# Metabolomics
# High resolution characterization of human metabolism and biochemical status on habitual diet and ketogenic diet

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# Abstract

**Introduction and background**
Ketogenic diet (KD) consisting of mostly fat, adequate protein and very low carbohydrate consumption induces metabolic modifications where the main fuel switches from glucose to fat degradation. The diet is used in the treatment of several inborn errors of metabolism and as treatment for refractory epilepsy. The diet has also become popular as a rapid weight reduction method. High intake of fat, particularly saturated fat, can lead to high levels of total and LDL cholesterol concentrations known to be associated with development of atherosclerosis. This raises a concern that KD increases the risk of cardiovascular disease. However, the biochemical effects of KD are insufficiently described, and previous studies have been conducted primarily using overweight participants.
**Objectives**
This study aimed to identify metabolic changes in normal weight subjects as a result of following a ketogenic diet for three weeks. In addition, comparison between the ketogenic and habitual diet metabolomes of subjects with low and high relative LDL-C change after KD intervention was conducted.
**Subjects and methods**
In 2011-2012, a six-weeks, randomized controlled cross-over trial investigating the effect of three weeks on ketogenic diet (<20 g carbohydrates) was conducted in healthy normal weight subjects (N=30), mainly students and employees at the Department of Nutrition research at University of Oslo. As a continuation, comprehensive global metabolomics analyses of plasma with high-performance liquid chromatography coupled to mass spectrometry (LC-MS) was performed in this thesis to identify and quantify as many metabolites as possible in the biological samples.
**Results**
An average of 1085 and 419 features were detected in positive and negative ionization mode, respectively. Differential analysis using volcano plot showed that 48 features in positive ionization mode and 32 compounds in negative ionization mode were detected in significantly higher levels in samples taken after KD versus habitual diet with a log2 fold change >1. Significantly higher levels of ketone bodies, lipids, acylcarnitines, trihomomethionine, acetylglycine, GABA and hydroxyvaleric were detected. Hydroxyvaleric acid had a greater increase in the group with the highest relative LDL-C increase compared to the lowest relative LDL-C increase group. Furthermore, citric acid, malic acid and alpha-ketoglutaric acid as intermediated in the citric acid cycle were significantly upregulated after KD. Levels of the amino acids threonine, asparagine, tryptophan, proline, b-alanine, leucine, arginine, methionine, glutamine and alanine were significantly decreased, while valine was elevated 43 % after 3 weeks on KD. Univariate correlation matrix showed a significant positive correlation ($p<0.03$) between the amount of amino acid histidine and change in LDL-C with a correlation coefficient of 0.44 (CI 0.06, 0.71). Lower plasma levels of stachydrine, methyl indole 3-acetate, caffeine and N-acetylvaline was observed after KD.
**Conclusion**
Applying global metabolomics in a randomized controlled study has the potential to detect differences in the metabolome after dietary intervention, and further increase our knowledge of the biochemical effects and consequences of a KD. In particular, we found alterations in a diverse set of compounds. However, further investigations are needed to understand the mechanisms behind these changes and to determine long-term effects of following a KD.

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# Abbreviations

| | |
|---|---|
| ApoB | apolipoprotein B |
| ApoA1 | apolipoprotein A1 |
| CVD | cardiovascular disease |
| CM | chylomicrone |
| Da | dalton |
| ESI | electrospray ionization |
| GLUT1 | glucose transporter protein type 1 |
| HDL | high-density lipoprotein |
| HDL-C | HDL-cholesterol |
| IDL | intermediate-density lipoprotein |
| IEM | inborn error of metabolism |
| KD | ketogenic diet |
| LDL | low-density lipoprotein |
| LDL-C | LDL cholesterol |
| LDL-R | LDL receptor |
| LC-MS | liquid chromatography-mass spectrometry |
| MeOH | methanol |
| MS | mass spectrometry |
| *m/z* | mass-to-charge ratio |

| | |
|---|---|
| MCT | medium chain triglyceride |
| OUS | Oslo university hospital |
| PCA | principal component analysis |
| Rt | retention time |
| TC | total cholesterol |
| TG | triglycerides |
| V1 | visit 1 |
| V2 | visit 2 |
| V3 | visit 3 |
| VLDL | very-low-density lipoprotein |

