3 g of amino acids per day. This value is increased during liver disorders and can result in crystallizing leucine and tyrosine [6].

Table 4-8 Amino acid content of Protein90Plus as declared by the manufacturer

Amino Acid	Content [g/100 g Protein]
Isoleucine	5.3
Leucine	9.2
Lysine	7.6
Methionine	2.7
Phenylalanine	4.8
Threonine	4.4
Tryptophane	1.3
Valine	6.6
Arginine	3.3
Cysteine	0.6
Histidine	2.8
Tyrosine	5.0
Alanine	3.0
Asparicacid	7.0
Glutamicacid	20
Glycine	1.7
Proline	9.2
Serine	5.7

Metabolomics profiling of plasma amino acids after an oral glucose tolerance test provided information about changes in metabolism, resulting from such a dietary challenge [125, 126]. Plasma amino acid profiles were also found to be altered in disease states, such as diabetes, cardiovascular disease or myocardial infarction [127-129]. In urine, fingerprints of amino acids were identified for individuals in order to characterize metabolic changes [130]. In diabetes type 2 patients, urinary metabolic profiles identified changes in amino acids in the disease state [131] and after drug treatment [132]. So far, no study on amino acid profiling after a high-protein challenge has been undertaken. Metabolomics analysis of plasma and urine samples from the protein intervention study could provide valuable information about changes in metabolic pathways.

Predominant amino acids in the protein supplement given to the volunteers, according to the manufacturer, are glutamic acid, proline, leucine, lysine and asparicacid. Therefore, the protein powder is rich in essential amino acids, however, the major amino acid fraction is non-essential. Amino acids carrying sulfur groups like cysteine and methionine are of less abundance (Table 4-8).

Amino acids and some of their derivatives were analyzed in order to determine whether a rise in protein breakdown products or changes in amino acid metabolism in general can be observed, either in plasma or urine. Using LC-MS/MS 35 amino acids and derivatives could be quantitatively determined in plasma with concentrations >1 µmol/l and 36 in the urine samples. Parameters in urine are usually displayed in relation to the corresponding creatinine concentration, assuming that creatinine levels are stable. As creatinine significantly decreased in urine after protein supplementation, the amino acid values were normalized to the osmolarity that was constant.

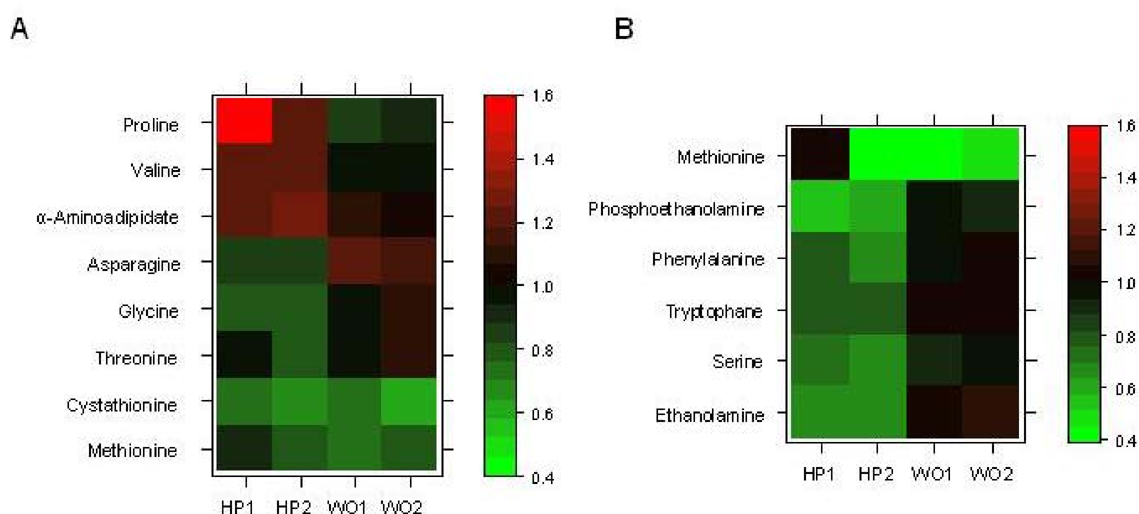

Figure 4-13 Changes in amino acid concentrations in plasma and urine

Statistically significantly altered amino acid values in plasma (A) and urine (B) after protein supplementation. Displayed are the heatmaps of the expression profiles of the values in HP1, HP2, WO1 and WO2 as compared to the mean baseline values. Statistical analysis was done using one-way ANOVA.

Some of the analyzed amino acids showed considerable within- and between-subject variability during the baseline. This basal noise had to be considered for determination of significant alterations during the protein intervention (data not shown). 8 out of the 35 amino acids quantified in plasma were statistically significantly altered according to ANOVA analysis, when comparing the mean baseline values to the amino acid levels after supplementation (Figure 4-13A). Proline, α-aminoadipidate and valine increased after protein supplementation in plasma, whereas methionine, threonine, asparagine, glycine and cystathionine decreased. Methionine and cystathionine further decreased after finishing the supplementation, whereas asparagine increased. Proline and α-aminoadipidate also showed subject

variability as stated before. 6 of the 36 amino acids quantified in urine (normalized with osmolarity) were significantly decreased after protein supplementation (Figure 4-13B) according to the ANOVA analysis. Phenylalanine, tryptophane, serine, phosphoethanolamine and ethanolamine returned to basal levels after finishing the supplementation, whereas methionine further decreased. Of these, tryptophane, phenylalanine and phosphoethanolamine showed subject variability at baseline.

Except for these amino acids, no major changes in concentrations in plasma and urine could be detected. None of the significantly affected amino acids belongs to or is directly linked to the urea cycle. Proline is the amino acid that showed the strongest increase during the intervention. As high amounts of proline are included in the protein supplement (see Table 4-8), this strong increase could directly result from massive proline intake and a low plasma clearance rate. This would also explain the decline of proline levels during the wash-out phase. The only BCAA that responded to the high amount of protein was valine with a significant increase that also declined during the wash-out phase. The sulfur-containing amino acids methionine and cystathionine showed lowered levels during the protein intervention in plasma, methionine also in urine, and these findings were unexpected. Methionine was the only amino acid that was significantly altered, both in plasma and in urine, and in both samples methionine levels decreased during the intervention and remained at lowered levels also after the supplementation period.

Results from the amino acid analysis with all changes in plasma and urine amino acid profiles are displayed in Figure 4-14 according to their participation in metabolic pathways. The results are based on ANOVA analysis and show the fold changes combined with their p-values. Increased amino acid levels are shown in red and decreased values in green. The left side of each box represents the impact on plasma levels, the right side the corresponding urinary changes. Subject variability was not included in this analysis.
The most obvious finding is that the majority of altered amino acids in fasting plasma and urine decreased in levels despite the protein supplementation. Only few amino acids such as serine, glycine, ornithine, ethanolamine, threonine and methionine were altered in the same direction in plasma and urine. Methionine values were most affected in plasma and in urine. Methionine is the only precursor of homocysteine that is considered to be an independent risk marker for cardiovascular diseases. As homocysteine concentrations were close to the detection limit, no obvious alterations could be detected. Homocysteine is formed from methionine via S-adenosylmethionine (SAM). It is further metabolized either through cystathionine formation or re-methylated under the conditions of a low methionine supply [21, 133]. Serine is the thiol-group acceptor in homocysteine removal in the first way, forming cysteine and homoserine. Decreased fasting methionine concentrations might therefore also result in decreased homocysteine levels. In parallel to methionine levels, serine concentrations were also significantly lowered during increased protein intake in plasma as in urine. The biogenic

amine derived from serine, ethanolamine, is another acceptor of the methyl group from SAM. Our findings indicate that a re-methylation of homocysteine is unlikely to occur under the condition of low plasma methionine levels as there is a lack of the methyl-group acceptor ethanolamine. Transsulfuration to cystathionine and further degradation to cysteine and α-aminobutyric acid seems more likely for homocysteine elimination as cysteine and α-aminobutyricacid showed increased levels. Both degradation products can also be used for energy production in the citric acid cycle [134]. Several studies also reported a decrease in plasma homocysteine levels after high-protein meals [21, 133-135]. Supplementation with serine was found to lower homocysteine concentrations by stimulation of cystathionine synthesis in a dietary intervention study. A high-casein diet induced increased homocysteine elimination through increased enzyme activities of homocysteine-converting enzymes like cystathionine β-synthase and betaine-homocysteine S-methyltransferase and therefore also stimulated cystathionine-related homocysteine metabolism [133]. This would then result in an increase in cysteine and its degradation to pyruvate and an increase in α-ketobutyrate, which is then also used for energy production in the citric acid cycle. Both derivatives, cystine and α-aminobutyrate, were slightly elevated in plasma. What remains not understood is the apparent down-regulation of the whole pathway as the protein supplement provided as well additional methionine for homocysteine formation. Methionine supplementation itself was found to increase plasma homocysteine levels. Therefore, it is speculated that other components in protein-rich diets could also alter plasma homocysteine levels [134]. Obviously, methionine levels react to the increased protein intake with a distinct and sustained change, and it would be interesting to assess, when methionine levels reach the baseline again. The long-lasting decrease of plasma and urine methionine levels is pointing at a long-term beneficial effect of protein-rich meals in the protection of the cardiovascular system by reduced homocystein levels. Surprisingly, the amino acids cystine and taurine, which participate in sulfur metabolism, were both found at decreased levels during high-protein supplementation as well. The major part of amino acid-derived sulfur, however, is excreted in urine as sulfuric acid.

Whereas urea was significantly increased both in plasma and urine, ammonia levels did not change. Ammonia is transported in the blood incorporated into glutamine, glutamate and alanine. Alanine is mainly used for gluconeogenesis in the liver, whereas glutamine can be metabolized in different organs. After separation of neurotoxic ammonia from glutamine and formation of glutamate and α-ketoglutarate, ammonia is eliminated in the form of urea. The urea cycle in the liver occupies mitochondria and the cytoplasm. Ammonia is initially fixed in carbamylphosphate, which is transformed by coupling to ornithine and formation of citrulline. The second amino group in urea is introduced through coupling to aspartate, and after separation of fumarate arginine is formed. Cleavage of arginine forms urea, and ornithine is recovered. Urea is less toxic than ammonia and is eliminated from blood by the kidneys. Arginine is also a precursor of creatine and nitric oxide [6]. During the protein supplementation several amino acids of the urea cycle appeared at elevated

concentrations in plasma, such as citrulline, aspartate and arginine. Ornithine was the only urea-cycle-linked amino acid with lowered levels after protein ingestion. The reason for increased urea synthesis is substrate availability but also increased *de novo* enzyme synthesis in the urea cycle [136]. Glucagon is believed to play an important role in the control of plasma amino acid levels and urea synthesis, as postprandial glucagon levels raise significantly after ingestion of high-protein meals. Insulin may also play a role in urea formation through its ability to lower blood amino acid concentrations [136]. After high-protein intake insulin secretion was shown to be increased [16, 137]. However, glucagon and insulin levels were not examined during the study period.

Branched-chain amino acids play an important role in muscle protein synthesis. They are mainly metabolized in the skeletal muscle, as there is a low activity of the enzyme aminotransferase in the liver, which is necessary for degradation of these substrates. In muscle, the brached-chain amino acids provide amino groups for the formation of glutamate, glutamine and alanine. Valine and isoleucine are directly metabolized to succinyl-CoA, which is part of the citric acid cycle and provides α-ketoglutarate for glutamate synthesis. Glutamate is converted to glutamine after binding ammonia and excreted into the blood. Alanine is formed through the enzyme alanine-aminotransferase after glucose degradation and released from the muscle. It can be transaminated to pyruvate in the liver, and newly formed glucose is again released into blood and is therefore available for the muscle [6]. Leucine has an outstanding role in protein biosynthesis. Supplementation of this amino acid showed an increased muscle recovery after exercise. This is caused by phosphorylation events and the insulin signaling cascade involved in control of protein synthesis [9]. Leucine was found to be elevated in animals during recovery after food restriction and in humans after exercise. Therefore, increased leucine oxidation would also augment the production of alanine and glutamine as substrates for gluconeogenesis [9]. BCAAs were reported to be associated with insulin resistance in diabetes type 2 through an increased catabolism [127]. Plasma levels of leucine/isoleucine could predict fasting insulin and insulin-resistance during an oral glucose tolerance test [125]. Plasma concentrations of the the branched-chain amino acids leucine and isoleucine were slightly increased during the supplementation phase in our study, whereas those of valine were even significantly increased. Plasma alanine levels however only slightly rose and even decreased in urine. These findings would suggest that the protein supplementation promotes muscle protein biosynthesis as well as increased insulin sensitivity. These effects probably are small after all, as the increase was not significant for all BCAAs. In contrast, postprandial data on plasma BCAA concentrations might differ from the results after overnight fasting, as they would be expected to be significantly elevated after high-protein meals. Muscle protein metabolism was found to respond to exercise and diet [138, 139].

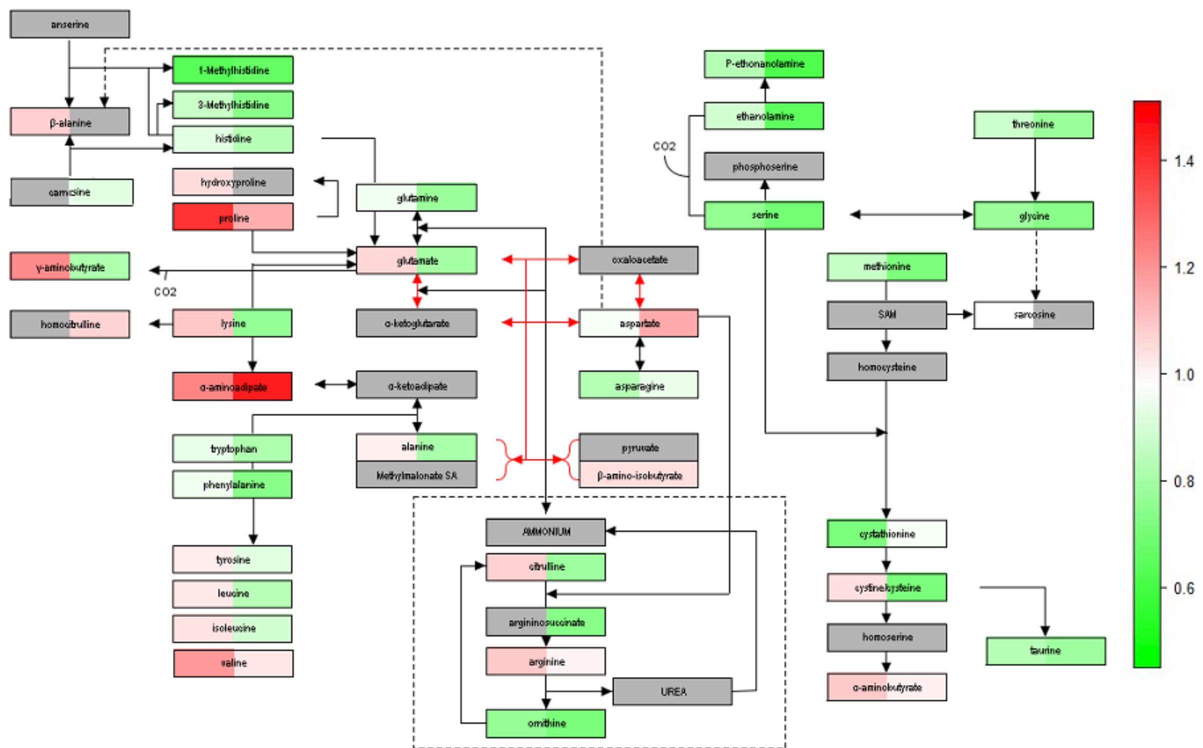

Figure 4-14 Amino acid pathways altered through protein supplementation

Graphical display of the impact of the altered amino acids on amino acid metabolism using PathVisio (BMC Bioinformatics 2008). The fold increase of the amino acids in plasma (left) and urine (right) is shown in red, the decrease in green. Changes are based on p-values derived from the amino acid analysis in plasma and urine.

3-Methylhistidine is a by-product during the breakdown of actin and myosin in muscle. It is often measured in urine to determine muscle protein catabolism [6]. The amio acid is derived from histidine, which is a precursor of the mediator histamine, and is degraded through formation of glutamate. 3-Methylhistidine and histidine levels were slightly reduced in plasma and urine, whereas 1-methylhistidine was detected at significantly lower concentrations. 1-Methylhistidine is another methylation product of histidine and is a component of the dipeptide anserine [139]. Its concentration in urine was found to be directly related to the intake of protein in the diet, but only if the protein was ingested in the form of beef. Anserine is found in the brain and skeletal muscle of mammals [139-141] Unfortunately, anserine levels were below the analytical detection limit both in plasma and urine. Another dipeptide, carnosine, was also measured in high concentrations in the skeletal muscle of mammals [140, 142]. Carnosine is a crosslinking product of histidine and β-alanine and was reported to inhibit glycation. The anti-crosslinking activity of the components that form the dipeptide carnosine was the highest one for the unmodified imidazole ring, but also β-alanine was showing this effect. β-Alanylation of histidine might thereby be modulating the reactivity of the imidazole group or might also be responsible for the interception of β-alanine. Carnosine was postulated to have a positive effect in retarding aging and in the progression of Alzheimer's disease [143]. Its concentration could unfortunately not be detected in plasma and did essentially not change in urine. A reduction of muscle

breakdown products supports the hypothesis that the protein supplementation is increasing muscle protein biosynthesis or at least reduces its breakdown.

Proline is metabolized to glutamate for needs of gluconeogenesis and can also be converted to hydroxyproline that is involved in collagene synthesis. Hydoxyproline is released after degradation of connective tissue and is either oxidized or eliminated in urine. Hydroxyproline levels in urine are used as an indicator to measure changes in the metabolism of connective tissue [6]. Proline was found to be significantly elevated in plasma during the intervention phase and also slightly elevated in urine. This also resulted in the moderate increase of hydroxyproline concentrations in plasma. Hydroxyproline in urine was below detection limit.

The amino acid derivative α-aminoadipic acid appeared with elevated levels in plasma and urine. This amino-derivative of an α-keto acid is derived from the essential amino acids lysine and tryptophane, both with decreased concentrations in urine. In contrast, lysine levels were only slightly increased in plasma. α-Aminoadipic acid can also be formed after degradation of collagen-derived 5-hydroxylysine, which was not measured [6]. An elevation of α-aminoadipic acid levels could therefore indicate an increased metabolic degradation of lysine and tryptophane, with the keto acids converted into acetyl-CoA and introduced into the citric acid cycle and in part appearing in α-aminoadipic acid.

A correlation matrix between the responses in amino acids, either only in plasma or only in urine or between plasma and urine, is shown in Figure 4-20. Clusters coloured in blue indicate that there are significant correlations within these groups of amino acids. At first sight it can be noticed that within plasma only few clusters can be identified, whereas in urine many amino acids correlate in response to the high-protein diet. A comparison between parameters in plasma *versus* urine clearly shows that there is hardly any correlation between these two samples.
In plasma, there are two main amino acid clusters. They can be clearly seen in the cluster image as the blue regions in the lower left square and the upper right square. Interestingly, there is some correlation between amino acids of the same functional groups. The branched-chain amino acids valine, leucine and isoleucine are clustering as well as threonine and serine. Aromatic amino acids that strongly correlate in plasma are tyrosine and α-aminoadipic acid. These amino acids show positive correlation (data not shown). α-Aminoadipic acid also clusters with ornithine and lysine in plasma. Further correlations can be found for the amino acids 1-methyl-L-histidine and ethanolamine that correlate negatively with valine, proline and lysine in plasma.
In urine, the number of amino acids that correlate to each other, considering their fold-changes and p-values, is much higher. This is associated with significantly reduced levels during protein supplementation for most amino acids, and therefore correlations are in most cases positive. The branched-chain amino acids leucine and isoleucine can be taken together in one cluster as well as citrulline and homocitrulline. The majority of correlations are found amongst those that carry acidic or

amide groups. 1-methyl-L-histidine, 3-methyl-L-histidine, phenylalanine, histidine and tyrosine also correlated with significant p-values.

When comparing plasma and urine, only very few clusters can be identified. These findings address the question of how the elimination of amino acids is regulated. Obviously, increased plasma levels do not correlate with high urinary concentrations. Amino acids compete with each other for different transport systems for uptake into cells and blood and for enzymes during transamination and oxidation. Their different affinities for those systems could be responsible for the rank orders found in urinary amino acids.

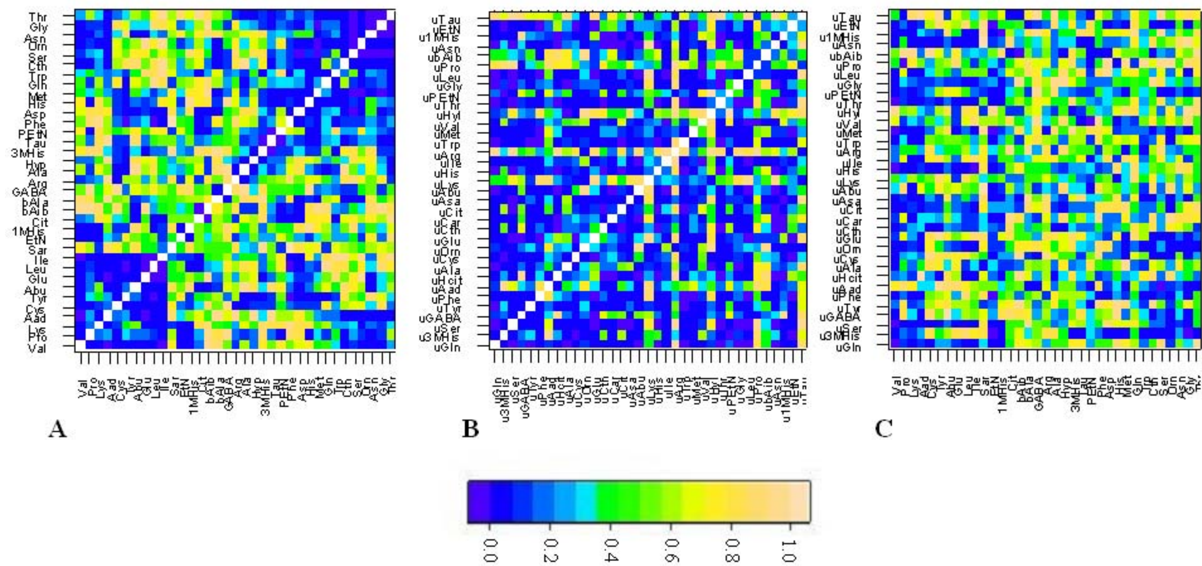

Figure 4-15 Amino acid correlation amongst plasma and urine samples

P-values correlation matrix for plasma (A), urine (B) and plasma and urine in comparison (C). Results are based on the mean amino acid levels during the high-protein intervention compared to the mean baseline values. Urine amino acids are normalized by osmolarity. Amino acid clusters are shown in blue whereas correlations based on higher p-values are displayed using green to yellow colours.

