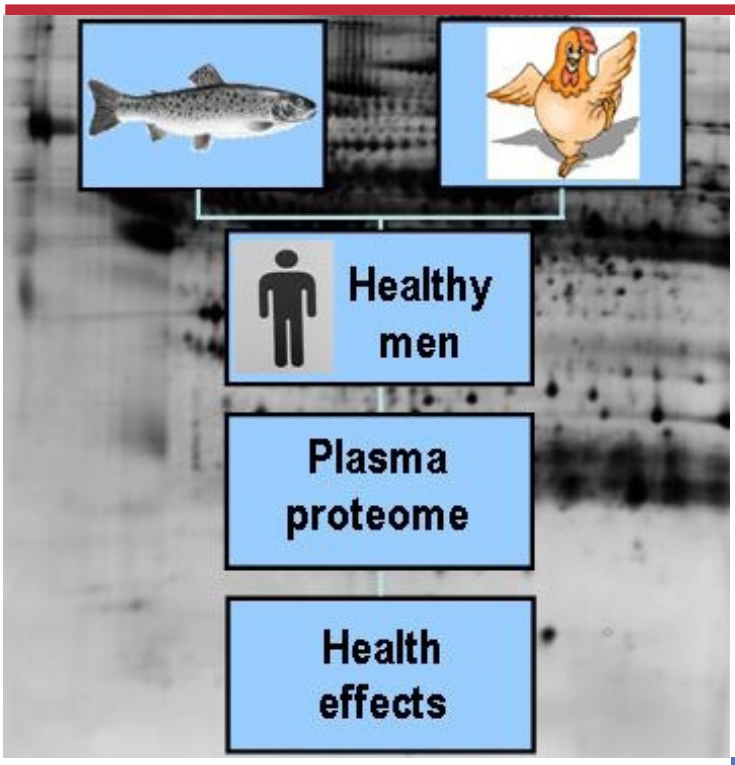
Proteomics in Nutritional Research

A 2-DE based study of diet induced changes in the human plasma proteome



Maria Rentsch
PhD Thesis
2011

DTU Food
National Food Institute

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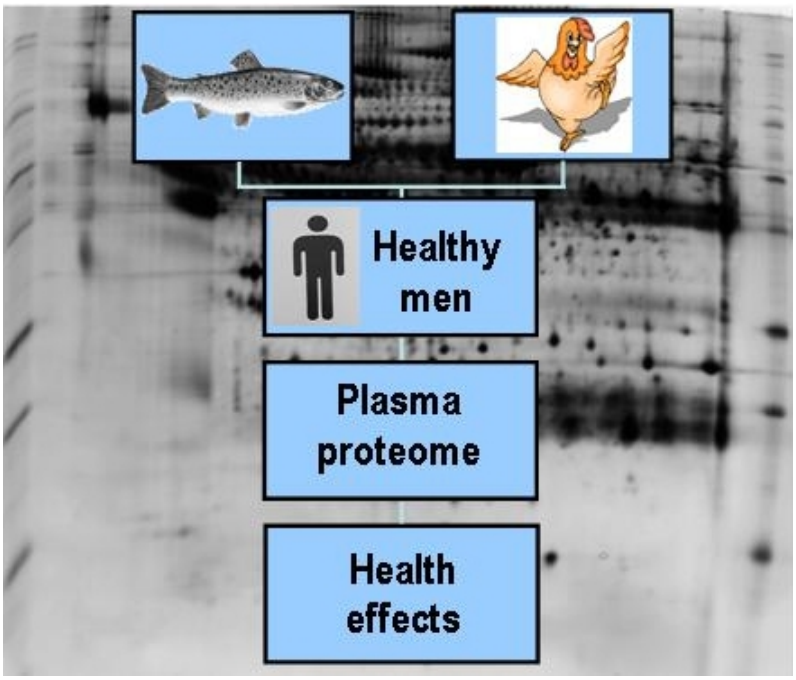
PhD Thesis

by

Maria L. Rentsch



Division of Industrial Food Research
National Food Institute
Technical University of Denmark

June 2011

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Preface

This PhD study was initiated in January 2007 and is based on human blood plasma from a single meal study and an intervention study conducted at the Department of Human Nutrition, Faculty of Life Sciences, University of Copenhagen (KU). The experimental work was carried out at the National Food Institute, Technical University of Denmark (DTU) and at the Department of Biochemistry and Biology, Southern University of Denmark (SDU).

During my three years as PhD student, my main workplace has been DTU in Lyngby, where I learned a lot about sample preparation, how to run two dimensional electrophoresis, data processing and statistical analyses. I also spent half a year at Department of Human Nutrition, KU, where I was the main co-ordinator of the human single meal study. At this place, I gained valuable knowledge about how to conduct human nutritional studies including recruitment of subjects, planning and performing the study. Moreover, with assistance from Rene Lametsch, Department of Food Science, Faculty of Life Sciences, University of Copenhagen, I also got insights into mass spectrometry based identification of proteins.

This thesis consists of two parts; part I describes the health beneficial effects of fish intake in relation to the association between n-3 LCPUFA and decreased risk of cardiovascular disease. Part II describes the applied proteomic methodology including sample preparation, two-dimensional electrophoresis, data analysis and mass spectrometry. The present PhD project was supported by grants from the Danish Agency for Science, Technology and Innovation, and the Danish Food Industry Agency.

Acknowledgements

During my time as a PhD student I have been privileged to be surrounded by highly competent and scientifically skilled people. First, I would like to give thanks to my two supervisors who helped me develop scientifically and supported me during these three years of research. Flemming Jessen, for his great expertise within 2-DE proteomics, and Lotte Lauritzen for sharing her knowledge and enthusiasm about nutritional research and health benefits of n-3 polyunsaturated fatty acids. Warm thanks also to my project manager, Henrik H. Nielsen, for always having an open door and to René Lametsch for his excellent supervision on mass spectrometry.

I am also grateful to a number of people who helped with the practical work. Thanks to BioMar A/S for raising the trout for the human trials and to Jeanette U. Møller for slaughtering and packaging the trout from the fish farm. Thanks to the kitchen staff for preparing the meals and also to Elin Skytte who sampled blood from the subjects and measured the plasma triglyceride content. A special thanks to Hanne Jacobsen, who taught me how to run 2-DE, to notice every little detail during the experimental work with 2-DE gels and for always being positive and helpful. Thanks to Andrea Lorentzen for her great devotion and expertise within mass spectrometry.

Also special thanks to my colleagues at the Division of Seafood Research, DTU for good laughs and constructive discussions. Last but absolutely not least, I am grateful to my family and friends who are the greatest support I can imagine.

Enclosed Papers 

This thesis is based on the following three papers:

- Paper I: Rentsch ML, Lametsch R, Lauritzen L, Bügel SH, Jessen F. A pilot study of the postprandial changes in human plasma proteome profiles after intake of mixed meals (*Prepared for submission to British Journal of Nutrition*)
- Paper II: Rentsch ML, Lametsch R, Lauritzen L, Bügel SH, Jessen F. Consumption of farmed trout affects the human plasma proteome profiles in healthy men (*Manuscript*)
- Paper III: Rentsch, ML, Jessen, F. Proteomic analysis of inter-individual variability of protein patterns in plasma from healthy subjects (*Manuscript*)

Summary

Substantial evidence indicates an association between diet and incidence of chronic diseases. Thus, the development of biomarkers of predicting outcome for food based studies is essential given insights into the metabolic and cellular response induced by nutrients. Moreover, these biomarkers will provide knowledge on the early diet induced changes of proteins that precede health benefits or the onset of disease. However, the link between diet and disease is complex and difficult to unravel.

The main methodology used in the present PhD study was proteome analysis (two-dimensional gel electrophoresis), which is the study of the protein composition in an organism at a given time. Proteome analysis of human blood plasma offers a strong approach to increase the knowledge of the early dynamic changes in proteins induced by dietary components. Furthermore, plasma proteins are a circulating representation of body tissue thus reflecting the overall metabolic state of an individual.

Based on a single meal study and an intervention study in healthy subjects, the aim was to investigate acute and long-term effects of meal intake on the plasma proteome profiling. We further aimed to examine the influence on consumers health of replacing a marine based feed with one of vegetable origin for farmed trout. We also assesed the largest variance component in the 2-DE data set from the single meal study including different experimental parameters.

The results showed that single meal intake of trout compared to poultry elicited postprandial responses in proteins linked to the biological processes lipid transport, coagulation and complement cascade, acute phase response as well as cofactor and vitamin metabolism. No differences were seen in the postprandial triacylglycerol response after intake of trout compared to poultry. A daily meal of farmed trout fed either on pure marine diet or pure vegetable diet, or a reference meal of chicken for eight weeks induced changes in abundance of proteins implicated in the various biological processes haeme transport and catabolism, regulation of vasoconstriction, blood haemostasis, complement cascade, antioxidative defence, cellular component movement, glycolysis and vitamin transport. However, only a single protein identified as complement c1r differed significantly in the marine fed trout compared to the vegetable fed trout. The individual variation represented the largest variance component exceeding experimental parameters as fasting or postprandial sampling, meal type (poultry, marine fed trout and vegetable fed trout) and time (weeks).

This thesis demonstrated that 2-DE based proteomics is a powerful tool to detect meal induced changes in plasma proteins. The individual variation is a significant factor for comparative proteomic studies and needs to be taken into account in the experimental design of human studies and interpretation of data. Furthermore, we

have demonstrated that cross-over studies using an individual as its own control and measure analyte variations over time is an important experimental design for studying human plasma proteins due to large biological variation between individuals.

Resumé

Proteomteknologi i ernæringsforskning – et 2-DE baseret studie af kostinducerede ændringer i plasmaproteiner hos mennesker

Der er belæg for, at der er en sammenhæng mellem kost og forekomst af kroniske sygdomme. Udvikling af biomarkører til at forudsige effekter i forbindelse med ernæringsstudier er derfor af væsentlig betydning, idet der herved opnås en forståelse for de metaboliske og cellulære respons, som induceres af næringsstoffer. Desuden vil disse biomarkører give en indsigt i de tidlige kostinducerede proteinændringer, som går forud for sundhedsgavnlige effekter eller sygdomsfrembrud. Sammenhængen mellem kost og sygdom er dog kompliceret og vanskelig at forklare.

Den anvendte metode i dette PhD studie er primært proteomanalyse (todimensionel gelelektroforese), som er studiet af ændringer og mønstre i proteinekspression i en organisme på et givent tidspunkt. Proteomanalyse baseret på blodplasma fra mennesker er en velegnet metode til at øge kendskabet til de tidlige dynamiske kostinducerede ændringer, da cirkulerende plasmaproteiner repræsenterer vævet i et individ og dermed afspejler den generelle metaboliske tilstand.

Formålet med dette studie var at undersøge akutte og langtidseffekter på proteomprofilen i plasma efter indtagelse af måltider på baggrund af et enkelt måltidsstudie samt et interventionsstudie med raske forsøgspersoner. Desuden undersøgte vi betydningen af at erstatte marint baseret ørredfoder med vegetabilsk i forhold til forbrugernes sundhed. Derudover vurderede vi hvilke eksperimentelle parametre som bidrog til den største variation i et 2-DE datasæt fra enkelt-måltidsstudiet.

Resultaterne viste, at indtagelse af et enkelt måltid af ørred i forhold til fjerkræ udløste forskellige postprandielle respons i proteiner, som er involverede i følgende biologiske processer som lipid transport, koagulation- og komplement-kaskade, akut fase respons samt cofaktor og vitamin metabolisme. Der blev ikke detekteret forskel i det postprandielle triglycerid respons efter indtagelse af ørred sammenlignet med fjerkræ. Et dagligt måltid bestående af enten marint fodret ørred, vegetabilsk fodret ørred eller fjerkræ (reference) gennem otte uger inducerede ændringer i forekomsten af proteiner involveret i forskellige biologiske processer så som hæm-transport og katabolisme, regulering af vasokonstriktion, blod homeostasis, komplement kaskade, antioxidativt forsvar, bevægelse af cellulære komponenter, glykolyse og vitamin transport. Der var dog kun et enkelt protein, komplement c1r, som ændrede sig signifikant efter indtagelse af den marint fodrede ørred sammenlignet med den vegetabilske. Individ-forskelle bidrog til den største variation og overgik eksperimentelle parametre som faste eller postprandielle prøver, måltidstype (fjerkræ, marint fodret ørred og vegetabilsk fodret ørred) og tid (uger).

Denne tese viste, at 2-DE baseret proteom-teknologi er et vigtig redskab til at detektere måltids-inducerede ændringer i plasma proteiner. Individ-forskelle er af

stor betydning for proteomstudier og bør derfor tages i betragtning i forbindelse med eksperimentelt design af humane studier og fortolkning af data. Vi har endvidere vist, at anvendelse af overkrydsningsstudier, hvor hver forsøgsperson er sin egen kontrol, er et vigtigt eksperimentelt design i studiet af plasma proteomet pga. store biologiske forskelle mellem individer.

List of abbreviations

2-DE	Two-dimensional electrophoresis
ALA	Alpha linolenic acid
ANOVA	Analysis of variance
CA	Carrier ampholyte
CHD	Coronary heart disease
CVD	Cardiovascular heart disease
DIGE	Difference gel electrophoresis
DTT	Dithiotreitol
FDR	False discovery rate
IPG	Immobilised pH gradient
LA	Linoleic acid
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization Time-of-Flight
MS	Mass spectrometry
n-3 LCPUFA	n-3 long-chain polyunsaturated fatty acids
PCA	Principal component analysis
pI	Isoelectric point
PLSR	Principal least squares regression
PUFA	Polyunsaturated fatty acids
RCT	Randomised controlled trials
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAG	Triacylglycerol
VLDL	Very low density lipoprotein

1 Introduction

During the last decades, a considerable effort has been made in discovering, quantifying and validating biomarkers. However, new and better biomarkers are urgently needed to improve health and disease diagnosis in the general population. The development of early, sensitive and accurate biomarkers for predicting outcome of food based studies is becoming more and more important given substantial evidence indicating an association between diet and incidence of chronic diseases [Hu and Willett 2002; Van et al., 2008]. Yet, the link between diet and chronic disease is complex and difficult to unravel [de Roos B. 2009].

The main methodology used in the present PhD study is proteome analysis based on two-dimensional gel electrophoresis. This technique is powerful for the high resolution separation of proteins in a complex mixture, thus providing a snapshot of the protein composition in an organism at a given time. From the complex protein pattern it is possible to extract information of proteins, the key actors in virtually all biological processes in the human body, changing in response to various biological stimuli and responses such as diet or disease [de Roos B. and McArdle 2008]. In this way, it is possible to increase the understanding of the dynamic changes in proteins induced by dietary components, which may serve as candidate biomarkers of health and thereby reveal early indications of disease risk [de Roos B. and McArdle 2008; de Roos 2008]. However, the use of proteome studies in nutritional science is still in its youth, but new investigations using this approach will improve our molecular understanding of dietary induced health effects in humans and provide knowledge on the complex mechanisms of action [Kussmann and Affolter 2009].

Human blood plasma represents an attractive and promising source for biomarker discovery since it is easily accessible and is a circulating representation of all body tissue reflecting the overall metabolic state of an individual [Anderson and Anderson 2002; Jacobs et al., 2005; Nedelkov et al., 2005]. Despite its clinical importance and huge amounts of investigations, major challenges exist for plasma proteomic based biomarker discovery due to the huge dynamic range of plasma proteins comprising more than 10 orders of magnitude [Anderson and Anderson 2002; Jacobs et al., 2005]. Furthermore, the human biological variability is a major obstacle in clinical studies because it may mask the discovery of candidate biomarkers and influence the proteins in a way that might enhance or degrade its performance as biomarkers.

In Denmark, farmed fish contributes increasingly to the overall fish consumption and the rainbow trout (*Oncorhynchus mykiss*), is a significant specie due to a high annual production. Regular fish consumption and fish oil supplementation are associated with human health benefits, particular with regard to decreased risk of cardiovascular disease (CVD) [Kris-Etherton et al., 2002]. These beneficial effects have been ascribed to the high content of the n-3 long chain polyunsaturated

fatty acids (n-3 LCPUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in fish [Breslow 2006; Bucher et al., 2002; Kris-Etherton et al., 2002; Wang et al., 2006a]. A further understanding of the underlying mechanisms of the beneficial effects of fish intake will thus play a significant role for human health due to the detection of early metabolic changes that precede the onset of CVD.

2 Part I: Beneficial health effects of fish intake

2.1 Fish and health

Fish is considered one of the main components of a healthy diet. In Denmark, official dietary guidelines concerning fish are expressed in one of the Eight Dietary Advices from the Ministry of Food, Agriculture and Fisheries. The Danish public is recommended to consume 200-300 grams of fish pr. week based on an evaluation of the positive nutritional aspects relative to the potential toxic aspects. However, a major cause of concern is the fact that fish is consumed in relatively small amounts in western diets [Simopoulos 2006] including Denmark, in which fish consumption is lower than recommended [Pedersen et al., 2010].

The interest and understanding of the health effects of fish and fish oil originate from studies in populations consuming diets rich in n-3 polyunsaturated fatty acids (n-3 LCPUFA). In the 1970s, Danish researchers reported beneficial lipid profiles and a greatly lower incidence of CVD in coastal Greenlandic Eskimos consuming a high fat diet rich in n-3 LCPUFA from fish and marine mammals as part of their native lifestyle, compared to an equivalent Danish population [Bang and Dyerberg 1972; Kromann and Green 1980]. Similar effects of fish consumption were seen in the Japanese population. The high fish intake in Japan, compared to Japanese men living in Hawaii and California, was associated with a very low rate of myocardial infarction and death from coronary heart disease (CHD). The incidence in Japan was half that observed in Hawaii, and the highest rate was reported in Japanese people living in California [Robertson et al., 1977]. These initial studies raised considerable interest and led to the subsequent research interest on the role of n-3 LCPUFA in preventing and treating diseases, particular CVD, which is the leading course of morbidity and mortality worldwide [Torrejon et al., 2007].

2.2 Polyunsaturated fatty acids metabolism and dietary sources

The n-3 and n-6 fatty acids are two families of polyunsaturated fatty acids (PUFA). Alpha linolenic acid (ALA) and linoleic acid (LA), the precursors of n-3 and n-6 fatty acids respectively, cannot be *de novo* synthesised or inter-converted in the human body and is termed nutritionally essential fatty acids. A sufficient intake from food or other sources is essential for humans. In the body, ALA and LA are converted and elongated to longer chained unsaturated fatty acid derivates (see Figure 1) and the intermediates can either be incorporated into phospholipids or become substrates for further elongation and desaturation processes [Russo 2009]. ALA is mainly derived from plants e.g. linseed and canola oil and major dietary sources of LA are maize, sunflowerseed, safflowerseed and rapeseed oil, which are used in many processed food products such as bread, cakes, biscuits and crisps [Russo 2009; Schmitz and Ecker 2008].

ALA can be converted in the liver to n-3 LCPUFA including the bioactive eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [Russo 2009]. According to a recent review [Burdge and Calder 2005], chronically increased ALA consumption raised the EPA concentration but an insufficient conversion to DHA in plasma and cell pools was seen. Overall, ALA appears to be a limited source of n-3 LCPUFA [Burdge and Calder 2005]. Fish, especially oily fish such as mackerel, sardine, herring, salmon and trout, is a rich source of EPA and DHA. One should note, that the content of these fatty acids also can vary considerable within species dependent on season, fishing area as well as age and gender [Calder 2004; Racine and Deckelbaum 2007]. EPA and DHA are incorporated into phospholipids in most cell membranes in varying proportions and are released by phospholipases. They are essential for many body functions in man such as growth and reproduction [Lauritzen et al., 2001; Wathes et al., 2007]. Moreover, they play a significant role in health status and can act via multiple pathways affecting cell membrane function, eicosanoid metabolism and gene expression [Arab 2003; Racine and Deckelbaum 2007]. EPA can be metabolised to eicosanoids of the prostaglandin-3, thromboxane-3 and leukotrine-5 series [Schmitz and Ecker 2008]. The longer chained n-6 fatty acid arachidonic acid (AA) is derived from eggs, lard and meat [Arab 2003; Russo 2009] and is the main precursor in eicosanoid synthesis resulting in prostaglandin-2, thromboxane-2 and leukotrine-4 series. The difference between n-3 and n-6 PUFA derived eicosanoids is that mediators derived from EPA and DHA exert most anti-inflammatory effects whereas those derived from AA show mostly pro-inflammatory effects [Arab 2003; Schmitz and Ecker 2008].