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# 1 Introduction

## 1.1 Ketogenic Diet

### Ketogenic diet as treatment for disease

Ketogenic diet (KD) is a collective name for a diet consisting of mostly fat, adequate protein and very low carbohydrate consumption. The diet is called “ketogenic” because of the elevated production of ketone bodies. The diet was used as a treatment for epilepsy in the 1920s (1-3). Furthermore, KD is used in the treatment for several inborn errors of metabolism (IEMs) (4). IEMs are a large group of rare monogenetic diseases, usually caused by deficiency or defects of enzymes involved in anabolic or catabolic biochemical pathways, thereby affecting the levels of metabolites in cells, tissues and body fluids (5). Among them are glucose transporter protein type 1(GLUT1) deficiency syndrome and pyruvate dehydrogenase deficiency, where KD is the primary treatment available (4). The biological mechanisms by which KD works for epilepsy are still unknown (6), except for GLUT1 and pyruvate dehydrogenase deficiency where KD contributes to an alternative energy source (7).

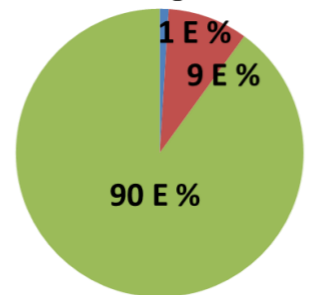
### Low carbohydrate revolution

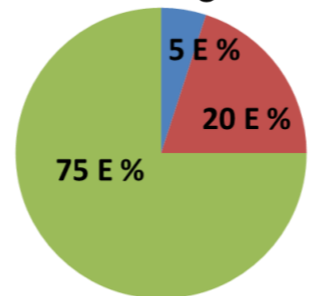
Later on the diet has been popular in the hope of achieving better health or a rapid weight loss (8, 9). The prevalence of obesity has been increasing worldwide over the past several decades in both children and adults (10). Obesity is associated with metabolic disorders like type-2-diabetes, cardiovascular disease (CVD), hypertension, cancers, osteoarthritis and the development of metabolic syndrome. Metabolic syndrome includes large waist circumference, dyslipidemia, elevated fasting glucose levels and elevated blood pressure (10). In 1972, Robert C. Atkin published his book *Dr. Atkin`s Diet revolution.* Atkin stated that a low intake of carbohydrates, mainly sugars and starches would be beneficial for health and for achieving better weight control. Atkin`s diet has been “One of the most popular fad diets in the United States” (11), and thus starting a low-carbohydrate revolution. High-fat foods such as vegetable oils, full-fat dairy products, margarine, mayonnaise, avocado and nuts represent a considerable proportion of the diet (12, 13). Moderate amounts of meat, fish, eggs and cheese are necessary to secure an adequate intake of protein, while bread, potato, pasta and rice are not compatible with a KD due to the high levels of carbohydrate. A moderate amount of other

carbohydrate sources such as vegetables, fruits and berries is accepted as they are important nutrient sources contributing with essential vitamins and minerals (14).

### 1.1.1 Variants of ketogenic diet

There are different variants of KD. How to manage the diet due to different levels of carbohydrate intake is the main difference that distinguish the diets. **Figure 1** illustrate the percentage distribution of the daily calorie intake of each macronutrient in a classic and modified KD compared to Nordic nutrition recommendations. Classic, modified and medium-chain triglyceride KD are most commonly used in Norway (13).




- **Classic ketogenic diet**
  The classic KD is based on a ratio of grams of fat to grams of carbohydrate and protein combined (1). All meals have the same ketogenic ratio and are calculated with a 0.1 gram accuracy. A 2:1 ketogenic ratio equates to a meal that contains twice as many grams of fat as protein and carbohydrate combined. This signifies that the higher ketogenic ratio, the more fat in the diet.

- **Modified ketogenic diet**
  The modified KD limits carbohydrates to 10-20 g daily which corresponds to a slice of whole grain bread (15), but no limitations on protein intake and nearly 75 % of the daily calorie intake from fat. This KD is also known as the modified Atkins diet, as it is a modification of the diet that Robert C. Atkin established in 1972.