4.4.4. Analysis of the Plasma Proteome after Protein Supplementation

The body protein is distributed with around 40% in muscle, 30% in skin and blood and the remaining 30% in other organs. Blood carries several proteins of hepatic origin. Synthesized in liver hepatocytes, these proteins are secreted into blood. Body proteins undergo constant synthesis and breakdown. This depends on the amino acid pool that provides the building blocks of protein anabolism. Amino acids are provided either through the diet or through muscle protein degradation in the human body [144]. It would therefore be expected that excessive intake of dietary proteins could also alter blood protein levels.

To assess this, plasma was isolated from whole blood after centrifugation, and plasma samples were depleted of the 12 most-abundant proteins using antibody depletion followed by concentration of the flow-through from the spin columns. 100 µg of protein was loaded onto each gel, and gels were stained with Coomassie. One gel for each of the ten volunteers was taken for each of the seven time points. Before assessing the effect of the protein supplementation, the three baseline time points were compared to each other to measure the biological noise in the samples, and samples from the intervention period were also compared to those of the wash-out period.

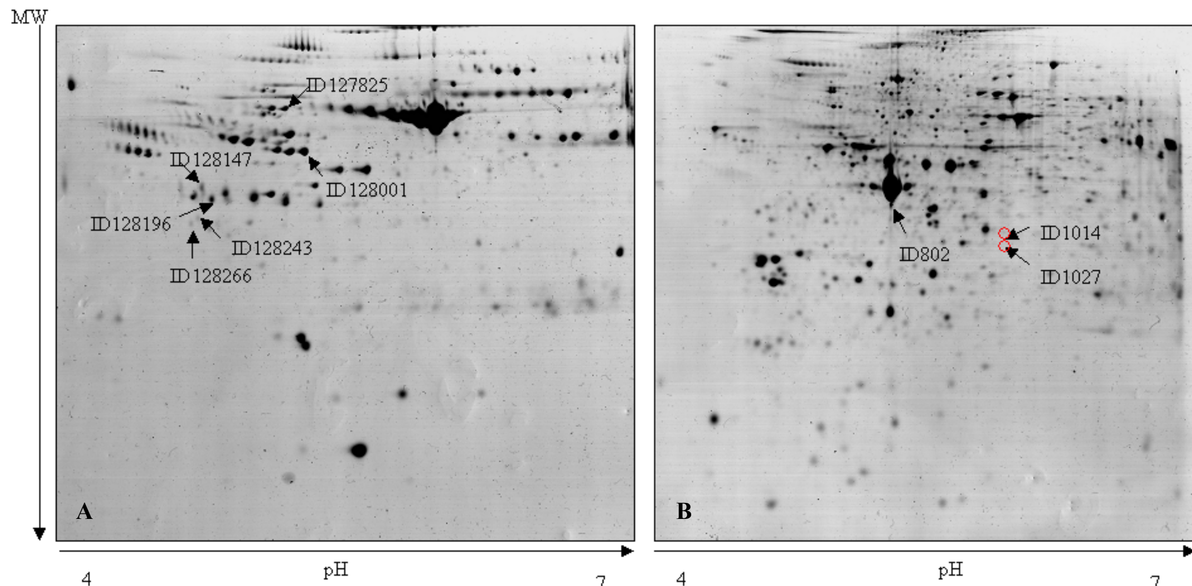

Figure 4-16 Gels of baseline plasma and PBMC samples from the intervention study

Representative 2D gel of plasma (A) and PBMC cell lysate (B) showing changes in spot densities at baseline levels during the first time points (BL1, BL2 and BL3) of the protein intervention study. 100 µg of depleted plasma and 200 µg of PBMC sample was applied to 18 cm IPG strips pH 4-7 and run on 21.5% acrylamide, using either the discontinuous buffer system for plasma or the continuous buffer system for PBMC in the second dimension. Staining was carried out with Coomassie. One gel from each of the 10 volunteers was taken per time point. BL2 and BL3 were compared to BL1 with the Delta2D software. Altered spots were identified using the proteome maps, and identified spots are shown with arrows and numbers. The circles indicate faint spots that could not be identified with mass spectrometry.

Overall, 777 spots were detected in the automatic mode applied in the gel analysis program. 707 of them (91%) appeared as non-significantly regulated spots in the statistical analysis. After a manual editing, only six spots changed according to spot volumes during the baseline phase. The relation between BL1 and BL2 resulted in four significantly regulated spots (Fig. 4-16A, Table 4-9). Two of them could be identified with the plasma proteome map, the other two are below detection limit for MS based analysis.

Table 4-9 Regulated 2D spots in the plasma baseline of the trial

Spot ID	Regulation	t-Test [%]	Spot No. Database	Protein Name
			BL2_BL1	
ID128196	2.55	99.89	106	Zinc-alpha-2-glycoprotein
ID128243	0.48	99.87	Not identified	Not identified
ID128266	0.47	99.79	Not identified	Not identified
ID128147	0.45	99.98	12	Haptoglobin
			BL3_BL1	
ID127825	2.24	99.99	55	Alpha-1B-glycoprotein
ID128001	0.50	99.20	46	Vitamin D-binding protein

List of regulated spots derived from the Delta2D gel analysis. Given is the spot ID, the regulation factor based on the quotient of the mean spot volumes, the t-test for significance and the spot identification from the plasma database. 10 gels per time point (1 for each volunteer) were compared to BL1.

Identified proteins that were altered during the baseline phase in plasma are zinc-alpha-2-glycoprotein and haptoglobulin. Zinc-alpha-2-glycoprotein is a novel adipokine that is produced in brown and white adipocytes and can be found in body fluids like serum or sweat. This protein was reported to act as a biomarker for the detection of Alzheimer's disease and several cancer types [110, 145]. Haptoglobulin is an acute-phase protein that is mainly synthesized in the liver and secreted into blood. Its function is in binding of hemoglobin for degradation and in prevention of iron from excretion through the kidneys. Haptoglobulin values are measured in blood for diagnosis of hemolysis indicated by decreased haptoglobulin concentrations [146]. Between BL1 and BL3, two proteins appeared to be altered. These are alpha-1B-glycoprotein and vitamin D-binding protein. The function of alpha-1B-glycoprotein from the immunoglobulin superfamily is not known yet, however, it seems to be associated with the development of pancreatic cancers [114]. Vitamin D-binding (DBP) protein is responsible for the transport of vitamin D and actin. It controls bone development, can also bind fatty acids and is a modulator in immune response and inflammatory processes. Changes in DBP were found to be related to altered insulin levels [147]. The spot of DBP that was found to be regulated is a rather prominent one, and therefore this spot could be saturated in the 2D gel analysis and might not provide any reliable information about its regulation.

As gel variations have not been assessed for gels derived from the same individual, it cannot be determined whether the observed variances are due to only analytical variation or derive from biological origin. However, according to the relatively low number of changed proteins, the plasma proteome seems to be relatively stable at baseline. Inter-individual changes in particular are expected to be higher than any other biological or analytical noise.

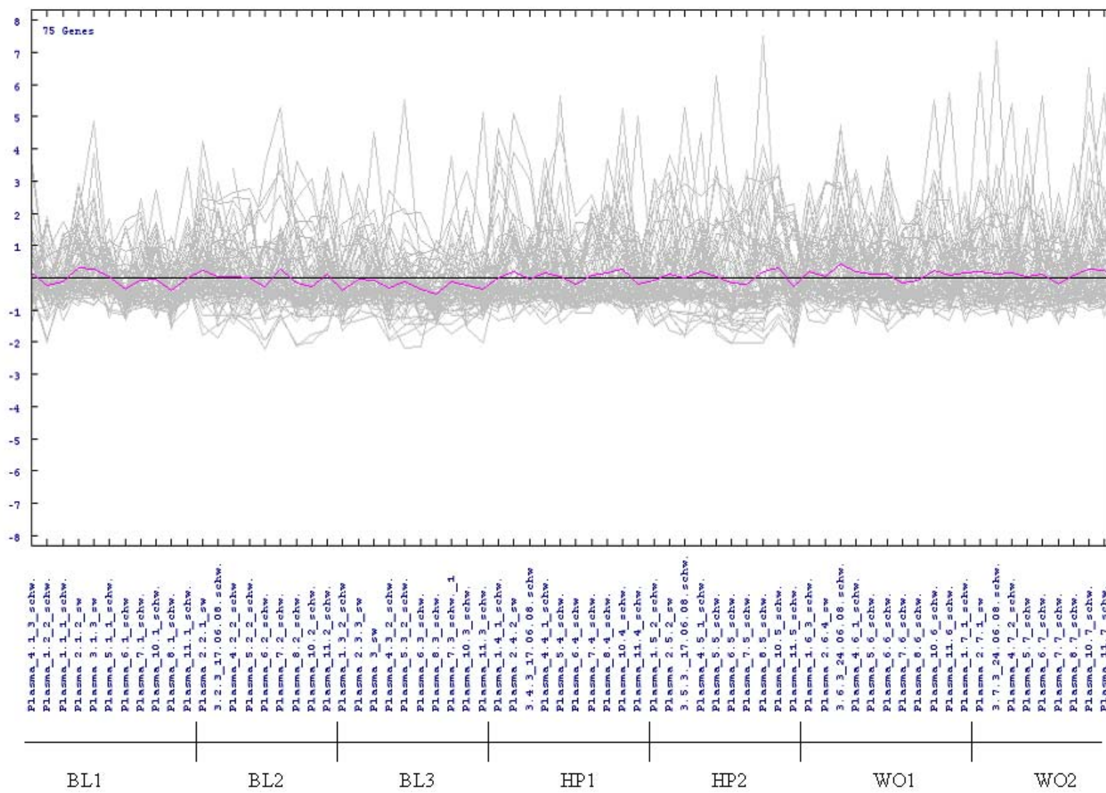

Figure 4-17 Expression profiles of all significant spots in plasma

Expression profiles of all plasma gels during the intervention phase of the trial showing regulation factors of all significant spots and the mean regulation (purple line). Regulation is based on the mean spot densities of all baseline gels. 75 spots (named as genes) were significantly altered based on ANOVA.

During the high-protein intervention gels from the various time points within the supplementation (HP1 and HP2) and those from the wash-out period (WO1 and WO2) were compared to the mean baseline values. Automatic gel analysis identified 75 spots with significantly altered densities, which is 10% of the total spot number. As displayed in Figure 4-17, the expression profiles of those significant spots clearly show the basal variation during BL1, BL2 and BL3, where the mean expression profiles differ from the mean baseline spot densities. However, only few spots in individual gels appeared regulated more than two-fold, and those appeared during the protein supplementation with peak levels reached at HP2. During the early wash-out period at WO1, the variability of expression profiles was less compared to all other time points and the profiles. When taking the mean of the baseline expression levels, significantly regulated spots during the intervention period are mostly represented with increased protein levels. A principal component analysis of all significant spots as well as hierarchical clustering did not yield any particular clustering of the samples (data not shown).

Manual editing decreased the number of significantly regulated spots to 9, most of them were up-regulated. The majority of spots were found upregulated at HP2 during the intervention phase (Table 4-10). Two of the identified proteins appeared with increased plasma concentrations at HP1 and at even higher levels at HP2. During the wash-out period none of the proteins that showed alterations during the high-protein phase appeared regulated, and at WO2 no altered proteins could be identified. The spot pattern of a representative 2D gel is shown in Figure 4-18.

Table 4-10 Regulated protein spots in the plasma high-protein and wash-out phase of the trial

Spot ID	Regulation	t-Test [%]	Spot No. Database	Protein Name
			HP1_BL	
ID127793	2.10	99.79	90	Afamin
ID127837	2.09	96.02	58	Hemopexin
ID127750	2.04	99.86	85	Serotransferrin
			HP2_BL	
ID127750	3.94	100.00	85	Serotransferrin
ID127945	3.89	97.33	78	Beta-2-glycoprotein 1
ID127821	2.74	100.00	60	Hemopexin
ID128186	2.30	99.88	105	Zinc-alpha-2-glycoprotein
ID127689	0.43	100.00	72	Complement factor B
			WO1_BL	
ID128048	2.28	99.81	45	Vitamin D-binding protein
ID128635	2.05	99.54	4	Apolipoprotein A-I

List of regulated spots derived from the Delta2D gel analysis. Given is the spot ID, the regulation factor based on the quotient of the mean spot volumes, the t-test for significance and the spot identification from the plasma database. 10 gels per time point (1 for each volunteer) were compared to the baseline.

One of the proteins identified with higher levels during high-protein intake was hemopexin, which is mainly synthesized in the liver but also in other cells of tissues. This protein is responsible for heme transport to the liver for breakdown and iron recovery. It is proposed to carry further functions, including antioxidant protection, defense against bacteria and promoting regeneration of nerves. Hemopexin may play a role in diseases like Alzheimer's and atherosclerosis but is also involved in the aging process [148]. Serum levels reflect the iron status of the human body. Decreased concentrations in blood are an indicator for severe hemolysis when heme-binding capacities are exceeded. Therefore, serum hemopexin levels can be used to assess a hemolytic anemia in clinical diagnosis. Increased hemopexin levels were found in the acute-phase response of inflammatory psychiatric disorders but also in diseases like diabetes mellitus and some cancer types. Hemopexin concentrations can also rise due to a heme overload, when the concentration of free heme in plasma is drastically increased [148]. Heme degradation is leading to an increase in plasma bilirubin levels. As during the high-protein diet phase plasma bilirubin levels were not increased (Table 4-5), this is probably not the case. In addition to pathophysiological conditions, an elevation of hemopexin levels was reported after physical training, possibly linked to hemopexin also produced in the skeletal muscle [148, 149].

Serotransferrin, also named transferrin, is reported to be a negative acute-phase protein, which means that after inflammation protein levels are decreased. It is synthesized in the liver and takes part in iron transport in blood. During iron deficiency, liver disorders and pregnancy, transferrin is found at high concentrations in blood. Lowered levels could indicate chronic inflammation, hemolysis or iron overload [144]. Serotransferrin was observed to be significantly increased during both intervention time points, but protein levels declined again thereafter during the wash-out period. Transferrin is one of the high-abundant proteins that is depleted from plasma during the pre-fractionation. As depletion is not 100%, the remaining protein can still be detected. Regulation of this protein is only observed, assuming that depletion is at constant rates over the whole analysis period of all samples.

Another protein that occurred in plasma at higher levels during the intervention phase is afamin, also named alpha-albumin. As this protein can bind vitamin E, it is expected to be altered due to dietary effects. In ovarian cancer patients showed lowered serum levels of afamin, indicating its application as a disease biomarker [112]. As other proteins from the albumin family, it also responds negatively in the acute-phase. During the high-protein intake phase afamin only responded positively at HP1 but not at HP-2 or the wash-out phase, suggesting a more artificial change. Decreased levels of complement factor B appeared also only at one time point (HP2). This protein is activating the alternative complement pathway that is part of the immune response. It is involved in proliferation and inhibition of activated lymphocytes in the immune response. Serum levels increase significantly during inflammation [150].

Beta-2-glycoprotein 1, also known as apolipoprotein H, is an abundant plasma protein that is binding cardiolipin and can inhibit the release of serotonin from platelets. Thereby, it interacts in coagulation through the inhibition of platelet aggregation. It can furthermore protect cells against atherosclerosis and apoptosis caused by nitric oxide. Effects in inhibition of angiogenesis were also reported [151]. An increase of beta-2-glycoprotein 1 levels, as observed at HP2, therefore could indicate a suppression of coagulation, improved hemodynamics and certain protection from atherosclerosis. An up-regulation of zinc-alpha-2-glycoprotein levels was also observed at HP2. This protein appears to be involved in control of lipolysis and has been associated with high-protein meals, resulting in satiety effects and weight loss [17]. During the wash-out period the protein level declined again. Further studies are needed to assess, whether zinc-alpha-2-glycoprotein alterations are rather due to normal biological variation, or whether it indeed significantly responds to the diet. Vitamin D-binding protein appeared at higher levels during the wash-out phase, but has also been found at changed levels during the baseline, and therefore seems more altered by general variations in its levels. Apolipoprotein A-I was significantly up-regulated only during WO1 without changes in baseline levels. Apolipoprotein A-I, as a component of HDL, is reported to decrease the risk of atherosclerosis [152]. In addition, it has to be noted that about 98% of total apolipoprotein A-I should have been depleted during the plasma

prefractionation process. Changes in concentration levels of this protein could also be caused by lowered binding capacity of the depletion column after several runs and irregular binding of the polyclonal antibodies.

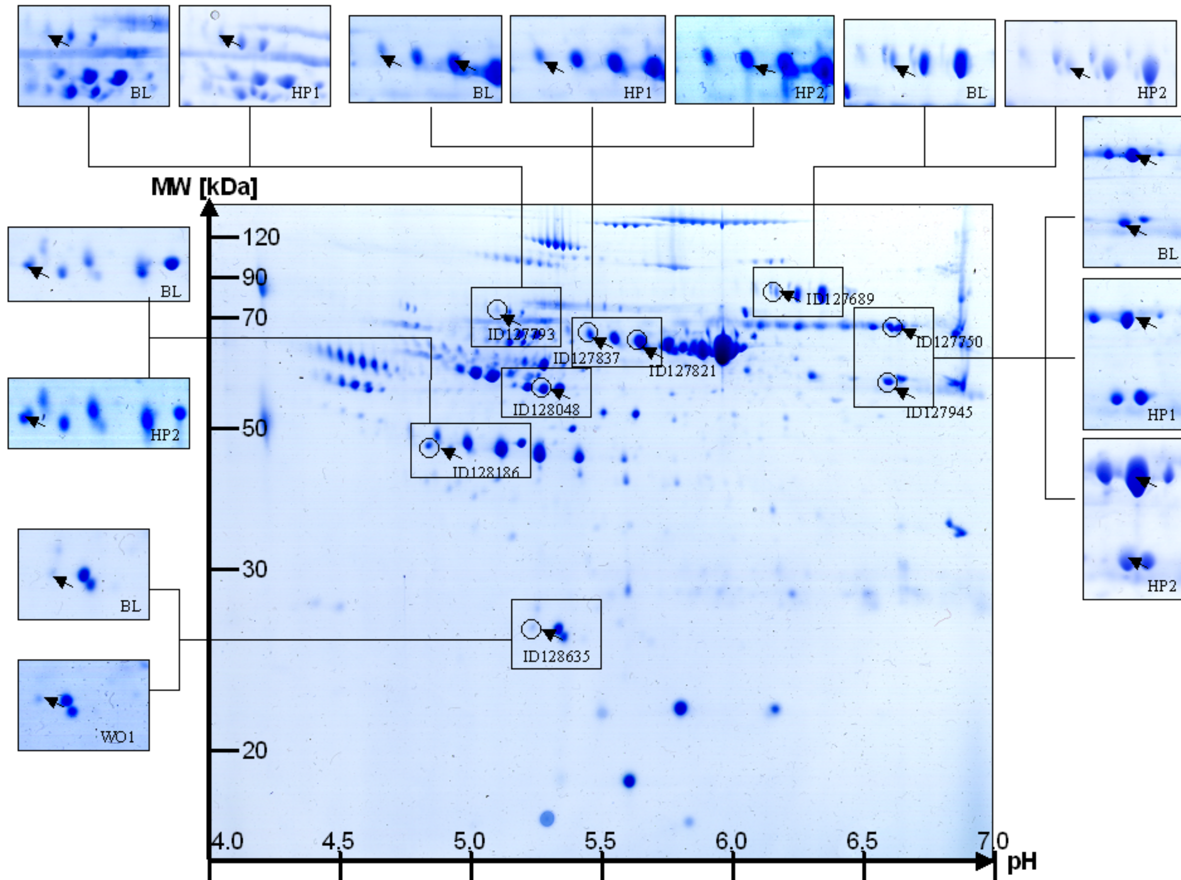

Figure 4-18 Altered spots in plasma during the trial

Representative 2D gel of depleted plasma and spot alterations from the trial comparing the high-protein phase (HP1 + HP2) and the wash-out phase (WO1) with the baseline (BL). 100 µg of depleted plasma was applied to 18 cm IPG strips pH 4-7 and run on 21.5% acrylamide using the discontinuous buffer system in the second dimension. Staining was carried out with Coomassie. One gel from each of the 10 volunteers was taken per time point. All baseline time points were taken together and compared to the high-protein and wash-out phase with the Delta2D software.

4.4.5. Analysis of the PBMC Proteome after Protein Supplementation

For PBMC isolation blood was collected in vacutainer tubes. After centrifugation the PBMC containing phase could be removed directly from the other phases, and proteins were isolated after cell lysis. 200 µg of PBMC protein solution was applied to each gel.

7% (53) of all identified spots (758) appeared as significantly altered during baseline after automatic gel analysis. After manual editing and elimination of false spot identities the baseline variation of PBMC spots appeared relatively small and resulted in only one protein spot that was altered significantly between BL1 and BL2 and two spots between BL1 and BL3 (Fig. 4-16B, Table 4-11). As those spots appeared very faint on the gels, they could not be identified and found in the PBMC database. Only one spot could be identified with additional MS analysis as guanine nucleotide-binding protein alpha-2 subunit. According to the SwissProt database this protein belongs to the G-protein family that is involved in transmembrane signaling. Based on genome data this family includes about 700-800 different proteins. Due to their diversity they regulate a huge spectrum of different processes. In lymphocytes, they were found to interact in cell proliferation and differentiation [153]. Higher levels of a guanine nucleotide-binding protein might thus point at an increased proliferation rate of PBMC.

Table 4-11 Regulated 2D spots in the PBMC baseline samples of the trial

Spot ID	Regulation	t-Test [%]	Spot No. Database	Protein Name
			BL2_BL1	
ID802	2.16	97.32	Not identified	Guanine nucleotide-binding protein
			BL3_BL1	
ID1014	0.48	99.03	Not identified	Not identified
ID1027	0.48	97.79	Not identified	Not identified

List of regulated spots derived from the Delta2D gel analysis. Given is the spot ID, the regulation factor based on the quotient of the mean spot volumes, the t-test for significance and the spot identification from the PBMC database. 10 gels per time point (1 for each volunteer) were compared to BL1.