The mechanisms whereby n-3 PUFA directly affects gene expression in different tissue are complex and involve multiple processes, and the mechanisms remain unclear [Deckelbaum et al., 2006; Sampath and Ntambi 2004]. N-3 PUFA has been shown to modulate gene expression by regulating different transcription factors such as sterol-regulatory element binding protein (SREBP) and peroxisome proliferator-activated receptor (PPAR). Both transcription factors are responsible for regulating genes involved in lipid homeostasis; SREBP is a key regulator of fatty acid, triacylglycerol and cholesterol synthesis and PPAR plays a role in lipid oxidation and thermogenesis [Price et al., 2000; Sampath and Ntambi 2004].

2.3 Dietary ratio of n-6/n-3 PUFA

Since hepatic conversion of n-3 and n-6 PUFA to longer chained fatty acids shares the same series of elongating and desaturating enzymes, a competition exists between those two families for biochemical metabolism of fatty acids with an excess of one leading to a decrease in the conversion of the other [Schmitz and Ecker 2008]. Particular, the delta-6 desaturase is the rate-limiting enzyme and ALA and LA compete for this enzyme in the synthesis of longer chained PUFA [Emken et

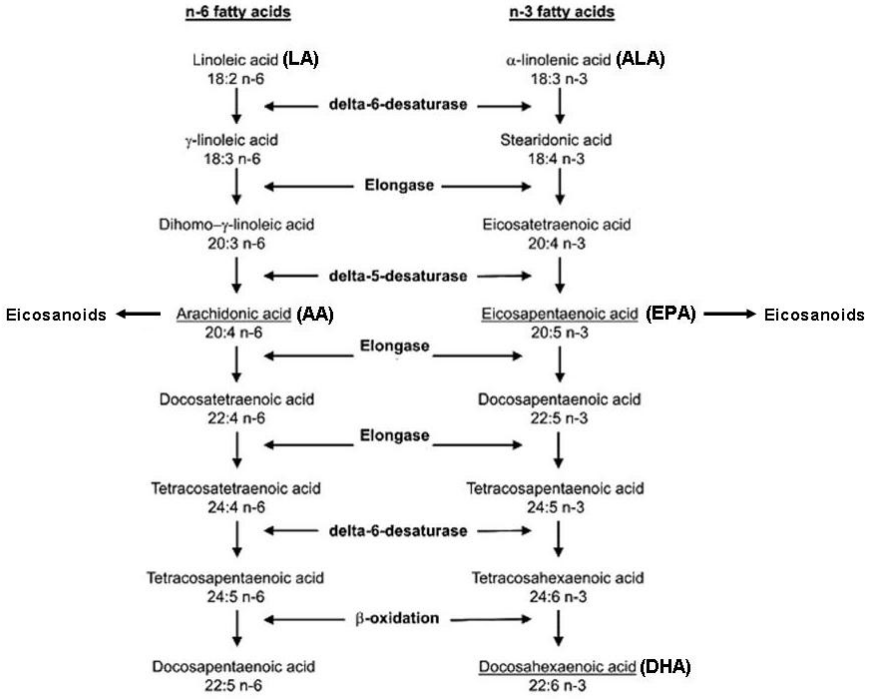


Figure 1: **Biochemical pathways for the metabolism of n-3 and n-6 PUFA.** Modified from [Schmitz and Ecker 2008]. LA: linoleic acid, AA: arachidonic acid, ALA: a-linolenic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.

al., 1994]. Thus, the dietary n-3/n-6 PUFA ratio may have implications for functional outcomes e.g. via changes in cell membrane properties, intracellular signalling, binding of n-3 LCPUFA to transcription factors or changes in bioactive compounds [Calder 2007]. These outcomes are of importance for human health, particularly in the pathogenesis of chronic diseases such as CVD [Russo 2009].

Major changes in our diet have taken place during the evolution from hunter-gatherers to modern agriculture and a westernised life style, particularly in the type and amount of essential fatty acids. During the last 150 years, a dramatic increase is seen in human consumption of vegetable seed oils rich in n-6 fatty acids with a parallel decrease in n-3 fatty acids. Thus, in the present western diet, the ratio of n-6/n-3 PUFA can be as high as 20:1 compared to the diet of our ancestors probably being 1:1 or 2:1 [Simopoulos 2000; Simopoulos 2002]. A high intake of n-6 compared to n-3 fatty acids will favour mediators of primarily pro-inflammatory, pro-thrombotic and pro-aggregatory roles compared to those derived from n-3 fatty acid metabolisms favouring the pathogenesis of chronic diseases (reviewed in [Simopoulos 2002]). Epidemiological studies have shown correlations between the n-6/n-3 PUFA ratio in human diets and CVD. Thus, a lower ratio is needed for prevention and management of CVD diseases, and the optimal ratio is

varying with the disease of consideration [Simopoulos 2006]. However, it should be noted, that beneficial effects of n-6 intake have been reported such as the well described potential of LA to lower both total and LDL cholesterol in the blood and to increase HDL-cholesterol [Kris-Etherton and Yu 1997].

2.4 N-3 LCPUFA and cardiovascular disease

Evidence from a meta-analysis based on randomised controlled trials and cohort studies has shown that intake of fish or fish oil is associated with decreased risk of cardiovascular disease and mortality both in CVD patients as well as people of CVD risk [Bucher et al., 2002; Wang et al., 2006a]. In contrast, a meta-analysis by Hooper et al., 2006 showed no effect of n-3 PUFA on cardiovascular events. The reason for this inconsistency concerning the n-3 PUFA beneficial effects is likely to be attributable to confounding factors such as health status of participants, especially since the Hooper analysis also included studies with healthy people, whereas the Wang analysis were only based on studies with CVD patients. Furthermore, the included studies differ in duration of follow-up time and most importantly type of n-3 PUFA as the Hooper meta-analysis also focused on studies that supplied alpha-linolenic acid, as well as source (dietary or supplemental) and dose administered. These effects have been ascribed to the high content of n-3 LCPUFA in fish [Bucher et al., 2002; Kris-Etherton et al., 2002; Wang et al., 2006a]. Numerous effects of n-3 LCPUFA in relation to health have been reported and some of the proposed mechanisms for the protective role of these fatty acids against cardiovascular disease include lowered blood pressure [Geleijnse et al., 2002], altered lipid profile; especially lowering of triacylglycerol [Harris 1996], reduced trombotic tendency [Robinson and Stone 2006], anti-inflammatory effects [Zampelas et al., 2005], anti-arrhythmic effects including reduction in heart rate [Mozaffarian et al., 2005] and improved vascular function [Chin and Dart 1995]. The plasma triacylglycerol (TAG) lowering effects of n-3 LCPUFA are well established and a reduction up to 30% is reported with effects seen in both normolipidemic and hypertriglyceridemic patients, but with greater efficacy at higher triglyceride baseline levels [Harris 1996]. Fish oil seems to have a small, dose-dependent effect on blood pressure in hyper-cholesteromics and stable hypertensive people, though no effects are seen on healthy subjects [Morris et al., 1993]. A meta-analysis has shown that fish oil reduces heart rate particular in trials with a long treatment duration and with higher baseline heart rate [Mozaffarian et al., 2005]. Thus, beneficial effects are mainly seen in older subjects with already high values of blood pressure and heart rate.

Cohort studies have reported beneficial effects of n-3 LCPUFA, whereas others studies have failed to detect these effects. The Nurses Health study including 85,000 women based on validated questionnaires reported a strong inverse association of fish/n-3 LCPUFA intake and the incidence of all cause mortality through-

out a 16-year follow-up period. Furthermore, the fatty acid-induced protection appeared to be stronger for CHD death than for non-fatal myocardial infarction [Hu et al., 2002]. In contrast, the US Physicians Health Study demonstrated no effects on total myocardial infarction, non-sudden cardiac death or total cardiovascular mortality in healthy men [Morris et al., 1995]. Likewise, the Health Professionals six year follow up Study found no overall association between fish or n-3 LCPUFA intake and the risk of coronary disease in men initially free of CVD, but a non-significant trend toward lower risk of fatal CHD with increasing fish consumption was seen [Ascherio et al., 1995].

The Gizzi-Prevenzione trial, a large secondary prevention trial, has investigated the effect of dietary supplementation with n-3 LCPUFA in 11,000 patients with a recent myocardial infarction. It was found that daily supplementation of one gram of n-3 LCPUFA for two years reduced the occurrence of main cardiovascular end points such as cardiovascular death, nonfatal myocardial infarction and stroke by 20%, coronary or cardiac death and sudden death by 30%, and finally all fatal events by 20% [Valagussa et al., 1999]. In the Japanese five year EPA supplementation study (JELIS) conducted in more than 18,000 hypercholesterolemic patients, documentation of a 19% reduction in major coronary events in the secondary prevention subgroup was seen [Yokoyama et al., 2007]. Results from the 30-year follow up of the Chicago Western Electric Study supported the evidence that fish consumption favourably affects CHD mortality. Men, free of CVD, who consumed at least 35 g of fish daily, had a 40% lower risk of fatal CHD, especially non-sudden death from myocardial infarction [Daviglus et al., 1997].

In a recent meta-analysis, it was described that the effect of fish and fish oil intake in terms of cardiac death is non-linear. Consumption of one to two servings of fish pr.week, especially fish with a high content of n-3 LCPUFA, reduces the risk of coronary death by 36% and total mortality by 17%. Moreover, little additional protective effects were seen with increased fish intake above this level [Mozaffarian and Rimm 2006].

Taken together, the above mentioned studies primarily support the growing body of evidence that strongly suggests that consumption of n-3 LCPUFA has beneficial effects on cardiovascular health. Thus, the American Heart Association, which aims to improve diet and lifestyle to prevent CVD, recommends the general population to eat fish, preferable a fatty fish meal at least twice a week and for those people with known coronary heart disease to consume one g of EPA/DHA pr. day preferably from fish or dietary supplement [Lichtenstein et al., 2006]. In addition to providing EPA and DHA, however, it should be noted that fish consumption may also lead to a decrease in consumption of other foods high in saturated fat and trans-fatty acids.

2.5 Fish protein

Although the main focus of beneficial effects of fish intake according to prevention and treatment of CVD has been related to the high content of n-3 LCPUFA, fish is also an excellent source of other nutrients including high-quality proteins, vitamin A, D and B, minerals and trace elements, especially iodine and selenium. During a fish meal a significant amount of protein is consumed. Interestingly, the possibility that fish protein, particularly amino acids, may contribute to the beneficial health effects has emerged recently. Yet, only few studies have examined the positive health effects of fish protein intake on health markers in humans as well as in animal models of CVD. Ait-Yahia and colleagues have shown that a dietary intervention of purified fish protein lowered blood pressure and decreased very low density lipoprotein (VLDL)-TAG in hypertensive rats [Ait et al., 2005]. Additionally, Boukortt et al. have shown a similar beneficial effect on blood pressure after an intervention consisting of fish protein compared to casein in hypertensive rats [Boukortt et al., 2004]. Furthermore, in normotensive rabbits, dietary fish protein induced a decrease in VLDL-TAG and a concomitant increase in high density lipoprotein-cholesterol and lipoprotein lipase activity [Bergeron et al., 1991; Bergeron et al., 1992]. These results are of importance, since high blood pressure and unhealthy lipid composition are contributors to development of CVD. Other studies in rats have demonstrated that intake of protein from defatted cod fillets resulted in improved glucose tolerance and insulin sensitivity [Lavigne et al., 2000; Tremblay et al., 2005]. A single cross-over study carried out in insulin-resistant men and women has compared the effect of a four-week intervention with proteins from cod compared with other animal proteins concerning their effects on insulin sensitivity [Ouellet et al., 2007]. The consumed diets differed only in terms of protein source. The intake of cod protein improved insulin sensitivity and had a strong tendency to better the beta cell function. Moreover, data from this study showed that intake of cod protein decreased plasma C-reactive protein levels [Ouellet et al., 2008]. These findings may contribute to prevention of type II diabetes by reducing the metabolic complications related to insulin sensitivity.

2.6 Fish farming and sustainability

In recent years, people in developed countries have been encouraged to increase their intake of EPA and DHA from fatty fish to improve health and prevent development of major chronic diseases, especially CVD (see section n-3 LCPUFA and cardiovascular disease). Given this overall interest in n-3 fatty acid consumption, the decreased supplies will not be able to meet the increasing demand due to the rapid worldwide decline of fish stocks. Even though the global catches of fish have been on decline since the 1980s, the number of collapsed stocks has been increasing exponentially since the 1950s consequently leading to the extinctions of marine populations in the worlds oceans [Jenkins et al., 2009]. Thus, the aquaculture

sector aims at relieving the pressure on wild stocks of fish and thus compensates for the shortfall of fish harvest by commercially raising farmed fish for human consumption. Thereby, it has become the fastest growing food production sector of the world and by 2010, more than 85% of global fish oil production is estimated to be consumed in aquaculture feeds [Barlow 2000]. However, the issue is whether the aquaculture industry really enhances or diminishes the available fish supply. In aquaculture industry, carnivore fish species are grown on diets containing significant amounts of fish meal and fish oil. These predators require approximately 2.5-5 times as much fish biomass as is produced and the carnivorous fish farming is thus still perceived as a net fish consumer rather than a producer, which has raised concern about the long-term sustainability of these industries [Naylor et al., 2000]. In that connection, great improvement has been made, since feed conversion ratio values (ratio of the weight of feed added to the weight of fish produced) around one is reported for several fish species [Mente et al., 2006].

Consequently, since wild fish is a finite value, substantial effort has been spent on evaluating different feeding regimes as alternatives to fish meal and fish oils e.g. ingredients of plant origin. The effect of a change in feeding regime from marine to vegetable based origin such as soy bean, lupin, peas and sunflower have been proposed. Nevertheless, complete replacement of fish oil and protein in feed for carnivore farmed fish faces several barriers. A change in feeding regime may influence flesh and eating quality of the fish as well as nutritional properties e.g. the content of n-3 LCPUFA. Thereby, the following consumption of these fish may affect end-consumers health. Furthermore, plant ingredients contain different anti-nutritional factors like protease inhibitors, phytates, lectins, anti-vitamins and tannins, which present in varying degrees may have adverse effects in fish in relation to diet utilisation and digestibility and thus growth performance [Francis et al., 2001]. Studies have shown that rainbow trout fed a diet composed of plant based protein and oil was significantly affected according to sensory attributes of the fillets such as smell, taste, texture, and colour compared to a marine based diet [de Francesco et al., 2004; Skonberg et al., 1993]. In proteomic based studies of liver proteins from rainbow trout, dietary replacement of fish meal with plant proteins has shown to affect a number of metabolic pathways demonstrating the complex nature of gene expression responses to dietary manipulations [Martin et al., 2003; Vilhelmsson et al., 2004].

3 Part II: Proteomics

3.1 Biomarkers and blood proteomics

A biomarker is a measurable indicator of a specific biological state, pathological process and progress or pharmacological response to a therapeutic intervention [Rifai et al., 2006; van Ommen et al., 2009]. The biomarker discovery process aims at identifying proteins being up and down-regulated in association with a specific biological process or disease state compared to healthy individuals [Kussmann and Affolter 2009]. During the last decades, a considerable effort has been made in discovering, quantifying, verifying and validating biomarkers. However, new and better biomarkers are urgently needed to improve health and disease diagnosis. The development of useful, sensitive and accurate biomarkers for predicting outcome in food based studies is becoming more and more important given substantial evidence indicating an association between diet and incidence of chronic diseases [Hu and Willett 2002; Van et al., 2008]. Ideally, a biomarker of health should be able to reflect the subtle but relevant molecular changes of food interventions in health status that precede the onset of disease [van Ommen et al., 2009]. These early biomarkers will give insights into the metabolic and cellular responses induced by a nutrient and how it affects homeostasis in specific tissue within the whole organism. Moreover, they will provide knowledge on the early diet induced changes of proteins that precede health benefits or the onset of disease.

Human blood plasma represents an attractive and promising source for biomarker discovery due to several reasons. Blood is easily accessible in humans [Wang et al., 2006b] and the plasma harbours tens of thousands of proteins that are synthesised, secreted or lost from a variety of cells and tissue [Anderson and Anderson 2002; Jacobs et al., 2005]. Thus, the plasma proteome is a circulating representation of all body tissue reflecting the overall metabolic state of an individual [Anderson and Anderson 2002; Jacobs et al., 2005; Nedelkov et al., 2005]. Despite its clinical importance and huge amounts of investigations, major challenges exist for plasma proteomic based biomarker discovery. Firstly, the complexity and the sheer dynamic range of plasma protein concentrations, secondly, the relatively low abundance of many proteins which are of biological and disease-specific interest and thirdly, the inter-individual biological variation both in terms of physiological and pathological variation between humans [Rifai et al., 2006]. Even though the number of proteins detected in human plasma or serum is increasing rapidly, a paradoxically decline is observed in the number of new protein biomarkers approved for use in clinical diagnostics [Anderson 2005a]. During the last decade, the rate of introduction of new protein analytes approved by the US Food and Drug administration has fallen to one biomarker pr. year [Rifai et al., 2006]. In fact, only a handful of proteins are currently established in clinical use [Anderson and Anderson 2002].

3.2 History of proteomics

The term "proteomics" was first introduced in 1995 and defined as the large scale characterisation of the entire protein complement of a cell line, tissue or an organism [Anderson and Anderson 1996; Wasinger et al., 1995; Wilkins et al., 1996]. Initially, the term was used to describe the study of the expressed proteins of a genome using two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) for separating and identifying proteins. The scope of proteomics has broadened considerably during the last decade and at present includes protein expression profiling, protein function, protein modifications, protein localisation and compartmentalisation and protein-protein interactions [Graves and Haystead 2002]. This rapid expansion of proteomics with increasing application to answer biological questions is clearly reflected in the number of published papers and a literature search conducted in Medline using the query "proteomics" gives 23849 hits. These vast amounts of experimental data being gathered reveal that the proteome is more complex than the genome. The total number of genes in the human genome is 24,000-30,000. If a given cell express 10,000 genes, the number of proteome components in that cell may exceed 100,000 since one gene gives rise to a variety of proteins through mechanisms extending from alternative splicing, proteolytic processing to a wide range of posttranslational modifications [Kim et al., 2004].

Two-dimensional gel electrophoresis (2-DE) is still one of the most widely used approaches in proteomics for identification of changes in proteins of tissue and bio fluids [de Roos B. 2009]. The method is powerful for the high resolution separation of proteins in a complex mixture, thus providing a snapshot of the protein composition in an organism at a given time under a given condition. The classical 2-DE approach used in proteomics today based on the concept of combining isoelectric focusing (IEF) and SDS-PAGE was initially introduced in the pre-omics era in the mid-seventies by O'Farrell [O'Farrell 1975] and Klose [Klose 1975], who began the mapping of proteins from E. Coli and mouse, respectively. They made unforeseen contributions to the research field of proteomics and the application of 2-DE to study cellular and tissue proteins from a global point of view [Klose 2009; O'Farrell 2008]. In the first dimension, the IEF step, proteins are separated according to electric charge (pI), whereas in the second dimension including the SDS-PAGE, separation of proteins is based on their molecular weights. The robustness of the methods has dramatically increased in nowadays mainly due to the introduction of immobilised pH gradients improving the resolution and reproducibility of protein separation in the first dimension [Bjellqvist et al., 1982] and a drastically increased sensitivity of detected proteins by the introduction of silver staining [Merril et al., 1981]. Moreover, the major breakthrough was the development of micro-sequencing technologies in the beginning of the nineties involving the identification of proteins of interest at first by Edman based sequencing following the development and advances of MS and MS/MS based identification of proteins [Aebersold and Mann 2003]. This greatly improved the quality of data

obtained from 2-DE. More recently, the introduction of fluorescent staining has further provided higher sensitivity and larger dynamic range compared to silver staining [Steinberg et al., 2000]. Especially, the development of Difference Gel Electrophoresis (DIGE), first described by Unlu et al., has greatly improved the quantification of proteins in 2-D gels and allows for detection of subtle changes in protein abundance [Unlu et al., 1997]. The inter-gel variation is substantially eliminated by running three samples, marked with different fluorophores, on the same gel.