- **Medium-chain triglyceride ketogenic diet**
  The medium-chain triglyceride KD is similar to the classic diet except that 30- 60 % of the daily calorie intake from fat is specified to be medium chain triglyceride (MCT) fat (1). MCT fat provides more ketones per calorie of energy than long chain triglycerides (LCT) (1).

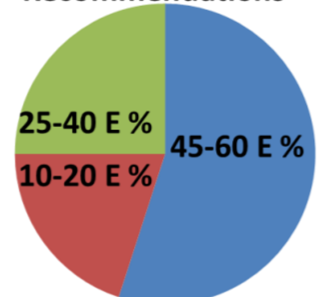



**Figure 1** Pie chart that illustrates the percentage amounts of macronutrients in a classic and modified ketogenic diet compared to Nordic Nutrition Recommendations.

Changing the diet from a recommended Nordic diet (16) where 45-60 % of the energy requirements comes from carbohydrates to a KD low on carbohydrate results in physiological

changes. The KD induces metabolic modifications associated with a state of starvation where the main fuel switches from glucose to fat degradation. This induces “physiological ketosis”.

### 1.1.2 Physiology of ketosis

In a normal physiological state the beta cells of the pancreatic islets secrete the peptide hormone insulin in response to a carbohydrate rich meal. Insulin is an important regulator of the metabolism of carbohydrates, fat and protein. The glucose level in the blood after a ketogenic meal is low, thus the secretion of insulin is reduced. This results in reduced absorption of glucose from the blood into the liver. The lower level of glucose in the liver reduces the production of and hence the availability of oxaloacetate in the liver. Oxaloacetate is a product of glucose and amino acid degradation and reacts with acetyl CoA to produce citrate for ATP generation in the citric acid cycle. Increased release of fatty acids from the adipose tissue and subsequent transport into the liver where the fatty acids are metabolized leads to production of Acetyl CoA. Carnitine is essential in transport of long-chain fatty acids into mitochondria for oxidation (1).