During the intervention phase the majority of protein significantly altered spots appeared with up-regulated levels, as shown in the expression profiles of Figure 4-19. 212 spots appeared as significantly altered spots, representing 28% of all identified spots on the gels (wihout manual editing). The mean expressions changed to positive profiles immediately after protein supplementation, whereas during the baseline the mean expression levels varied around the zero line with only very few variations. During the early wash-out period the profiles fell to negative values and reached the zero line again at WO2. The highest positive expression levels were found at HP2, the lowest levels appeared during the baseline at BL3. The expression profiles during the wash-out period were rather homogenous when compared to the other time points. Also for PBMC samples no clustering could be observed (data not shown).

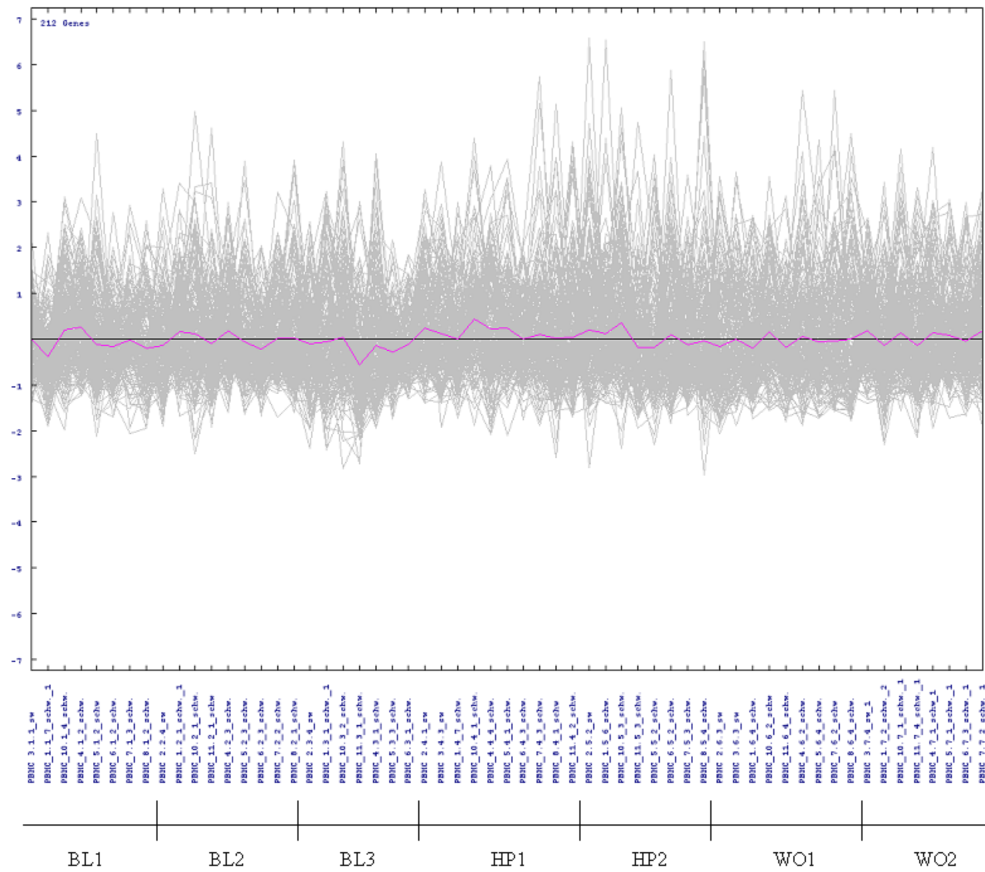

Figure 4-19 Expression profiles of all significant spots in PBMC

Expression profiles of all PBMC gels during the intervention phase of the trial showing regulation factors of all significant spots and the mean regulation (purple line). Regulation is based on the mean spot densities of all baseline gels. 212 spots (named as genes) were significantly altered based on ANOVA.

Manual editing of the identified spots resulted in an overall number of 14 regulated spots. During the high-protein intervention phase fewer proteins were affected as compared to the wash-out period, where more alterations were observed. None of the proteins that were changed during the early protein supplementation at HP1 appeared as affected at HP2 as well. Several proteins could not be identified in the PBMC database, as they appeared too faint on the gels and had to be mass-analyzed additionally (Fig. 4-20, Table 4-12).

Almost all proteins that appeared at higher concentrations during the high-protein phase possess structural functions. Lamin-B1 belongs to the lamin family that form a network of filaments in the cell nucleus membrane [154, 155]. So far, the function of lamin proteins is mainly unknown. Lamin levels were found to be reduced in apoptotic human breast cancer cells as a result of proteolytic cleavage of these proteins during apoptosis [156]. Higher levels of lamin-B1, as observed during the high-protein intake phase, therefore could point at an increased cell differentiation and proliferation of lymphocytes. Lamin-B1 is the only membrane protein that was affected by the high-protein dose.

Variations of lamin-B1 levels were observed in a study that investigated changes in the monocyte proteome in a general control population [28]. Beta-actin was also found in significantly elevated concentrations in response to the protein intake. This protein is ubiquitously expressed in cells and can interact in cell motility processes. Higher levels of beta-actin could also indicate an up-regulation of cell proliferation [157]. As beta-actin levels decreased again during the wash-out phase, this effect was reversible.

Table 4-12 Regulated 2D spots in the PBMC high-protein and the wash-out phase of the trial

Spot ID	Regulation	t-Test [%]	Spot No. Database	Protein Name
			HP1_BL	
ID343	2.98	100.00	Not identified	Lamin-B1
ID1124	2.88	100.00	Not identified	Beta-actin
ID154	2.46	99.77	176	Alpha-actinin-1
ID1052	2.36	99.98	49	Beta-actin
			HP2_BL	
ID658	2.13	99.75	Not identified	Alpha-centractin
ID763	0.45	99.61	75	Serum albumin
			WO1_BL	
ID276	2.26	100.00	3	Gelsolin
ID248	2.10	99.96	2	Gelsolin
ID802	2.09	99.02	Not identified	Guanine nucleotide-binding protein
ID848	2.01	99.99	89	PDZ and LIM domain protein 1
ID1052	0.49	98.34	49	Beta-actin
ID428	0.47	99.28	Not identified	Serum albumin
ID475	0.30	99.09	130	Glucose-6-phosphate 1-dehydrogenase
ID462	0.30	99.07	Not identified	Fibrinogen beta chain
			WO2_BL	
ID1031	2.07	96.91	50	Gamma-actin
ID475	0.37	95.24	130	Glucose-6-phosphate 1-dehydrogenase

List of regulated spots derived from the Delta2D gel analysis. Given is the spot ID, the regulation factor based on the quotient of the mean spot volumes, the t-test for significance and the spot identification from the PBMC database. 10 gels per time point (1 for each volunteer) were compared to the baseline.

Alpha-actinin-1 is cross-linking actin filaments in muscle and non-muscle cells. In contrast to muscle, non-muscle alpha-actinin is inhibited by the prescence of calcium [158]. Alpha-actinin levels were found to be elevated as well. Another protein that is expressed at higher concentrations at HP2 is alpha-centractin, also belonging to the actin family and a component of the dynactin complex. It is located in the centrosome, where it is integrated in the process of mitosis and nuclear migration and might play a role in linking intracellular organelles to the dynactin complex [159]. Increased levels of these structural proteins could also indicate increased cell proliferation in the PBMC population.
Serum albumin that shows reduced levels during acute immune responses [144] was found in proteome analysis of the PBMC. Albumin occurrence in PBMC is due to its binding, adherence or possibly also uptake from the blood, which cannot be prevented by PBMC isolation from whole blood by the various washing steps and therefore is a methodological artefact.

Most proteins with altered levels during the wash-out phase also belong to the group of structural proteins. The predominant protein that was found at increased concentrations during the early wash-out period is gelsolin, a protein interacting in cell motility and signal transduction in cytoskeletal re-modeling [160]. It is found in the cytosol of macrophages and many other cells [161]. Gelsolin was shown to be involved in the MAPK-pathway response in monocytes, when induced by anti-beta-2-glycoprotein 1 antibodies [162]. Plasma gelsolin levels were also reported to be lowered in several disease states, such as acute respiratory syndrome, sepsis, major trauma and liver injury [163]. Our findings suggest an enhanced actin re-organization in PBMC in response to the high protein intake. Elevated beta- and gamma-actin concentrations support this as well. PDZ and LIM domain protein 1, also known as elfin, is an adapter protein responding to oxidative stress. It binds to alpha-actinin-1 and associates with actin filaments in activated platelets [164, 165]. It occurs in platelets but not in PBMC, and therefore its occurrance in the PBMC proteome is due to a platelet contamination or adhesion of platelets to the PBMC cell surface during the PBMC isolation process.

One protein that appeared at lowered levels during both wash-out time points is glucose-6-phosphate 1 dehydrogenase, an oxidoreductase and the rate-limiting enzyme in the pentose phosphate pathway of carbohydrate metabolism. It generates NADPH which is used in reductive biosynthesis reactions, such as the biosynthesis of fatty acids and cholesterol. One function of NADPH is to prevent the cell from oxidative stress from peroxides through glutathione reduction. High expression levels of the enzyme were reported in white adipose tissue of obese animals and over-expression in adipocytes with increased lipogenesis [166]. Lowered tissue glucose-6-phosphate 1 dehydrogenase activity was found after a high-protein, carbohydrate-free diet in rats, and activities even remained at lowered levels after the intervention was finished [167]. Lowered levels of glucose-6-phosphate 1 dehydrogenase would lead to a diminished production of riboses through the pentose phosphate pathway. Thus, nucleotide synthesis could be affected and lymphocyte proliferation no longer supported [168]. Less NADPH production could also lead to an increase in oxidative stress of PBMC after the intervention phase. As the changes occured only during the wash-out phase, it is not clear whether it is associated with the high protein intake. Fibrinogen beta chain was also decreased in the wash-out phase. This protein is involved in blood coagulation and is reported to occur at elevated levels during the acute-phase response [169]. Increased plasma concentrations have been found in chronic, low-grade inflammatory diseases, such as atherosclerosis [170]. In lymphocytes, this protein could be involved in inflammatory processes through synthesis of pro-inflammatory cytokines [169].

Analysis of the PBMC proteome was rather difficult, as spot patterns on 2D gels varied strongly between gels. This resulted in difficulties in comparing the gel images from different volunteers. The relatively low number of regulated spots representing the inter-individual baseline therefore has to be considered with care. These variations in spot patterns probably also led to a relatively low number of

identified proteins with altered levels after high-protein intake. A reason for this could be that PBMC isolation and preparation is crucial for maintaining protein content. So, even only little variation in the preparation process could cause major differences in protein analysis. Especially the variation between subjects was reported as a problem in gene expression analysis [20]. However, the number of significantly regulated spots clearly increased during the protein intervention, representing as a surrogate the response of the PBMC proteome to high protein intake with more overt changes than in plasma.

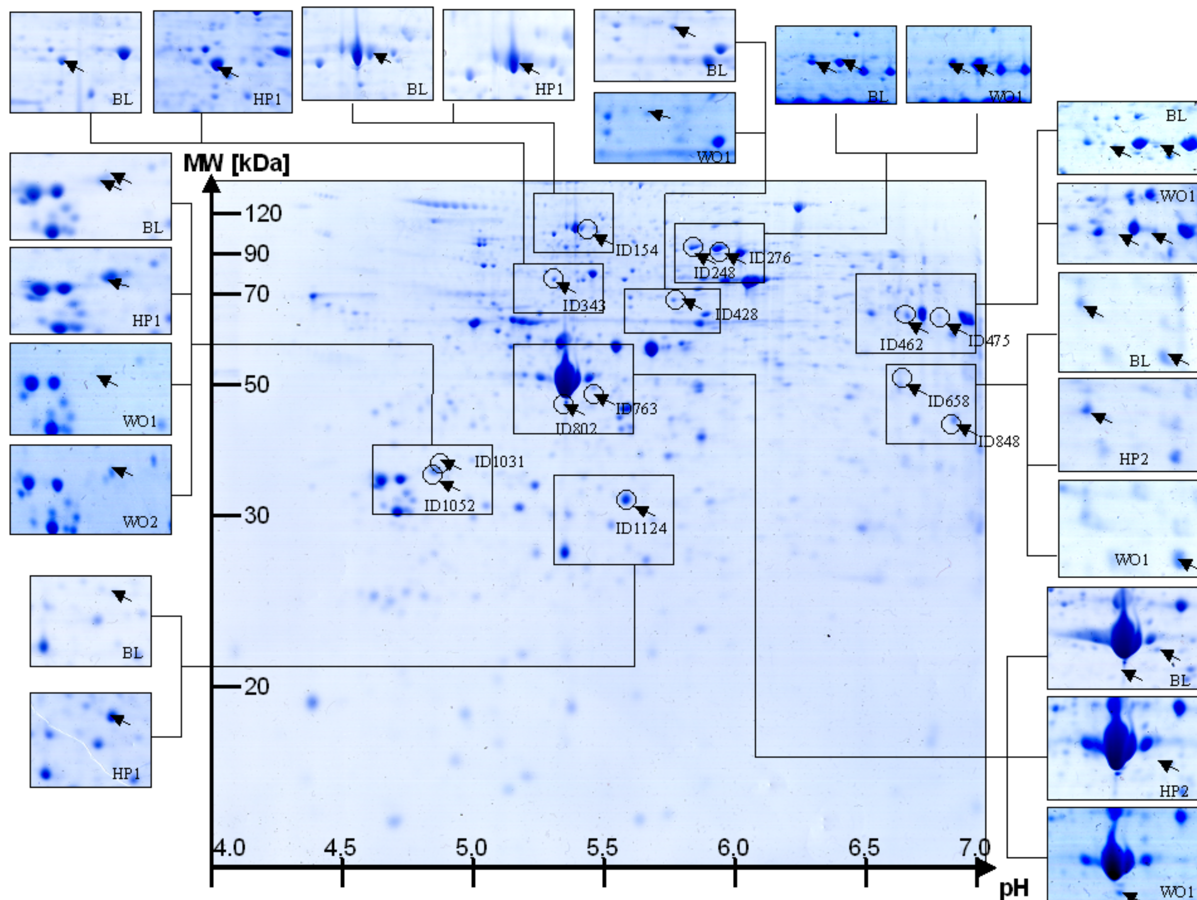

Figure 4-20 Altered spots in PBMC during the trial

Representative 2D gel of PBMC cell lysate and spot alterations from the trial comparing the high-protein phase (HP1 + HP2) and the wash-out phase (WO1+ WO2) with the baseline (BL). 200 µg of PBMC cell lysate was applied to 18 cm IPG strips pH 4-7 and run on 21.5% acrylamide using the continuous buffer system in the second dimension. Staining was carried out with Coomassie. One gel from each of the 10 volunteers was taken per time point. All baseline time points were taken together and compared to the high-protein and wash-out phase with the Delta2D software.

5. General Remarks and Conclusions

5.1. Proteomics Applications in Human Intervention Studies

Changes in protein levels due to dietary interventions are often very small and are consequently difficult to assess. Proteomics analysis requires the separation of complex protein mixtures prior to protein identification. This is done by electrophoretic and chromatographic approaches using differences in their physical and chemical properties. However, these properties are also responsible for some of the difficulties in proteomics analysis. The first difficulty is the huge concentration range that is represented especially in sample sources like plasma. Therefore, additional steps like depletion and pre-fractionation are needed to reduce complexity. Solubility is another problem. Proteins tend to precipitate at their p*I* due to reduced solubility. This results in smears instead of distinct spots in 2D gels. Hydrophobic proteins or membrane proteins are highly insoluble which requires special treatment for these proteins in addition. As proteins also have high adsorption energies, they can attach to separation matrices like column material and may result in loss of protein or retarded elution times in chromatographic approaches. The three-dimensional structure of proteins also contributes to the retention time. Unfolding during the separation could result in oxidation of thiol groups. This clearly shows that each technology has to overcome several difficulties that are linked with separation of proteins as complex macromolecules.

Proteomics is the only choice when changes in protein levels should be monitored simultaneously. The technology is used for screening experiments and helps to identify responses in humans to drug treatment, disease states or dietary intervention. Whenever only certain parameters are of interest the use of other technologies should be considered in order to save time and get more reliable results. Quick measurement of distinct proteins can be done with ELISA tests if proper antibodies are available. LC-MS/MS technologies can also be applied in a similar way in order to filter just for those parameters that are of interest. As high-resolution 2D PAGE can be considered the method of choice for the separation and display of complex protein mixtures, this technique was used for the generation of plasma and PBMC proteome databases. Separation of protein samples of plasma and PBMC using 2D electrophoresis appears to be a valid method, as shown in our experiments. Through the separation based on p*I* and MW, additional information about the physical properties of proteins can be gained in contrast to other chromatographic approaches. Quantification is done by a direct comparison of spot densities in stained 2D gels. Commonly used stains are compatible with mass spectrometry and therefore allow direct protein identification without complex clean-up steps. The dynamic range of detection depends on the staining procedure, with classical colloidal staining that can detect 50-100 ng of protein up to fluorescence staining that is able to display 0.25-5 ng of protein. Fluorescence staining requires additional equipment for detection and further gel handling. Thus, the choice of staining method depends on the budget that is available, as fluorescence stains are very expensive. Using

Coomassie as protein stain with its rather low sensitivity did not result in the discovery of novel biomarkers of interest during our studies. The dynamic concentration range that is covered by this staining technique is very small and includes only few proteins that have the potential to act as markers. 2D electrophoresis is very susceptible to external factors like temperature differences, variations in buffer and gel composition and handling of the procedure. A major drawback of classical 2D electrophoresis in general is the complex analysis procedure which is very time and labor consuming. This makes its use in high-throughput analysis in large intervention studies almost impossible. Increased automation is therefore required here.

The second factor that plays an important role in sensitivity of the proteome analysis is the detection limit of the MS analysis. In order to maximize the detection range of proteins, either highly sensitive MS equipment is needed or highly effective enrichment has to done before MS analysis. Reducing complexibility by sample pre-processing or pre-separation would also further increase the number of proteins that can be detected in a plasma sample. It was not tested whether antibody-based depletion with the method used resulted in a significant loss of low-abundant proteins as bound to depleted ones. A complete analysis of the bound protein fraction in antibody-based depletion could overcome this problem to capture all proteins in plasma. However, the separation after the depletion showed additional and distinct spots on the gels that could easily be analysed by MS. A promising alternative seems to be depletion using the MARS column (see chapter 1.5.) that can even be applied on-line with HPLC separation and MS identification of the proteins. Multiple pre-fractionation steps could further improve the yield and results. However, reproducibility is usually decreased with increasing number of analysis steps undertaken. To overcome problems like unspecific binding and loss of proteins in antibody-based depletion, separation using free-flow electrophoresis appears a valid alternative. It is highly reproducible and results in no loss of proteins when all fractions are analyzed. Another advantage of this electrophoretic method is the automation of the process (chapter 1.5.). Other proteomics approaches such as LC-MS/MS including multi-dimensional plasma separation steps or modern multiplex MS technologies would be a valuable alternative in order to receive reliable data together with high-throughput analysis.

5.2. The “Stability” of the Plasma and PBMC Proteomes

The “stability” of plasma and PBMC proteomes depends on many factors that can introduce analytical and biological variation. The inner- and inter-lab variance of the 2D method for plasma and blood cells using the standard protocols for each laboratory was assessed within our EU project with four different laboratories. This clearly demonstrated the limited reproducibility of 2D gel electrophoresis. Even within the same laboratory under identical conditions and protocols a 12-16% variation in spot densities could be observed for PBMC and plasma after manual editing of the results. This variability was only due to technical factors that result from sample handling, temperature variability in the second dimension, differences in the current during the first dimension and other factors. A

comparison of 2D gels between four laboratories showed that the variation is about 23-30% for plasma and PBMC. Therefore, different equipment and different handling of the samples brings in another factor that clearly influences the spot patterns. Changes that result from technical factors such as differences in currents, temperature and buffer composition are almost impossible to eliminate completely. In order to minimize these effects and the variability through different handling of various persons performing the analysis, electrophoresis should always be carried out by the same person following a standardized protocol. If possible, an internal standard should also be included, as done using the DIGE technology (for details see chapter 1.5.). It was observed that depending on the person who was undertaking the analysis, spot patterns could differ considerably, and PBMC analysis is more prone to artefacts than plasma. Therefore, it is questionable whether PBMC proteomics really provides valuable results that represent real changes in metabolism.

Biological variation finally is an additional huge problem on top of the technical and analytical problems. Within the same individual, several proteins appeared to be altered when analyzed at different time points under the same conditions. As hormonal changes and other effects like infections, nutrition and lifestyle can influence body proteins, this is not surprising. Proteomes compared between volunteers clearly vary in their spot patterns and spot densities, which results in the difficulty to filter those changes from those derived solely from the intervention. A homogeneous study group and as possible a similar lifestyle can minimize these differences. Analysis of a sufficient number of samples or volunteers and replicates will also help to obtain results of good quality and statistical significance. Ideally, proteome analysis for one project is always done by the same person under the same conditions.

5.3. Application of 2D Maps of Plasma and PBMC in Biomarker Discovery

As 2D electrophoresis allows the visualization of hundreds of protein spots at once on a single gel it is currently the preferred screening process in proteomics application in biomarker discovery. Because it is easier to obtain samples from body fluids, they are superior to biopsies as a biomarker discovery material. Blood plasma is reported to contain a huge amount of proteins that may serve as markers for changes in interorgan metabolism, as blood is in contact with all organs and tissues. PBMC contribute to immune responses in the body and may therefore provide valuable information about status and function of the immune system. Both samples contain proteins that may reveal a biomarker quality for prediction of diseases, and also for assessing the response to nutritional interventions.

The present work therefore concentrated on plasma and PBMC as an easily accessible sample source. A proteomics platform was developed to screen for changes especially of protein markers of interest after dietary interventions. Protocols for sample preparation and protein separation using 2D electrophoresis were optimized and used to establish standard protocols and “clickable” proteome maps from 2D electrophoresis of plasma and PBMC. Identification of proteins in these 2D maps was

done using MADI-TOF analysis of all visible spots on the gels. All information about each identified protein including the protein name, mass spectrometry data, the theoretical p*I* and MW and potential functions was collected in a database. This now allows a convenient comparison of gels and direct protein identification in further intervention studies. Including the proteome maps in the software-based gel analysis immediately provides all details that are needed to interpret changes in spot densities and protein expression levels. As further MS analysis and search for information about pathway interactions and biological properties are no more necessary, using the proteome maps can save time and money. However, the most time-consuming step in the analysis is the electrophoresis process that still has to be gone through. In high-throughput analysis, the rate-limiting factor currently is the capacity of gels that can be loaded in the first and the second dimension. Further capacity problems occur when gels are stained for four days in the Coomassie solution. The overall economy of time is about three days out of an overall duration of 10 days. Taken together, the information that can be derived from the proteome maps may be helpful as long as the analysis is performed according to the standard protocols. The techniques that were used to build the databases however were not very sensitive. Thus, the number of identified proteins and also the number of putative marker proteins is relatively small and should be increased for more valuable information.