3.3 Sample preparation of human plasma for 2-DE

Sample preparation is of utmost importance and very critical to acquisition of high quality data from proteome analysis based on 2-DE. Preparation should be as simple as possible to increase reproducibility of the experiment. The main purpose of sample preparation is to solubilise the proteins making them suitable for 2-DE analysis, while minimising the loss of proteins during the procedure. The solubilisation process includes denaturation of the protein to break non-covalent bindings within and among the proteins, while maintaining the native charge and molecular weight of soluble proteins. Ideally, the perfect solubilisation mixture should freeze all the proteins in their exact state both in terms of amino acid composition and posttranslational modifications [Rabilloud 1999].

A large number of standard protocols are available, but these protocols have to be adapted and optimised for the type of sample e.g. body fluids, tissue extracts and microbial cells to be analysed. The following section will focus on the sample preparation procedures of human plasma, since it is used in the present PhD study and is a highly complicated specimen to analyse by 2-DE. Figure 2 illustrates the flow diagram of sample preparation of plasma. Blood is collected in tubes containing anticoagulants to prevent blood clotting. Different anticoagulants have different molecular mechanisms, which are reflected in the proteome profile. In a study by Hsieh et al., the addition of anticoagulants including heparin, EDTA and sodium citrate was shown to have an impact on the following plasma proteome profiling on the basis of whole MALDI-TOF spectra [Hsieh et al., 2006]. Moreover, the time-lag before separation of plasma from whole blood is very critical for the analytic stability and the following proteome profiling due to cellular elements; platelets in particular, can secrete a variety of cellular components. Therefore, in the present PhD study plasma was prepared directly after blood collection to minimise pre-analytical effects.

Addition of protease inhibitor cocktails have extensively been used in order to prevent degradation of proteins that otherwise will result in artefactual spots on the 2-D gel and loss of high molecular weighted proteins. However, protease inhibitors are not always recommended since they may modify protein structures and induce changes in the charge of the molecules [Kim and Kim 2007] and may interfere with

the MS based identification of proteins of interest. TCA/acetone mediated precipitation of proteins is useful to minimise protein degradation by proteases [Gorg et al., 2004]. In addition, it has been shown that urea, thiourea and CHAPS in combination are very effective inhibitors of proteolytic activity [Castellanos-Serra and Paz-Lago 2002].

The major problem concerning the visualisation of plasma proteins is the broad and dynamic range of proteins comprising more than 10 orders of magnitude [Anderson and Anderson 2002; Jacobs et al., 2005], which really poses a challenge for proteomics. The steep dynamic range begins with the core plasma protein, albumin, which comprises approximately 50% of plasma protein content (45mg/ml) and ends with circulating cytokines present at 1-10pg/ml [Anderson and Anderson 2002; Jacobs et al., 2005]. Therefore, sub-fractionation of plasma is a requisite to simplify the protein composition of the plasma sample and to reach the lesser abundant proteins. Strategies that often have been used to overcome this problem are to fractionate the proteome into smaller sub-sets. Multiple approaches have been used to simplify the plasma proteome including affinity, size and antibody based depletion of high abundance proteins [Kim and Kim 2007]. Removal by immunoaffinity capture is the most commonly used approach because of advantages of the highly selective depletion process and the low cross-reactivity to non-target proteins [Crosley et al., 2009; de Roos 2008; Jenkins et al., 2008]. In the present PhD study, plasma was fractionated by an IgY-12 antibody based spin column (Beckman Coulter, CA) in accordance with the manufacturers instructions to remove the top 12 high abundance proteins. These proteins make up less than 0.1% of the total number of proteins present in plasma, yet they comprise up to 95% of the total protein concentration in plasma [Huang et al., 2005]. Desrosiers and co-workers [Desrosiers et al., 2007] have compared the efficiency of IgY-12 column with two other columns removing two and six of the high abundance proteins, respectively. They showed that the IgY-12 column was most efficient in enriching less abundant proteins. This sub-fractionation of plasma will allow better resolution and higher sensitivity required to detect the lower abundant plasma proteins compared to crude plasma and thus increases the possibility to "dig" deeper into the human plasma proteome. However, the principal disadvantage of antibody based depletion includes its relatively high cost and low sample capacity. Moreover, a potential artefact to be aware of includes non-specific binding to the column caused by the ability of high abundant proteins, like albumin, to act as carrier protein for peptides and protein fragments [Desrosiers et al., 2007; Elrick et al., 2006]. The 2-DE based plasma protein profiling of human plasma before and after IgY-12 treatment depleted of the top 12 high abundance proteins is shown in Figure 3. It is clearly seen that the high abundance proteins contribute to a lot of smear in gel 3A. Removal of these proteins enables detection of the lower abundant proteins seen in gel 3B, and the result is a gel of distinct and separated spots.

2-DE based analysis of human plasma is rather difficult due to the many components in the sample that bind with proteins and possibly interfere during the

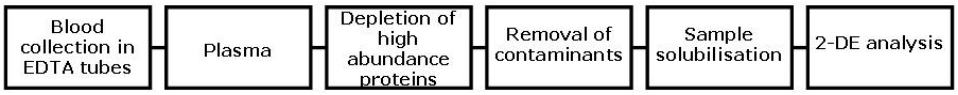


Figure 2: **Flow diagram of the sample preparation procedure of plasma for 2-DE analysis.**

analysis. Thus, removal of interfering substances is a prerequisite to 2-DE if their amounts exceed a critical interference threshold. Contaminants in the sample primarily include lipids, salts, polysaccharides and nucleic acids. Plasma turbidity is normally caused by the lipids (TAG, phospholipids and sterols) in the sample, which is highly present if sampling is conducted after meal intake. Lipids can interfere with the depletion procedure, the IEF current in the first dimension and the 2-DE resolution [Kim and Kim 2007]. The removal of lipids can be accomplished by the use of a solubilisation buffer containing a strong denaturing agent [Jiang et al., 2004]. During first dimensional electrophoretic separation, salt ions can interfere with the focusing of proteins and the time required for proteins to reach a steady state will be prolonged [Gorg et al., 2004; Kim and Kim 2007]. Desalting can be achieved by TCA and acetone precipitation, which was used in the present sample preparation of human plasma. However, this may result in protein losses due to incomplete precipitation and insufficient re-solubilisation of proteins. Polysaccharides and nucleic acids may interact with carrier ampholytes and proteins, which are capable of giving rise to streaky patterns in the 2-DE gel. A common method used to minimise the influence of these molecules, is to precipitate proteins based on TCA and acetone [Gorg et al., 2004].

Prior to 2-DE, the sample has to be reduced, denatured, disaggregated and solubilised to completely disrupt molecular interactions (disulfide bridges and non-covalent interactions including ionic bonds, hydrogen bonds and hydrophobic interactions) under conditions compatible with IEF. Addition of excess reducing agents is necessary for cleavage of intra and intermolecular disulfide bonds so proteins completely can unfold. Dithiothreitol (DTT) is one of the most frequently used reductants [Gorg et al., 2004]. However, one should be aware of that DTT is charged at alkaline pH and therefore is still not a perfect reducing agent. Some proteins of high cysteine content or high cysteine reactivity are not fully reduced by DTT, but addition of phosphines, which are uncharged reducing agents, can solve the problem [Rabilloud 1999]. It has been reported that tributyl phosphine may be more effective with protein samples that are difficult to solubilise [Herbert et al., 1998]. However, problems concerning stability and solubility of this reagent in aqueous solution are present.

Protein denaturation achieved by addition of chaoptropic agents like urea and thiourea is widely used in sample preparation for 2-DE due to their efficiency for solubilising proteins, as opposed to urea alone. Urea is quite efficient in disrupting hydrogen bonds while thiourea is more suitable for breaking hydrophobic interac-

tions. Additional detergents such as CHAPS are commonly included in the solubilisation mixture due to its efficiency for solubilising hydrophobic proteins [Gorg et al., 2004].

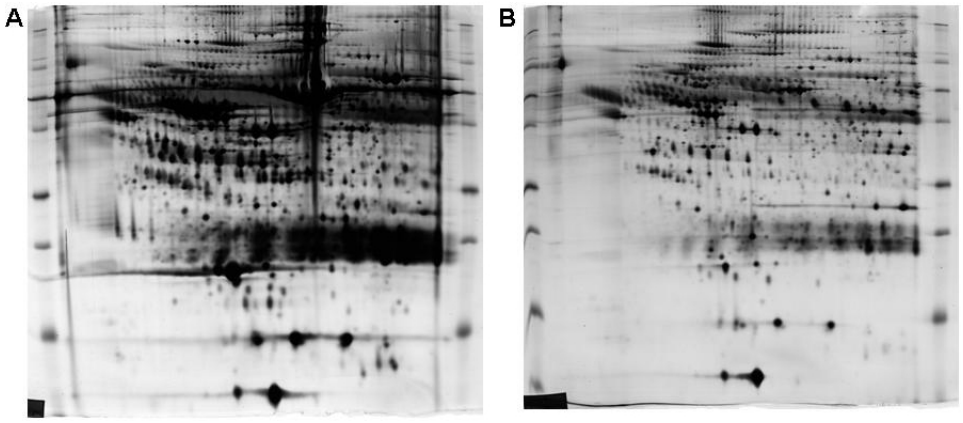


Figure 3: **2-DE based plasma protein profiling of human plasma before and after IgY-12 treatment.** In the first dimension, proteins are separated according to electric charge in an immobilised pH gradient of pH 4-7, whereas in the second dimension, proteins are separated according to their molecular weights in a 12% SDS-PAGE gel. A: Un-fractionated human plasma, B: Human plasma protein fraction after IgY treatment depleted of the 12 most abundant proteins.

3.4 First dimensional electrophoresis

The first dimension of 2-DE consists of isoelectric focusing, where proteins are separated on basis of their charge. The isoelectric point (pI) of a protein is the pH value of the proteins surroundings at which the protein has a zero net charge. Carrier-ampholyte (CA) generated pH gradient was originally used in the first 2-DE protocols introduced by O'Farrell and Klose in 1975 [Klose 1975; O'Farrell 1975], but the method suffers from several limitations including resolution, reproducibility, separation of very acidic and/or very alkaline proteins and sample loading capacity [Weiss and Gorg 2009]. In order to overcome the limitations of CA, immobilised pH gradients (IPG) was developed by Bjellqvist [Bjellqvist et al., 1982] and further improved by Gorg and colleagues [Gorg et al., 1988]. To achieve high resolution 2-DE patterns, the classical IEF protocol was modified taking into account that IPGs behave differently compared to CA generated pH gradients. This new separation technique greatly improved the resolution and it was the beginning of a long journey of stepwise improvements of the 2-DE technology. Consequently, IPG is nowadays the current method of choice in IEF of 2-DE in proteome analysis [Gorg et al., 2009]. The IPG gradient is immobilised due to covalent anchoring of the pH gradient to the acrylamide matrix during casting, which generates an

extremely stable pH gradient. This leads to true steady state IEF with high reproducibility due to constant zone position and pattern constancy and elimination of batch differences as observed with CA [Gorg et al., 1988; Righetti and Bossi 1997]. IPG can be cast in different ranges between pH 2.5-pH 12 in both narrow or wide ranges [Gorg et al., 2004] and the choice of range is dependent on sample complexity and scientific purpose. Temperature during IEF has a significant effect on the 2-DE pattern, since the position of protein spots on the gel vary along the pH gradient, when different temperatures are applied. Thus, to achieve high reproducibility of 2-DE gels, it is very important that IEF is conducted under a defined temperature, where 20°C has been shown to provide optimal conditions [Gorg et al., 1991].

3.5 Second dimensional electrophoresis

Following the separation of proteins by first dimensional IEF, the second dimension is carried out by SDS-PAGE in an electric field, where proteins are separated according to their molecular weights. SDS is an anionic detergent and disrupts hydrogen bonds, blocks hydrophobic interactions and partly unfolds the protein. It gives the proteins the same net negative charge per unit mass overwhelming the native charge of sample proteins. Thus, during electrophoresis the denatured SDS polypeptides are separated in the gel solely based on their molecular mass.

SDS-PAGE in second dimension can be performed on horizontal or vertical systems. The horizontal system is suited for pre-cast commercial gels but has limited capacity of only a single gel, whereas the vertical system used in the present PhD study is the system of choice for multiple runs in parallel, particularly for large-scale analysis which requires simultaneous batch electrophoresis to increase through-put and maximise reproducibility [Anderson and Anderson 1978]. The most commonly used buffers for 2-DE is the discontinuous buffer system by Laemmli [Laemmli 1970]. Altering the amount of acrylamide will affect pore size and thus the sieving properties of the polyacrylamide gel. Nevertheless obtaining high-quality separation of the entire molecular weight range of the sample proteins remains an art, as differences can arise from a number of factors during the process. The result of 2-DE analysis is a protein map of spots that are assigned their x and y coordinates. Proteins are recognised according to their specific coordinates and each spot represents one to few proteins depending on the complexity of the sample. High resolution 2-DE can potentially resolve up to 10,000 protein spots simultaneously [Zimny-Arndt et al., 2009] and from the complex protein profile pattern it is possible to extract information about changes in protein expression levels, isoforms or posttranslational modifications.

3.6 Visualisation of proteins

After conducting 2-DE, the separated proteins in the gel have to be visualised, as the main purpose of differential proteomics is to study the changes in expression level/amount of proteins. It is important that the visualisation approach meets the acquirements of high sensitivity (low detection limit), high linear dynamic range (quantitative accuracy) and compatibility of post-electrophoretic identification methodologies such as mass spectrometry. However, currently no staining method for proteome analysis fulfils all these criteria [Gorg et al., 2004]. The visualisation of proteins can be obtained by application of various staining techniques including organic dye, silver stain, reverse stain, fluorescent stain, radio-labelling and chemiluminescent stain [Patton 2002]. Conventional Coomassie Brilliant Blue is one of the most widely used dyes for 2-DE protein staining due to low cost, ease of use and good compatibility with downstream protein identification methods such as mass spectrometry. The principal limitation of this dye is insufficient detection sensitivity, hence it is not able to stain low abundance proteins. It is based on end-point staining with a detection limitation of 10-100 ng protein per spot. Additionally, the dye provides a linear dynamic range of 10-30 fold of protein concentration [Weiss et al., 2009; Wu et al., 2005].

Silver staining is a frequently used approach for visualisation of 2-DE separated proteins. It is sensitive and permits detection of the low nanogram level (0.5-1 ng of protein) but with a restricted linear dynamic range of only 10 fold concentrations. In contrast to Coomassie, silver stain is a quite complex procedure with carefully controlled multi time-steps and staining development must be stopped at some arbitrary time point in order to avoid saturation effect [Wu et al., 2005]. Consequently, gel-to-gel reproducibility can be problematic. The most sensitive silver stains are MS incompatible since aldehydes in the fixatives irreversible cross-link polypeptide chains. However, alternative silver staining protocols have been developed that are compatible with MS based protein identification [Shevchenko et al., 1996; Wu et al., 2005]. Generally, the modified technique is poorer in terms of detection sensitivity and background staining is less uniform [Patton 2002].

Fluorescence staining of proteins is gaining popularity due to high sensitivity, broad dynamic range and ease of use. On the other hand, the cost of the method is quite high due to expensive chemicals and instruments needed for the detection of fluorophores. The most common used fluorescence stain is non-covalently bound fluorescence dye, such as Sypro Ruby, a ruthenium based metal chelate stain that binds to basic amino acids in proteins and was originally developed for detection of proteins in SDS-PAGE gels and proteins blotted to membranes [Wu et al., 2005]. The detection sensitivity (1 ng of protein) is as high as silver staining and the linear dynamic range is at least three orders of magnitude exceeding Coomassie Brilliant Blue and silver stain in terms of performance. Furthermore, it allows one-step end-point staining and it can thus be performed over night without overdeveloping and

concomitantly with low background staining [Ahnert et al., 2004; Patton 2002]. Finally, most procedures are compatible with protein identification methods such as mass spectrometry [Gorg et al., 2004].

In the present PhD study, three different types of staining to visualise human plasma proteins were compared; Coomassie Briliant Blue, Sypro Ruby and silver staining shown in Figure 4, respectively. By manual inspection, it is clearly demonstrated that the approach based on silver staining is by far the most sensitive staining technique to visualise the proteins. However, one should be aware of the drawbacks of this staining method mentioned above. The low sensitivity of Coomassie Brilliant Blue is also clearly confirmed even despite the fact that the sample is four times as concentrated. Therefore, the staining of plasma proteins in the enclosed papers is carried out by use of silver staining.

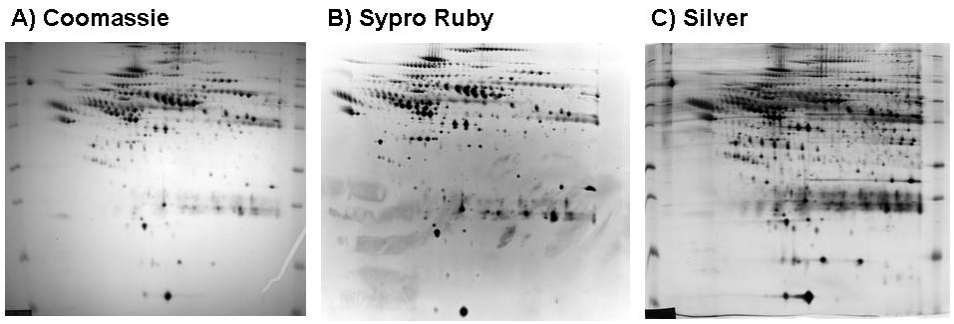


Figure 4: **Visualisation of proteins.** Plasma proteins are visualised on 2-DE gels by use of (A) Coomassie, (B) Sypro Ruby and (C) silver staining.

3.7 Image analysis

After protein staining, it is of vital importance that protein spots are aligned accurately to ensure high quality data from proteome experiments. One of the key limitations for 2-DE based studies is the lack of a rapid, robust and reproducible method for gel image analysis in terms of detecting, matching and quantifying spots. To facilitate rapid and accurate image analysis with relatively high throughput, a number of commercial software packages are available for image analysis of 2-DE gels e.g. Delta 2D, Melanie, PDQuest, Image Master 2D Elite and Progenesis [Raman et al., 2002]. These are based on different detection and matching principles and have a broad range of options, capability and price. However, all software packages use the same traditional workflow involved in differential image comparisons analysis; (i) spot detection, (ii) gel-to-gel matching and (iii) spot quantification [Raman et al., 2002]. Yet, manual editing during the spot detection is nearly impossible to avoid due to sample complexity with several thousands of spots on a gel and mismatches must be carefully checked and edited e.g. deletion of false protein spots and correction of spot shape. Even a very experienced

person will not be able to produce gels with similar spot patterns. Dissimilarity of spot positions in the 2-DE gel may be caused by variations in pH of running buffer, incomplete polymerisation, current leakage, air bubbles in gels and influence of high abundant proteins on the pH gradient in IPG gels caused by their own locally concentrated buffer capacity [Berth et al., 2007]. Slight variations in protein load pr. gel and staining efficiency can have a considerable impact on the raw spot volumes. Hence, spot volume normalisation, a necessary step in 2-DE data acquisition to minimize the analytical variation caused by gel-to-gel variations, is very important for quantification of spot volumes.

Thus, it is important to stress, that even with the best performing program it is not possible to extract good results from bad gels. Thus, 2-DE gels of good quality are a prerequisite for high-quality image analysis and it is important to avoid experimentally caused artefacts (background, noise and streaking), which will influence the spot detection and quantification process.

3.8 Data analysis

In 2-DE based proteomics, large amounts of high dimensionality data are produced due to the detection of hundreds or even thousands of protein spots. Generally, data is divided in two groups, treatment versus control group, and spots are commonly tested individually by use of univariate statistics such as the Students t-test to reveal spots changing in abundance between the two groups [Biron et al., 2006]. The variation in spot volumes is heterogeneous; some spots differ highly in volume, while others show only small differences. Consequently, data deviating from normal distribution is not uncommon. Often, data analysis is based on Students t-test but the normal distribution of data is frequently assumed and not tested [Wilkins et al., 2006]. Overall, inappropriate use of Students t-test or analysis of variance (ANOVA) may result in a number of false positives spots, which is the case in several proteomic studies, and this needs to be taken into consideration in the evaluation of the data. Multiple testing correction methods exist (Bonferroni correction and False Discovery Rate (FDR)) to adjust the values of protein spots based on Students t-test or ANOVA to keep the overall rate of error as low as possible. An extension of FDR is the q-value, which is the expected proportion of false positives incurred among a set of significant findings changing in expression [Storey and Tibshirani 2003]. For each given p-value, a q-value can be reported and an overall estimate for the proportion of protein species changing in abundance.