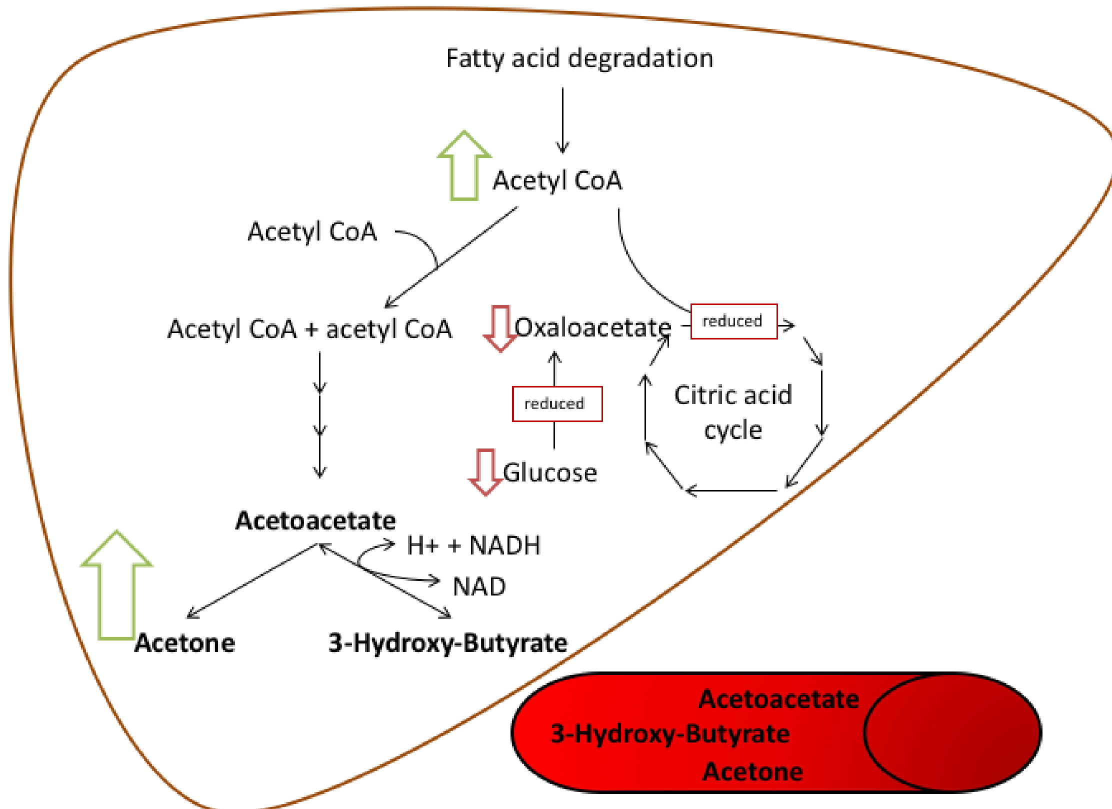



**Figure 2 ketogenesis** simplified production of ketone bodies in the liver in response to ketogenic diet

Lower levels of oxaloacetate combined with elevated levels of Acetyl CoA that exceed the amount needed for citrate synthesis lead to shunting of Acetyl CoA to production of ketone bodies, ketogenesis, resulting in a metabolic state of ketosis (17). This is illustrated in **figure 2**. Glucose levels remains stable due to synthesis from glucogenic amino acids and glycerol liberated via lysis of triglycerides (1, 18, 19).

## Ketone bodies

Ketone bodies are water soluble molecules that contain hydrogen, carbon and oxygen. Acetoacetate, beta-hydroxybutyrate and acetone are three well known endogenous ketone bodies used to produce adenosine triphosphate (ATP) as a result of metabolic degradation. Ketones can be measured in urine and blood (1). Circulating levels of ketone bodies vary across the populations due to variations in basal metabolic rate, hepatic glycogen stores and mobilization of amino acids from muscle proteins (20).

Ketolysis is the metabolic degradation of ketone bodies illustrated in **figure 3**. The ketone bodies are exported to the circulation system for uptake by the mitochondria of extrahepatic organs, where they are converted to acetyl coA and enters the citric acid cycle to generate ATP.

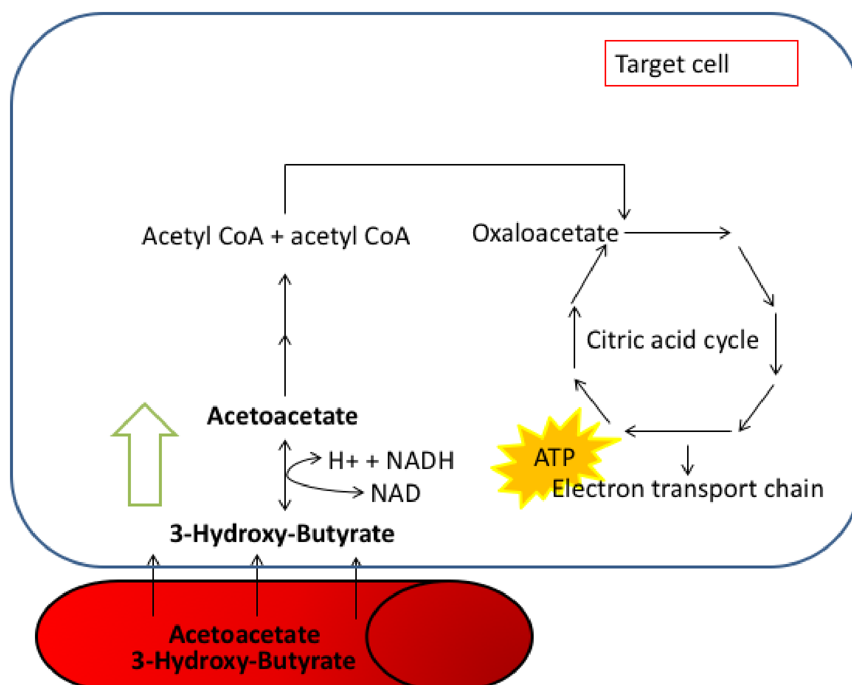



**Figure 3 ketolysis** simplified presentation of metabolic degradation of ketone bodies in a target cell generating ATP

So, two hallmarks of a KD is the reduction in blood sugar levels and the elevation of ketone levels in the blood (17). However, these changes can cause undesirable consequences.