Taken together, plasma and PBMC proteomics as established here and as done in many other laboratories shows a high susceptibility to technical variation when applied to these samples and that is difficult to control even with SOPs. In addition, the natural variation in protein levels adds another layer of variance that makes it extremely difficult to obtain robust, reproducible, and meaningful data.

5.4. High-Protein Supplementation and Its Impact

Despite enormous changes reported for postprandial amino acid levels after protein supplementation [9], fasting plasma and urinary concentrations of amino acids assessed here, each after an overnight fast, seemed to be affected only to a small extent by the high protein intake. The observed changes in amino acid profiles did not correspond to the amount of amino acids provided in the protein supplement, and therefore alterations were of metabolic origin. There were some remarkable differences between the response of plasma and urine amino acid levels to the intervention.

The human protein intervention study was undertaken to apply the developed in-house databases for assessing putative changes in plasma and PBMC proteins. Clinical chemistry parameters, amino acids in plasma and urine and the proteomes of plasma and PBMC were thus analyzed. Repeated measurements of all parameters after overnight fasting before the protein supplementation gave some insights into basal variations. All volunteers described a strong satiety effect immediately after intake of the first dose of the protein supplement. During the following meals a reduced food intake was observed. There were no reports among the participants about obstipation and other discomfort that could be related to the protein supplementation.

Dietary supplementation with high amounts of proteins leads to a postprandial increase in plasma amino acids and also amino acid oxidation in metabolism. As expected, volunteers responded to the high-protein intake with a strong increase in plasma and urine urea levels. Amino acids from the urea cycle did not increase significantly in their levels (only slightly) and no enzymes related to urea production could be identified in the proteome analysis. Besides the urea cycle, methionine and homocysteine metabolism appeared to be affected by the protein supplementation with a decrease of several plasma and urinary amino acids. This is in accordance with previous findings that reported a decrease in plasma homocysteine concentrations after high-protein intake, which could contribute to the prevention of atherosclerosis and cardiovascular disease. Amino acid analysis overall yielded only a small number of affected parameters in plasma and urine, considering a distinct within- and between-subject variability during the baseline. The majority of the affected amino acids in plasma displayed lowered levels during the supplementation phase, whereas in urine, only decreased concentrations could be found. There was essentially no correlation between amino acid profiles in plasma and urine levels, suggesting a prime role in kidney tubular handling of amino acids under those conditions determining the patterns and concentrations found here.

The plasma proteome showed higher baseline variation than the PBMC proteome. In both sample sources the majority of proteins that were found displayed increased levels during the protein supplementation period, and may be taken as a surrogate response with an overall promoted protein synthesis resulting from higher protein intake. The plasma proteomics data point at an increased synthesis of hepatic proteins, especially those which respond inversely in the acute-phase and those involved in iron transport and metabolism. Analysis of the proteome of PBMC and in this respect in immune cells revealed a rise in selected structural proteins during high protein intake. This could result from a higher proliferation rate that reversed again after the intervention phase. Among all significantly changed proteins only glucose-6-phosphate dehydrogenase 1 could be related to metabolism.

In summary, the high protein intake was well tolerated. A high protein turnover as revealed by increased urea production and excretion is not associated with major and sustained changes in plasma amino acid levels and only minor alterations in plasma and PBMC proteomes that seem only to indicate increased protein anabolic functions.

5.5. Future Perspectives

To improve the sensitivity of 2D electrophoresis it is suggested to apply fluorescent stains as they can enhance spot detection sensitivity by a factor of 1000. Variations in the spot patterns can be minimized with fluorescent DIGE stains combined with an internal standard. As software exists that can directly compare gels that are stained using DIGE, spot analysis in this case is very convenient but is also the most cost intensive alternative available. Highly sensitive staining techniques require an MS system

that has a low detection limit. Enrichment in proteins for MS analysis would otherwise result in time-consuming sample preparation steps and in further loss of protein. Moreover, effective MS/MS systems are available for peptide sequencing now to capture also those proteins that could not be identified with peptide mass fingerprinting as done here.

As novel plasma pre-separation systems are developed, the quality of plasma proteomics analysis can be further improved. So far, each of the applications that are on the market has its limitations. The depletion columns that were applied during our studies, for example, could be re-used up to 100 times. However, the antibodies lost part of their binding capacities after several runs, resulting in an increase of high-abundant proteins in the samples. Single-use systems would prevent this but increase costs. As proteins and peptides of interest might be removed together with the high-abundant proteins, it is questionable whether depletion is the best choice. Other approaches like free-flow electrophoresis could reduce sample complexity and thus improve the quality of the results without loss of any analytes from the sample. However, this method is rather labor-intensive. If new feasible possibilites for plasma pre-processing are up-coming in the future, they should be considered for plasma proteome analysis. These approaches might be helpful to enlarge the number of visible spots on 2D gels. However, 2D electrophoresis is clearly limited in its use for intervention studies. Multi-dimensional chromatography coupled to a highly sensitive MS system will probably improve plasma proteome analysis. This would also allow automation of sample treatment and high-throughput analysis.

Whether proteomics applications in general are useful in detecting protein changes in response to acute or chronic dietary treatments in human volunteer studies remains to be seen. To overcome technical and biological variation, huge cohorts and repeated measurements are needed, and this will drive costs for personnel and materials. However, combining proteomics with transcriptomics and metabolomics approaches may reveal more coherent changes – even in smaller cohorts - that also allow interpretation of the changes in the context of biological networks.

6. Appendix

6.1. Abbreviations

2D	two-dimensional
2DE	two-dimensional electrophoresis
4D	four-dimensional
AMT	Accurate Mass and Time Tag Technology
apo	apolipoprotein
ATP	adenosine triphosphate
BCAA	branched-chain amino acids
BL	baseline
BMI	body mass index
BSA	bovine serum albumin
BUN	bound urea nitrogen
BW	body weight
CBB	Coomassie Brilliant Blue
CHAPS	3-[[3-cholamidopropyl]dimethyl-ammonio]-1-propanesulfonate
CPT	cell preparation tubes
CRE	creatinine
Cy2	fluorescence dye, emission 508 nm
Cy3	fluorescence dye, emission 570 nm
Cy5	fluorescence dye, emission 670 nm
Da	dalton
DBP	vitamin D-binding protein
DIGE	Differential Gel Electrophoresis
DNA	desoxyribonucleaic acid
DPD	Differential Peptide Display
DTT	dithiotreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electrospray Ionization
EU	European Union
FAO	Food and Agriculture Organization
ff.	following
FFE	Free-Flow Electrophoresis
Fig.	figure
FT-ICR-MS	Fourier Transform Ion Resonance Cyclotron Mass Spectrometer

HDL	high-density lipoprotein
HP	high-protein
HPLC	High Performance Liquid Chromatography
HPPP	Human Plasma Proteome Project
HUPO	Human Proteome Organization
ICAT	Isotope-Coded Affinity Tag
ID	identity
IEF	isoelectric focusing
Ig	immunoglobulin
IPG	immobilized pH gradient
IT	Ion Trap
iTRAQ	Isobaric Tag for Relative and Absolute Quantitation
LC	Liquid Chromatography
LDL	low-desity lipoprotein
M	molar
MALDI	Matrix-Assisted Laser Desorption Ionization
MAPK	mitogen-activated protein kinase
MARS	Multiple Affinity Removal System
MicroSol	Microscale Solution
mRNA	messenger deoxyribonucleic acid
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS/MS/MS	triple mass spectrometry
MudPIT	Multi-Dimension Protein Identification Technology
MW	molecular weight
NADPH	nicotinamide adenosine dinucleotide phosphate
NH_3	ammonia
NMR	Nuclear Magnetic Resonance
n.n.	not identified
NuGO	European Nutrigenomics Organisation
PAGE	Polyacryle Gel Electrophoresis
PBMC	peripheral blood mononuclear cells
PDCAAS	protein digestibility corrected amino acid source
p*I*	isoelectric point
PNGase F	peptide N glucosidase F
PRIDE	Proteomics Identification Database
PTM	post-translational modification

qTOF	Quadrupole Time-of-Flight
Qtrap	Quadrupole Ion Trap
RDA	recommended daily allowance
RNA	ribonucleic acid
RP	reversed-phase
RSD	relative standard deviation
SAM	S-adenosylmethionine
SCX	strong cation exchange
SD	standard deviation
SDS	sodium dodecyl sulfate
SELDI	Surface-Enhanced Laser Desorption Ionization
SNP	single nucleotide polymorphism
SO_4^{2-}	sulfate
TNF-α	tumor necrosis factor alpha
TOF	Time-of-Flight
TRIS	tris(hydroxymethyl)-aminomethan
vs.	*versus*
WAX	weak anion exchange
WO	wash-out

6.2. Tables

Table 6-1 Proteins included into the plasma and PBMC databases

Protein Name	Function	Biological Process	Cellular Origin	Tissue Specificity
14-3-3 Protein epsilon 14-3-3 Protein gamma 14-3-3 Protein zeta/delta	Adapter protein in signaling, host-virus intraction	Signaling	Cytoplasm, melanosome	
78 kDa Glucose-regulated protein	Assembly of multimeric protein complexes in ER, anti-apoptotic, response to glucose-starvation, ATP and protein binding	Others	ER lumen, melanosome	
Actin-like protein 2	Cell motility, ATP-binding, regulation of actin-polymerization	Structural	Cytoplasm, cytoskeleton	
Actin-like protein 3	Actin polymerization	Structural	Cytoplasm, cytoskeleton	
Actin-related protein 2/3 complex subunit 5	Regulation of actin polymerization	Structural	Cytoplasm, cytoskeleton	
Afamin	Transport protein	Transport	Secreted	
Alpha-1-antichymotrypsin	Inhibition of angiotensin-2 formation	Immune response	Secreted	Plasma, liver
Alpha-1-antitrypsin	Inhibitor of serine proteases, blood coagulation	Coagulation	Secreted	Plasma
Alpha-1B-glycoprotein	Glycoprotein, secreted	Inflammation	Secreted	Plasma
Alpha-2-HS-glycoprotein	Endocytosis, bone mineralization	Structural	Secreted	Liver, bone matrix, plasma
Alpha-actinin-1	F-actin cross-linking protein, calcium-binding	Structural	Cytoplasm, cytoskeleton	
Angiotensinogen	Regulation of volume and mineral balance of body fluids	Others	Secreted	Plasma, liver
Antithrombin-III	Coagulation, serine protease inhibitor	Coagulation	Secreted	Plasma
Apolipoprotein A-I	Transport of cholesterol from tissues to the liver	Lipid Metabolism	Secreted	Plasma , liver, small intestine
Apolipoprotein A-IV	Chylomicrons, VLDL secretion and catabolism	Metabolism	Secreted	Intestine, plasma
Beta-2-glycoprotein 1	Binds negatively charged ligands, prevents intrinsic coagulation	Coagulation	Secreted	Plasma, liver
Beta-actin	Cell motility, ATP-binding	Structural	Cytoplasm, cytoskeleton	
C4b-binding protein alpha chain	Controls complement activation	Inflammation	Secreted	Plasma (chylomicrons)
CD5 antigen-like	Immune system, inhibitor of apoptosis	Immune response	Secreted	Spleen, lymph node, thymus, bone marrow
Chloride intracellular channel protein 1	Chloride ion channel, ion transport	Transport	Nucleus, nucleus membrane, cytoplasm, cell membrane	
Coagulation factor XIII A chain	Coagulation	Coagulation	Secreted, cytoplasm	Plasma
Complement C1s subcomponent	Serine protease, complement pathway	Inflammation	Extracellular	
Complement component C6 Complement component C9 Complement factor B	Complement alternate pathway, immune response, innate immunity, cytolysis	Immune response	Secreted, cell membrane	
Coronin-1A	Component of the cytoskeleton of highly motile cells, cell motion, actin-binding	Structural	Cytoplasm, cytoskeleton, membrane	Brain, thymus, spleen, bone marrow, lymph node
Coronin-1C	Cytokinesis, motility, and signal transduction	Signaling	Cytoskeleton	
Elongation factor 1-gamma	Anchoring the complex to other cellular components, protein biosynthesis	Others	Cytoplasm	Kidney, intestine, pancreas, stomach, lung, brain, spleen, liver

Protein Name	Function	Biological Process	Cellular Origin	Tissue Specificity
Eukaryotic initiation factor 4A-I	mRNA binding to ribosome, protein biosynthesis	Others	Cytoplasm	
F-actin capping protein subunit alpha-1 F-actin capping protein subunit alpha-2 F-actin capping protein subunit beta	Blocking the exchange of fast-growing subunits, actin-binding	Structural	Cytoplasm, cytoskeleton	
Fibrinogen beta chain Fibrinogen gamma chain	Coagulation	Coagulation	Secreted	Plasma
Fibronectin	Involved in cell adhesion, cell motility, opsonization, wound healing, and maintenance of cell shape	Structural	Extracellular	Plasma, liver, urine
Gamma-actin	Cell motility, ATP-binding	Structural	Cytoplasm, cytoskeleton	
Gelsolin	Calcium-regulated, actin-modulating protein	Structural	Secreted, cytoplasm, cytoskeleton	Phagocytic cells, platelets, fibroblasts, nonmuscle cells, smooth and skeletal muscle cells
Glucose-6-phosphate 1-dehydrogenase	Produces pentose sugars for nucleic acid synthesis, carbohydrate metabolism	Metabolism	Cytoplasm, plasma mambrane	Lymphoblasts, granulocytes, sperm
Glutathione S-transferase P	Conjugation of reduced glutathione, anti-apoptotic, metabolism	Metabolism	Cytoplasm	
Glutathione transferase omega-1	Glutathione-transferase activity, metabolism	Metabolism	Cytoplasm	Ubiquitous
Haptoglobin	Hemoglobin and iron binding protein	Inflammation	Secreted	Liver, plasma
Heat shock 70 kDa protein	Chaperone	Chaperone	Cytoplasm, melanosome	Ubiquitous
Heat shock 70 kDa protein 1	Chaperone, anti-apoptotic	Chaperone	ER, mitochondrion, nucleus	Testis
Heat shock protein HSP 90-alpha Heat shock protein HSP 90-beta	Molecular chaperone, stress response	Chaperone	Cytoplasm, melanosome	
Hemopexin	Heme binding, iron recovery	Transport	Secreted	Plasma, liver
Ig kappa chain C region Ig mu chain C region	Immune response	Immune response	Extracellular	
Keratin, type I cytoskeletal 10	Heterotetramer of two type I and two type II keratins, structural	Structural	Intermediate filament	Suprabasal cell layers
Kininogen-1	Inhibition of thiol proteases and thrombocyte aggregation, coagulation	Coagulation	Secreted	Plasma
Leukocyte elastase inhibitor	Regulates the activity of neutrophil proteases, protease inhibitor	Structural	Cytoplasm	
L-lactate dehydrogenase B chain	Pyruvate fermentation to lactate, oxidation reduction	Metabolism	Cytoplasm	
Moesin	Connections cytosceleton-plasma membrane, leukocyte adhesion, receptor binding	Structural	Cytoplasm, cytoskeleton, cell membrane	Ubiquitous
Myosin light polypeptide 6	Regulatory light chain of myosin	Signaling	Myosin complex	
Myosin regulatory light chain 2	Smooth muscle and nonmuscle cell contractile activity, protein binding	Signaling	Myosin complex	

Protein Name	Function	Biological Process	Cellular Origin	Tissue Specificity
Myosin-9	Cytokinesis, cell shape, and specialized functions	Structural	Cytoplasm, myosin complex, nucleus	Kidney, leucocytes
Parvalbumin alpha	Involved in muscle relaxation, calcium-binding	Signaling	Others	Muscle
PDZ and LIM domain protein 1	Adapter, response to oxidative stress, zinc-binding	Structural	Cytoplasm, cytoskeleton	Heart, skeletal muscle, not PBMC
Peroxiredoxin-6	Redox regulation of the cell, lipid degradation	Metabolism	Cytoplasm, lysosome, cytoplasmic vesicle	
Plasma retinol-binding protein	Transport of retinol from the liver, interacts with transthyretin	Transport	Secreted	
Plastin-2	Actin-binding protein, calcium-binding	Structural	Cytoplasm	Spleen and other lymph node-containing organs
Pleckstrin	Kinase C substrate of platelets, signaling	Signaling	Intracellular	Platelets
Protein disulfide-isomerase A3 Protein disulfide-isomerase A6	Rearrangement of -S-S- bonds in proteins, signal transduction, redox homeostasis	Signaling	ER lumen, melanosome	
Purine nucleoside phosphorylase	Glycosyltransferase, DNA modification	Others	Cytoplasm	
Rab GDP dissociation inhibitor beta Rho GDP-dissociation inhibitor 1	Regulates the GDP/GTP exchange reaction of most Rab proteins, protein transport, signal transduction	Signaling	Cytoplasm, membrane	Ubiquitous
Serotransferrin	Iron transport	Transport	Secreted	Liver, plasma
Serum albumin	Regulation of osmotic pressure, transport	Transport	Secreted	Plasma
Serum amyloid P-component	Interaction with DNA, scavenger molecule	Structural	Secreted	Serum, urine
T-complex protein 1 subunit beta	Molecular chaperone	Chaperone	Cytoplasm	
Transthyretin	Thyroid hormone-binding protein	Transport	Secreted	Liver, choroid plexus
Tropomyosin alpha-3 chain	Muscle contraction, stabilizing cytoskeleton, actin binding	Structural	Cytoplasm, cytoskeleton	Muscle
Tropomyosin alpha-4 chain	Muscle contraction, stabilizing cytoskeleton, actin binding	Structural	Cytoplasm, cytoskeleton	Muscle
Tubulin alpha-ubiquitous chain Tubulin beta chain Tubulin beta-1 chain	Major constituent of microtubules, cell motion, protein polymerization	Structural	Microtubule	Spleen, thymus, immature brain
Vimentin	Class-III intermediate filaments, host-virus interaction	Structural	Intermediate filament	Fibroblasts, lymphocytes
Vinculin	Cell adhesion	Structural	Cytoplasm, cytoskeleton, cell membrane	Muscle
Vitamin D-binding protein	Vitamin D binding, prevents actin polymerization	Transport	Secreted	
Vitronectin	Cell-to-substrate adhesion , protease-inhibiting activity	Inflammation	Secreted	Plasma
WD repeat protein 1	Disassembly of actin filaments	Structural	Cytoplasm, cytoskeleton	
Zinc finger protein	Cell shape, cytoskeleton	Structural	Cytoplasm, cytoskeleton	
Zinc-alpha-2-glycoprotein	Stimulates lipid degradation	Lipid Metabolism	Secreted	Blood plasma, seminal plasma, urine, saliva, sweat, epithelial cells, liver

Details about proteins included into the plasma and PBMC database. Given is their function, cellular origin, tissue specificity and involvement in biological processes according to SwissProt.