Using univariate statistics alone, it is impossible to reveal all the complex interactions in protein expression profiling, and essential data information as well as data structure is lost in the analysis. In multivariate statistical analysis e.g. principal component analysis (PCA) and Partial Least Squares Regression (PLSR), all spots are analysed simultaneously and this method offers a strong approach for the evaluation of 2-DE maps to get an overview of the main variation and structure in data

[Jensen et al., 2008; Karp and Lilley 2007]. PCA is a multivariate pattern recognition method breaking down the data matrix into systematic variation and noise. PCA results in the decomposition of raw data into scores, describing the relationship between samples, and into loadings showing the relationship between variables [Marengo et al., 2007]. The variation is described by principal components (PC1, PC2, PC3 etc,), where the first PC explains the maximum possible variance in the data set and each successive component account for the remaining residual variance. The PCs are then calculated hierarchically, so experimental noise and random variation are contained in the last PCs [Marengo et al., 2007; Marengo et al., 2008]. Of course, this is only true, if experimental noise and random variations represent a minor contribution compared to the systematic variation in data. An example of PCA analysis of data from 2-DE based proteomics is demonstrated in Figure 5. The proteome data is obtained from the single meal study in which human plasma samples were collected before meal intake and during the postprandial phase. From the 2-DE based data, it is demonstrated that samples are grouped, to a more or less extent, according to each subject and the inter-individual variation thus represents a major variance component in the data.

Another very useful multivariate statistical approach is PLSR. By use of this regression method it is possible to identify the relation of variation between two sets of data (x and y data matrix), e.g. spots compared to other characteristics of the samples. The result of the PLSR analysis is a prediction model, where the variation in the total spot dataset (x-variables) is used to explain the variation in other measurements or related information of the same samples (y-variables).

3.9 Mass spectrometry based identification of proteins of interest

The classical 2-DE based approach coupled with spot identification by mass spectrometry has become the most widely used approach in proteomics to effectively identifying the protein spots of tissue, cells and bio fluids [Fuchs et al., 2005; Roepstorff 1997]. Various proteome maps with identified proteins originating from different phyla, tissue and bio fluids are available on the internet at the Swiss-2D-PAGE site (www.expasy.org/swiss-2dpage/viewer) with a constantly increasing number of identified protein spots. A huge development of MS based technologies both in relation to instruments and computational protein analysis techniques has enhanced the sensitivity and through-put of identification of 2-DE separated proteins [Gygi and Aebersold 2000; Poutanen et al., 2001]. Furthermore, the sequencing of several genomes, including the human genome, has been a great advantage in MS based identification of proteins [Roepstorff 1997].

Mass spectrometry enables structural information such as exact peptide masses and amino acid sequences, which makes it possible to identify proteins of interest [Graves and Haystead 2002]. Different methods have been developed for identification of proteins from 2-DE gels. Basically, fingerprints of proteins are created by

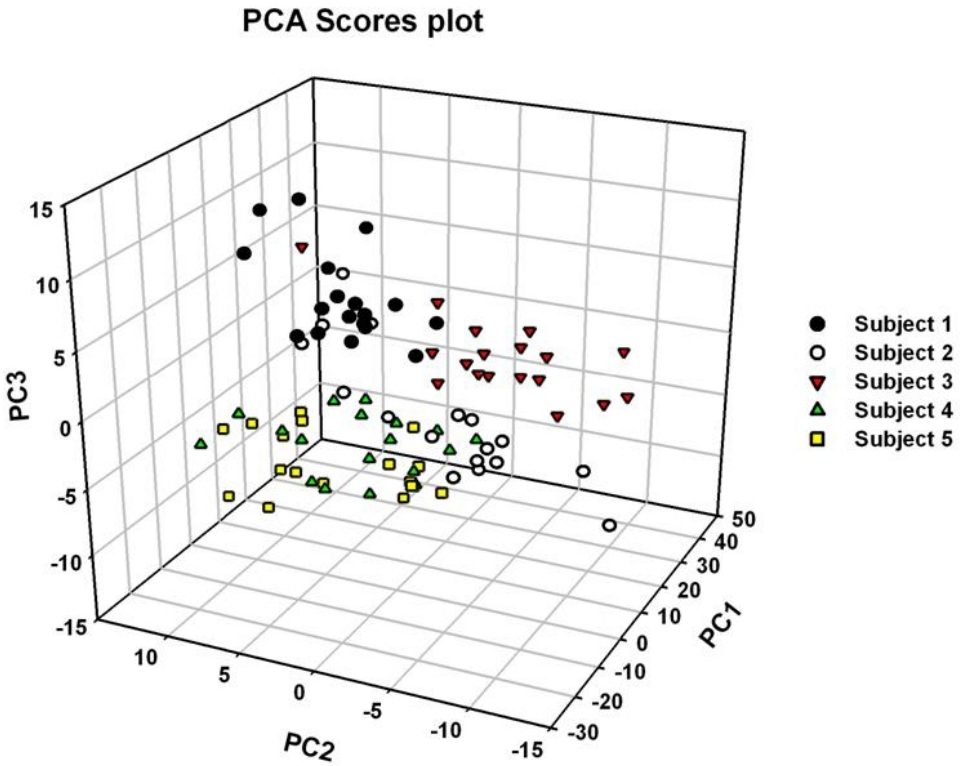


Figure 5: **PCA Scores plot of 2-DE separated human plasma proteins derived from the single meal study.** The PCA scores plot displays the first three principal components in which PC1 (23%), PC2 (8%) and PC3 (6%) account for 37% of the total variation in the data set.

in-gel digestion of proteins by a specific protease e.g. trypsin following a recording of a mass spectrometric peptide map with information of experimental peptide mass-to-charge value. Comparing the experimentally obtained mass against a theoretical obtained mass data from an already identified protein in a database leads to protein identification. The work presented in this thesis is based on MS/MS results obtained from Maldi/Tof-Tof mass analyser, which is a classical approach and commonly used for identification of proteins from 2-DE. Maldi-MS is a sensitive method and normally used to analyse relative simple peptide mixtures, like in this case a protein excised from a 2-DE gel, contrary to liquid-chromatography electro spray ionisation MS systems, which are ideal for analysis of complex samples [Abersold and Mann 2003]. Additionally, Maldi-MS is more tolerant to contaminants like salts and small amounts of detergents, which may appear in the excised protein spots from the 2-DE gel [Domon and Aebersold 2006]. Having determined the mass-charge value and intensity of all the peptides in the MS spectrum, the mass spectrometer proceeds to obtain sequence information about these peptides by further fragmentising single selected peptide ions. The MS/MS spectrum is

the result of an ensemble of one particular precursor ion fragmented at different amide bonds and short amino acid sequence tags are generated for the individual peptides. These partial sequences are compared to the original peptide masses of all known proteins within the database, which greatly improves specificity [Steen and Mann 2004]. Various peptide mass search programs are available on the Internet e.g. Mascot, MS-fit and PeptideSearch [Kim et al., 2004] and the complexity of the algorithms used to search sequence databases is program dependent. When conducting large-scale studies with following identification of proteins by database searching, one should use an organism with sequenced genome; so that all possible peptides are known and time consuming *de novo* sequencing is thus not mandatory. The Mascot MS/MS-search (www.matrixscience.com) is used for database searching, where different MS variables e.g. instrument, taxonomy, database, enzyme and modifications etc. are typed in prior to the search. Figure 6 shows a Mascot report from a database search using a Maldi-Tof/Tof spectrum of a trypsin-digested spot from a 2-D gel containing human plasma proteins. In this case, peptides gaining a Mascot score greater than 27 are found significant ($p<0.05$). However, it should be noticed that the significance limit is database dependant due to number of entries. A single protein was significantly identified as retinol-binding protein 4 with a score of 281, which is highly trustworthy. The retinol-binding protein hit has a sequence coverage of 29%, which is defined as the ratio of the portion of the protein sequence covered by the matched peptides to the whole length protein sequence.

The accuracy of the mono-isotopic mass is limited by the instrument used. Today, by use of modern instruments, mass accuracy lower than 50 ppm is routine [Barnes and Kim 2004]. In paper I, protein identifications are based on 2-5 peptides with a mean mass accuracy of 7 ppm. Ideally, one would expect to observe a signal for all peptides and whole sequence coverage of the protein. However, this is hardly ever the case. Some proteins had an experimental molecular mass, estimated from the spot position on the 2-DE gel, differing from the theoretical molecular mass. This indicates that the analysed proteins were a fragment of their respective full length proteins. Furthermore, this was clearly documented in the mascot report by looking at the original amino acid sequence, where the matched peptides were found for instance at the C-terminal end of the sequence with resulting low sequence coverage, since the calculation is based on the full length protein. From time to time a spot contains a mixture of proteins. When a single protein in the spot is identified, additional proteins can be identified by doing a “second pass” search meaning that after a correct identification of the first protein, the unmatched peptides can be used in a new search. This feature is offered by the search engine Mascot.

A MATRIX SCIENCE **Mascot Search Results**

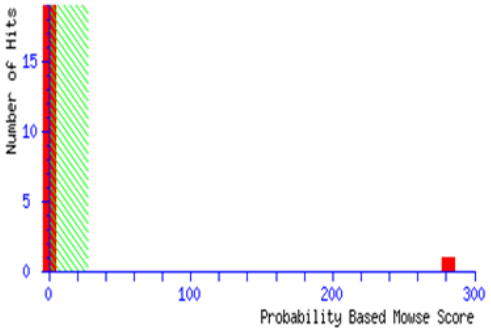
Database : SwissProt 57.12 (513877 sequences; 180750753 residues)

Taxonomy : Homo sapiens (human) (20401 sequences)

Timestamp : 5 Jan 2010 at 10:35:35 GMT

Protein hits : RET4_HUMAN Retinol-binding protein 4 OS=Homo sapiens GN=RBP4 PE=1 SV=3

Probability Based Mowse Score
Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 27 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. RET4_HUMAN Mass: 23337 Score: 281
Queries matched: 4

Retinol-binding protein 4 OS=Homo sapiens GN=RBP4 PE=1 SV=3

B MATRIX SCIENCE **Mascot Search Results**

Protein View

Match to: **RET4_HUMAN** Score: **281**
Retinol-binding protein 4 OS=Homo sapiens GN=RBP4 PE=1 SV=3

Nominal mass (M_r): **23337**; Calculated pI value: **5.76**
NCBI BLAST search of RET4_HUMAN against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **29%**

Matched peptides shown in **Bold Red**

```
     1 MKWVWALLLL AALGSGRAER DCRVSSFRVK ENFDKARFSG TWYAMAKKDP
    51 EGLFLQDNIV AEFSVDETGQ MSATAKGRVR LLNNWDVCAD MVGTFTDTED
   101 PAKFKMKYWG VASFLQKGND DHWIVDTDYD TYAVQYSCRL LNLDGTCADS
   151 YSFVFSRDPN GLPPEAQKIV RQRQEELCLA RQYRLIVHNG YCDGRSERNL
   201 L
```

Figure 6: **Mass spectrometry based identification of proteins.** MS/MS based Mascot search results of a spot exised from a 2-DE gel.

3.10 Experimental design and biological variability

Randomised controlled trials (RCT) are considered as gold standard in human studies, when evaluating the efficacy and safety of a treatment intervention [Silverman 2009]. In these trials, subjects are randomised into different parallel treatment groups and each group receives a specific treatment. They are simpler in experimental design and more feasible compared to cross-over studies [Lathyris et al., 2007]. In a cross-over study, each individual receives two or more treatments, but in a random order. These studies represent relatively complex study design and it can be difficult to apply for many research questions [Lathyris et al., 2007]. The particular strength of a cross-over study is that the different treatments are evaluated on the same subject, allowing comparison at the individual level rather than on the group level as seen in RCT. Since each subject receives both interventions and thus acts as its own control, the number of subjects required to produce similar precision is reduced considerably compared to parallel group trials [Elbourne et al., 2002]. Furthermore, this study design can eliminate biological variation, since variation in repeated responses within a subject is usually smaller than that seen between subjects. Also, the number of replicate 2-DE gels is reduced. In contrast, it should be noted that a cross-over design mediates a risk of a carry-over effect, occurring when the treatment given in the fist period has an effect that is carried over in the subsequent treatment period [Elbourne et al., 2002]. However, a wash-out period between the different treatments may reduce the effect of carry-over. Another noteworthy aspect is the treatment period interactions, which may introduce bias into the outcome of the study.

In nutritional studies, the metabolic changes caused by a dietary intervention are often small and subtle when working with subjects who are generally healthy and in metabolic homeostasis. In addition, the effect of a dietary treatment is often smaller than the biological variation in metabolic responses between individuals [Rezzi et al., 2007]. Factors like sex, age, genetic background, environment, health status and lifestyle may contribute to this variability [Anderson and Anderson 2002; Nedelkov 2008; Winkler et al., 2008]. Consequently, this poses a challenge for nutritional experimental design when evaluating dietary effects in humans and care should be taken to minimise this source of heterogeneity in a given study. One way to cope with this variation is to conduct carefully controlled studies with defined diets where each subject participate both in the control and the test group i.e. cross-over study design. Furthermore, the criteria for selecting participants are also of importance in terms of reducing inter-individual variation. Pre-study screening of participants including measurements of biological parameters such as body mass index, blood pressure and clinical measurements as blood lipid status and glycaemia is essential for participant selection. Moreover, additional knowledge about the participants including age, habitual dietary and lifestyle habits may contribute to the selection process. According to proteomic based biomarker discovery, the biological variation may affect a given protein in a way that might

enhance or degrade its performance as a biomarker. To date, the extent of the inter-individual variability of protein expression remains unclear. One of the main challenges in proteomic based nutritional research is to separate the changes in protein expression profiling induced by a well-determined food intervention from the so-called confounding factors such as biological variability. Only few studies have demonstrated the presence of inter-individual variability in different proteomes e.g. plasma, platelets, monocytes, peripheral blood mononuclear cells, saliva, urine, cerebrospinal fluid and liver [Hu et al., 2005; Nedelkov et al., 2005; Nedelkov 2008; Quintana et al., 2009; Winkler et al., 2008; Zerefos et al., 2006; Zhang et al., 2006].

4 Aim of the study

The overall objective of this PhD study was to investigate the impact of meal intake on human plasma proteins assessed by proteomics. Both acute effects and long-term effects were evaluated based on a single meal study and a human intervention trial, respectively.

The specific aims of the study were:

- To examine the postprandial changes in the plasma proteome and lipid profile of elderly healthy men after single meal intake of trout (marine based feed) and poultry. Furthermore, it was warranted to investigate whether a two-dimensional gel based approach was a valuable tool with respect to detection of the postprandial induced changes in human plasma proteins (Paper I).
- To investigate the long-term effects of a change in feeding regime of farmed trout from marine origin to vegetable based diet on consumers health. The effects were assessed by measurements of plasma proteome profiles in healthy adult males (Paper II).
- To study the extent of biological variation between subjects with respect to protein abundance assessed by measurements of the plasma proteome profiles in healthy elderly men (Paper III).

5 Overview of experimental design

The experimental part of the present PhD thesis is based on a single meal study and an intervention trial conducted in healthy elderly men. The results from these studies are presented in the three enclosed papers (see appendix). The following section will briefly describe the experimental design of the two human studies to give an overview of the experiments, which have been addressed in the papers.

5.1 Single meal study

The single meal study was a randomised single-blinded dietary cross-over study including five healthy men in the age of 54-70 years. In random order, the subjects received three test meals with poultry (control), marine fed trout (high of n-3 PUFA) or vegetable fed trout (high of n-6 PUFA) separated by one-week of interval. Blood was sampled before the meal intake (baseline), four times during the postprandial phase (1.5, 3, 4.5 and 6 h after meal ingestion) and after 24 h (fasting blood sample) for proteome analysis and MS-based identification of proteins of interest as well as TAG analysis (see Figure 7 and 8). Details concerning the

participants, the meal content and the methods are described thoroughly in paper I and paper III.

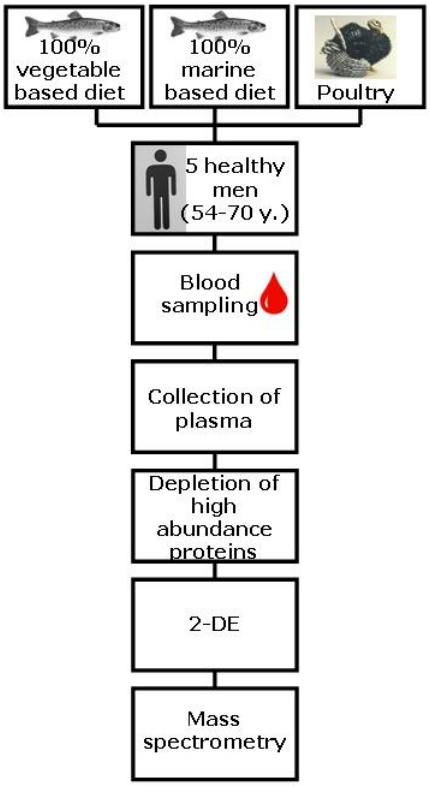


Figure 7: **Experimental design and methods conducted in the single meal study.**

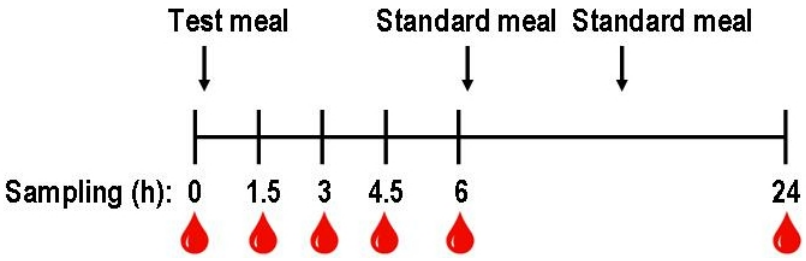


Figure 8: **Time line of single meal study.** Blood was sampled before the meal intake, four times during the postprandial phase following a 24 h fasting sample. A standard lunch and dinner were served in order to control the macronutrient and energy intake during the test day.

5.2 Human intervention trial

The human eight week intervention trial was a three-grouped parallel randomised study including 68 healthy men in the age of 40-70 years. Subjects were randomly assigned to one of the three groups eating either one daily meal of trout fed by a pure marine based diet, one daily meal of trout fed by a pure vegetable based diet or a daily meal of poultry (control meal). Blood was collected at baseline (week 0) and at the end of the intervention (week 8) for plasma proteome analysis and MS-based identification of proteins of interest (see Figure 9). 30 of the 68 subjects (10 from each intervention group) were randomly selected and their samples were used for proteomic analysis. Details concerning the participants, the meal content and the methods are described in paper II.

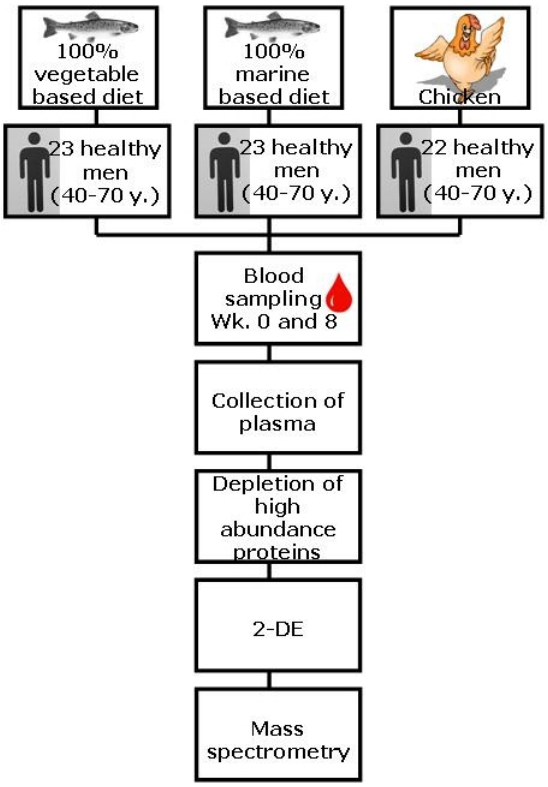


Figure 9: **Experimental design and methods conducted in the human intervention study.**

6 Main results and discussion

During the experimental work with human plasma from the single meal study and the intervention study, various choices were made in the laboratory regarding sample processing and 2-DE with the aim of obtaining most stable and reliable plasma profiles. Several steps in the proteomic procedure appear critical for and might contribute to variation. Pre-analytical variables such as sample collection, storage and handling may have profound effects on the composition of the plasma sample and these could confound the proteomic data analysis. Therefore, it is important to quantify the sources of variation inherent in the proteomic method and improve them to the extent possible in order to reduce artefacts that could bias the results and further obscure the detection of the dietary induced changes in the human plasma protein profiles.