### 1.1.3 Side effects of the ketogenic diet

Constipation, vomiting, diarrhea, headache, fatigue and weight loss are common side effects reported on a classic and modified KD (21). Furthermore, there is an increased risk for nutritional deficiencies since the diet limits food groups such as fruit and starchy vegetables. Thus, multi-vitamin supplementation is recommended, especially among those who are not aware of their own nutrient intake. Hypoglycemic episodes can occur during the first weeks before the metabolism adapts to ketone bodies and fat as primary sources of energy (1). The ketosis during the first couple of weeks on the diet lowers the pH in the blood and may cause a mild metabolic acidosis (12). A severe metabolic acidosis, ketoacidosis, can occur in persons with diabetes mellitus and is a life-threatening condition.

Additionally, elevated total cholesterol and LDL cholesterol have been reported as adverse effects of the KD (21-25).

### 1.1.4 Raised lipid levels in a ketogenic diet

Raised lipids levels are common in people on KD (21-24). Elevation of total cholesterol, lipoproteins, total apolipoprotein B (apoB) and apolipoprotein A1 (apoA-1) was reported among children on KD for six months, but there was no description regarding type of fat intake in the report (22). Studies on adults on KD report similar finding (26, 27).

#### Cholesterol and lipoprotein

Cholesterol is an essential component of cell membranes and is a precursor of bile acids and steroid hormones (10). Cholesterol is effectively transported in the blood stream packed within lipoproteins that delivers lipids to cells and tissue. Lipoproteins consist primarily of cholesteryl esters and triglycerides in their core and phospholipid, free cholesterol and apolipoproteins (apo) on the surface (28). Lipoproteins can be classified based on their densities. In order of increasing density; Chylomicrones (CM), very low-density lipoprotein (VLDL), intermediate density lipoproteins (IDL), low-density lipoproteins (LDL) and high-

density lipoproteins (HDL). ApoB-containing lipoproteins (VLDL, IDL and LDL) in plasma are the main transporters for both exogenous and endogenous cholesterol from the liver to peripheral tissues (10, 29). ApoB100 is the main structural apolipoprotein of LDL with a single molecule of apoB-100 per LDL particle. The HDL contains apoA-1 as its major apolipoprotein and transport the cholesterol from peripheral tissues to the liver, termed reverse cholesterol transport (10). Triglycerides are formed from exogenous fatty acids and endogenously either produced by the liver or released from adipose tissue (10). As a rule of thumb, the following values have been classified as optimal; A total cholesterol < 5 mmol/L, LDL <3mmol/L, triglycerides <2 mmol/l and HDL >1 mmol/l for men and <1.3 for women (30).

Elevated total cholesterol and LDL cholesterol (LDL-C) are known risk factors for developing cardiovascular disease. This raises a concern that a KD with a high intake of fat increases the risk of atherosclerosis.

### 1.1.5 Cardiovascular disease and atherosclerosis risk

Atherosclerosis is a complex and degenerative disease of the vascular endothelium where plaques inside the arterial walls reduce the internal vessel diameter and inhibit the blood supply to vital organs, in particular when plaques rupture and cause platelet aggregation and coagulation dramatically reducing or blocking blood flow, causing chronic and acute cardiovascular disease (CVD)(31). CVD is a class of diseases affecting the heart and circulatory system and is the major cause of death and morbidity in the Norwegian population (32). Important metabolic risk factors for developing CVD includes high blood pressure, high fasting blood glucose and high cholesterol, all of which are modifiable risk factors (33, 34). In addition, an unhealthy diet is one of the modifiable risk factors that causes the most deaths in Norway (32). Furthermore, age, gender and genetics contribute in addition as non-modifiable risk factors.