Table 6-2 Identified proteins in the plasma database

Spot #	SwissProt #	Protein Name	Score	Sequence Coverage	MW [kDa]	p*I*
1	P02766	Transthyretin	96	55	16.0	5.4
2	P02753	Plasma retinol-binding protein	83	40	23.3	5.7
3	P02647	Apolipoprotein A-I	132	46	30.8	5.5
4	P02647	Apolipoprotein A-I	98	42	30.8	5.5
5	P02743	Serum amyloid P-component	87	31	25.5	6.1
6	P02647	Apolipoprotein A-I	79	36	30.8	5.5
8	P01834	Ig kappa chain C region	61	48	11.8	5.5
9	P01834	Ig kappa chain C region	88	80	11.8	5.5
10	P01834	Ig kappa chain C region	58	48	11.8	5.5
12	P00738	Haptoglobin	76	25	11.8	10.1
13	P06727	Apolipoprotein A-IV	209	49	45.4	5.2
14	O43866	CD5 antigen-like	138	49	39.6	5.2
15	P02679	Fibrinogen gamma chain	164	56	52.1	5.3
16	P02679	Fibrinogen gamma chain	168	50	52.1	5.3
17	P02679	Fibrinogen gamma chain	73	39	52.1	5.3
19	P02765	Alpha-2-HS-glycoprotein	55	22	40.1	5.4
20	P02765	Alpha-2-HS-glycoprotein	80	22	40.1	5.4
21	P02765	Alpha-2-HS-glycoprotein	79	19	40.1	5.4
22	P02765	Alpha-2-HS-glycoprotein	70	22	40.1	5.4
23	P01011	Alpha-1-antichymotrypsin	115	33	47.8	5.2
24	P01011	Alpha-1-antichymotrypsin	139	41	47.8	5.2
25	P01011	Alpha-1-antichymotrypsin	146	40	47.8	5.2
26	P01011	Alpha-1-antichymotrypsin	106	45	47.8	5.2
27	P01011	Alpha-1-antichymotrypsin	73	31	47.8	5.2
28	P01042	Kininogen-1	74	15	73.0	6.4
29	P01042	Kininogen-1	128	20	73.0	6.4
30	P01042	Kininogen-1	132	20	73.0	6.4
31	P01042	Kininogen-1	124	25	73.0	6.4
32	P01042	Kininogen-1	113	20	73.0	6.4
34	Q5JSP0	Zinc finger protein	65	12	80.2	5.7
35	P01019	Angiotensinogen	70	15	53.4	5.9
36	P01019	Angiotensinogen	113	35	53.4	5.9
37	P01019	Angiotensinogen	85	18	53.4	5.9
38	P01008	Antithrombin-III	69	23	53.0	6.3
39	P01008	Antithrombin-III	149	35	53.0	6.3
40	P01009	Alpha-1-antitrypsin	196	46	46.9	5.3
41	P02774	Vitamin D-binding protein	116	33	54.5	5.3
42	P01009	Alpha-1-antitrypsin	216	44	46.9	5.3
43	P01009	Alpha-1-antitrypsin	311	62	46.9	5.3
44	P01009	Alpha-1-antitrypsin	74	27	46.9	5.3
45	P02774	Vitamin D-binding protein	161	39	54.5	5.3
46	P02774	Vitamin D-binding protein	259	49	54.5	5.3
49	P01019	Angiotensinogen	85	32	53.4	5.9
50	P01008	Antithrombin-III	148	40	53.0	6.3
51	P01008	Antithrombin-III	62	17	53.0	6.3
53	P04217	Alpha-1B-glycoprotein	81	25	54.8	5.5
54	P04217	Alpha-1B-glycoprotein	145	29	54.8	5.5
55	P04217	Alpha-1B-glycoprotein	164	29	54.8	5.5
56	P04217	Alpha-1B-glycoprotein	92	25	54.8	5.5
57	P02790	Hemopexin	131	37	52.4	6.6
58	P02790	Hemopexin	138	38	52.4	6.6
59	P02790	Hemopexin	115	31	52.4	6.6
60	P02790	Hemopexin	90	30	52.4	6.6
61	P02790	Hemopexin	94	29	52.4	6.6
62	P02790	Hemopexin	60	22	52.4	6.6
63	P02768	Serum albumin	274	44	71.3	5.9

Spot #	SwissProt #	Protein Name	Score	Sequence Coverage	MW [kDa]	p*I*
64	P02768	Serum albumin	143	39	71.3	5.9
65	P02768	Serum albumin	229	35	71.3	5.9
66	P02790	Hemopexin	101	24	52.4	6.6
67	P02768	Serum albumin	235	37	71.3	5.9
68	P02768	Serum albumin	269	51	71.3	5.9
69	P04003	C4b-binding protein alpha chain	105	27	69.0	7.9
70	P06396	Gelsolin	117	30	86.0	5.9
71	P00751	Complement factor B	65	15	86.8	6.7
72	P00751	Complement factor B	93	20	86.8	6.7
73	P00751	Complement factor B	70	23	86.8	6.7
74	P00751	Complement factor B	75	16	86.8	6.7
75	P02749	Beta-2-glycoprotein 1	99	35	39.6	9.5
76	P02749	Beta-2-glycoprotein 1	62	33	39.6	9.5
77	P02675	Fibrinogen beta chain	56	11	56.6	9.3
78	P02749	Beta-2-glycoprotein 1	122	42	39.6	9.5
79	P02675	Fibrinogen beta chain	143	36	56.6	9.3
80	P02675	Fibrinogen beta chain	179	45	56.6	9.3
81	P02790	Hemopexin	92	28	52.4	6.6
82	P04003	C4b-binding protein alpha chain	91	30	69.0	7.9
83	P01871	Ig mu chain C region	93	23	50.2	6.4
84	P02787	Serotransferrin	161	22	79.3	7
85	P02787	Serotransferrin	229	36	79.3	7
86	P02787	Serotransferrin	89	22	79.3	7
88	P09871	Complement C1s subcomponent	137	33	78.2	4.7
90	P43652	Afamin	71	21	71.0	5.6
91	P43652	Afamin	124	18	71.0	5.6
92	P43652	Afamin	127	30	71.0	5.6
93	P02774	Vitamin D-binding protein	220	48	54.5	5.3
95	P13671	Complement component C6	109	15	108.4	6.3
96	P13671	Complement component C6	123	18	108.4	6.3
97	P13671	Complement component C6	93	19	108.4	6.3
98	P04004	Vitronectin	56	17	55.1	5.5
99	P04004	Vitronectin	91	23	55.1	5.5
100	P25311	Zinc-alpha-2-glycoprotein	90	27	34.1	5.5
104	P00738	Haptoglobin	130	25	45.9	10.1
105	P25311	Zinc-alpha-2-glycoprotein	71	28	34.1	5.5
106	P25311	Zinc-alpha-2-glycoprotein	197	50	34.1	5.5
108	P00738	Haptoglobin	63	18	45.9	10.1
109	P06727	Apolipoprotein A-IV	229	52	45.4	5.2
112	P00738	Haptoglobin	73	23	45.9	10.1
113	P02748	Complement component C9	124	28	64.6	5.3
114	P02748	Complement component C9	145	25	64.6	5.3
115	P01871	Ig mu chain C region	139	23	50.2	6.4
116	P01871	Ig mu chain C region	121	23	50.2	6.4
117	P01871	Ig mu chain C region	121	30	50.2	6.4
118	P01871	Ig mu chain C region	106	31	50.2	6.4
119	P01871	Ig mu chain C region	139	23	50.2	6.4

Data from analysed and identified protein spots of plasma including SwissProt identification, MASCOT score (scores above 54 are significant), sequence coverage and the theoretical molecular weight and isoelectric point.

Table 6-3 Identified proteins in the PBMC database

Spot #	SwissProt #	Protein Name	Score	Sequence Coverage	MW [kDa]	p*I*
1	P18206	Vinculin	228	32	124.29	5.4
2	P06396	Gelsolin	124	27	86.04	5.86
3	P06396	Gelsolin	140	23	86.04	5.86
4	P00488	Coagulation factor XIII A chain	136	32	83.73	5.7
5	P02768	Serum albumin	214	45	71.32	5.9
6	P02768	Serum albumin	366	50	71.32	5.9
7	P02768	Serum albumin	294	50	71.32	5.9
8	P02768	Serum albumin	116	30	71.32	5.9
12	P02787	Serotransferrin	222	38	79.28	7
13	P02787	Serotransferrin	166	27	79.28	7
14	P26038	Moesin	67	20	67.89	6
15	P02787	Serotransferrin	185	31	79.28	7
16	P02787	Serotransferrin	151	25	79.28	7
17	P26038	Moesin	93	15	67.89	6
18	P11142	Heat shock 70 kDa protein	104	24	71.08	5.2
20	P08107	Heat shock 70 kDa protein 1	138	25	70.28	5.4
23	P30101	Protein disulfide-isomerase A3	185	41	57.146	5.9
25	P02679	Fibrinogen gamma chain	177	47	52.11	5.3
26	P02679	Fibrinogen gamma chain	149	56	52.11	5.3
27	P61158	Actin-like protein 3	197	46	47.8	5.5
28	P60709	Beta-actin	139	44	42.05	5.2
29	P63261	Gamma-actin	135	46	42.11	5.2
30	P60709	Beta-actin	111	42	42.05	5.2
31	P06727	Apolipoprotein A-IV	194	50	45.37	5.2
32	P02679	Fibrinogen gamma chain	201	54	52.11	5.3
33	P30101	Protein disulfide-isomerase A6	193	50	48.49	4.8
35	P07437	Tubulin beta chain	199	49	50.1	4.6
36	P01009	Alpha-1-antitrypsin	190	38	46.88	5.3
37	P01009	Alpha-1-antitrypsin	124	26	46.88	5.3
38	P11021	78 kDa Glucose-regulated protein	115	28	72.4	4.9
42	P62258	14-3-3 Protein epsilon	102	32	29.33	4.6
43	P67936	Tropomyosin alpha-4 chain	147	44	28.62	4.5
44	P06753	Tropomyosin alpha-3 chain	73	20	32.86	4.5
46	P61981	14-3-3 Protein gamma	76	46	28.46	4.7
47	P31946	14-3-3 Protein zeta/delta	56	18	27.9	4.6
48	P63104	14-3-3 Protein zeta/delta	142	56	27.9	4.6
49	P60709	Beta-actin	90	29	42.05	5.2
50	P63261	Gamma-actin	120	38	42.11	5.2
51	O00299	Chloride intracellular channel protein 1	160	60	27.25	4.9
53	P02647	Apolipoprotein A-I	118	42	30.76	5.5
54	P02647	Apolipoprotein A-I	173	54	30.76	5.5
55	P19105	Myosin regulatory light chain 2	54	40	19.84	4.5
56	P19105	Myosin regulatory light chain 2	63	50	19.84	4.5
59	P60660	Myosin light polypeptide 6	65	33	17.09	4.4
61	P02647	Apolipoprotein A-I	135	45	30.76	5.5
62	P09211	Glutathione S-transferase P	60	38	23.57	5.3
64	P78417	Glutathione transferase omega-1	61	16	27.83	6.3
67	P30041	Peroxiredoxin-6	147	61	25.13	6
68	P47756	F-actin capping protein subunit beta	137	42	31.62	5.2
69	P07195	L-lactate dehydrogenase B chain	126	35	36.9	5.7
70	P47755	F-actin capping protein subunit alpha-2	138	55	33.16	5.5
73	P52907	F-actin capping protein subunit alpha-1	102	41	33.07	5.4
75	P02768	Serum albumin	164	44	71.32	5.9
76	P02768	Serum albumin	190	24	71.32	5.9
77	P02768	Serum albumin	76	22	71.32	5.9
78	P08567	Pleckstrin	82	32	40.47	9.3

Spot #	SwissProt #	Protein Name	Score	Sequence Coverage	MW [kDa]	p*I*
79	Q9ULV4	Coronin-1C	73	18	53.9	6.7
80	P08567	Pleckstrin	73	32	40.47	9.3
83	P30740	Leukocyte elastase inhibitor	107	30	42.83	5.9
87	P35579	Myosin-9	84	7	227.65	5.4
88	P61160	Actin-like protein 2	137	38	45.02	6.3
89	O00151	PDZ and LIM domain protein 1	136	43	36.51	6.6
90	P26641	Elongation factor 1-gamma	91	16	50.43	6.3
92	P50395	Rab GDP dissociation inhibitor beta	237	56	51.09	10.1
93	P31146	Coronin-1A	65	18	51.68	6.3
95	P78371	T-complex protein 1 subunit beta	130	30	57.79	6
96	P78371	T-complex protein 1 subunit beta	113	31	57.79	6
100	P02675	Fibrinogen beta chain	185	50	56.58	9.3
101	P02768	Serum albumin	74	15	71.32	5.9
102	P02675	Fibrinogen beta chain	216	43	56.58	9.3
103	P02675	Fibrinogen beta chain	197	42	56.58	9.3
105	O75083	WD repeat protein 1	143	34	66.84	6.2
106	O75083	WD repeat protein 1	119	35	66.84	6.2
109	P02766	Transthyretin	58	32	15.99	5.4
110	P20472	Parvalbumin alpha	59	52	12.05	4.8
111	O15511	Actin-related protein 2/3 complex subunit 5	59	45	16.37	5.3
117	P08670	Vimentin	133	38	53.68	4.9
118	P08670	Vimentin	125	37	53.68	4.9
119	P68363	Tubulin alpha-ubiquitous chain	90	31	50.8	4.8
120	P02768	Serum albumin	221	47	71.32	5.9
121	P02768	Serum albumin	190	48	71.32	5.9
122	P02768	Serum albumin	280	47	71.32	5.9
123	P02768	Serum albumin	166	40	71.32	5.9
125	P02751	Fibronectin	83	7	266.03	5.4
126	P02751	Fibronectin	71	6	266.03	5.4
127	P02751	Fibronectin	65	5	266.03	5.4
129	P11413	Glucose-6-phosphate 1-dehydrogenase	133	33	59.68	6.4
130	P11413	Glucose-6-phosphate 1-dehydrogenase	136	31	59.68	6.4
131	P13645	Keratin, type I cytoskeletal 10	56	15	59.71	5
132	P52565	Rho GDP-dissociation inhibitor 1	89	31	23.25	4.9
145	P78417	Glutathione transferase omega-1	110	43	27.83	6.3
146	P00491	Purine nucleoside phosphorylase	129	52	32.33	6.5
147	P00491	Purine nucleoside phosphorylase	179	65	32.33	6.5
149	P13796	Plastin-2	206	43	70.82	10.1
154	P60842	Eukaryotic initiation factor 4A-I	96	31	46.35	5.2
156	Q9H4B7	Tubulin beta-1 chain	86	36	50.87	4.9
157	Q9H4B7	Tubulin beta-1 chain	120	24	50.87	4.9
158	P68363	Tubulin alpha-ubiquitous chain	108	35	50.8	4.8
160	Q9H4B7	Tubulin beta-1 chain	153	40	50.87	4.9
161	P02768	Serum albumin	128	25	71.32	5.9
167	P02768	Serum albumin	108	37	71.32	5.9
168	P02768	Serum albumin	176	33	71.32	5.9
170	P12814	Alpha-actinin-1	102	16	103.56	10.1
173	P08238	Heat shock protein HSP 90-beta	104	23	83.55	4.8
174	P07900	Heat shock protein HSP 90-alpha	193	35	85.01	4.8
175	P12814	Alpha-actinin-1	391	56	103.56	10.1
176	P12814	Alpha-actinin-1	257	48	103.56	10.1
177	P12814	Alpha-actinin-1	133	26	103.56	10.1
178	P12814	Alpha-actinin-1	96	12	103.56	10.1
179	P12814	Alpha-actinin-1	54	9	103.56	10.1

Data from analysed and identified protein spots of PBMC including SwissProt identification, MASCOT score (scores above 54 are significant), sequence coverage and the theoretical molecular weight and isoelectric point.

Table 6-4 Characteristics of the 11 volunteers included into the trial

Volunteer	Age [years]	Height [m]	Weight [kg]	BMI [kg/cm2]	Sports/Week	Creatinine [mg/dl]
1	27	1.8	87	26.85	6-7 hrs	0.97
2	29	1.73	62	20.72	-	0.87
3	25	1.76	65	20.98	4-5 hrs	0.86
4	27	1.77	72	22.98	-	1.1
5	18	1.8	66	20.37	11 hrs	0.92
6	26	1.76	70	22.60	-	0.96
7	25	1.85	102	29.80	7 hrs	0.93
8	30	1.88	77	21.79	1 h	0.86
9	46	1.67	89	31.91	1 h	0.71
10	26	1.77	76	24.26	-	0.88
11	41	1.88	75	21.22	-	0.92

Table 6-5 Regulated spots in plasma during the baseline of the trial

Label	ID Database	Mean Volume BL1	Coefficient of Var. BL1	Mean Volume BL2	Coefficient of Var. BL2	Ratio BL2/BL1	t-Test
ID128196	106	0.15	15.83	0.37	46.44	2.55	99.89
ID128243	n.n.	0.06	34.60	0.03	47.84	0.48	99.87
ID128266	n.n.	0.06	38.37	0.03	46.43	0.47	99.79
ID128147	12	0.19	31.70	0.09	39.89	0.45	99.98
Label	**ID Database**	**Mean Volume BL1**	**Coefficient of Var. BL1**	**Mean Volume BL3**	**Coefficient of Var. BL3**	**Ratio BL3/BL1**	**t-Test**
ID127825	55	0.21	71.56	0.48	12.41	2.25	99.99
ID128001	46	1.11	36.08	0.55	70.54	0.50	99.20

List of regulated spots in plasma during the baseline (BL2 + BL3 vs. BL1) in the trial. Labels are given in the Delta2D software as well as mean volumes and coefficients of variation. IDs are derived from the PBMC database. All values are derived from 10 gels for each time point (1 gel for each volunteer). n.n.: protein could not be identified.

Table 6-6 Regulated spots in plasma during the high-protein intake phase of the trial

Label	ID Database	Mean Volume BL	Coefficient of Var. BL	Mean Volume HP1	Coefficient of Var. HP1	Ratio HP1/BL	t-Test
ID127793	90	0.04	53.64	0.08	72.11	2.10	99.79
ID127837	58	0.19	115.20	0.39	89.91	2.09	96.02
ID127750	86	0.21	74.85	0.44	46.76	2.04	99.86
Label	**ID Database**	**Mean Volume BL**	**Coefficient of Var. BL**	**Mean Volume HP2**	**Coefficient of Var. HP2**	**Ratio HP2/BL**	**t-Test**
ID127750	85	0.21	74.85	0.84	35.29	3.94	100.00
ID127945	78	0.03	100.06	0.11	166.16	3.89	97.33
ID127821	60	0.23	81.33	0.63	39.25	2.74	100.00
ID128186	105	0.36	89.05	0.84	54.39	2.30	99.87
ID127689	72	0.57	28.65	0.24	68.20	0.43	100.00

List of regulated spots in plasma during the high-protein phase (HP1 + HP2 vs. BL) in the trial. Labels are given in the Delta2D software as well as mean volumes and coefficients of variation. IDs are derived from the PBMC database. All values are derived from 10 gels for HP1 and HP2 and 30 gels for BL (1 gel per volunteer for each time point; BL1 + BL2 + BL3 taken together as BL).

Table 6-7 Regulated spots in plasma during the wash-out phase of the trial

Label	ID Database	Mean Volume BL	Coefficient of Var. BL	Mean Volume WO1	Coefficient of Var. WO1	Ratio WO1/BL	t-Test
ID128048	45	0.24	109.92	0.54	33.08	2.28	99.81
ID128635	4	0.09	81.57	0.18	58.90	2.05	99.54

List of regulated spots in plasma during the wash-out period (WO1 vs. BL) in the trial. Labels are given in the Delta2D software as well as mean volumes and coefficients of variation. IDs are derived from the PBMC database. All values are derived from 10 gels for WO1 and 30 gels for BL (1 gel per volunteer for each time point; BL1 + BL2 + BL3 taken together as BL).

Table 6-8 Regulated spots in PBMC during the baseline period of the trial

Label	ID Database	Mean Volume BL1	Coefficient of Var. BL1	Mean Volume BL2	Coefficient of Var. BL2	Ratio BL2/BL1	t-Test
ID802	n.n.	0.16	32.03	0.35	56.41	2.16	97.32
Label	**ID Database**	**Mean Volume BL1**	**Coefficient of Var. BL1**	**Mean Volume BL3**	**Coefficient of Var. BL3**	**Ratio BL3/BL1**	**t-Test**
ID1014	n.n.	0.03	39.09	0.01	52.04	0.48	99.03
ID1027	n.n.	0.04	33.48	0.02	87.88	0.48	97.79

List of regulated spots in PBMC during the baseline (BL2 + BL3 vs. BL1) in the trial. Labels are given in the Delta2D software as well as mean volumes and coefficients of variation. IDs are derived from the PBMC database. All values are derived from 10 gels for each time point (1 gel for each volunteer). n.n.: protein could not be identified.

Table 6-9 Regulated spots in PBMC during the high-protein intake phase of the trial

Label	ID Database	Mean Volume BL	Coefficient of Var. BL	Mean Volume HP1	Coefficient of Var. HP1	Ratio HP1/BL	t-Test
ID343	149	0.09	40.01	0.28	37.01	2.98	100.00
ID1124	n.n.	0.15	41.26	0.43	38.34	2.88	100.00
ID154	176	0.04	52.89	0.10	78.51	2.46	99.77
ID1052	49	0.13	55.41	0.30	53.43	2.36	99.98
Label	**ID Database**	**Mean Volume BL**	**Coefficient of Var. BL**	**Mean Volume HP2**	**Coefficient of Var. HP2**	**Ratio HP2/BL**	**t-Test**
ID658	n.n.	0.05	81.00	0.10	38.35	2.13	99.75
ID763	75	0.37	46.68	0.17	42.68	0.45	99.61

List of regulated spots in PBMC during the high-protein phase (HP1 + HP2 vs. BL) in the trial. Labels are given in the Delta2D software as well as mean volumes and coefficients of variation. IDs are derived from the PBMC database. All values are derived from 10 gels for HP1 and HP2 and 30 gels for BL (1 gel per volunteer for each time point; BL1 + BL2 + BL3 taken together as BL). n.n.: protein could not be identified.

Table 6-10 Regulated spots in PBMC during the wash-out phase of the trial

Label	ID Database	Mean Volume BL	Coefficient of Var. BL	Mean Volume WO1	Coefficient of Var. WO1	Ratio WO1/BL	t-Test
ID276	3	0.21	51.50	0.48	37.74	2.26	100.00
ID248	2	0.06	56.40	0.13	43.61	2.10	99.96
ID802	n.n.	0.27	61.34	0.57	76.32	2.09	99.02
ID848	89	0.18	64.35	0.36	15.14	2.01	99.99
ID1052	49	0.13	55.41	0.06	64.03	0.49	98.34
ID428	n.n.	0.22	51.03	0.11	53.11	0.47	99.28
ID475	130	0.32	72.72	0.10	37.39	0.30	99.09
ID462	n.n.	0.41	73.17	0.12	31.99	0.30	99.07
Label	**ID Database**	**Mean Volume BL**	**Coefficient of Var. BL**	**Mean Volume WO2**	**Coefficient of Var. WO2**	**Ratio WO2/BL**	**t-Test**
ID1031	50	0.12	96.52	0.24	71.72	2.07	96.91
ID475	130	0.06	82.57	0.02	44.77	0.37	95.24

List of regulated spots in PBMC during the wash-out period (WO1 + WO2 vs. BL) in the trial. Labels are given in the Delta2D software as well as mean volumes and coefficients of variation. IDs are derived from the PBMC database. All values are derived from 10 gels for WO1 and WO2 and 30 gels for BL (1 gel per volunteer for each time point; BL1 + BL2 + BL3 taken together as BL). n.n.: protein could not be identified.

Table 6-11 Identification of regulated protein spots in PBMC not listed in the database

Spot ID	SwissProt #	Protein Name	Score	Sequence Coverage	MW [kDa]	p*I*
ID343	P20700	Lamin-B1	150	40	66.65	5.1
ID1124	P60709	Beta-actin	72	16	42.05	5.2
ID658	P61163	Alpha-centractin	85	5	42.70	6.4
ID802	P50148	Guanine nucleotide-binding protein	110	43	41.00	5.0
ID428	P02768	Serum albumin precursor	163	36	71.32	5.9
ID462	P02675	Fibrinogen beta chain	83	26	56.6	9.3

Mass spectrometric analysis of regulated spots in PBMC during the protein intervention study. Analysis was undertaken using MALDI-TOF-MS/MS, spots are identified at least 3 times. Given is the spot ID from the Delta2D analysis, the SwissProt ID, the protein name, score, sequence coverage and theoretical molecular weight and p*I* as found in the MASCOT database.