6.1 Preparation of human plasma samples

Sample preparation of plasma often includes addition of protease inhibitors (PI) in order to preserve the protein population from proteolytic cleavage that otherwise may result in artificial protein spots and loss of high molecular weight proteins on the 2-DE gel. However, the use of PI is challenging since differences in 2-DE patterns both with and without PI are documented. Schuchard and colleagues have shown that several human plasma protein trains on 2-DE gels were shifted toward a higher isoelectric point following addition of a PI cocktail [Schuchard et al., 2005]. In contrast, Hulmes et al. have demonstrated that inclusion of PI in plasma samples improved sample qualities and gave reproducible results based on classical 2-DE image analysis, 2D-PAGE quality scoring and enzyme immunoassay [Hulmes et al., 2004]. Furthermore, samples without PI cocktail varied significantly from control samples. In the present study, the benefits versus drawbacks of inclusion of PI described in the literature were balanced and it was decided not to use PI in the preparation for the human plasma samples. In that connection, different initiatives were taken during sample preparation to reduce proteolysis. Following the Human Plasma Proteome Project recommendations [Omenn et al., 2005], blood was collected in vacutainers containing EDTA anticoagulant, whose chelating action may be advantageous. Moreover, plasma was prepared directly after venipuncture and the applied TCA protein precipitation as well as urea, thiourea and chaps in the reswelling solution are useful for minimising proteolytic activity [Castellanos-Serra and Paz-Lago 2002; Gorg et al., 2004]. Still, it must be kept in mind that proteolysis will presumably occur, since it is rather difficult to completely inactivate all proteases. Consequently, a number of spots on the 2-D gel may reflect protein fragments as a result of proteolysis. However, according to the tables of MS/MS based protein identification (paper I and II) this seems not to be the case. On the basis of the mass spectrum and the experimental MW and PI compared to

the theoretical values, it was indicated that only few (four out of 37) of the different significant spots changing in abundance were fragments of the reported proteins.

Another variable such as temperature during blood centrifugation may affect the plasma profiles since several handling procedures have been shown to influence the proteome profiling [Hsieh et al., 2006; Hulmes et al., 2004]. The blood samples from the volunteers were centrifuged at room temperature, although protein stability is better at lower temperatures, but his temperature was chosen to avoid cold activation of platelets [Banks et al., 2005]. This is of high importance since platelets play a central role in thrombosis, haemostasis and inflammation, which are essential processes implicated in CVD [Gawaz et al., 2005] and of great importance when evaluating health beneficial effects of fish consumption. In the light of the foregoing reflections, there is without doubt a need for standardising methods of collection, processing, and storage of plasma samples to minimise the effect of pre-analytical variables on plasma proteome profiling and be able to detect biomarkers in an unbiased fashion.

6.2 Depletion of high abundant proteins

Multiple strategies have been used to simplify the plasma proteome due to the vast disparity in protein abundance of human plasma [Kim and Kim 2007] in which removal of high abundance proteins by immunoaffinity capture is increasingly being recognised as the most effective sample preparation strategy for plasma proteomics [de Roos 2008]. In that connection, a study by Whiteaker and colleagues, who examined eight different pre-fractionation approaches of human serum, demonstrated that immunoaffinity subtraction of the high abundant proteins was most effective and reproducible [Whiteaker et al., 2007]. On the other hand, this method is not free of troubles. In the present study, plasma was fractionated by use of the commercial available IgY-12 proteome partitioning column from Beckman Coulter which captures the 12 most abundant plasma proteins. The principal rationale for using this immunodepletion strategy was to lower the detection limit on the 2-DE gels and make the lower abundant proteins accessible to global proteome profiling, since the majority of potential disease/health biomarkers may be present at extremely low concentrations in plasma. Although use of the IgY-12 column greatly improved the resolution of the gels, the lower abundant proteins were still out of reach and the identified proteins were mainly common plasma proteins involved in blood key functions.

It is important to note that some of the identified proteins were IgY-target proteins which should have been retained by the column, although it was expected that the remaining amount of these proteins was proportional to their original amounts. On the basis of this result and the fact that the column is used for all samples, variations in column absorption efficiency may occur. Although the column could deplete 100 samples according to the manufacturers product descriptions, it has

been shown that after 25 samples the protein content of the flow-though fraction increased suggesting decreased efficiency for retaining the high abundant target proteins [de Roos et al., 2008]. Therefore, consistent depletion conditions may not be guaranteed. To shed light on this matter, a linear regression analysis of nine selected (four non-significant and five significant IgY-target spots from paper I was conducted. The proteomic data of these nine spots was listed in order according to the date that the sample was fractionated by the IgY-column. The regression slope was found significant in seven (two non-significant and five significant IgY-spots (636, 859, 936, 937 and 940) out of nine examined spots. Conclusively, these spots are doubtful since a time dependent variation in column absorption efficiency is indicated and the results of these spots should thus be interpreted with caution.

The potential loss of inadvertent capture of non-targeted proteins due to the ability of high abundant proteins, like albumin, to act as carrier proteins [Desrosiers et al., 2007; Elrick et al., 2006] is also of concern, since some minor proteins may possibly remain bound to the column and will be lacking in the flow-through fraction loaded on the 2-D gel. This may remove potential biomarker candidates during the depletion process. Huang et al. have addressed this concern by examining the flow-through fraction and bound/eluted fraction after IgY-depletion. They found that proteins identified in the bound/eluted fraction consisted mainly of the targeted abundant proteins except for four non-targeted proteins [Huang et al., 2005]. Equivalent to that study, Tu and colleagues demonstrated that the bound fraction associated with immunodepletion contained a number of non-targeted proteins [Tu et al., 2010]. Taken together, in quantitative plasma proteomic studies based on immunoaffinity capturing, it is important to keep in mind the risk of not only the potential non-selective loss of protein candidates but also incomplete absorption ability of the column as suggested by the present results.

6.3 Visualisation of spots

Silver staining is a frequently used approach for visualisation of protein spots on the 2-D gel due to its positive features such as excellent sensitivity below one ng of protein and relative low cost for reagents [Weiss et al., 2009]. In this study, proteins were stained by use of silver staining as described in [Wulff et al., 2008] and the excellent sensitivity was demonstrated in comparison with Sypro Ruby fluorescent stain and Coomassie Brilliant Blue where silver staining clearly visualised most spots on the 2-D gel. Thus, this staining technique increased the possibility to visualise as many proteins of potential use as specific biomarkers. However, one of the drawbacks by the stain is the limited linear dynamic range which is important, since spot saturation effects impede normalisation and accurate quantifications of spots. Moreover, gel-to-gel variations are not negligible due to several solution changes and other carefully timed steps. In addition, fluorescence staining is an attractive approach since it offers reliable quantification over a wide linear dynamic

range combined with a high sensitivity of spot detection [Wu et al., 2005]. Yet, the dyes are very expensive and a costly fluorescent scanner is required, which was not available at the time the staining procedure took place.

6.4 Technical variation and the significance of the IgY-12 fractionation on the proteomic data

The number of replicates is often restricted in 2-DE studies due to labour and cost. Biological replicates (different measurements within the same experimental group) and technical replicates (repeated measurements of the same biological sample) can be used in proteomic studies. Reliable sample preparation and lots of experience with 2-DE based proteomics ought to minimise the technical variation. In this study biological replication was carried out which is better than technical replication, if a limited number of gels can be made [Horgan 2007]. Furthermore, if technical replicates were used, it will be occurring at the expense of biological replication which is not recommendable [Horgan 2007].

It is important to shed light on the technical variation since high 2-DE reproducibility is an essential prerequisite for obtaining high quality data and further identify potential protein biomarkers. For that reason, six technical replicate gels of IgY-12 fractionated plasma from a single subject were run according to the applied 2-DE procedure to investigate the scope of the technical variation in data, i.e. the contribution of the IgY-12 column (data not shown). The mean coefficient of variation (CV) of all spots (921 spots, 6 gels) in the replicate dataset was 25% in which 45% of all the spots had CV values less than 20%. Moreover, it was clearly seen that spots with a high variation ($>$ three fold changes) were primarily low spot volumes or located in the blurred area of the gel (Fig. 10A). Furthermore, six gels of pooled IgY-12 preparations of human plasma samples from the same subject as mentioned before was run to illustrate to what extent the IgY-column contributes to variation. Analysis of pooled IgY-12 plasma samples (dataset of 921 spots, data not shown) resulted in a mean CV of 18% in which 69% of the spots had CV values less than 20%. In that connection, results from a former experimental variation study of a single human plasma sample prepared eight times by top-6 high abundance protein depletion following 2D DIGE reported that 50% of all spots had less than 10% CV [Corzett et al., 2006]. By comparison, we could report that 11% of the total number of spots in our replicate study had less than 10% CV. However, it should be noted, that the DIGE technique reduces the inter-gel variations and thus overcomes the limitation of traditional 2-DE since three samples are loaded on a single gel.

To get insights into the technical variation of spots distinctly separated on the 2-D gel, 25 randomly picked spots of both large and small volumes were selected (Fig. 10B). The mean CV of the 25 spots was 11% (range CV 5-17%) in the replicate gels. In addition, nine selected (four non-significant and five significant) IgY-target spots identified in paper I had a mean CV of 12% and 7% in the replicate study

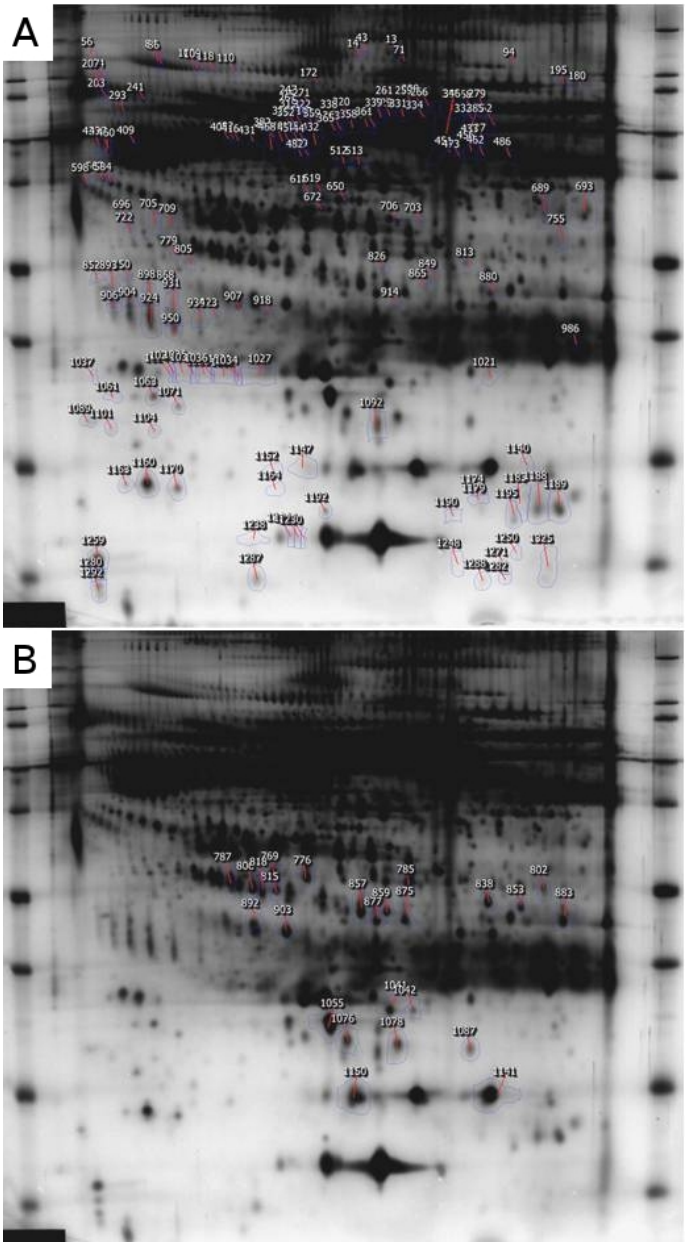


Figure 10: **Representative 2-DE gel from the replicate study.** A: The spatial distribution of spot volumes larger than three fold changes. B: The spatial distribution of 25 randomly selected spots.

and the pooled study, respectively. Based on the results presented above, it was indicated that the IgY-12 column contributes to variation in the proteomic data, however, only to a lesser extent and did therefore not contribute the main variation in the data.

Table 1: Overview of the main results

	Paper I	Paper II	Paper III
Study	Human single meal study	Human intervention study	Human single meal study
Aim	Investigation of acute effects (24 h) of marine fed trout compared to a reference meal of poultry.	Investigation of long-term effects (8 weeks) of trout fed by different feeds: 1) Marine based feed (M) 2) Vegetable based feed (V) compared to a reference meal of chicken (C).	Evaluation of the variation in 2-DE plasma proteome patterns based on experimental parameters: 1) Fast and postprandial sampling 2) Meal type (P, M and V) 3) Sampling week 4) Subject
Primary results	**Postprandial effects of:** Poultry versus baseline; change in abundance of 7 different proteins involved in lipid metabolism, complement and coagulation cascade, acute phase response, cofactor and vitamin metabolism. Marine fed trout versus baseline; change in abundance of 4 different proteins involved in lipid metabolism, complement and coagulation cascade, and acute phase response. **Meal effects at concurrent time points:** Marine fed trout versus poultry; change in abundance of 7 different proteins involved in lipid metabolism, complement and coagulation cascade, and acute phase response.	**Meal effects of:** Trout (M or V) versus chicken; Change in abundance of10 different proteins implicated in haeme transport and catabolism, regulation of vasoconstriction, blood homeostasis, complement cascade, antioxidative defence, cellular component movement, glycolysis and vitamin transport. Vegetable versus marine fed trout: Only a single protein involved in complement activation changed in abundance.	**Inter-individual variation:** The largest variance component observed was between subjects. **Meal variations:** Vegetable fed trout grouped separately from marine fed trout and poultry within subjects.

6.5 Acute postprandial effects of trout and poultry

To give an overview of the main findings from the present PhD project, aim and main results from the two human studies are summarised in Table 1. Firstly, we aimed at examining the acute postprandial changes in the plasma proteome and lipid profile of elderly healthy men after single meal intake of trout and poultry. Furthermore, it was warranted to investigate whether the 2-DE based approach was an applicable tool to detect the postprandial changes in plasma proteins. Since most individuals spend a large portion of their daytime in the postprandial state, it is essential to investigate how dietary components affect the biochemical and physiological processes within the human body. In healthy subjects, n-3 LCPUFAs have been reported to reduce the magnitude of the postprandial TAG response [Aviram et al., 1986; Zampelas et al., 1994], but this was not demonstrated in the single meal study (Fig.1, paper 1). The change in plasma TAG concentration following intake of trout and poultry resulted in a bell-shaped postprandial response, which is in congruence with previous findings [Callow et al., 2002; Heath et al., 2003; Overgaard et al., 2008]. All subjects demonstrated well-controlled lipid homeostasis which is an important reflection of their metabolic efficiency, but no significant difference was observed between the two TAG responses. The amount of fat in the test meal and the inter-individual variability in the response, probably caused by differences in metabolism may be a contributing factor for the lack of TAG lowering effect of trout compared to poultry. In contrast, single meal studies in healthy subjects have demonstrated a reduced postprandial lipaemia following fish oil intake in comparison with a fat load primarily containing saturated fat [Aviram et al., 1986; Zampelas et al., 1994]. However, the given fat loads were far greater than those consumed in the present trout meal. Furthermore, it should be noted, that only five subjects were included in the study but the applied cross-over design, in which the different treatments are evaluated on the same subject, reduces the number of subjects required to produce similar precision compared to parallel design [Elbourne et al., 2002].

After single meal intake of trout and poultry, we were able to detect postprandial changes during 24 h in human plasma proteins (Table 1, paper I). By use of fractionated plasma depleted of the top 12 abundant proteins, the resolution of the 2-DE gel images was greatly improved which increased the possibility to quantify the lower abundant proteins. Image analysis of silver stained 2-DE gels resulted in the detection of 1157 spots separated according to charge and molecular weight. Of these, a total of 17 spots were found to change significantly in abundance in the postprandial state relative to baseline (pre-prandial state) after either the trout or poultry meal. None of the spots changed in response to both meals, which indicates that the two type of meals elicited different responses in plasma. By comparison of trout and poultry at concurrent time points, 14 spots were significantly changed. Based on mass spectrometry, the significant spots reflected proteins categorised to be involved in different biological processes as lipid

transport, coagulation and complement cascade, acute phase response as well as cofactor and vitamin metabolism. However, it should be noted, that some of the significant changed spots were IgY-target spots, which should have been retained by the column. Therefore, these proteins must be interpreted with caution. The meal induced fold changes after trout and poultry intake relative to baseline (table 1, paper 1) were small. Thus, one might speculate if the variation caused by the IgY-column fractionation may have added substantially to these results. This seems not to be the case since the meal induced fold changes of the significant spots were higher than the CVs of the same spots in the replicate data set with the exception of spot 750 (ficolin 3, a non-IgY- target protein) and 937 (haptoglobin, an IgY-target protein). These spots had a higher CV% in the replicate data set and the meal response of those two spots may be doubtful. Furthermore, significant baseline variations in spot volumes of 859 and 923 was seen and the meal response of trout compared to poultry of these spots shown in table 2 should be interpreted with caution since the baseline variation may confould the shown meal effects. The postprandial response of trout and poultry compared to baseline variations of the non-significant spot 818 is shown in Fig.11. It is demonstrated that the postprandial response exceeded baseline variations in four out of five subjects in spite of the postprandial change in spot volume was not signicant. Taken together, the meal induced changes of the plasma proteins detected in the present study are true and it is furthermore important to keep in mind that dietary compounds often have a subtle, but relevant effects on biomarkers of health [de Roos and McArdle 2008].

Ficolin-3, a non-IgY-target protein, was found significant at time 6 h as well as 24 h after poultry intake compared to baseline (table 1 in paper I). This protein is chosen as an example to illustrate the error structure of a significant non-IgY-target protein due to the isoforms present, even though the CV of spot 750 was higher in the replicate data compared to the postprandial induced changes. However, the high CV was probably due to a very low spot volume in a single replicate gel. The postprandial time course of the ficolin-3 isoforms, spot 750 and 745, is shown in Fig. 12, in which a parallel and gradual decline in volume intensity of both spots is seen. Despite the high risk of false positives in multiple testing [Biron et al., 2006], the time course of these spots clearly indicates that the postprandial change was not appeared by chance. This is supported by the fact that both isoforms decreased over time, even though the earlier time points were not significantly changed, and the affected pathway was also relevant according to meal intake.