High blood cholesterol, especially LDL-C is a causal factor in the pathophysiology of atherosclerosis and is associated with increased risk of CVD (10, 29). Lipids, primarily cholesterol-rich apoB containing lipoproteins and cholesteryl esters, and cells of the immune system are major contributors in the atherogenic process. In short, lipid accumulation in intima causes endothelial dysfunction as one of the first steps in the development of

atherosclerosis. This recruits monocytes from the bloodstream that migrate into the intima of the arterial wall where they differentiates into macrophages that ingests oxidized LDL and further progress to form foam cells (31, 35).

### 1.1.6 Role of fat quality for development of atherosclerosis

Total fat includes saturated fat, monounsaturated fat, omega-3 polyunsaturated fat and omega-6 polyunsaturated fat (10). National dietary guidelines in Norway focus on a diet and an eating pattern that prevents chronic disease and promotes health (36). The guidelines recommend a diet with 25-40 E % total fat, <10 E% saturated fat, 10-20 E % monounsaturated fat, 5-10 E% polyunsaturated fat (36). High intake of fat, particularly saturated fat in the diet, is associated with development of health problems. Large amounts of saturated fat in the diet can lead to high levels of total and LDL cholesterol concentrations associated with the development of atherosclerosis (29). The plasma cholesterol raising effects of saturated fat, particularly myristic (C14:0) and palmitic (16:0) acids, have been well established (10). However, the evidence for how a diet rich in saturated fat can cause atherosclerotic vascular disease is lacking despite several studies that have attempted to address this question. Replacing saturated fat with polyunsaturated fatty acids improves the lipid profile and reduces the cardiovascular disease risk (36-38). Thus, replacing foods from animal sources rich in saturated fat with foods high in mono- and polyunsaturated fat e.g. vegetable oils are preferable.

The distribution between saturated and unsaturated fat in a KD can be of high importance, especially regarding changes in the lipid profile. There is a lack of knowledge of the long-term physiological changes of adherence to KD. Previous studies have been conducted primarily using overweight participants (39, 40). Losing weight will change and improve the metabolic profile (10). But what is the effect of KD on normal weight individuals?

### 1.1.7 Effect of Ketogenic diet on LDL cholesterol and gene expression in normal-weight, young adults: A randomized controlled study

The low carbohydrate high fat (LCHF) study was conducted by Retterstøl et al in 2011/2012. A total of 30 normal-weight, young adults mainly students and employees at the Department

of Nutrition research at the University of Oslo completed the study. The participants were randomly allocated to a LCHF diet or habitual diet for three weeks. The group assigned to LCHF diet had a plasma LDL-C increase from 2.2 ±0.4 mmol/l at baseline to 3.1±0.8 mmol/l after intervention. The group continuing their habitual diet had no difference in plasma LDL-C levels (2.5±0.8 mmol/l) p<0,001 after the three weeks on the continued habitual diet.

In the LCHF study specific assays were used to identify and quantify metabolites of interest coupled to cholesterol pathways, lipid profile and peripheral blood mononuclear cells gene expression. But what about everything else? What about all the possible information related to the hundreds and thousands of metabolites that we do not detect with specific assays?

## 1.2 Metabolomics

Metabolomics is the systematic study of low molecular weight molecules (metabolites), typically less than 1500 Da, in a biological sample (41). Carbohydrates, amino acids, nucleotides and lipids are substrates, intermediates and end products of metabolism that can be investigated using metabolomics to complement genomics, transcriptomics, proteomics, and other omics studies as illustrated in **figure 4 (42)**. Metabolomics provides information closer to the phenotype. The phenotype refers to the physical and biochemical characteristics of an organism that is determined by its genotype and the influence of a myriad of environmental factors over time directly upon the organism and on the expression of its genes (43). The metabolome can be defined as the entire collection of metabolites in a biological sample (44). Metabolomics can be divided into a targeted or an untargeted, global approach (45). Specific assays are used in targeted metabolomics to identify and quantify specific metabolites of interest in a biochemical pathway. A global approach aims to investigate as many metabolites as possible in a biological sample(45).