Table 6-12 Plasma amino acids in the trial

Analyte	BL1	BL2	BL3	HP1	HP2	WO1	WO2	HP *vs.* BL
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	p-Value
L-Glutamine	613.1 ± 59.0	630.5 ± 53.4	595.6 ± 63.1	606.5 ± 61.3	581.5 ± 74.7	587.7 ± 46.9	612.8 ± 44.7	0.253
L-Alanine	336.1 ± 73.4	329.2 ± 78.5	335.4 ± 81.2	346.5 ± 65.2	331.0 ± 58.4	356.1 ± 69.7	356.3 ± 52.8	0.757
Glycine	218.9 ± 5.02	215.9 ± 23.1	207.3 ± 43.9	172.4[c] ± 20.0	164.0[c] ± 21.1	209.2 ± 15.1	228.0 ± 35.6	<0.001
L-Valine	226.9 ± 7.07	227.6 ± 26.0	214.4 ± 23.0	269.9[c] ± 21.1	266.5[c] ± 32.3	221.2 ± 24.0	216.3 ± 17.1	<0.001
L-Lysine	163.8 ± 3.04	154.1 ± 24.1	145.9 ± 23.0	179.6[b] ± 27.1	161.7 ± 19.0	145.3 ± 18.0	148.5 ± 20.0	0.028
L-Proline	186.5 ± 35.8	201.1 ± 60.7	207.3 ± 62.5	315.3[c] ± 88.6	243.0[b] ± 49.0	172.1 ± 36.9	183.5 ± 37.0	<0.001
L-Threonine	121.7 ± 1.07	115.2 ± 11.0	113.5 ± 19.0	115.0 ± 19.0	92.7[c] ± 15.0	111.3 ± 8.0	126.0 ± 22.1	0.010
L-Leucine	134.1 ± 21.0	121.0 ± 44.8	126.8 ± 19.0	134.9 ± 14.0	137.6 ± 16.0	133.7 ± 12.1	129.4 ± 12.1	0.253
L-Serine	113.0 ± 37.7	102.5 ± 18.0	107.8 ± 41.9	82.7[a] ± 9.1	76.2[b] ± 10.0	94.4 ± 14.1	99.0 ± 15.1	0.002
L-Histidine	83.80 ± 1.10	80.03 ± 1.05	74.82 ± 9.09	76.64 ± 1.06	72.28[a] ± 8.09	74.85 ± 1.06	78.44 ± 1.10	0.023
L-Ornithine	90.52 ± 6.08	62.49 ± 1.10	69.15 ± 9.54	59.78 ± 5.04	53.45[b] ± 1.09	56.67[a] ± 1.08	54.58[a] ± 1.09	0.002
L-Asparagine	63.05 ± 7.03	60.71 ± 1.08	58.23 ± 1.09	52.41[a] ± 9.11	49.94[b] ± 1.10	72.02[b] ± 1.03	68.63[a] ± 0.10	<0.001
L-Isoleucine	68.48 ± 1.09	69.13 ± 1.06	66.96 ± 1.12	67.84 ± 1.06	75.31 ± 1.10	75.38[a] ± 1.06	69.76 ± 1.08	0.162
L-Cystine	79.27 ± 1.12	84.63 ± 3.01	82.70 ± 1.12	90.08 ± 5.26	84.25 ± 1.11	86.36 ± 1.12	91.57[b] ± 1.12	0.028
Taurine	52.25 ± 2.09	47.39 ± 1.05	49.57 ± 7.68	43.48 ± 1.06	38.89[a] ± 1.07	43.27 ± 1.06	48.86 ± 1.09	0.009
L-Tyrosine	63.50 ± 5.25	61.09 ± 1.05	64.50 ± 3.96	68.23 ± 1.11	61.59 ± 1.09	61.27 ± 1.07	66.25 ± 1.10	0.489
L-Phenylalanine	61.34 ± 2.12	61.46 ± 1.08	60.48 ± 1.12	59.60 ± 1.10	57.85 ± 1.10	58.72 ± 1.08	61.38 ± 1.11	0.162
L-Arginine	49.79 ± 5.90	70.75 ± 3.95	58.55 ± 5.38	66.47 ± 6.77	62.68 ± 4.74	64.99 ± 4.94	72.68[a] ± 3.24	0.253
L-Tryptophane	53.20 ± 7.03	50.52 ± 1.05	51.40 ± 1.06	50.58 ± 1.06	47.87 ± 1.07	52.04 ± 1.06	52.25 ± 1.06	0.162
ß-Alanine	24.45 ± 5.00	22.25 ± 1.03	24.88 ± 1.03	25.36 ± 1.03	26.18 ± 3.05	28.38[b] ± 4.08	26.70 ± 1.06	0.162
L-Citrulline	29.37 ± 1.05	29.90 ± 1.04	28.05 ± 1.06	31.22 ± 1.03	31.70 ± 1.04	27.54 ± 1.04	30.59 ± 1.04	0.013
L-Methionine	26.55 ± 1.04	25.39 ± 1.01	23.66 ± 1.04	23.62 ± 1.02	20.45[c] ± 1.02	18.87[c] ± 1.01	19.63[c] ± 1.02	0.003
L-Glutamicacid	26.76 ± 4.47	23.19 ± 4.79	28.41 ± 5.58	29.26 ± 3.84	28.24 ± 0.94	28.45 ± 8.63	27.33 ± 6.88	0.348
L-α-Amino-n-Butyricacid	18.56 ± 1.05	17.62 ± 6.00	13.99 ± 1.02	16.03 ± 1.03	19.93 ± 1.04	17.22 ± 1.05	17.74 ± 1.02	0.368
Ethanolamine	9.10 ± 1.07	1.08 ± 0.75	1.08 ± 0.94	1.07[a] ± 1.02	1.07[a] ± 1.01	1.08 ± 0.52	9.03 ± 0.97	0.002
Hydroxyproline	8.02 ± 1.01	1.10 ± 1.04	1.06 ± 1.01	1.07 ± 1.01	1.10 ± 1.04	1.08 ± 1.02	1.10 ± 1.05	0.520
1-Methyl-L-Histidine	1.05 ± 1.03	8.00 ± 1.08	1.04 ± 1.02	1.01 ± 0.50	4.01 ± 1.02	1.05 ± 1.04	1.04 ± 1.03	0.084
3-Methyl-L-Histidine	1.04 ± 0.94	1.04 ± 0.66	1.03 ± 0.62	1.03 ± 0.82	1.03[a] ± 1.01	1.04 ± 1.00	1.04 ± 1.04	0.018
o-Phospho-ethanolamine	1.02 ± 1.10	1.02 ± 0.49	1.02 ± 1.01	1.02 ± 0.65	1.01 ± 0.77	1.02 ± 0.31	1.02 ± 0.76	0.061
γ-Amino-n-Butyricacid	1.02 ± 0.88	0.84 ± 0.74	0.23 ± 0.18	1.01 ± 0.78	1.01 ± 0.41	1.01 ± 0.25	1.01 ± 0.78	0.078
Sarcosine	1.01 ± 0.33	1.01 ± 0.27	1.01 ± 0.98	1.01[a] ± 1.01	1.01[a] ± 0.99	1.01 ± 0.71	1.01 ± 0.43	0.977
Cystathione	1.01 ± 0.42	2.07 ± 0.61	1.01 ± 0.46	1.01 ± 0.56	1.01[a] ± 0.48	1.01[a] ± 0.52	1.08[c] ± 0.24	<0.001
L-Asparicacid	4.04 ± 1.04	1.01 ± 0.54	1.04 ± 7.04	1.02 ± 0.69	1.02 ± 1.01	1.01 ± 0.54	2.07 ± 0.64	0.385
L-α-Amino-Adipicacid	0.62 ± 0.56	1.00 ± 0.56	0.53 ± 0.50	1.01 ± 0.46	1.01 ± 0.34	1.01 ± 0.32	1.12 ± 0.20	<0.001
D,L-ß-Amino-Isobutyricacid	1.01 ± 0.90	1.01 ± 1.00	0.87 ± 0.81	1.01 ± 0.52	1.01 ± 0.35	1.01 ± 0.54	1.01 ± 0.52	0.977

Amino acids in plasma measured with LC-MS/MS. Mean values of the 10 volunteers and the standard deviation are given in µmol/l. The p-value is given for the high-protein time points compared to the baseline based on ANOVA analysis. Values with superscripts (a,b,c) indicate significant differences (a p<0.05, b p<0.01, c p<0.001) of the group compared to the baseline group (BL1+BL2+BL3). n.n.: amino acid concentration below the detection limit (<1 µmol/l).

Table 6-13 Urinary amino acids in the trial

Analyte	BL1	BL2	BL3	HP1	HP2	WO1	WO2	HP *vs.* BL
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	p-Value
L-Glutamine	0.78 ± 0.39	0.73 ± 0.23	0.74 ± 0.17	0.55^{a} ± 0.13	0.54^{a} ± 0.11	0.68 ± 0.18	0.72 ± 0.25	0.003
L-Alanine	0.32 ± 0.13	0.38 ± 0.18	0.40 ± 0.13	0.30 ± 0.11	0.27 ± 0.11	0.39 ± 0.15	0.46 ± 0.24	0.058
Glycine	1.01 ± 1.06	1.01 ± 0.94	1.01 ± 0.60	1.01 ± 0.58	1.05^{a} ± 0.49	1.01 ± 0.61	1.01 ± 0.48	0.024
L-Valine	0.07 ± 0.03	0.06 ± 0.02	0.07 ± 0.02	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.02	0.06 ± 0.01	0.551
L-Lysine	0.11 ± 0.06	0.14 ± 0.09	0.10 ± 0.05	0.09 ± 0.05	0.08 ± 0.03	0.10 ± 0.05	0.10 ± 0.05	0.034
L-Proline	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.102
L-Threonine	0.18 ± 0.07	0.17 ± 0.08	0.17 ± 0.06	0.14 ± 0.04	0.11^{a} ± 0.03	0.16 ± 0.04	0.18 ± 0.07	0.024
L-Leucine	0.05 ± 0.03	0.05 ± 0.01	0.05 ± 0.01	0.04^{a} ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.04 ± 0.01	0.073
L-Serine	0.65 ± 0.35	0.57 ± 0.14	0.60 ± 0.16	0.41^{b} ± 0.12	0.37^{b} ± 0.08	0.54 ± 0.14	0.58 ± 0.17	0.003
L-Histidine	1.01 ± 0.56	1.01 ± 0.50	1.09 ± 0.33	0.96 ± 0.34	0.90 ± 0.24	1.01 ± 0.35	1.04 ± 0.42	0.041
L-Ornithine	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.01	0.01^{a} ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.014
L-Asparagine	0.17 ± 0.07	0.18 ± 0.10	0.15 ± 0.03	0.12 ± 0.03	0.17 ± 0.05	0.18 ± 0.05	0.17 ± 0.07	0.845
L-Isoleucine	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.326
L-Cystine	0.15 ± 0.08	0.14 ± 0.03	0.16 ± 0.03	0.11^{a} ± 0.02	0.10^{b} ± 0.03	0.17 ± 0.06	0.16 ± 0.05	0.005
Taurine	0.50 ± 0.38	0.76 ± 0.73	0.78 ± 0.55	0.54 ± 0.40	0.58 ± 0.23	0.58 ± 0.26	0.54 ± 0.41	0.532
L-Tyrosine	0.16 ± 0.06	0.14 ± 0.04	0.16 ± 0.04	0.15 ± 0.04	0.13 ± 0.02	0.15 ± 0.04	0.16 ± 0.04	0.326
L-Phenylalanine	0.10 ± 0.03	0.10 ± 0.03	0.11 ± 0.03	0.08^{a} ± 0.02	0.07^{c} ± 0.02	0.10 ± 0.03	0.10 ± 0.04	<0.001
L-Arginine	0.04 ± 0.02	0.04 ± 0.04	0.04 ± 0.01	0.05 ± 0.03	0.03 ± 0.01	0.04 ± 0.02	0.04 ± 0.02	0.597
L-Tryptophane	0.12 ± 0.05	0.10 ± 0.03	0.12 ± 0.03	0.09^{a} ± 0.02	0.08^{a} ± 0.02	0.11 ± 0.03	0.11 ± 0.03	0.005
D,L-ß-Amino-isobutyricacid	0.17 ± 0.19	0.18 ± 0.21	0.13 ± 0.14	0.09 ± 0.05	0.13 ± 0.13	0.18 ± 0.20	0.22 ± 0.32	0.447
L-Citrulline	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.130
L-Methionine	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.01^{c} ± 0.00	0.01^{c} ± 0.00	0.01^{c} ± 0.00	0.001
L-Glutamicacid	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.02^{a} ± 0.00	0.02^{a} ± 0.00	0.03 ± 0.01	0.02 ± 0.01	0.014
L-α-Amino-n-Butyricacid	0.02 ± 0.02	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	0.678
Ethanolamine	0.69 ± 0.28	0.66 ± 0.12	0.79 ± 0.18	0.46^{c} ± 0.13	0.44^{c} ± 0.11	0.71 ± 0.20	0.76 ± 0.23	<0.001
Arginino-succiniacid	0.05 ± 0.02	0.04 ± 0.01	0.05 ± 0.02	0.03^{a} ± 0.01	0.03^{b} ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.003
1-Methyl-L-Histidine	1.01 ± 1.00	1.01 ± 1.01	0.78 ± 0.71	0.09 ± 0.07	0.36 ± 0.24	0.77 ± 0.72	0.44 ± 0.35	0.144
3-Methyl-L-Histidine	0.40 ± 0.11	0.47 ± 0.11	0.45 ± 0.08	0.33 ± 0.09	0.34^{b} ± 0.07	0.51^{b} ± 0.15	0.43 ± 0.10	0.001
o-Phospho-ethanolamine	0.07 ± 0.05	0.07 ± 0.02	0.08 ± 0.04	0.04^{b} ± 0.01	0.04^{a} ± 0.02	0.07 ± 0.02	0.07 ± 0.04	0.002
γ-Amino-n-Butyricacid	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01^{c} ± 0.00	0.01 ± 0.00	0.120
L-Carnosine	0.04 ± 0.02	0.08 ± 0.07	0.03 ± 0.01	0.03 ± 0.02	0.03 ± 0.03	0.04 ± 0.03	0.03 ± 0.01	0.845
L-Asparicacid	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.678
Cystathione	0.07 ± 0.06	0.04 ± 0.01	0.04 ± 0.03	0.03 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.04	0.878
L-α-Amino-adipicacid	0.05 ± 0.03	0.05 ± 0.04	0.04 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.05 ± 0.01	0.03 ± 0.01	0.024
Homocitrulline	0.04 ± 0.03	0.06 ± 0.04	0.04 ± 0.02	0.04 ± 0.03	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	0.539
δ-Hydroxylysine	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.539

Amino acids in urine corrected by osmolarity measured with LC-MS/MS. Mean values of the 10 volunteers and the standard deviation are given in mM/(Osm/kg). The p-value is given for the high-protein time points compared to the baseline based on ANOVA analysis. Values with superscripts $^{(a,b,c)}$ indicate significant differences (^{a} $p<0.05$, ^{b} $p<0.01$, ^{c} $p<0.001$) of the group compared to the baseline group (BL1+BL2+BL3). n.n.: amino acid concentration below the detection limit (<1 µmol/l).

7. References

1. **de Roos B** (2009) Nutrition proteomics and biomarker discovery. *Expert Rev Proteomics* 6: p. 349-351
2. **Scalbert A, Brennan L, Fiehn O, Hankemeier T, Kristal BS, van Ommen B, Pujos-Guillot E, Verheij E, Wishart S, and Wopereis S** (2009) Mass-spectrometry-based metabolomics: limitations and recommendations for future progress with particular focus on nutrition research. *Metabolomics* 5: p. 435-458
3. **van Ommen B, Keijer J, Kleemann R, Elliott R, Drevon CA, McArdle H, Gibney M, and Müller M** (2008) The challenges for molecular nutrition research 2: quantification of the nutritional phenotype. *Genes Nutr* 3: p. 51-59
4. **Rezzi S, Ramadan Z, Fay LB, and Kochhar S** (2007) Nutritional metabonomics: applications and perspectives. *J Proteome Res* 6: p. 513-525
5. **Afman L, and Müller M** (2006) Nutrigenomics: from molecular nutrition to prevention of disease. *J Am Diet Assoc* 106: p. 569-576
6. **Löffler G, Petrides, P. E.** (1998) Biochemie und Pathobiochemie. *Verlag S.* 6. Auflage. Berlin, Heidelberg, New York
7. **Tomé D, and Bos C** (2000) Dietary protein and nitrogen utilization. *J Nutr* 130: p. 1868S-1873S
8. **Lacroix M, Bos C, Léonil J, Airinei G, Luengo C, Daré S, Benamouzig R, Fouillet H, Fauquant J, Tomé D, and Gaudichon C** (2006) Compared with casein or total milk protein, digestion of milk soluble proteins is too rapid to sustain the anabolic postprandial amino acid requirement. *Am J Clin Nutr* 84: p. 1070-1079
9. **Layman DK** (2004) Protein quantity and quality at levels above the RDA improves adult weight loss. *J Am Coll Nutr* 23: p. 631S-636S
10. **Campbell B, Kreider RB, Ziegenfuss T, La Bounty P, Roberts M, Burke D, Landis J, Lopez H, and Antonio J** (2007) International Society of Sports Nutrition position stand: protein and exercise. *J Int Sports Soc Nutr* Sep 26: p. 8
11. **Bilsborough S, and Mann N** (2006) A review of issues of dietary protein intake in humans. *Int J Sport Nutr Exerc Metab* 16: p. 129-152
12. **Ulusoy E, and Eren B** (2006) Histological changes of liver glycogen storage in mice (Mus musculus) caused by high-protein diets. *Histol Histopathol* 21: p. 925-930
13. **Lowery LM, and Devia L** (2009) Dietary protein safety and resistance exercise: what do we really know? *J Int Soc Sports Nutr* Jan12: p. 3
14. **van Loon LJC, Saris WHM, Kruijshoop M, and Wagenmakers AJM** (2000) Maximizing postexercise muscle glycogen synthesis: carbohydrate supplementation and the application of amino acid or protein hydrolysate mixtures. *Am J Clin Nutr* 72: p. 106-111
15. **Promintzer M, and Krebs M** (2006) Effects of dietary protein on glucose homeostasis. *Curr Opin Clin Nutr Metab Care* 9: p. 463-468
16. **Linn T, Santosa B, Grönemeyer D, Aygen S, Scholz N, Busch M, and Bretzel RG** (2000) Effect of long-term dietary protein intake on glucose metabolism in humans. *Diabetologia* 43: p. 1257-1265
17. **Potier M, Darcel N, and Tomé D** (2009) Protein, amino acids and the control of food intake. *Curr Opin Clin Nutr Metab Care* 12: p. 54-58
18. **Tannous dit El Khoury D, Obeid O, Azar ST, and Hwalla N** (2006) Variations in postprandial ghrelin status following ingestion of high-carbohydrate, high-fat and high-protein meals in males. *A Nutr Metab* 50: p. 260-269
19. **Promitzer M, Krebs, M.** (2006) Effects of dietary protein on glucose homeostasis. *Curr Opin Clin Nutr Metab Care* 9: p. 463-468
20. **van Erk MJ, Blom WA, van Ommen B, and Hendriks HF** (2006) High-protein and high-carbohydrate breakfasts differentially change the transcriptome of human blood cells. *Am J Clin Nutr* 84: p. 1233-1241
21. **Verhoef P, van Vliet T, Olthof MR, and Katan MB** (2005) A high-protein diet increases postprandial but not fasting plasma total homocysteine concentrations: a dietary controlled, crossover trial in healthy volunteers. *Am J Clin Nutr* 82: p. 553-558

22. **Astrup A** (2005) The satieting power of protein-a key to obesity prevention? *Am J Clin Nutr* 82: p. 1-2
23. **Poortmans JR, and Dellalieux O** (2000) Do regular high protein diets have potential health risks on kidney function in athletes? *Int J Sport Nutr Exerc Metab* 10: p. 28-38
24. **Anderson NL, and Anderson NG** (2002) The human plasma proteome; history, character, and diagnosis prospects. *Mol Cell Proteomics* 1: p. 845-867
25. **Brand J, Haslberger T, Zolg W, Pestlin G, and Palme S** (2006) Depletion efficiency and recovery of trace markers from a multiparameter immunodepletion column. *Proteomics* 6: p. 3236-3242
26. **Ramirez-Boo M, Garrido JJ, Ogueta S, Calvete JJ, Gómez-Díaz C, and Moreno Á** (2006) Analysis of porcine peripheral blood mononuclear cells proteome by 2-DE and MS: Analytical and biological variability in the protein expression level and protein identification. *Proteomics* 6: p. S215-S225
27. **Schulz M, Dotzlaw H, Mikkat S, Eggert M, and Neeck G** (2007) Proteomic analysis of peripheral blood mononuclear cells: selective protein processing observed in patients with rheumatoid arthritis. *J Prot Res* 6: p. 3752-3759
28. **Hryniewicz-Jankowska A, Choudhary PK, and Goodman SR** (2007) Variation in the monocyte proteome. *Exp Biol Med* 232: p. 967-976
29. **Kussmann M, Raymond F, and Affolter M** (2006) OMICS-driven biomarker discovery in nutrition and health. *J Biotechnol* 124: p. 758-787
30. **Graves PR, and Haystead TA** (2003) Proteomics and the molecular biologist., in *Handbook of the Proteomic Methods*, Inc. HP, Totowa, NJ: p. 3-16.
31. **Anderson NL, and Anderson NG** (1998) Proteome and proteomics: New technologies, new concepts, and new words. *Electrophoresis* 19: p. 1853-1861
32. **Trayhurn P** (2000) Proteomics and nutrition- a science for the first decade of the new millennium. *Br J Nutr* 83: p. 1-2
33. **Gromov PS, Ostergaard M, Gromova I, and Celis JE** (2002) Human proteomic databases: a powerful resource for functional genomics in health and disease. *Prog Biophys Mol Biol* 80: p. 3-22
34. **Bouwens M, Afman LA, and Müller M** (2007) Fasting induces changes in peripheral blood mononuclear cell gene expression profiles related to increases in fatty acid β-oxidation: functional role of peroxisome proliferator-activated receptor a in human peripheral blood mononuclear cells. *Am J Clin Nutr* 86: p. 1515-1523
35. **Fuchs D, Vafeiadou K, Hall WL, Daniel H, Williams CM, Schroot JH, and Wenzel U** (2007) Proteomic biomarkers of peripheral blood mononuclear cells obtained from postmenopausal women undergoing an intervention with soy isoflavones. *Am J Clin Nutr* 86: p. 1369-1375
36. **Fuchs D, Piller R, Linseisen J, Daniel H, and Wenzel U** (2007) The human peripheral blood mononuclear cell proteome responds to a dietary flaxseed-intervention and proteins identified suggest a protective effect in atherosclerosis. *Proteomics* 7: p. 3278-3288
37. **de Roos B, Duthie SJ, Polley ACJ, Mulholland F, Bouwman FG, Heim C, Rucklidge GJ, Johnson IT, Mariman EC, Daniel H, and Elliott RM** (2008) Proteomics methodological recommendations for studies involving human plasma, platelets, and peripheral blood mononuclear cells. *J Proteome Res* 7: p. 2280-2290
38. **Nedelkov D, Kiernan UA, Niederkofler EE, Tubbs KA, and Nelson RW** (2005) Investigating diversity in human plasma proteins. *Proc Natl Acad Sci USA* 102: p. 10852-10857
39. **Jacobs JM, Adkins JN, Qian WJ, Liu T, Shen Y, Camp II DG, and Smith RD** (2005) Utilizing human blood plasma for proteomic biomarker discovery. *J Proteome Res* 4: p. 1073-1085
40. **Hsieh SY, Chen RK, Pan YH, and Lee HL** (2006) Systematical evaluation of the effects of sample collection procedures on low-molecular-weight serum/plasma proteome profiling. *Proteomics* 6: p. 3189-3198
41. **Omenn GS** (2004) The Human Proteome Organization Plasma Proteome Project pilot phase: reference specimens, technology platform comparisons, and standardized data submissions and analyses. *Proteomics* 4: p. 1235-1240