A 2-DE based approach is powerful for the high resolution separation of proteins in a complex mixture as plasma [Zimny-Arndt et al., 2009]. However, the major limitation of the 2-DE gel based approach is the rather time consuming steps including sample preparation, 2-DE, staining, image analysis, statistical analysis of data and protein identification. The visualisation of proteins can be obtained by application of various staining techniques as organic dye, silver stain, reverse stain, fluorescent stain, radio-labelling and chemiluminescent stain [Patton 2002]. All the 2-DE results from the enclosed papers are based on a sensitive silver staining

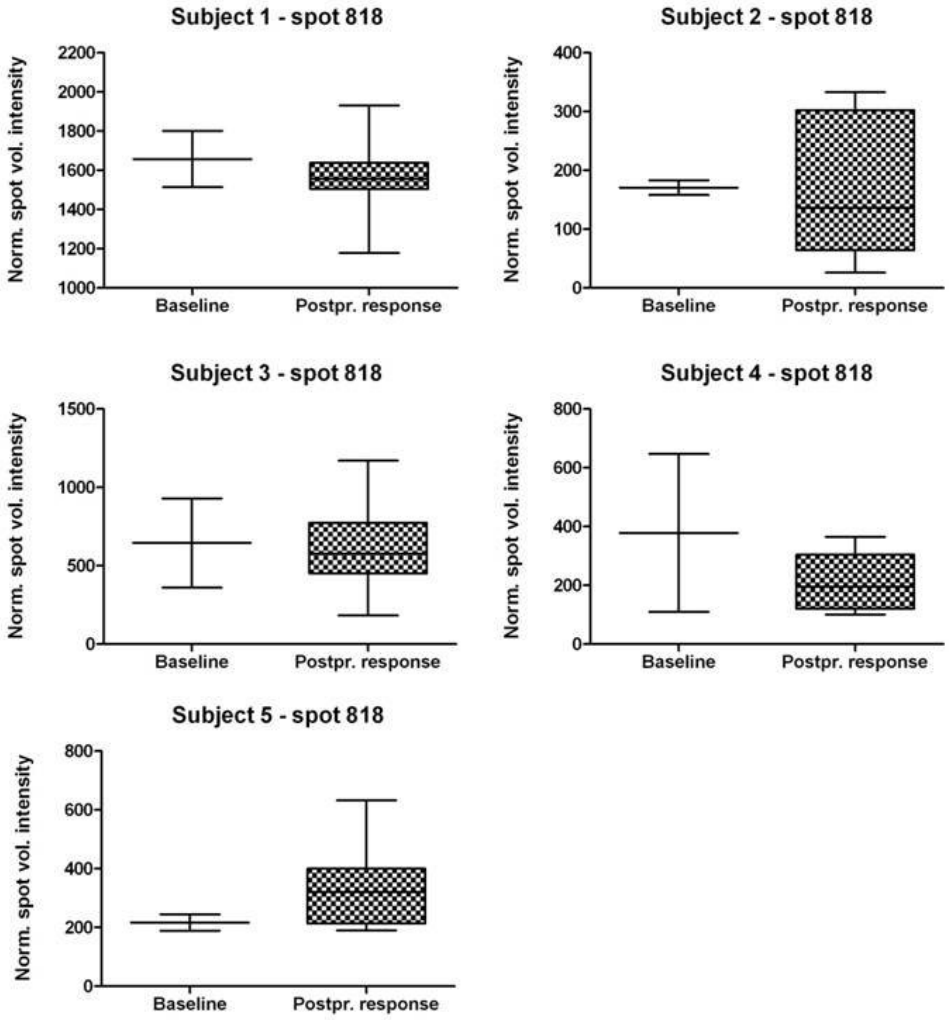


Figure 11: **The postprandial response of trout and poultry compared to baseline variations of the non-significant spot 818 in subject 1-5.** In four out of five subjects, the postprandial response exceeded the baseline variations.

method, which detected several hundreds of proteins. However, it should be noted, that the staining method has a restricted dynamic range and spot saturation may thus appear [Ahnert et al., 2004; Patton 2002]. Consequently, this can give rise to bias during baseline subtraction due to the biological variation in spot intensity may exceed the detectable analytical staining range.

Statistical analyses of the 2-DE data in paper I included both univariate and multivariate data analysis. Multivariate PLS regression was used as an additional statistical tool to reveal all possible proteins associated with meal induced changes. As expected, there was a large overlap of selected spots when compared to the mul-

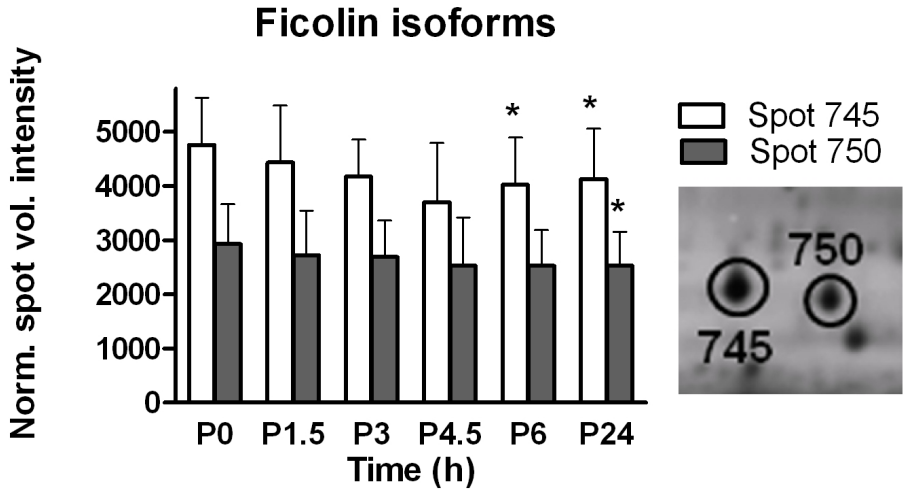


Figure 12: **The postprandial response of ficolin-3 isoforms.** The postprandial time course of spot 745 and 750 identified as ficolin-3 shows a parallel and gradual decline in spot volume intensity. The insert shows the distribution of the two spots on the gel.

tivariate method, but some spots were only significantly selected by use of PLS. Thus, in order to find all relevant spots in the data analysis it is suggested to use univariate statistics combined with multivariate methods [Jensen et al., 2008]. Conclusively, despite methodological limitations of the IgY-12 column and the 2-DE, this proteomic approach has provided insigths into the effects of meal intake on plasma proteins. A further understanding of the metabolic mechanisms of protein targets of nutrients is important contributions to knowledge of food based outcomes in terms of health and disease.

6.6 Long-term effects of trout fed a marine or a vegetable based feed

Reduced availability of wild fish is a major challenge for the aquaculture sector. Therefore, different feeding regimes of plant origins are evaluated as alternatives to fish bone meal and fish oil. However, the impact of a change in feed composition on human health is largely unknown. Thus, we secondly aimed at investigating whether a change in feed composition of farmed trout affected health of consumers assessed by plasma proteome analysis. A parallel eight week dietary intervention study was conducted in adult males as described in paper II, in which subjects consumed a daily meal of farmed trout raised either on pure marine diet or pure vegetable diet, or a reference meal of chicken. Plasma proteins, depleted of the 12 high abundant proteins, revealed a total of 681 distinct separated high-quality spots

visualised by silver staining. Based on delta spot volumes (baseline subtracted spots), a total of 23 spots changed significantly in abundance when comparing the three intervention groups of marine fed trout, vegetable fed trout and chicken. Of these, 18 spots were identified by mass spectrometry and the proteins were linked to the various biological processes haeme transport and catabolism, regulation of vasoconstriction, blood haemostasis, complement cascade, antioxidative defence, cellular component movement, glycolysis and vitamin transport (Table 2, paper II). Nine spots were significantly changed in abundance after intake of marine fed trout compared to chicken and ten spot after intake of vegetable fed trout compared to chicken. Two of the affected spots were changed after both types of trout meals. Only a single spot identified as complement c1r was increased in the group consuming the vegetable fed trout compared to the marine fed trout. This protein is involved in the complement system which is a major component of host defence. The system initiates a cascade of highly regulated activation steps culminating in the membrane attack complex resulting in cytolysis [Porter and Reid 1978]. However, inappropriate activation of the complement system can also contribute to inflammation seen in various pathological conditions [Morgan and Harris 2003]. This finding is of significance since inflammation plays a central role in the development of atherosclerosis. Indeed, if future replacement of fish oil with vegetable oils becomes inevitably, it is important to know more about the nutritional consequences of such replacement for the consumer. From a nutritional point of view, vegetable oils rich in ALA would be of choice in feed for farmed trout, since salmonids are capable of converting ALA to its more biological active forms of n-3 PUFA, EPA and DHA [Sargent and Tacon 1999].

6.7 Inter-individual variation

The biological variation of the human population is a major obstacle in clinical studies and biomarker discovery. Factors like sex, age, genetic background, health status and lifestyle may contribute to this variability [Anderson and Anderson 2002; Nedelkov 2008; Winkler et al., 2008]. Proteomic studies need to address the biological variation in protein expression between individuals in the general population before application of biomarkers in clinical research. This variation between individuals may influence the protein in a way that might enhance or degrade its performance as a biomarker. To date, the knowledge of the quantitative differences in plasma protein expression across the general population is limited and only few studies have investigated the inter-individual variability of different proteomes. Hence, the need for a better understanding within this research field is urgently needed. Thus, we thirdly aimed at investigating the extent of the biological variation between subjects with respect to protein abundance assessed by measurements of the plasma proteome profiles in healthy elderly men. By use of the 2-DE data set (a total of 483 distinct separated high-quality spots) from the single meal study following applications of multivariate methods, PCA and PLSR, we

revealed the individual differences between subjects (paper III). Multivariate data analysis is a valuable tool since it offers a strong approach for evaluation of 2-DE based protein profiles to get an overview of the main variation and structure in data [Jensen et al., 2008; Karp and Lilley 2007]. PCA analysis was performed to get an overview of how the experimental parameters including fasting versus postprandial sampling, meal type, sampling week and subject contributed to the main variation in the data set (Fig.1, paper III). Results from the PCA scores plot revealed that the variation between subjects represented the largest variance component exceeding the other measured experimental parameters. By use of PLS regression of the total proteome data set (Table 1, paper III), a large number of spots were demonstrated to be differentially expressed between subjects. Furthermore, analysing the data with a longitudinal approach revealed an effect of the meals. PCAs on the subjects one by one showed that postprandial samples taken after intake of the trout fed a vegetable based feed grouped separately from samples taken after intake of the other two meals (Fig.2, paper III). In four of five subjects there was a grouping according to meal, however, of different extent (distance between groups) in the subjects. Due to the detection of the significant IgY-target spots shown in paper I, it is essential to examine whether the IgY-12 column may have added the present results concerning the groping of samples due to individual variation. The distribution of the known IgY-target spots was examined and they were found to be randomly distributed in the loading plot from the PCA analysis of the experimental parameters (Fig.1 paper III) and also in the PCA loading plot of the spots that were differentially expressed between subjects (Fig.4, paper III). Furthermore, excluding the IgY-target spots from the data matrix resulted in the similar and clear grouping of the individuals in the PCA scores plot. Conclusively, these results indicate that the IgY-12 fractionation has not contributed to the present results concerning inter-individual variability.

The inter-individual variability can of course not be avoided but has to be taken into consideration in the experimental study design, analytical proteomic strategy and interpretation of the obtained results. Many authors have acknowledged the inter-individual variability in humans, however, the extent of variation remains unclear and is an important factor to try and quantify. From the results achieved in paper III, we have demonstrated that cross-over studies using an individual as its own control and measure analyte variations over time is an important experimental design for studying human plasma proteins due to large biological variation between individuals. In this regard, these findings contribute to the further understanding of the human proteome variability improving the biomarker discovery process being able to discover new biomarkers that reflect early functional changes of importance.

Biomarkers measured in homeostatic perturbations such as challenge test may be more valuable compared to the same biomarkers based on homeostatic conditions (fasting state) [van Ommen et al., 2009]. We obtained perturbations with a metabolic challenge in the human body in the form of a meal following postprandial measurements (the single meal study). This is a highly relevant challenge

since much of the 24 h day is spent in the non-fasting state. The value of challenge test is demonstrated in a recent cohort investigation, the Womens Health Study, examining the fasting compared to non-fasting TAG levels in relation to CVD risk [Bansal et al., 2007]. Postprandial plasma TAG was shown to be associated with increased cardiovascular events while the fasting TAG showed little relationship. Thus, postprandial measures may be more robust indicators of disease risk and the great variability of postprandial levels between individuals provides important information about an individuals metabolism. However, these challenge tests warrant further investigations as indicators of the metabolic state to detect the accurate disease risk.

7 Conclusions

Based on the 2-DE proteomic investigation of dietary induced changes in the human plasma profiles from the single meal study and the intervention study, we conclude that:

- Consumption of single meals of poultry or trout elicited acute postprandial changes in abundance of several plasma proteins implicated in the different biological processes lipid transport, complement and coagulation cascade, acute phase response as well as cofactor and vitamin metabolism. Intake of trout and poultry elicited no differences in the postprandial bell-shaped TAG response. A 2-DE based approach, however in refined ways, may be a valuable tool with respect to detection of the postprandial induced changes in human plasma proteins.
- An eight week dietary intervention with chicken and trout fed either a marine based feed or a vegetable based feed revealed a change in abundance of plasma proteins involved in the biological processes haeme transport and catabolism, regulation of vasoconstriction, blood haemostasis, complement cascade, antioxidative defence, cellular component movement, glycolysis and vitamin transport. Furthermore, the change in feed composition of trout had a lower impact on the plasma protein profiling in contrast to the differences in response after intake of trout compared to chicken.
- The inter-individual variability represented the largest observed variance component exceeding fasting or postprandial sampling, meal type (poultry, marine and vegetable fed trout) and sampling week). Thus, the inter-individual variability is a significant factor for comparative proteomic studies and needs to be taken into account in the experimental design of human studies and interpretation of data. Furthermore, cross-over design in which each individual acts as its own control and longitudinal sampling on the basis of a physiological challenge (e.g. meal intake) seems more informative than parallel study design.

These conclusions are summerised in Figure 13.

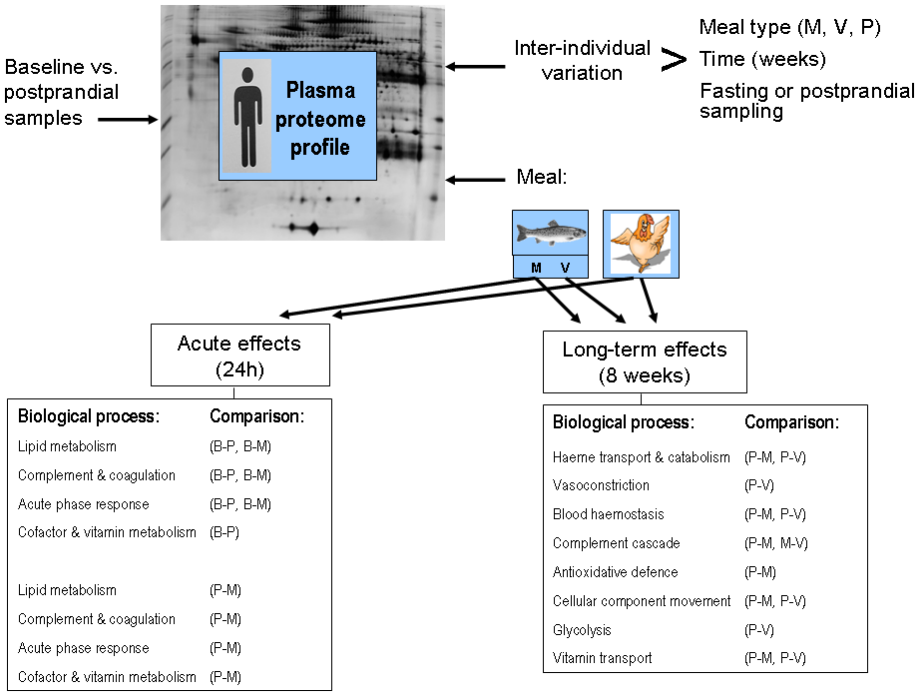


Figure 13: **Overview of conclusions.** B = baseline, P = poultry, M = marine fed trout and V = vegetable fed trout.

8 Future perspectives

This thesis has contributed to the clarification of several research questions and concurrently generated new issues and perspectives, which require further investigation.

In relation to diet induced changes in human plasma proteins, we need to increase our understanding of the underlying molecular mechanisms that are in play to improve health and prevent the onset of disease. We have shown a measurable effect on a number of proteins implicated in various pathways of importance for chronic disease such as CVD. A further understanding within this research field will have a large impact on public health. Specifically, development of proteomic tools based on the identification of new plasma proteomic biomarkers that identify metabolic changes in specific tissue and organ is needed to clarify the complex mechanisms of action. These novel biomarkers, compared to already known risk markers, will allow early detection of the onset of disease or, ideally, the pre-disease state.

In the present study, we have examined the impact of mixed meals on human plasma proteins. The effects of single nutrient components, protein, lipid and carbohydrate and their interactions on metabolism will be interesting to investigate. In future nutritional studies, we should focus on the experimental design to increase the sensitivity of detecting and identifying biological changes related to dietary induced health effects. We have demonstrated that cross-over studies using an individual as its own control and measure analyte variations over time are a very sensitive experimental design.

Based on the results from the dietary intervention study with differently fed trout, new studies should be performed to further evaluate the influence of the change in feeding regimes for farmed trout in relation to the nutritional properties of the fish especially with focus on n-3 LCPUFA content. This is an important issue to clarify due to the well-documented beneficial effects of these fatty acids.

9 2-DE based plasma proteomics: Lessons learned

Based on 2-DE proteomics, we were able to detect and identify a number of potential candidate proteins in human plasma that were affected after intake of trout compared to poultry both according to acute and long-term effects. The candidate proteins are related to pathways involved in transportation of food components, lipid modulating processes, immune function and anti-oxidative defence. The present approach, though optimised with respect to methodology and design offers a potential for revealing nutritional biomarkers, also supported by the fact that proteins found significant in the present study are involved in likely pathways related to dietary consumption.

During the work with plasma proteomics, several barriers were encountered including the extreme dynamic range of plasma proteins spanning at least ten orders of magnitude and the resulting requirement for fractionation to reveal even a modest number of proteins. Depletion of the high abundant proteins, which comprise the vast majority of total protein mass, greatly improved the resolution of the gels, but the lower abundant proteins were still out of reach. However, in quantitative plasma proteomic studies based on immunoaffinity capturing, it is important to keep in mind the risk of not only the potential non-selective loss of protein candidates but also incomplete absorption ability of the column as suggested by the present results. Furthermore, the present 2-DE approach has limitations. 2-DE is laborious due to multiple analytical steps which may increase the risk of bias. Gel to gel variations often occurs and the applied silver staining has a limited dynamic range. Moreover, multiple testing increases the risk of false positives. On the contrary, 2-DE enable the simultaneous analysis of hundreds of proteins, access to posttranslational modifications and a relative quantification on the 2-DE images. Pre-analytical factors such as sample collection, storage and handling as well as analytical factors can have profound effects on the outcome of a discovery study. In addition, the biological variation of the subjects may also contribute to bias. One way to cope with this inter-individual variation is to conduct carefully controlled studies e.g. cross-over studies, in which each subjects participate both in the control and test group, due to the variation within a subject is usually smaller than that seen between subjects.

Clearly, proteomics studies do not lead to biomarkers that directly can be applied in clinical diagnostics; the journey from discovery to practical implementation in clinical diagnostics is still long and uncertain. 2-DE based proteomics should be considered as a screening tool that offers a well-established approach to find new biomarkers of plasma - the circulating representation of all body tissue and thus the most comprehensive human proteome. These candidate proteins must be further verified and validated in large-scale studies required to determine the key parameters for diagnostics test such as biological variability, sensitivity and specificity

according to target state, and the contribution in various multi biomarker panels [Anderson 2005b]. Despite the high potential of plasma proteomics for biomarker discovery and vast amount of intensified research and investment, a paradoxically decline is observed in the number of new biomarkers approved for clinical diagnostics [Anderson and Anderson 2002]. The reason for the shortage of new biomarkers is probably related to the high false discovery rate of biomarkers in proteomics technologies arisen from biological and technical variability in combination with a lack of robust methods for biomarker verification in clinical large sample sets [Anderson 2005; Rifai et al., 2006; Carr and Anderson 2008]. Currently, there is no ideal, extensive biomarker discovery platform for plasma proteomics. A standard biomarker pipeline is urgently needed that relieves the bottleneck between biomarker discovery and clinical validation.

The emerging technology of mass spectrometry targeted protein quantification has increasingly becoming important for biomarker development in clinical research [Pan et al., 2009; Anderson 2005]. The absolute quantification relies on the use of stable isotopes labelled reference peptides/proteins which acts as internal standards, enabling protein quantification by comparing the signals from the exogenously labelled and the corresponding unlabelled peptide. In a recent study, mass spectrometry-based multiple reaction monitoring of proteins revealed the absolute quantification of 45 endogenous proteins in human plasma based on trypsin digests without prior affinity depletion or enrichment [Kuzyk et al., 2009]. Future proteomics technologies should to be able to detect biomarker candidates in the presence of high abundance proteins to avoid depletion of candidate proteins due to non-specific binding to high abundant proteins. Development of standard, robust sample preparation strategies and more sophisticated technologies like the targeted MS methods in combination with novel data processing methods are expected to provide a smoother path from science to practice than exists today.

References

[Aebersold and Mann, 2003] Aebersold, R. and Mann, M. (2003). Mass spectrometry-based proteomics. *Nature*, 422(6928):198–207.

[Ahnert et al., 2004] Ahnert, N., Patton, W., and Schulenberg, B. (2004). Optimized conditions for diluting and reusing a fluorescent protein gel stain. *Electrophoresis*, 25(15):2506–2510.

[Ait et al., 2005] Ait, Y. D., Madani, S., Prost, J., Bouchenak, M., and Belleville, J. (2005). Fish protein improves blood pressure but alters hdl2 and hdl3 composition and tissue lipoprotein lipase activities in spontaneously hypertensive rats. *Eur.J.Nutr.*, 44(1):10–17.

[Anderson, 2005a] Anderson, L. (2005a). Candidate-based proteomics in the search for biomarkers of cardiovascular disease. *J.Physiol*, 563(Pt 1):23–60.

[Anderson, 2005b] Anderson, N. (2005b). The roles of multiple proteomic platforms in a pipeline for new diagnostics. *Mol.Cell Proteomics*, 4(10):1441–1444.

[Anderson and Anderson, 1978] Anderson, N. and Anderson, N. (1978). Analytical techniques for cell fractions. xxii. two-dimensional analysis of serum and tissue proteins: multiple gradient-slab gel electrophoresis. *Anal.Biochem.*, 85(2):341–354.

[Anderson and Anderson, 1996] Anderson, N. and Anderson, N. (1996). Twenty years of two-dimensional electrophoresis: past, present and future. *Electrophoresis*, 17(3):443–453.

[Anderson and Anderson, 2002] Anderson, N. and Anderson, N. (2002). The human plasma proteome: history, character, and diagnostic prospects. *Mol.Cell Proteomics*, 1(11):845–867.

[Arab, 2003] Arab, L. (2003). Biomarkers of fat and fatty acid intake. *J.Nutr.*, 133 Suppl 3:925S–932S.

[Ascherio et al., 1995] Ascherio, A., Rimm, E., Stampfer, M., Giovannucci, E., and Willett, W. (1995). Dietary intake of marine n-3 fatty acids, fish intake, and the risk of coronary disease among men. *N.Engl.J.Med.*, 332(15):977–982.

[Aviram et al., 1986] Aviram, M., Brox, J., and Nordoy, A. (1986). Acute effects of dietary cod liver oil and cream on plasma lipoproteins. *Ann.Nutr.Metab*, 30(2):143–148.

[Bang and Dyerberg, 1972] Bang, H. and Dyerberg, J. (1972). Plasma lipids and lipoproteins in greenlandic west coast eskimos. *Acta Med.Scand.*, 192(1-2):85–94.

[Banks et al., 2005] Banks, R., Stanley, A., Cairns, D., Barrett, J., Clarke, P., Thompson, D., and Selby, P. (2005). Influences of blood sample processing on low-molecular-weight proteome identified by surface-enhanced laser desorption/ionization mass spectrometry. *Clin.Chem.*, 51(9):1637–1649.

[Bansal et al., 2007] Bansal, S., Buring, J., Rifai, N., Mora, S., Sacks, F., and Ridker, P. (2007). Fasting compared with nonfasting triglycerides and risk of cardiovascular events in women. *JAMA*, 298(3):309–316.

[Barlow, 2000] Barlow, S. (2000). Fish meal and fish oil: Sustainable feed ingredients for aquafeeds. *Global Aquaculture Advocate*, 4:85–88.

[Barnes and Kim, 2004] Barnes, S. and Kim, H. (2004). Nutriproteomics: identifying the molecular targets of nutritive and non-nutritive components of the diet. *J.Biochem.Mol.Biol.*, 37(1):59–74.

[Bergeron et al., 1992] Bergeron, N., Deshaies, Y., and Jacques, H. (1992). Dietary fish protein modulates high density lipoprotein cholesterol and lipoprotein lipase activity in rabbits. *J.Nutr.*, 122(8):1731–1737.

[Bergeron et al., 1991] Bergeron, N., Deshaies, Y., Lavigne, C., and Jacques, H. (1991). Interaction between dietary proteins and lipids in the regulation of serum and liver lipids in the rabbit. effect of fish protein. *Lipids*, 26(9):759–764.

[Berth et al., 2007] Berth, M., Moser, F., Kolbe, M., and Bernhardt, J. (2007). The state of the art in the analysis of two-dimensional gel electrophoresis images. *Appl.Microbiol.Biotechnol.*, 76(6):1223–1243.

[Biron et al., 2006] Biron, D., Brun, C., Lefevre, T., Lebarbenchon, C., Loxdale, H., Chevenet, F., Brizard, J., and Thomas, F. (2006). The pitfalls of proteomics experiments without the correct use of bioinformatics tools. *Proteomics.*, 6(20):5577–5596.

[Bjellqvist et al., 1982] Bjellqvist, B., Ek, K., Righetti, P., Gianazza, E., Gorg, A., Westermeier, R., and Postel, W. (1982). Isoelectric focusing in immobilized ph gradients: principle, methodology and some applications. *J.Biochem.Biophys.Methods*, 6(4):317–339.

[Boukortt et al., 2004] Boukortt, F., Girard, A., Prost, J., it Yahia, D., Bouchenak, M., and Belleville, J. (2004). Fish protein improves the total antioxidant status of streptozotocin-induced diabetes in spontaneously hypertensive rat. *Med.Sci.Monit.*, 10(11):BR397–BR404.

[Breslow, 2006] Breslow, J. (2006). n-3 fatty acids and cardiovascular disease. *Am.J.Clin.Nutr.*, 83(6 Suppl):1477S–1482S.

[Bucher et al., 2002] Bucher, H., Hengstler, P., Schindler, C., and Meier, G. (2002). N-3 polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials. *Am.J.Med.*, 112(4):298–304.

[Burdge and Calder, 2005] Burdge, G. and Calder, P. (2005). Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reprod.Nutr.Dev.*, 45(5):581–597.

[Calder, 2004] Calder, P. (2004). n-3 fatty acids and cardiovascular disease: evidence explained and mechanisms explored. *Clin.Sci.(Lond)*, 107(1):1–11.

[Calder, 2007] Calder, P. (2007). Immunomodulation by omega-3 fatty acids. *Prostaglandins Leukot.Essent.Fatty Acids*, 77(5-6):327–335.

[Callow et al., 2002] Callow, J., Summers, L., Bradshaw, H., and Frayn, K. (2002). Changes in ldl particle composition after the consumption of meals containing different amounts and types of fat. *Am.J.Clin.Nutr.*, 76(2):345–350.

[Carr and Anderson, 2008] Carr, S. and Anderson, L. (2008). Protein quantitation through targeted mass spectrometry: the way out of biomarker purgatory? *Clin.Chem.*, 54(11):1749–1752.

[Castellanos-Serra and Paz-Lago, 2002] Castellanos-Serra, L. and Paz-Lago, D. (2002). Inhibition of unwanted proteolysis during sample preparation: evaluation of its efficiency in challenge experiments. *Electrophoresis*, 23(11):1745–1753.

[Chin and Dart, 1995] Chin, J. and Dart, A. (1995). How do fish oils affect vascular function? *Clin.Exp.Pharmacol.Physiol*, 22(2):71–81.

[Corzett et al., 2006] Corzett, T., Fodor, I., Choi, M., Walsworth, V., Chromy, B., Turteltaub, K., and Cutchen-Maloney, S. (2006). Statistical analysis of the experimental variation in the proteomic characterization of human plasma by two-dimensional difference gel electrophoresis. *J.Proteome.Res.*, 5(10):2611–2619.

[Crosley et al., 2009] Crosley, L., Duthie, S., Polley, A., Bouwman, F., Heim, C., Mulholland, F., Horgan, G., Johnson, I., Mariman, E., Elliott, R., Daniel, H., and de, R. 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(2):95–102.

[Daviglus et al., 1997] Daviglus, M., Stamler, J., Orencia, A., Dyer, A., Liu, K., Greenland, P., Walsh, M., Morris, D., and Shekelle, R. (1997). Fish consumption and the 30-year risk of fatal myocardial infarction. *N.Engl.J.Med.*, 336(15):1046–1053.

[de Francesco et al., 2004] de Francesco, M., Parisi, G., Medale, F., Lupi, P., Kaushik, S., and Poli, B. (2004). Effect of long-term feeding with a plant protein mixture based diet on growth and body/fillet quality traits of large rainbow trout (oncorhynchus mykiss). *Aquaculture*, 236(1-4):413–429.

[de Roos, 2008] de Roos, B. (2008). Proteomic analysis of human plasma and blood cells in nutritional studies: development of biomarkers to aid disease prevention. *Expert.Rev.Proteomics.*, 5(6):819–826.

[de Roos et al., 2008] 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). Proteomic methodological recommendations for studies involving human plasma, platelets, and peripheral blood mononuclear cells. *J.Proteome.Res.*, 7(6):2280–2290.

[de Roos B., 2009] de Roos B. (2009). Nutrition proteomics and biomarker discovery. *Expert.Rev.Proteomics*, 6(4):349–351.

[de Roos B. and McArdle, 2008] de Roos B. and McArdle, H. (2008). Proteomics as a tool for the modelling of biological processes and biomarker development in nutrition research. *Br.J.Nutr.*, 99 Suppl 3:S66–S71.

[Deckelbaum et al., 2006] Deckelbaum, R., Worgall, T., and Seo, T. (2006). n-3 fatty acids and gene expression. *Am.J.Clin.Nutr.*, 83(6 Suppl):1520S–1525S.

[Desrosiers et al., 2007] Desrosiers, R., Beaulieu, E., Buchanan, M., and Beliveau, R. (2007). Proteomic analysis of human plasma proteins by two-dimensional gel electrophoresis and by antibody arrays following depletion of high-abundance proteins. *Cell Biochem.Biophys.*, 49(3):182–195.

[Domon and Aebersold, 2006] Domon, B. and Aebersold, R. (2006). Mass spectrometry and protein analysis. *Science*, 312(5771):212–217.

[Elbourne et al., 2002] Elbourne, D., Altman, D., Higgins, J., Curtin, F., Worthington, H., and Vail, A. (2002). Meta-analyses involving cross-over trials: methodological issues. *Int.J.Epidemiol.*, 31(1):140–149.

[Elrick et al., 2006] Elrick, M., Walgren, J., Mitchell, M., and Thompson, D. (2006). Proteomics: recent applications and new technologies. *Basic Clin.Pharmacol.Toxicol.*, 98(5):432–441.

[Emken et al., 1994] Emken, E., Adlof, R., and Gulley, R. (1994). Dietary linoleic acid influences desaturation and acylation of deuterium-labeled linoleic and linolenic acids in young adult males. *Biochim.Biophys.Acta*, 1213(3):277–288.

[Francis et al., 2001] Francis, G., Makkar, H., and Becker, K. (2001). Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture*, 199(3-4):197–227.

[Fuchs et al., 2005] Fuchs, D., Winkelmann, I., Johnson, I., Mariman, E., Wenzel, U., and Daniel, H. (2005). Proteomics in nutrition research: principles, technologies and applications. *Br.J.Nutr.*, 94(3):302–314.

[Gawaz et al., 2005] Gawaz, M., Langer, H., and May, A. (2005). Platelets in inflammation and atherogenesis. *J.Clin.Invest*, 115(12):3378–3384.

[Geleijnse et al., 2002] Geleijnse, J., Giltay, E., Grobbee, D., Donders, A., and Kok, F. (2002). Blood pressure response to fish oil supplementation: metaregression analysis of randomized trials. *J.Hypertens.*, 20(8):1493–1499.

[Gorg et al., 2009] Gorg, A., Drews, O., Luck, C., Weiland, F., and Weiss, W. (2009). 2-de with ipgs. *Electrophoresis*, 30 Suppl 1:S122–S132.

[Gorg et al., 1991] Gorg, A., Postel, W., Friedrich, C., Kuick, R., Strahler, J., and Hanash, S. (1991). Temperature-dependent spot positional variability in two-dimensional polypeptide patterns. *Electrophoresis*, 12(9):653–658.

[Gorg et al., 1988] Gorg, A., Postel, W., and Gunther, S. (1988). The current state of two-dimensional electrophoresis with immobilized ph gradients. *Electrophoresis*, 9(9):531–546.

[Gorg et al., 2004] Gorg, A., Weiss, W., and Dunn, M. (2004). Current two-dimensional electrophoresis technology for proteomics. *Proteomics*, 4(12):3665–3685.

[Graves and Haystead, 2002] Graves, P. and Haystead, T. (2002). Molecular biologist's guide to proteomics. *Microbiol.Mol.Biol.Rev.*, 66(1):39–63.

[Gygi and Aebersold, 2000] Gygi, S. and Aebersold, R. (2000). Mass spectrometry and proteomics. *Curr.Opin.Chem.Biol.*, 4(5):489–494.

[Harris, 1996] Harris, W. (1996). n-3 fatty acids and lipoproteins: comparison of results from human and animal studies. *Lipids*, 31(3):243–252.

[Heath et al., 2003] Heath, R., Karpe, F., Milne, R., Burdge, G., Wootton, S., and Frayn, K. (2003). Selective partitioning of dietary fatty acids into the vldl tg pool in the early postprandial period. *J.Lipid Res.*, 44(11):2065–2072.

[Herbert et al., 1998] Herbert, B., Molloy, M., Gooley, A., Walsh, B., Bryson, W., and Williams, K. (1998). Improved protein solubility in two-dimensional electrophoresis using tributyl phosphine as reducing agent. *Electrophoresis*, 19(5):845–851.

[Hooper et al., 2006] Hooper, L., Thompson, R., Harrison, R., Summerbell, C., Ness, A., Moore, H., Worthington, H., Durrington, P., Higgins, J., Capps, N., Riemersma, R., Ebrahim, S., and Davey, S. G. (2006). Risks and benefits of omega 3 fats for mortality, cardiovascular disease, and cancer: systematic review. *BMJ*, 332(7544):752–760.

[Horgan, 2007] Horgan, G. (2007). Sample size and replication in 2d gel electrophoresis studies. *J.Proteome.Res.*, 6(7):2884–2887.

[Hsieh et al., 2006] Hsieh, S., Chen, R., Pan, Y., and Lee, H. (2006). Systematical evaluation of the effects of sample collection procedures on low-molecular-weight serum/plasma proteome profiling. *Proteomics*, 6(10):3189–3198.

[Hu et al., 2002] Hu, F., Bronner, L., Willett, W., Stampfer, M., Rexrode, K., Albert, C., Hunter, D., and Manson, J. (2002). Fish and omega-3 fatty acid intake and risk of coronary heart disease in women. *JAMA*, 287(14):1815–1821.

[Hu and Willett, 2002] Hu, F. and Willett, W. (2002). Optimal diets for prevention of coronary heart disease. *JAMA*, 288(20):2569–2578.

[Hu et al., 2005] Hu, Y., Malone, J., Fagan, A., Townsend, R., and Holtzman, D. (2005). Comparative proteomic analysis of intra- and interindividual variation in human cerebrospinal fluid. *Mol.Cell Proteomics*, 4(12):2000–2009.

[Huang et al., 2005] Huang, L., Harvie, G., Feitelson, J., Gramatikoff, K., Herold, D., Allen, D., Amunngama, R., Hagler, R., Pisano, M., Zhang, W., and Fang, X. (2005). Immunoaffinity separation of plasma proteins by igy microbeads: meeting the needs of proteomic sample preparation and analysis. *Proteomics*, 5(13):3314–3328.

[Hulmes et al., 2004] Hulmes, D., Bethea, D., Ho, K., Huang, S., Ricci, D., Opiteck, G., and Hefta, S. (2004). An investigation of plasma collection, stabilization, and storage procedures for proteomic analysis of clinical samples. *Clinical Proteomics Journal*, 1:17–30.

[Jacobs et al., 2005] Jacobs, J., Adkins, J., Qian, W., Liu, T., Shen, Y., Camp, D., and Smith, R. (2005). Utilizing human blood plasma for proteomic biomarker discovery. *J.Proteome.Res.*, 4(4):1073–1085.

[Jenkins et al., 2009] Jenkins, D., Sievenpiper, J., Pauly, D., Sumaila, U., Kendall, C., and Mowat, F. (2009). Are dietary recommendations for the use of fish oils sustainable? *CMAJ.*, 180(6):633–637.

[Jenkins et al., 2008] Jenkins, R., Kitteringham, N., Greenough, C., and Park, B. (2008). Albumin and immunoglobulin depletion of human plasma. *Methods Mol.Biol.*, 425:15–25.

[Jensen et al., 2008] Jensen, K., Jessen, F., and Jorgensen, B. (2008). Multivariate data analysis of two-dimensional gel electrophoresis protein patterns from few samples. *J.Proteome.Res.*, 7(3):1288–1296.

[Jiang et al., 2004] Jiang, L., He, L., and Fountoulakis, M. (2004). Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. *J.Chromatogr.A*, 1023(2):317–320.

[Karp and Lilley, 2007] Karp, N. and Lilley, K. (2007). Design and analysis issues in quantitative proteomics studies. *Proteomics*, 7 Suppl 1:42–50.

[Kim et al., 2004] Kim, H., Page, G., and Barnes, S. (2004). Proteomics and mass spectrometry in nutrition research. *Nutrition*, 20(1):155–165.

[Kim and Kim, 2007] Kim, M. and Kim, C. (2007). Human blood plasma preparation for two-dimensional gel electrophoresis. *J.Chromatogr.B Analyt.Technol.Biomed.Life Sci.*, 849(1-2):203–210.

[Klose, 1975] Klose, J. (1975). Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. a novel approach to testing for induced point mutations in mammals. *Humangenetik.*, 26(3):231–243.

[Klose, 2009] Klose, J. (2009). From 2-d electrophoresis to proteomics. *Electrophoresis*, 30 Suppl 1:S142–S149.

[Kris-Etherton et al., 2002] Kris-Etherton, P., Harris, W., and Appel, L. (2002). Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation*, 106(21):2747–2757.

[Kris-Etherton and Yu, 1997] Kris-Etherton, P. and Yu, S. (1997). Individual fatty acid effects on plasma lipids and lipoproteins: human studies. *Am.J.Clin.Nutr.*, 65(5 Suppl):1628S–1644S.

[Kromann and Green, 1980] Kromann, N. and Green, A. (1980). Epidemiological studies in the upernavik district, greenland. incidence of some chronic diseases 1950-1974. *Acta Med.Scand.*, 208(5):401–406.

[Kussmann and Affolter, 2009] Kussmann, M. and Affolter, M. (2009). Proteomics at the center of nutrigenomics: comprehensive molecular understanding of dietary health effects. *Nutrition*, 25(11-12):1085–1093.

[Kuzyk et al., 2009] Kuzyk, M., Smith, D., Yang, J., Cross, T., Jackson, A., Hardie, D., Anderson, N., and Borchers, C. (2009). Multiple reaction monitoring-based, multiplexed, absolute quantitation of 45 proteins in human plasma. *Mol.Cell Proteomics*, 8(8):1860–1877.

[Laemmli, 1970] Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage t4. *Nature*, 227(5259):680–685.

[Lathyris et al., 2007] Lathyris, D., Trikalinos, T., and Ioannidis, J. (2007). Evidence from crossover trials: empirical evaluation and comparison against parallel arm trials. *Int.J.Epidemiol.*, 36(2):422–430.

[Lauritzen et al., 2001] Lauritzen, L., Hansen, H., Jorgensen, M., and Michaelsen, K. (2001). The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. *Prog.Lipid Res.*, 40(1-2):1–94.

[Lavigne et al., 2000] Lavigne, C., Marette, A., and Jacques, H. (2000). Cod and soy proteins compared with casein improve glucose tolerance and insulin sensitivity in rats. *Am.J.Physiol Endocrinol.Metab*, 278(3):E491–E500.

[Lichtenstein et al., 2006] Lichtenstein, A., Appel, L., Brands, M., Carnethon, M., Daniels, S., Franch, H., Franklin, B., Kris-Etherton, P., Harris, W., Howard, B., Karanja, N., Lefevre, M., Rudel, L., Sacks, F., Van, H. L., Winston, M., and

Wylie-Rosett, J. (2006). Diet and lifestyle recommendations revision 2006: a scientific statement from the american heart association nutrition committee. *Circulation*, 114(1):82–96.

[Marengo et al., 2007] Marengo, E., Robotti, E., and Bobba, M. (2007). Multivariate statistical tools for the evaluation of proteomic maps: recent achievements and applications. *Current Proteomics*, 4:53–66.

[Marengo et al., 2008] Marengo, E., Robotti, E., and Bobba, M. (2008). 2d-page maps analysis. *Methods Mol.Biol.*, 428:291–325.