42. **Omenn GS, States DJ, Adamski M, Blackwell TW, Menon R, Hermjakob H, Apweiler R, Haab BB, Simpson RJ, Eddes JS, Kapp EA, Moritz RL, Chan DW, Rai AJ, Admon A, Aebersold R, Eng J, Hancock WS, Hefta SA, Meyer H, Paik YK, Yoo JS, Ping P, Pounds J, Adkins J, Qian X, Wang R, Wasinger V, Wu CY, Zhao X, Zemg R, Archakov A, Tsugita A, Beer I, Pandey A, Pisano M, Andrews P, Tammen H, Speicher DW, and Hanash SM** (2005) Overview of the HUPO Plasma Proteome Project: Results from the pilot phase with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database. *Proteomics* 5: p. 3226-3245

43. **Rai AJ, Gelfand CA, Haywood BC, Warunek DJ, Yi J, Schuchard MD, Mehigh RJ, Cockrill SL, Scott GB, Tammen H, Schulz-Knappe P, Speicher DW, Vitzthum F, Haab BB, Siest G, and Chan DW** (2005) HUPO Plasma Proteome Project specimen collection and handling: Towards the standardization of parameters for plasma proteome samples. *Proteomics* 5: p. 3262-3277

44. **Tanaka Y, Akiyama H, Kuroda T, Jung G, Tanahashi K, Sugaya H, Utsumi J, Kawasaki H, and Hirano H** (2006) A novel approach and protocol for discovering extremely low-abundant proteins in serum. *Proteomics* 6: p. 4845-4855

45. **Harper RG, Workman SR, Schützner S, Timpermann AT, and Sutton JN** (2004) Low-molecular-weight human serum proteome using ultrafiltration, isoelectric focussing, and mass spectrometry. *Electrophoresis* 25: p. 1299-1306

46. **Lee HJ, Lee EY, Kwon MS, and Paik YK** (2006) Biomarker dicsovery from the plasma proteome using multidimensional fraction proteomics. *Curr Opin Chem Biol* 10: p. 42-49

47. **Björhall K, Miliotis T, and Davidsson P** (2005) Comparison of different depletion strategies for improved resolution in proteomic analysis of human serum samples. *Proteomics* 5: p. 307-317

48. **Ahmed N, Barker G, Oliva K, Garfin D, Talmadge K, Georgiou H, Quinn M, and Rice G** (2003) An approach to remove albumin for the proteomic analysis of low abundance biomarkers in human serum. *Proteomics* 3: p. 1980-1987

49. **Steel LF, Trotter MG, Nakajima PB, Mattu TS, Gonye G, and Block G** (2003) Efficient and specific removal of albumin from human serum samples. *Mol Cell Proteomics* 2: p. 262-270

50. **Adkins JN, Varnum SM, Auberry KJ, Moore RJ, Angell NH, Smith RD, Springer DL, and Pounds JG** (2002) Toward a human blood serum proteome. *MCP Papers in Press* 1: p. 947-955

51. **Echan LA, Tang HY, Ali-Khan N, Lee KB, and Speicher DW** (2005) Depletion of multiple high-abundance proteins improves protein profiling capacities of human serum and plasma. *Proteomics* 5: p. 3292-3303

52. **Zolotarjova N, Martosella J, Nicol G, Bailey J, Boyes BE, and Barrett WC** (2005) Differences among techniques for high-abundant protein depletion. *Proteomics* 5: p. 3304-3313

53. **Huang HL, Stasyk T, Morandell S, Mogg M, Schreiber M, Feuerstein I, Huck CW, Stecher G, Bonn GK, and Huber LA** (2005) Enrichment of low-abundant serum proteins by albumin/immunoglobulin G immunoaffinity depletion under partly denaturing conditions. *Electrophoresis* 26: p. 2843-2849

54. **Marshall J, Jankowski A, Furesz S, Kireeva I, Barker L, Dombrovsky M, Zhu W, Jacks K, Ingratta L, Bruin J, Kristensen E, Zhang R, Stanton E, Takahashi M, and Jackowski G** (2004) Human serum proteins preseparated by electrophoresis or chromatography followed by tandem mass spectrometry. *J Proteome Res* 3: p. 364-382

55. **Wasinger VC, Locke VL, Raftery MJ, Larance M, Rothemund D, Liew A, Bate I, and Guilhaus M** (2005) Two-dimensional liquid chromatography/tandem mass spectrometry analysis of Gradiflow™ fractionated native human plasma. *Proteomics* 5: p. 3397-3401

56. **Görg A, Boguth G, Köpf A, Reil G, Parlar H, and Weiss W** (2002) Sample prefractionation with Sephadex isoelectric focusing prior to narrow pH range two-dimensional gels. *Proteomics* 2: p. 1652-1657

57. **Tang HY, Ali-Khan N, Echan LA, Levenkova N, Rux JJ, and Speicher DW** (2005) A novel four-dimensional strategy combining protein and peptide separation methods enables

detection of low-abundance proteins in human plasma and serum proteomes. *Proteomics* 5: p. 3329-3342

58. **Barnea E, Sorkin R, Ziv T, Beer L, and Admon A** (2005) Evaluation of prefractionation methods as a preparatory step for multidimensional based chromatography of serum proteins. *Proteomics* 5: p. 3367-3375
59. **Baumann S, Ceglarek U, Fiedler GM, Lembcke J, Leichtle A, and Thiery J** (2005) Standardized approach to proteome profiling of human serum based on magnetic bead separation and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *Clin Chem* 51: p. 973-980
60. **O'Farrell PH** (1975) High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250: p. 4007-4021
61. **Görg A, Weiss W, and Dunn MJ** (2004) Current two-dimensional electrophoresis technology for proteomics. *Proteomics* 4: p. 3665-3685
62. **Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, and Weiss W** (2000) The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 21: p. 1037-1053
63. **Görg A, Obermaier C, Boguth G, and Weiss W** (1999) Recent developments in two-dimensional gel electrophoresis with immobilized pH gradients: Wide pH gradients up to pH 12, longer separation distances and simplified procedures. *Electrophoresis* 20: p. 712-717
64. **Hanash SM** (2003) Disease proteomics. *Nature* 422: p. 226-232
65. **Starita-Geribaldi M, Roux F, Garin J, Chevallier D, Fénichel P, and Pointis G** (2003) Development of narrow immobilized pH gradients covering one pH unit for human seminal plasma proteomic analysis. *Proteomics* 3: p. 1611-1619
66. **Fuchs D, Winkelmann I, Johnson IT, Mariman E, Wezel U, and Daniel H** (2005) Proteomics in nutrition research: principles, technologies and applications. *Br J Nutr* 94: p. 302-314
67. **Chromy BA, Gonzales AD, Perkins J, Choi MW, Corzett MH, Chang BC, Corzett CH, and L. M-MS** (2004) Proteomic analysis of human serum by two-dimensional differential gel electrophoresis after depletion of high-abundant proteins. *J Proteome Res* 3: p. 1120-1127
68. **Qin S, Ferdinand AS, Richie JP, O'Leary MP, Mok SC, and C.-S. LB** (2005) Chromatofocusing fractionation and two-dimensional difference gel electrophoresis for low abundance serum proteins. *Proteomics* 5: p. 3183-3192
69. **Marouga R, David S, and Hawkins E** (2005) The development of the DIGE system: 2D fluorescence difference gel analysis technology. *Anal Bioanal Chem* 382: p. 669-678
70. **Nissum M, Kuhfuss S, Hauptmann M, Obermaier C, Sukop U, Wildgruber R, Weber G, Eckerskorn C, and Malmström J** (2007) Two-dimensional separation of human plasma proteins using iterative free-flow electrophoresis. *Proteomics* 7: p. 4218-4227
71. **Cho SY, Lee EY, Lee JS, Kim HY, Park JM, Kwon MS, Park YK, Lee HJ, Kang MJ, Kim JY, Yoo JS, Park SJ, Cho JW, Kim HS, and Paik YK** (2005) Efficient prefractionation of low-abundance proteins in human plasma and construction of a two-dimensional map. *Proteomics* 5: p. 3386-3396
72. **Moritz RL, Clippingdale AB, Kapp EA, Eddes JS, Ji H, Gilbert S, Connolly LM, and Simpson RJ** (2005) Application of 2-D free-flow electrophoresis/RP-HPLC for proteomic analysis of human plasma depleted of multi high-abundance proteins. *Proteomics* 5: p. 3402-3413
73. **Shen Y, Jacobs JM, Camp DGI, Fang R, Moore RJ, Smith RD, Xiao W, Davis RW, and Tompkins RG** (2004) Ultra-high-efficiency strong cation exchange LC/RPLC/MS/MS for high dynamic range characterization of the human plasma proteome. *Anal Chem* 76: p. 1134-1144
74. **He P, He HZ, Dai J, Wang Y, Sheng QH, Zhou LP, Zhang ZS, Sun YL, Liu F, Wang K, Zhang JS, Wang HX, Song ZM, Zhang HR, Zeng R, and Zhao X** (2005) The human plasma proteome: Analysis of Chinese serum using shotgun strategy. *Proteomics* 5: p. 3442-3453
75. **Adkins JN, Monroe ME, Auberry KJ, Shen Y, Jacobs JM, Camp II DG, Vitzthum F, Rodland KD, Zangar RC, Smith RD, and Pounds JG** (2005) A proteomic study of the

HUPO Plasma Proteome Project's pilot samples using an accurate mass and time tag strategy. *Proteomics* 5: p. 3454-3466

76. **Aebersold R, and Mann M** (2003) Mass spectrometry-based proteomics. *Nature* 422: p. 198-207
77. **Mann M, Hendrickson RC, and Pandey A** (2001) Analysis of proteins and proteomics by mass spectrometry. *Annu Rev Biochem* 70: p. 437-473
78. **Rai AJ, Stemmer PM, Zhang Z, Adam B, Morgan WT, Caffrey RE, Podust VN, Patel M, Lim L, Shipulina NV, Chan DW, Semmes OJ, and Eastwood Leung H** (2005) Analysis of Human Proteome Organization Plasma Proteome Project (HUPO PPP) reference specimens using surface enhanced laser desorption/ionization-time of flight (SELDI-TOF) mass spectrometry: Multi-institution correlation of spectra and identification of biomarkers. *Proteomics* 5: p. 3467-3474
79. **Banks RE, Stanley AJ, Cairns DA, Barrett JH, Clarke P, Thompson D, and Selby PJ** (2005) Influences of blood sample processing on low-molecular-weight proteome identified by Surface-Enhanced Laser Desorption/Ionization mass spectrometry. *Clin Chem* 51: p. 1637-1649
80. **Lahm HW, and Langen H** (2000) Mass spectrometry: A tool for the identification of proteins separated by gels. *Electrophoresis* 21: p. 2105-2114
81. **Kapp EA, Schütz F, Connolly LM, Chakel JA, Meza JE, Miller CA, Fenyo D, Eng JK, Adkins JN, Omenn GS, and Simpson RJ** (2005) An evaluation, comparison, and accurate benchmarking of several publicly available MS/MS search algorithms: Sensitivity and specificity analysis. *Proteomics* 5: p. 3475-3490
82. **Ping P, Vondriska TM, Creighton CJ, Ganhi TKB, Yang Z, Menon R, Kwon MS, Cho SY, Drwal G, Kellmann M, Peri S, Suresh S, Gronborg M, Molina H, Chaerkady R, Rekha B, Shet AS, Gerszten RE, Wu H, Raftery M, Wasinger V, Schulz-Knappe P, Hanash SM, Paik Y, Hancock WS, States DJ, Omenn GS, and Pandey A** (2005) A functional annotation of subproteomes in human plasma. *Proteomics* 5: p. 3506-3519
83. **Muthusamy B, Hanumanthu G, Suresh S, Rekha B, Srinivas D, Karthick L, Vrushabendra BM, Sharma S, Mishra G, Chatterjee P, Mangala KS, Shivashankar HN, Chandrika KN, Deshpande N, Suresh M, Kannabiran N, Niranjan V, Nalli A, Prasad TS, Arun KS, Reddy R, Chandran S, Jadhav T, Julie D, Mahesh M, John SL, Palvankar K, Sudir D, Bala P, Rashmi NS, Vishnupriya G, Dhar K, Reshma S, Chaerkady R, Gandhi TK, Harsha HC, Mohan SS, Deshpande KS, Sarker M, and Pandey A** (2005) Plasma proteome database as a resource for proteomics research. *Proteomics* 5: p. 3531-3536
84. **Martens L, Hermjakob H, Jones P, Adamski M, Taylor C, States D, Gevaert K, Vandekerckhove J, and Apweiler R** (2005) PRIDE: The proteomics identifications database. *Proteomics* 5: p. 3537-3545
85. **Deutsch EW, Eng JK, Zhang H, King NL, Nesvizhskii AI, Lin B, Lee H, Yi EC, Ossola R, and Aebersold R** (2005) Human Plasma PeptideAtlas. *Proteomics* 5: p. 3497-3500
86. **Kleinert P, Kuster T, Arnold D, Jaeken J, Heizmann CW, and Troxler H** (2007) Effect of glycosylation on the protein pattern in 2-D-gel electrophoresis. *Proteomics* 7: p. 15-22
87. **Yang Z, Hancock WS, Chew TR, and Bonilla L** (2005) A study of glycoproteins in human serum and plasma reference standards (HUPO) using multilectin affinity chromatography coupled with RPLC-MS/MS. *Proteomics* 5: p. 3353-3366
88. **Richter R, Schulz-Knappe P, Schrader M, Ständker L, Jürgens M, Tammen H, and Forssmann WG** (1999) Composition of the peptide fraction in human blood plasma: database of circulating human peptides. *J Chromatogr B Biomed Sci Appl* 726: p. 25-35
89. **Tammen H, Schulte I, Hess R, Menzel C, Kellmann M, Mohring T, and Schulz-Knappe P** (2005) Peptidomic analysis of human blood specimens: Comparison between plasma specimens and serum by differential peptide display. *Proteomics* 5: p. 3414-3422
90. **Anderson NL, and Anderson NG** (1977) High resolution two-dimensional electrophoresis of human plasma proteins. *Proc Natl Acad Sci USA* 74: p. 5421-5425
91. **Anderson NL, and Anderson NG** (1991) A two-dimensional gel database of human plasma proteins. *Electrophoresis* 12: p. 883-906

92. **Anderson NL, Polanski M, Pieper R, Gatlin T, Tirumalai RS, Conrads TP, Veenstra TD, Adkins JN, Pounds JG, Fagan R, and Lobley A** (2004) The human plasma proteome. *Mol Cell Proteomics* 3: p. 311-326
93. **Li X, Gong Y, Wang Y, Wu S, Cai Y, He P, Lu Z, Ying W, Zhang Y, Jiao L, He H, Zhang Z, He F, Zhao X, and Qian X** (2005) Comparison of alternative analytical techniques for the characterisation of the human serum proteome in HUPO Plasma Proteome Project. *Proteomics* 5: p. 3423-3441
94. **Shen Y, Kim, J., Strittmatter, E. F., Jacobs, J. M., CampII, D. G., Fang, R., Tolié, N., Moore, R. J., Smith, R. D.** (2005) Characterization of the human blood plasma proteome. *Proteomics* 5: p. 4034-4045
95. **Faca V, Pitteri SJ, Newcomb L, Glukhova V, Phanstiel D, Krasnoselsky A, Zhang Q, Struthers J, Wang H, Eng J, Fitzgibbon M, McIntosh M, and Hanash S** (2007) Contribution of protein fractionation to depth of analysis of the serum and plasma proteomes. *J Prot Res* 6: p. 3558-3565
96. **Adamski M, Blackwell T, Menon R, Martens L, Hermjakob H, Taylor C, Omenn GS, and States D** (2005) Data management and preliminary data analysis in the pilot phase of the HUPO Plasma Proteome Project. *Proteomics* 5: p. 3246-3261
97. **Bradford MM** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: p. 248-254
98. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature* 227: p. 680-685
99. **Smyth GK** (2005) Limma: linear models for microarray data. *Bioinformatics and Computational Biology Solutions using R and Bioconductor*, edited by Gentleman R. CV, Dudoit S., Irizarry R., Huber W., Springer. p. 397 - 420.
100. **Harrell FJ** (2009) Hmisc: Harrel Miscellaneous
101. **Sarkar D** (2008) Lattice: Multivariate Data Visualization with R. Springer
102. **Spector P** (2008) Data manipulation with R. Springer
103. **Granger J, Siddiqui J, Copeland S, and Remick D** (2005) Albumin depletion of human plasma also removes low abundance proteins including the cytokines. *Proteomics* 5: p. 4713-4718
104. **Diaz D, Prieto A, Barcenilla H, Monserrat J, Prieto P, Sánchez MA, Reyes E, Hernandez-Fuentes MP, de la Hera A, Orfao A, and Alvarez-Mon M** (2004) Loss of lineage antigens is a common feature of apoptotic lymphocytes. *J Leuk Biol* 76: p. 609-615
105. **Webb JH** (2002) Studies on the interaction between vitamin K-dependent protein S and complemet regulator C4b-binding protein: localization of binding sites and identification of a possible function of the complex. *Scand J Clin Lab Invest Suppl* 237: p. 19-28
106. **Gillooly DJ, Simonse A, and Stenmark H** (2001) Cellular functions of phosphatidylinositol 3-phosphate and FYVE domain proteins. *Biochem J* 355: p. 249-258
107. **He QY, Lau GK, Zhou Y, Yuen ST, Lin MC, Kung HF, and Chiu JF** (2003) Serum biomarkers of hepatitis B virus infected liver inflammation: a proteomic study. *Proteomics* 3: p. 666-674
108. **Gangadharan B, Antrobus R, Dwek RA, and Zitzmann N** (2007) Novel serum biomarker candidates for liver fibrosis in hepatitis C patients. *Clin Chem.* 53: p. 1792-1799
109. **Cheung KJ, Tilleman K, Deforce D, Colle I, and van Vlierberghe H** (2009) The HCV serum proteome: a search for fibrosis protein markers. *J Viral Hepat* 16: p. 418-429
110. **Roher AE, Maarouf CL, Sue LI, Hu Y, Wilson J, and Beach TG** (2009) Proteomics-derived cerebrospinal fluid markers of autopsy-confirmed Alzheimer's disease. *Biomarkers* 14: p. 493-501
111. **Hye A, Lynham S, Thambisetty M, Causevic M, Campbell J, Byers HL, Hooper C, Rijsdijk F, Tabrizi SJ, Banner S, Shaw CE, Foy C, Poppe M, Archer N, Hamilton G, Powell J, Brown RG, Sham P, Ward M, and Lovestone S** (2006) Proteome-based plasma biomarkers for Alzheimer's disease. *Brain* 129: p. 3042-3050
112. **Jackson D, Craven RA, Hutson RC, Graze I, Lueth P, Tonge RP, Hartley JL, Nickson JA, Rayner SJ, Johnston C, Dieplinger B, Hubalek M, Wilkinson N, Perren TJ, Kehoe S, Hall GD, Daxenbichler G, Dieplinger H, Selby PJ, and Banks RE** (2007) Proteomic

profiling identifies afamin as a potential biomarker for ovarian cancer. *Clin Cancer Res* 13: p. 7370-7378

113. **Zeng Z, Hincapie M, Haab BB, Hanash S, Pitteri SJ, Kluck S, Hogan JM, Kennedy J, and Hancock WS** (2010) The development of an integrated platform to identify breast cancer glycoproteome changes in human serum. *J Chromatogr A* 1217: 3307-3315
114. **Tian M, Cui YZ, Song GH, Zong MJ, Zhou XY, Chen Y, and Han JX** (2008) Proteomic analysis identifies MMP-9, DJ-1 and A1BG as overexpressed proteins in pancreatic juice from pancreatic ductal adenocarcinoma patients. *BMC Cancer* 16: p. 241-252
115. **Berhane BT, Zong C, Liem DA, Huang A, Le S, Edmondson RD, Jones RC, Qiao X, Whitelegge JP, Ping P, and Vondriska TM** (2005) Cardiovascular-related proteins identified in human plasma by the HUPO Plasma Proteome Project Pilot Phase. *Proteomics* 5: p. 3520-3530
116. **Pieper R, Gatlin CL, Makusky AJ, Russo PS, Schatz CR, Miller SS, Su Q, McGrath AM, Estock MA, Parmar PP, Zhao M, Huang ST, Zhou J, Wang F, Esquer-Blasco M, Anderson NL, Taylor J, and Steiner S** (2003) The human serum proteome: display of nearly 3700 chromatopraphically separated protein spots on two-dimensional electrophoresis gels and identification of 325 distinct proteins. *Proteomics* 3: p. 1345-1364
117. **Zuckerman ST, and Kao WJ** (2008) LC/MS identification of 12 intracellular cytoskeletal and inflammatory proteins from monocytes adherent on surface-absorbed fibronectin-derived peptides. *J Biomed Mater Res A* 85: p. 513-529
118. **Fujii K, Nakano T, Kawamura T, Usui F, Bando Y, Wang R, and Nishimura T** (2004) Multidimensional protein profiling technology and its application to human plasma proteome. *J Proteome Res* 3: p. 712-718
119. **Eravci M, Fuxius S, Broedel O, Weist S, Eravci S, Mansmann U, Schluter H, Tiemann J, and Baumgartner A** (2007) Improved comparative proteome analysis based on two-dimensional gel electrophoresis. *Proteomics* 7: p. 513-523
120. **Hunsucker SW, and Duncan MW** (2006) Is protein overlap in two-dimensional gels a serious practical problem? *Proteomics* 6: p. 1374-1375
121. **Humayun MA, Elango R, Ball RO, and Pencharz PB** (2007) Reevaluation of the protein requirement in young men with the indicator amino acid oxidation technique. *Am J Clin Nutr* 86: p. 995-1002
122. **Busque SM, and Wagner CA** (2009) Potassium restriction, high protein intake, and metabolic acidosis increase the expression of the glutamine transporter SNAT3 (Slc38a3) in mouse kidney. *Am J Physiol Renal Physiol* 297: p. F440-450
123. **Sabboh H, Besson C, Tressol J-C, Rémésy C, and Demigné C** (2006) Excess casein in the diet is not the unique cause of low-grade metabolic acidosis : role of a deficit in the potassium citrate in a rat model. *Ann Nutr Metab* 50: p. 229-236
124. **Baum N, Dichoso CC, and Carlton CE** (1975) Blood urea nitrogen and serum creatinine: physiology and interpretations. *Urology* 5: p. 583-588
125. **Shaham O, Wei R, Wang TJ, Ricciardi C, Lewis GD, Vasan RS, Carr SA, Thadhani R, Gerszten RE, and Mootha VK** (2008) Metabolic profiling of the human response to a glucose challenge reveals distinct axes of insulin sensitivity. *Mol Syst Biol* 4: p. 214
126. **Wopereis S, Rubingh CM, van Erk MJ, Verheij ER, van Vliet T, Cnubben NH, Smilde AK, van der Greef J, van Ommen B, and Hendriks HF** (2009) Metabolic profiling of the response to an oral glucose tolerance test detects subtle metabolic changes. *PloS One* 4: p. e4525
127. **Newgard CB, An J, Muehlbauer MJ, Stevens RD, Lien LF, M. HA, Shah SH, Arlotto M, Slentz CA, Rochon J, Gallup D, Illkayeva O, Wenner BR, Yancy WSJ, Eisenson H, Musante G, Surwit RS, Millington DS, Butler MD, and Svetkey LP** (2009) A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab* 9: p. 311-326
128. **Lewis GD, Wei R, Liu E, Yang E, Shi X, Martinovic M, Farrell L, Asnani A, Cyrille M, Ramanathan A, Shaham O, Berriz G, Lowry PA, Palacois IF, Taşan M, Roth FP, Min J, Baumgartner C, Keshishian H, Addona T, Mootha VK, Rosenzweig A, Carr SA, Fifer MA, Sabatine MS, and Gerszten RE** (2008) Metabolite profiling of blood from individuals

undergoing planned myocardial infarction reveals early markers of myocardial injury. *J Clin Invest* 118: p. 3503-3512

129. **Lewis GD, Asnani A, and Gerszten RE** (2008) Application of metabolomics to cardiovascular biomarker and pathway discovery. *J Am Coll Cardiol* 52: p. 117-123
130. **Assfalg M, Bertini I, Colangiuli D, Luchinat C, Schäfer H, Schütz B, and Spraul M** (2008) Evidence of different metabolic phenotypes in humans. *Proc Natl Acad Sci USA* 105: p. 1420-1424
131. **Salek RM, Maguire ML, Bentley E, Rubtos DV, Hough T, Cheeseman M, Nunez D, Sweatman BC, Haselden JN, Cox RD, Connor SC, and Griffin JL** (2007) A metabolomic comparison of urinary changes in type 2 diabetes in mouse, rat, and human. *Physiol Genomics* 29: p. 99-108
132. **van Doorn M, Vogels J, Tas A, van Hoogdalem EJ, Burggraaf J, Cohen A, and van der Greef J** (2006) Evaluation of metabolite profiles as biomarkers for the pharmacological effects of thiazolidinediones in type 2 diabetes mellitus patients and healthy volunteers. *Br J Clin Pharmacol* 63: p. 562-574
133. **Ohuchi S, Matsumoto Y, Morita T, and Sugiyama K** (2008) High-casein diet suppresses guanidinoacetic acid-induced hyperhomocysteinemia and potentiates the hypohomocysteinemic affect of serine in rats. *Biosci Biotechnol Biochem* 72: p. 3258-3264
134. **Stolzenberg-Solomon RZ, Miller III ER, Maguire MG, Selhub J, and Appel LJ** (1999) Association of dietary protein intake and coffee consumption with serum homocysteine concentrations in an older population. *Am J Clin Nutr* 69: p. 467-475
135. **Ohuchi S, Matsumoto Y, Morita T, and Sugiyama K** (2009) High casein diet decreases plasma homocysteine concentration in rats. *J Nutr Sci Vitaminol* 55: p. 22-30
136. **Hamberg O** (1997) Regulation of urea synthesis by diet protein and carbohydrate in normal man and in patients with cirrhosis. Relationship to glucagon and insulin. *Dan Med Bull* 44: p. 225-241
137. **Nuttall F. Q. G, M. C., Wald, J. L., Ahmed, M.** (1985) Plasma glucose and insulin profiles in normal subjects ingesting diets of varying carbohydrate, fat, and protein content. *J Am Coll Nutr* 4: p. 437-450
138. **Graham-Thiers PM, and Kronfeld DS** (2005) Amino acid supplementation improves muscle mass in aged and young horses. *J Anim Sci* 83: p. 2783-2788
139. **Gallagher D, Heymsfield SB, and Wang Z** (1999) Skeletal muscle markers. The role of protein and amino acids in sustaining and enhancing performance. Washington, DC. Natl. Acad. Press. 255
140. **Block WD, Hubbard RW, and Steele BF** (1965) Excretion of histidine and histidine derivatives by human subjects ingesting protein from different sources. *J Nutr* 85: p. 419-425
141. **Sakami W, and Wilson DW** (1944) Studies on methylhistidine II. A study of the metabolism of dl-1-methylhistidine in the albino rat. *JBC* 154: p. 223-225
142. **Derave W, Jones G, Jespel P, and Harris RC** (2008) Creatine supplementation augments skeletal muscle carnosine content in senescence-accelerated mice (SAMP8). *Rejuvenation Res* 11: p. 641-647
143. **Hobart LJ, Seibel I, Yeargans GS, and Seidler NW** (2004) Anti-crosslinking properties of carnosine: significance of histidine. *Life Sci* 75: p. 1379-1389
144. **Fuhrman PM, Charney P, and Mueller CM** (2004) Hepatic proteins and nutrition assessment. *J Am Diet Assoc* 104: p. 1258-1264
145. **Hassan I, A. W, Yadav S, Singh TP, and Ahmad F** (2008) Zinc α2-glycoprotein: a mutlidisciplinary protein. *Mol Cancer Res* 6: p. 892-906
146. **Engler R** (1981) Hemolysis and plasma proteins. *Ann Biol Clin* 39: p. 193-196
147. **Bertile F, Schaeffer C, Le Maho Y, Raclot T, and van Dorsselaer A** (2009) A proteomic approach to identify differentially expressed plasma proteins between the fed and prolonged fasted state. *Proteomics* 9: p. 148-158
148. **Delanghe JR, and Langlois MR** (2001) Hemopexin: a review of biological aspects and the role in laboratory medicine. *Clin Chim Acta* 312: p. 13-23
149. **Tolosano E, Fagoonee S, Morello N, Vinchi F, and Fiorito V** (2010) Heme scavenging and the other facets of hemopexin. *Antioxid Redox Signal* 12: 305-320

150. **Adinolfi M, and Lehner T** (1988) C9 and factor B as acute phase proteins and their diagnostic and prognostic value in disease. *Exp Clin Immunogenet* 5: p. 123-132
151. **Sakai T, Balasubramanian K, Maiti S, Halder JB, and Schroit AJ** (2007) Plasmin-cleaved β-2-glycoprotein 1 is an inhibitor of angiogenesis. *Am J Pathol* 171: p. 1659-1669
152. **Santos-Gallego CG, Ibanez B, and Badimon JJ** (2008) HDL-cholesterol: is it really good? Differences between apoA-I and HDL. *Biochem Pharmacol* 76: p. 443-452
153. **Wang K, and Wong YH** (2009) G Protein signaling controls the differentiation of multiple cell lineages. *BioFactors* 35: p. 232-238
154. **McKeon F** (1991) Nuclear lamin proteins: domains required for nuclear targeting, assembly, and cell-cycle-regulated dynamics. *Curr Opin Cell Biol* 3: p. 82-86
155. **Gerace L, and Burke B** (1988) Functional organization of the nuclear envelope. *Annu Rev Cell Biol* 4: p. 335-374
156. **Badock V, Steinhusen U, Bommert K, Wittmann-Liebold B, and Otto A** (2001) Apoptosis-induced cleavage of keratin 15 and keratin 17 in a human breast epithelial cell line. *Cell Death Differ* p. 3
157. **Ruan W, and Lai M** (2007) Actin, a reliable marker of internal control? *Clin Chim Acta* 385: p. 1-5
158. **Mangeat P, and Burridge K** (1984) Actin-membrane interaction in fibroblasts: what proteins are involved in this association? *J Cell Biol* 99: p. 95s-103s
159. **Holleran EA, Tokito MK, Karki S, and Holzbaur EL** (1996) Centractin (ARP1) associates with spectrin revealing a potential mechanism to link dynactin to intracellular organelles. *JBC* 135: p. 1815-1829
160. **Silacci P, Mazzolai L, Gauci C, Stergiopulos N, Yin HL, and Hayoz D** (2004) Gelsolin superfamily proteins: key regulators of cellular functions. *Cell Mol Life Sci* 61: p. 2614-2623
161. **Linder S, and Aepfelbacher M** (2003) Podosomes: adhesion hot-spots of invasive cells. *Trends Cell Biol* 13: p. 376-385
162. **Bohgaki M, Matsumoto M, Atsumi T, Kondo T, Yasuda S, Horita T, Nakayama KI, Okumura F, Hatakeyama S, and Koike T** (2009) Plasma gelsolin facilitates interaction between beta(2) glycoprotein I and alpha5beta1 integrin. *J Cell Mol Med*
163. **Bucki R, Levental I, Kulakowska A, and Janmey PA** (2008) Plasma gelsolin: function, prognostic value, and potential therapeutic use. *Curr Protein Pept Sci* 9: p. 541-551
164. **Harris BZ, and Lim WA** (2001) Mechanism and role of PDZ domains in signaling complex assembly. *J Cell Sci* 14: p. 3219-3231
165. **Bauer K, Kratzer M, Otte M, Luber de Quintana K, Hagmann J, Arnold GJ, Eckerkorn C, Lottspeich F, and Siess W** (2000) Human CLP36, a PDZ-domain and LIM-domain protein, binds to alpha-actinin-1 and associates with actin filaments and stress fibers in activated platelets and endothelial cells. *Blood* 96: p. 4236
166. **Park J, Rho HK, Kim KH, Choe SS, Lee YS, and Kim JB** (2005) Overexpression of glucose-6-phosphate dehydrogenase is associated with lipid dysregulation and insulin resistance in obesity. *Mol Cell Biol* 25: p. 5146-5157
167. **Brito SM, Moura MA, Kawashita NH, Festuccia WT, Garófalo MA, Kettelhut IC, and Migliorini RH** (2005) Adaptation to a high protein, carbohydrate-free diet induces a marked reduction of fatty acid synthesis and lipogenic enzymes in rat adipose tissue that is rapidly reverted by a balanced diet. *Can J Physiol Pharmacol* 83: p. 477-482
168. **Otton R, Graziola F, Hirata MH, Curi R, and Williams JF** (1998) Dietary fats alter the activity and expression of glucose-6-phosphate dehydrogenase in rat lymphoid cells and tissues. *Biochem Mol Biol Int* 46: p. 529-536
169. **Jensen T, Kierulf P, Sandset PM, Klingenberg O, Joø GB, Godal HC, and Skjønsberg OH** (2007) Fibrinogen and fibrin induce synthesis of proinflammatory cytokines from isolated peripheral blood mononuclear cells. *Thromb Haemost* 97: p. 822-829
170. **Paraskevas KI, Baker DM, Vrentzos GE, and Mikhailidis DP** (2008) The role of fibrinogen and fibrinolysis in peripheral arterial disease. *Thromb Res* 122: p. 1-12

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit

Human plasma and peripheral mononuclear cell (PBMC) proteome databasesfor biomarker discovery studies *in vivo*

selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen sind als solche kenntlich gemacht.

Diese Arbeit hat in gleicher oder ähnlicher Form noch keiner anderen Prüfungsbehörde vorgelegen.

München, den 04.05.2010

Carolin Heim

Erklärung

Teile dieser Arbeit wurden bereits veröffentlicht:

Heim C, Rubio-Aliaga I, Skurk T, Schmidt G, and Daniel H (2010) Effects of a high dietary high-protein intake in humans on fasting plasma and urinary amino acid profiles assessed by LC-MS/MS with iTRAQ-labeling. Eingereicht bei *Amino Acids*

Rubio-Aliaga I, de Roos B, Duthie SJ, Crosley LK, Mayer C, Horgan G, Colquhoun IJ, Le Gall G, Huber F, Kremer W, Rychlik M, Wopereis S, van Ommen B, Schmidt G, Heim C, Bouwman FG, Mariman EC, Mulholland F, Johnson IT, Polley AC, Elliott RM, and Daniel H (2010) Metabolomics of prolonged fasting in humans: from physiology to individual metabotypes. Eingereicht bei *Metabolomics*

de Roos B, Duthie S, Polley A, Mulholland F, Bouwman F, Heim C, Rucklidge G, Johnson I, Mariman E, Daniel H, and Elliott R (2008) Analytical variability and methodological recommendations for plasma, platelet and PBMC proteomics in human nutrition intervention studies. *J. Proteome Res.* 7: 2280-90

Lebenslauf

Carolin Heim
Josef-Frankl-Str. 60
80995 München
Tel. ++49 (0)89 45 21 74 11
carolin.heim@gmail.com

Geburtsname:	Carolin Wendl
Geburtstag:	5. April 1978
Geburtsort:	Karlsruhe
Familienstand:	verheiratet, 1 Kind (geb. 05.08.2008)
Nationalität:	deutsch

Schulbildung:

09/1984 - 07/1988	Erich- Kästner- Grundschule Ettlingenweier
09/1988 - 06/1997	Albertus- Magnus- Gymnasium Ettlingen
06/1997	Abitur

Universitäre Ausbildung:

10/1997 – 09/2000	Grundstudium Lebensmittelchemie, Technische Universität Karlsruhe
10/2000 – 08/2003	Hauptstudium Lebensmittelchemie, Technische Universität Dresden
08/2000	Diplom Lebensmittelchemie
12/2004 – 10/2010	Promotion, Technische Universität München, Institut für Ernährungsphysiologie

Praktische Tätigkeiten:

08/2001	Praktikum Forschungszentrum Karlsruhe, Institut für Toxikologie und Genetik
08/2002 – 10/2002	Praktikum BASF Plant Science Ltd. (Ludwigshafen), Abteilung Industriefettsäuren
10/2003 – 09/2004	Auslandspraktikum CSIRO Molecular Science (Melbourne, Australien), Biokatalysegruppe
seit 06/2010	Wissenschaftliche Mitarbeiterin Technische Universität München, Lehrstuhl für chemisch-technische Analyse und chemische Lebensmitteltechnologie

Publikationen

Originalarbeiten und Manuskripte:

Heim C, Rubio-Aliaga I, Skurk T, Schmidt G, and Daniel H (2010) Effects of a high dietary high-protein intake in humans on fasting plasma and urinary amino acid profiles assessed by LC-MS/MS with iTRAQ-labeling. Eingereicht bei *Amino Acids*

Rubio-Aliaga I, de Roos B, Duthie SJ, Crosley LK, Mayer C, Horgan G, Colquhoun IJ, Le Gall G, Huber F, Kremer W, Rychlik M, Wopereis S, van Ommen B, Schmidt G, Heim C, Bouwman FG, Mariman EC, Mulholland F, Johnson IT, Polley AC, Elliott RM, and Daniel H (2010) Metabolomics of prolonged fasting in humans: from physiology to individual metabotypes. Eingereicht bei *Metabolomics*

Crosley LK, Duthie SJ, Polley AC, Bouwman FG, Heim C, Mulholland F, Horgan G, Johnson IT, Mariman EC, Elliott RM, Daniel H, and de Roos B (2009) Variation in protein levels obtained from human blood cells and biofluids for platelet, peripheral blood mononuclear cell, plasma, urine and saliva proteomics. *Genes Nutr.* 4: 95-102

Baccini M, Bachmaier EM, Biggeri A, Boekschoten MV, Bouwman FG, Brennan L, Caesar R, Cinti S, Coort SL, Crosley K, Daniel H, Drevon CA, Duthie S, Eijssen L, Elliott RM, van Erk M, Evelo C, Gibney M, Heim C, Horgan GW, Johnson IT, Kelder T, Kleemann R, Kooistra T, van Iersel MP, Mariman EC, Mayer C, McLoughlin G, Müller M, Mulholland F, van Ommen B, Polley AC, Pujos-Guillot E, Rubio-Aliaga I, Roche HM, de Roos B, Sailer M, Tonini G, Williams LM, and de Wit N (2008) The NuGO proof of principle study package: a collaborative research effort of the European Nutrigenomics Organisation. *Genes Nutr.* 3: 147-151

de Roos B, Duthie S, Polley A, Mulholland F, Bouwman F, Heim C, Rucklidge G, Johnson I, Mariman E, Daniel H, and Elliott R (2008) Analytical variability and methodological recommendations for plasma, platelet and PBMC proteomics in human nutrition intervention studies. *J. Proteome Res.* 7: 2280-90

in Vorbereitung:

Heim C, Skurk T, Gelhaus B, and Daniel H (2010) Proteomic profiling of human plasma and PBMC after protein supplementation.

de Roos B, Elliott R, Mariman EC, Duthie SJ, Crosley LK, Polley AC, Mulholland F, Johnson IT, Bouwman FG, Heim C, Rubio-Aliaga I, Daniel H, and Mayer CD (2010) Incidental findings and outliers in nutrigenomics studies.

Bouwman FG, de Roos B, Rubio-Aliaga I, Crosley LK, Duthie SJ, Mayer C, Horgan G, Polley AC, Heim C, Coort SLM, Evelo CT, Mulholland F, Johnson IT, Elliott RM, Daniel H, and Mariman ECM (2010) 2D-electrophoresis and multiplex immunoassay proteomics analysis of different body fluids and cellular components reveal known and novel markers for prolonged fasting.

Posterpräsentationen:

Heim C, and Daniel H (2007) Generating *in-house* human plasma and PBMC proteome databases for biomarker discovery studies *in vivo*. NuGO Week 2007 in Oslo

de Roos B, Duthie S, Polley A, Mulholland F, Bouwman F, Heim C, Rucklidge G, Mariman E, Daniel H, and Elliott R (2007) NuGO WP5 PlasmaPlus study: analytical variability and methodological recommendations for plasma, platelet and lymphocyte proteomics in human samples. NuGO Week 2007 in Oslo

Wendl C, Wenzel U, and Daniel H (2005) Proteome-based biomarker discovery for flavonoid actions i*n vivo*. NuGO Week 2005 in Florenz

Wendl C, Fuchs D, Wenzel U, and Daniel H (2005) Proteome analysis of mononuclear cells and plasma for the discovery of biomarkers of action of dietary compounds *in vivo*. HUPO World Congress 2005 in München

Wendl C, Fuchs D, Wenzel U, and Daniel H (2005) A proteomics approach to examine the effects of a linseed intervention on proteins of human plasma and peripheral blood mononuclear cells. NuGO Introduction Course 2005 in Marseille

Wendl C, Böhl M, Weigel K, Sommer A, Henle T, and Schwenzer B (2003) Untersuchungen zum Einfluss von AGEs (Advanced Glycation Endproducts) auf die Expression gerinnungsrelevanter Proteine in HepG2- Zellen. Biotechnologie-Tag 2003 der Universität Leipzig

Danksagung

Die Erstellung dieser Arbeit wäre ohne Hilfe nicht möglich gewesen. Deshalb möchte ich mich bei all denjenigen bedanken, die zum Gelingen beigetragen haben. Mein besonderer Dank gilt:

- Frau Professor Daniel, die es mir ermöglicht hat, in ihrer Arbeitsgruppe das Thema zu bearbeiten. Mit ihrer Kompetenz und Kreativität hat sie immer wieder neue Denkanstöße gegeben. Die Freiheit, die sie mir während der Promotionszeit gelassen hat, hat es mir entscheidend ermöglicht, über den eigenen Tellerrand hinauszuschauen.
- Herrn Professor Haller für die Erstellung des Gutachtens und den Vorsitz der Prüfungskommission.
- Herrn Prof. Hauner für die Erstellung des Gutachtens.
- Uwe Wenzel für die Betreuung der Doktorarbeit bis zu seinem Weggang nach Giessen.
- Dagmar Fuchs und Isabel Winkelmann für die Einführung in die Proteomics.
- Barbara Gelhaus, die mir nicht nur bei der Analytik sehr zur Seite stand. Die Durchführung der Humanstudie wäre ohne sie undenkbar gewesen. Ihre moralische und fachliche Unterstützung war eine große Hilfe für mich.
- Gabriele Schmidt und Johanna Welzhofer für die Durchführung der Analytik der Aminosäuren und klinischen Parameter.
- Herrn Krause und seinen Mitarbeitern für die Analyse des Proteinpräparates und die Hilfe bei Fragen rund um den Computer.
- Den 11 „freiwilligen“ Probanden, die mit viel Begeisterung Tonnen von Proteinpulver geschluckt haben. Vielen Dank für Euer Mitwirken und mein größter Respekt davor, dass alle durchgehalten haben!
- Isabelle Frey, Katja Bühlmeyer, Annika Helbig und Dietmar Weitz dafür, dass ich so nett in die Arbeitsgruppe aufgenommen wurde und dass die Zeit mit Euch sowohl an der Uni als auch außerhalb eine sehr schöne und intensive war.
- Den „Buschfunk“-Mädels Jacqueline Benner, Katrin Lasch, Anne Näßl und Tanja Heidler für unterhaltsame Pausen und eine gute Laboratmosphäre.
- Baukje de Roos und Fran Mulholland für ihre Unterstützung und Diskussionsbereitschaft nicht nur im Rahmen der NuGO-Projekte.
- Der gesamten Arbeitsgruppe für eine schöne Zeit am Institut und die viele Unterstützung, die ich dort erfahren habe. Aber auch für sämtliche Feste, die für eine tolle Atmosphäre gesorgt haben und mir in guter Erinnerung bleiben werden.
- Jolyon White fürs Korrekturlesen.
- Meinem Mann Dominik für alles, was er während der letzten Jahre durchleben musste. Ohne seine tatkräftige und besonders moralische Hilfe wäre diese Arbeit nicht entstanden.