[Martin et al., 2003] Martin, S., Vilhelmsson, O., Medale, F., Watt, P., Kaushik, S., and Houlihan, D. (2003). Proteomic sensitivity to dietary manipulations in rainbow trout. *Biochim.Biophys.Acta*, 1651(1-2):17–29.

[Mente et al., 2006] Mente, E., Pierce, G., Santos, M., and Neofitou, C. (2006). Effect of feed and feeding in the culture of salmonids on the marine aquatic environment: a synthesis for european aquaculture. *Aquaculture International*, 14(5):499–522.

[Merril et al., 1981a] Merril, C., Dunau, M., and Goldman, D. (1981a). A rapid sensitive silver stain for polypeptides in polyacrylamide gels. *Anal.Biochem.*, 110(1):201–207.

[Merril et al., 1981b] Merril, C., Goldman, D., Sedman, S., and Ebert, M. (1981b). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science*, 211(4489):1437–1438.

[Morgan and Harris, 2003] Morgan, B. and Harris, C. (2003). Complement therapeutics; history and current progress. *Mol.Immunol.*, 40(2-4):159–170.

[Morris et al., 1995] Morris, M., Manson, J., Rosner, B., Buring, J., Willett, W., and Hennekens, C. (1995). Fish consumption and cardiovascular disease in the physicians' health study: a prospective study. *Am.J.Epidemiol.*, 142(2):166–175.

[Morris et al., 1993] Morris, M., Sacks, F., and Rosner, B. (1993). Does fish oil lower blood pressure? a meta-analysis of controlled trials. *Circulation*, 88(2):523–533.

[Mozaffarian et al., 2005] Mozaffarian, D., Geelen, A., Brouwer, I., Geleijnse, J., Zock, P., and Katan, M. (2005). Effect of fish oil on heart rate in humans: a meta-analysis of randomized controlled trials. *Circulation*, 112(13):1945–1952.

[Mozaffarian and Rimm, 2006] Mozaffarian, D. and Rimm, E. (2006). Fish intake, contaminants, and human health: evaluating the risks and the benefits. *JAMA*, 296(15):1885–1899.

[Naylor et al., 2000] Naylor, R., Goldburg, R., Primavera, J., Kautsky, N., Beveridge, M., Clay, J., Folke, C., Lubchenco, J., Mooney, H., and Troell, M.

(2000). Effect of aquaculture on world fish supplies. *Nature*, 405(6790):1017–1024.

[Nedelkov, 2008] Nedelkov, D. (2008). Population proteomics: investigation of protein diversity in human populations. *Proteomics*, 8(4):779–786.

[Nedelkov et al., 2005] Nedelkov, D., Kiernan, U., Niederkofler, E., Tubbs, K., and Nelson, R. (2005). Investigating diversity in human plasma proteins. *Proc.Natl.Acad.Sci.U.S.A*, 102(31):10852–10857.

[O'Farrell, 1975] O'Farrell, P. (1975). High resolution two-dimensional electrophoresis of proteins. *J.Biol.Chem.*, 250(10):4007–4021.

[O'Farrell, 2008] O'Farrell, P. (2008). The pre-omics era: the early days of two-dimensional gels. *Proteomics*, 8(23-24):4842–4852.

[Omenn et al., 2005] Omenn, G., States, D., Adamski, M., Blackwell, T., Menon, R., Hermjakob, H., Apweiler, R., Haab, B., Simpson, R., Eddes, J., Kapp, E., Moritz, R., Chan, D., Rai, A., Admon, A., Aebersold, R., Eng, J., Hancock, W., Hefta, S., Meyer, H., Paik, Y., Yoo, J., Ping, P., Pounds, J., Adkins, J., Qian, X., Wang, R., Wasinger, V., Wu, C., Zhao, X., Zeng, R., Archakov, A., Tsugita, A., Beer, I., Pandey, A., Pisano, M., Andrews, P., Tammen, H., Speicher, D., and Hanash, S. (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(13):3226–3245.

[Ouellet et al., 2007] Ouellet, V., Marois, J., Weisnagel, S., and Jacques, H. (2007). Dietary cod protein improves insulin sensitivity in insulin-resistant men and women: a randomized controlled trial. *Diabetes Care*, 30(11):2816–2821.

[Ouellet et al., 2008] Ouellet, V., Weisnagel, S., Marois, J., Bergeron, J., Julien, P., Gougeon, R., Tchernof, A., Holub, B., and Jacques, H. (2008). Dietary cod protein reduces plasma c-reactive protein in insulin-resistant men and women. *J.Nutr.*, 138(12):2386–2391.

[Overgaard et al., 2008] Overgaard, J., Porsgaard, T., Guo, Z., Lauritzen, L., and Mu, H. (2008). Postprandial lipid responses of butter blend containing fish oil in a single-meal study in humans. *Mol.Nutr.Food Res.*, 52(10):1140–1146.

[Pan et al., 2009] Pan, S., Aebersold, R., Chen, R., Rush, J., Goodlett, D., McIntosh, M., Zhang, J., and Brentnall, T. (2009). Mass spectrometry based targeted protein quantification: methods and applications. *J.Proteome Res.*, 8(2):787–797.

[Patton, 2002] Patton, W. (2002). Detection technologies in proteome analysis. *J.Chromatogr.B Analyt.Technol.Biomed.Life Sci.*, 771(1-2):3–31.

[Pedersen et al., 2010] Pedersen, A., Fagt, S., Groth, M., Christensen, T., Biltoft-Jensen, A., Matthiessen, J., Andersen, N., Krup, K., Hartkopp, H., Ygil, K., Hinsch, H.-J., Saxholt, E., and Trolle, E. (2010). Danskernes kostvaner 2003-2008. *DTU Fdevareinstituttet*, pages –.

[Porter and Reid, 1978] Porter, R. and Reid, K. (1978). The biochemistry of complement. *Nature*, 275(5682):699–704.

[Poutanen et al., 2001] Poutanen, M., Salusjarvi, L., Ruohonen, L., Penttila, M., and Kalkkinen, N. (2001). Use of matrix-assisted laser desorption/ionization time-of-flight mass mapping and nanospray liquid chromatography/electrospray ionization tandem mass spectrometry sequence tag analysis for high sensitivity identification of yeast proteins separated by two-dimensional gel electrophoresis. *Rapid Commun.Mass Spectrom.*, 15(18):1685–1692.

[Price et al., 2000] Price, P., Nelson, C., and Clarke, S. (2000). Omega-3 polyunsaturated fatty acid regulation of gene expression. *Curr.Opin.Lipidol.*, 11(1):3–7.

[Quintana et al., 2009] Quintana, M., Palicki, O., Lucchi, G., Ducoroy, P., Chambon, C., Salles, C., and Morzel, M. (2009). Inter-individual variability of protein patterns in saliva of healthy adults. *J.Proteomics*, 72(5):822–830.

[Rabilloud, 1999] Rabilloud, T. (1999). Solubilization of proteins in 2-d electrophoresis. an outline. *Methods Mol.Biol.*, 112:9–19.

[Racine and Deckelbaum, 2007] Racine, R. and Deckelbaum, R. (2007). Sources of the very-long-chain unsaturated omega-3 fatty acids: eicosapentaenoic acid and docosahexaenoic acid. *Curr.Opin.Clin.Nutr.Metab Care*, 10(2):123–128.

[Raman et al., 2002] Raman, B., Cheung, A., and Marten, M. (2002). Quantitative comparison and evaluation of two commercially available, two-dimensional electrophoresis image analysis software packages, z3 and melanie. *Electrophoresis*, 23(14):2194–2202.

[Rezzi et al., 2007] Rezzi, S., Ramadan, Z., Fay, L., and Kochhar, S. (2007). Nutritional metabonomics: applications and perspectives. *J.Proteome.Res.*, 6(2):513–525.

[Rifai et al., 2006] Rifai, N., Gillette, M., and Carr, S. (2006). Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat.Biotechnol.*, 24(8):971–983.

[Righetti and Bossi, 1997] Righetti, P. and Bossi, A. (1997). Isoelectric focusing in immobilized ph gradients: an update. *J.Chromatogr.B Biomed.Sci.Appl.*, 699(1-2):77–89.

[Robertson et al., 1977] Robertson, T., Kato, H., Rhoads, G., Kagan, A., Marmot, M., Syme, S., Gordon, T., Worth, R., Belsky, J., Dock, D., Miyanishi,

M., and Kawamoto, S. (1977). Epidemiologic studies of coronary heart disease and stroke in japanese men living in japan, hawaii and california. incidence of myocardial infarction and death from coronary heart disease. *Am.J.Cardiol.*, 39(2):239–243.

[Robinson and Stone, 2006] Robinson, J. and Stone, N. (2006). Antiatherosclerotic and antithrombotic effects of omega-3 fatty acids. *Am.J.Cardiol.*, 98(4A):39i–49i.

[Roepstorff, 1997] Roepstorff, P. (1997). Mass spectrometry in protein studies from genome to function. *Curr.Opin.Biotechnol.*, 8(1):6–13.

[Russo, 2009] Russo, G. (2009). Dietary n-6 and n-3 polyunsaturated fatty acids: from biochemistry to clinical implications in cardiovascular prevention. *Biochem.Pharmacol.*, 77(6):937–946.

[Sampath and Ntambi, 2004] Sampath, H. and Ntambi, J. (2004). Polyunsaturated fatty acid regulation of gene expression. *Nutr.Rev.*, 62(9):333–339.

[Sargent and Tacon, 1999] Sargent, J. and Tacon, A. (1999). Development of farmed fish: a nutritionally necessary alternative to meat. *Proc.Nutr.Soc.*, 58(2):377–383.

[Schmitz and Ecker, 2008] Schmitz, G. and Ecker, J. (2008). The opposing effects of n-3 and n-6 fatty acids. *Prog.Lipid Res.*, 47(2):147–155.

[Schuchard et al., 2005] Schuchard, M., Mehigh, R., Cockrill, S., Lipscomb, G., Stephan, J., Wildsmith, J., Valdes-Camin, R., Kappel, W., Rai, A., and Scott, G. (2005). Artifactual isoform profile modification following treatment of human plasma or serum with protease inhibitor, monitored by 2-dimensional electrophoresis and mass spectrometry. *Biotechniques*, 39(2):239–247.

[Shevchenko et al., 1996] Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal.Chem.*, 68(5):850–858.

[Silverman, 2009] Silverman, S. (2009). From randomized controlled trials to observational studies. *Am.J.Med.*, 122(2):114–120.

[Simopoulos, 2000] Simopoulos, A. (2000). Human requirement for n-3 polyunsaturated fatty acids. *Poult.Sci.*, 79(7):961–970.

[Simopoulos, 2002] Simopoulos, A. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed.Pharmacother.*, 56(8):365–379.

[Simopoulos, 2006] Simopoulos, A. (2006). Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications for chronic diseases. *Biomed.Pharmacother.*, 60(9):502–507.

[Skonberg et al., 1993] Skonberg, D., Rasco, B., and Dong, F. (1993). Effects of feeding high monounsaturated sunflower oil diets on sensory attributes of salmonid fillets. *Journal of Aquatic Food Product Technology*, 2(1):117–133.

[Steen and Mann, 2004] Steen, H. and Mann, M. (2004). The abc's (and xyz's) of peptide sequencing. *Nat.Rev.Mol.Cell Biol.*, 5(9):699–711.

[Steinberg et al., 2000] Steinberg, T., Lauber, W., Berggren, K., Kemper, C., Yue, S., and Patton, W. (2000). Fluorescence detection of proteins in sodium dodecyl sulfate-polyacrylamide gels using environmentally benign, nonfixative, saline solution. *Electrophoresis*, 21(3):497–508.

[Storey and Tibshirani, 2003] Storey, J. and Tibshirani, R. (2003). Statistical significance for genomewide studies. *Proc.Natl.Acad.Sci.U.S.A*, 100(16):9440–9445.

[Torrejon et al., 2007] Torrejon, C., Jung, U., and Deckelbaum, R. (2007). n-3 fatty acids and cardiovascular disease: actions and molecular mechanisms. *Prostaglandins Leukot.Essent.Fatty Acids*, 77(5-6):319–326.

[Tremblay et al., 2005] Tremblay, F., Jacques, H., and Marette, A. (2005). Modulation of insulin action by dietary proteins and amino acids: role of the mammalian target of rapamycin nutrient sensing pathway. *Curr.Opin.Clin.Nutr.Metab Care*, 8(4):457–462.

[Tu et al., 2010] Tu, C., Rudnick, P., Martinez, M., Cheek, K., Stein, S., Slebos, R., and Liebler, D. (2010). Depletion of abundant plasma proteins and limitations of plasma proteomics. *J.Proteome.Res.*, 9(10):4982–4991.

[Unlu et al., 1997] Unlu, M., Morgan, M., and Minden, J. (1997). Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis*, 18(11):2071–2077.

[Valagussa et al., 1999] Valagussa, F., Franzosi, M., Geraci, E., Mininni, N., Nicolosi, G., Santini, M., Tavazzi, L., Vecchio, C., Marchioli, R., Bomba, E., Chieffo, C., Maggioni, A., Schweiger, C., Tognoni, G., Barzi, F., Flamminio, A., Marfisi, R., Olivieri, M., Pera, C., Polidoro, A., Santoro, E., Zama, R., Pagliaro, L., Correale, E., Del Favero, A., Loi, U., Marubini, E., Campolo, L., Casari, A., Di Minno, G., Donati, M., Galli, M., Gattone, M., Garattini, S., Mancini, M., Marino, P., Santoro, G., Scardulla, C., Specchia, G., Cericola, A., Di Gregorio, D., Di Mascio, R., Levantesi, G., Mantini, L., Mastrogiuseppe, G., Tucci, C., Mocarelli, P., Baldinelli, R., Ceriotti, F., Colonna, A., Cortese, C., Fortunato, G., Franzini, C., Gonano, F., and Graziani, M. (1999). Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin e after myocardial infarction: results of the gissi-prevenzione trial. *Lancet*, 354(9177):447–455.

[Van et al., 2008] Van, H. L., McCoin, M., Kris-Etherton, P., Burke, F., Carson, J., Champagne, C., Karmally, W., and Sikand, G. (2008). The evidence for

dietary prevention and treatment of cardiovascular disease. *J.Am.Diet.Assoc.*, 108(2):287–331.

[van Ommen et al., 2009] van Ommen, B., Keijer, J., Heil, S., and Kaput, J. (2009). Challenging homeostasis to define biomarkers for nutrition related health. *Mol.Nutr.Food Res.*, 53(7):795–804.

[Vilhelmsson et al., 2004] Vilhelmsson, O., Martin, S., Medale, F., Kaushik, S., and Houlihan, D. (2004). Dietary plant-protein substitution affects hepatic metabolism in rainbow trout (oncorhynchus mykiss). *Br.J.Nutr.*, 92(1):71–80.

[Wang et al., 2006a] Wang, C., Harris, W., Chung, M., Lichtenstein, A., Balk, E., Kupelnick, B., Jordan, H., and Lau, J. (2006a). n-3 fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review. *Am.J.Clin.Nutr.*, 84(1):5–17.

[Wang et al., 2006b] Wang, J., Li, D., Dangott, L., and Wu, G. (2006b). Proteomics and its role in nutrition research. *J.Nutr.*, 136(7):1759–1762.

[Wasinger et al., 1995] Wasinger, V., Cordwell, S., Cerpa-Poljak, A., Yan, J., Gooley, A., Wilkins, M., Duncan, M., Harris, R., Williams, K., and Humphery-Smith, I. (1995). Progress with gene-product mapping of the mollicutes: Mycoplasma genitalium. *Electrophoresis*, 16(7):1090–1094.

[Wathes et al., 2007] Wathes, D., Abayasekara, D., and Aitken, R. (2007). Polyunsaturated fatty acids in male and female reproduction. *Biol.Reprod.*, 77(2):190–201.

[Weiss and Gorg, 2009] Weiss, W. and Gorg, A. (2009). High-resolution two-dimensional electrophoresis. *Methods Mol.Biol.*, 564:13–32.

[Weiss et al., 2009] Weiss, W., Weiland, F., and Gorg, A. (2009). Protein detection and quantitation technologies for gel-based proteome analysis. *Methods Mol.Biol.*, 564:59–82.

[Whiteaker et al., 2007] Whiteaker, J., Zhang, H., Eng, J., Fang, R., Piening, B., Feng, L., Lorentzen, T., Schoenherr, R., Keane, J., Holzman, T., Fitzgibbon, M., Lin, C., Zhang, H., Cooke, K., Liu, T., Camp, D., Anderson, L., Watts, J., Smith, R., McIntosh, M., and Paulovich, A. (2007). Head-to-head comparison of serum fractionation techniques. *J.Proteome.Res.*, 6(2):828–836.

[Wilkins et al., 2006] Wilkins, M., Appel, R., Van Eyk, J., Chung, M., Gorg, A., Hecker, M., Huber, L., Langen, H., Link, A., Paik, Y., Patterson, S., Pennington, S., Rabilloud, T., Simpson, R., Weiss, W., and Dunn, M. (2006). Guidelines for the next 10 years of proteomics. *Proteomics*, 6(1):4–8.

[Wilkins et al., 1996] Wilkins, M., Sanchez, J., Gooley, A., Appel, R., Humphery-Smith, I., Hochstrasser, D., and Williams, K. (1996). Progress with proteome

projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnol.Genet.Eng Rev.*, 13:19–50.

[Winkler et al., 2008] Winkler, W., Zellner, M., Diestinger, M., Babeluk, R., Marchetti, M., Goll, A., Zehetmayer, S., Bauer, P., Rappold, E., Miller, I., Roth, E., Allmaier, G., and Oehler, R. (2008). Biological variation of the platelet proteome in the elderly population and its implication for biomarker research. *Mol.Cell Proteomics.*, 7(1):193–203.

[Wu et al., 2005] Wu, J., Lenchik, N., Pabst, M., Solomon, S., Shull, J., and Gerling, I. (2005). Functional characterization of two-dimensional gel-separated proteins using sequential staining. *Electrophoresis*, 26(1):225–237.

[Wulff et al., 2008] Wulff, T., Jessen, F., Roepstorff, P., and Hoffmann, E. (2008). Long term anoxia in rainbow trout investigated by 2-de and ms/ms. *Proteomics.*, 8(5):1009–1018.

[Yokoyama et al., 2007] Yokoyama, M., Origasa, H., Matsuzaki, M., Matsuzawa, Y., Saito, Y., Ishikawa, Y., Oikawa, S., Sasaki, J., Hishida, H., Itakura, H., Kita, T., Kitabatake, A., Nakaya, N., Sakata, T., Shimada, K., and Shirato, K. (2007). Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (jelis): a randomised open-label, blinded endpoint analysis. *Lancet*, 369(9567):1090–1098.

[Zampelas et al., 2005] Zampelas, A., Panagiotakos, D., Pitsavos, C., Das, U., Chrysohoou, C., Skoumas, Y., and Stefanadis, C. (2005). Fish consumption among healthy adults is associated with decreased levels of inflammatory markers related to cardiovascular disease: the attica study. *J.Am.Coll.Cardiol.*, 46(1):120–124.

[Zampelas et al., 1994] Zampelas, A., Peel, A., Gould, B., Wright, J., and Williams, C. (1994). Polyunsaturated fatty acids of the n-6 and n-3 series: effects on postprandial lipid and apolipoprotein levels in healthy men. *Eur.J.Clin.Nutr.*, 48(12):842–848.

[Zerefos et al., 2006] Zerefos, P., Vougas, K., Dimitraki, P., Kossida, S., Petrolekas, A., Stravodimos, K., Giannopoulos, A., Fountoulakis, M., and Vlahou, A. (2006). Characterization of the human urine proteome by preparative electrophoresis in combination with 2-de. *Proteomics*, 6(15):4346–4355.

[Zhang et al., 2006] Zhang, X., Guo, Y., Song, Y., Sun, W., Yu, C., Zhao, X., Wang, H., Jiang, H., Li, Y., Qian, X., Jiang, Y., and He, F. (2006). Proteomic analysis of individual variation in normal livers of human beings using difference gel electrophoresis. *Proteomics*, 6(19):5260–5268.

[Zimny-Arndt et al., 2009] Zimny-Arndt, U., Schmid, M., Ackermann, R., and Jungblut, P. (2009). Classical proteomics: two-dimensional electrophoresis/maldi mass spectrometry. *Methods Mol.Biol.*, 492:65–91.

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