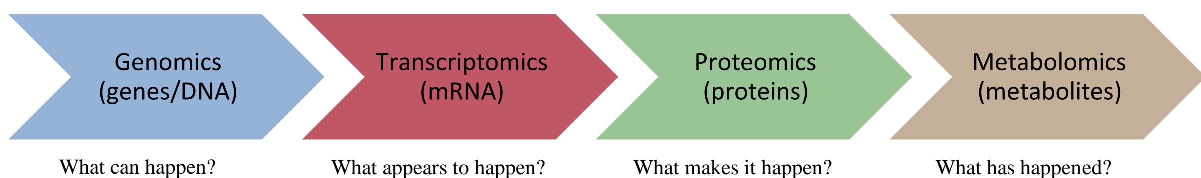



**Figure 4** The omics cascade. Metabolome is downstream of the genome, thus metabolomics provides information closer to the phenotype. The phenotype is the physical and biochemical characteristics of an organism that is the results of the combined effect of genome, lifestyle and environmental factors. The figure was adapted from (46).

### 1.2.1 Targeted analyses

Targeted analyses are specific assays used to identify and quantify selected metabolites or metabolite classes (47). These metabolites are substrates, intermediates, products or other substances involved in known biochemical pathways, usually endogenous, but many are exogenous. Targeted analyses are used in standard clinical chemistry for diagnostics of IEMs and other conditions (48). It is possible to analyze one or several compounds based on a hypothesis (47). However, the metabolism is complex and it is preferred to analyze as many compounds as possible if no clear hypothesis can be defined. Global metabolomics make this feasible.

### 1.2.2 Global metabolomics

Global metabolomics is an untargeted approach to identify and quantify as many metabolites as possible in a biological sample, thus being able to generate new hypotheses based on the findings (44, 45, 47). Untargeted assays using liquid chromatography-mass spectrometry (LC-MS) provide semi-quantitative data, where chromatographic peak areas are reported instead of absolute concentrations (49). Global metabolomics can be used to examine differences in the metabolic profile between a control and a treatment group (47). One single analytical method can not detect the entire collection of metabolites in a biological sample due to different properties of the metabolites, thus different strategies and methods are necessary to focus on different metabolite classes and to increase the number of metabolites detected.

### 1.2.3 LC-MS used in global metabolomics

The analytical platform used for this project was LC-MS with an electrospray ionization (ESI) source illustrated in **figure 5.** This is one of the most used analytical tools in global metabolomics (47, 50). The analytical platform and extraction method used will influence which metabolites that can be detected and measured.

A mass spectrometer (MS) is an analytical instrument applied to detect positively or negatively charged gas phase metabolites based on their mass-to-charge ratios *(m/z)* (51). Thousands of peaks referred to as metabolite features can usually be detected in a biological sample (47). Each feature consists of a measured mass-to-charge ratio *(m/z)* and a unique

retention time that is used to perform metabolomic profiling (45). Metabolites can be separated in time prior to mass spectrometry analysis to reduce the chance of matrix effects, e.g. ion suppression. Liquid chromatography (LC) is a technique to separate the compounds in a solution based on equilibrium between a solid stationary phase and a liquid mobile phase. A pump generates high pressure, allowing the liquid mobile phase to transport the sample compounds through a closed column containing the stationary phase (47). Each compound has its own unique properties and interacts differently with the solid and mobile phases chosen. The time from the sample is injected until the compound elutes from the LC column is referred to as the retention time of the compound (52).

**Figure 5** picture of the instrument used in untargeted metabolomics

The electrospray ionization (ESI) source is used to transform ions in a liquid phase to gas phase using electrical energy, before entering the MS (47, 53). Compounds in solution are differently charged (positively or negatively). The voltage applied determines whether the positively or negatively charged metabolites enter the MS, referred to as positive and negative ionization mode, respectively (47).

### 1.2.4 Untargeted metabolomics workflow

A common workflow to perform untargeted metabolomics using LC-MS starts with the experimental design and consist of collecting relevant samples, sample preparation, data acquisition, data processing and statistical analyses of the data generated to investigate trends and identify metabolites and metabolic pathways involved (45). This can generate hypotheses that may be validated with further quantitative measurements. **Figure 6** illustrates the untargeted metabolomics workflow.

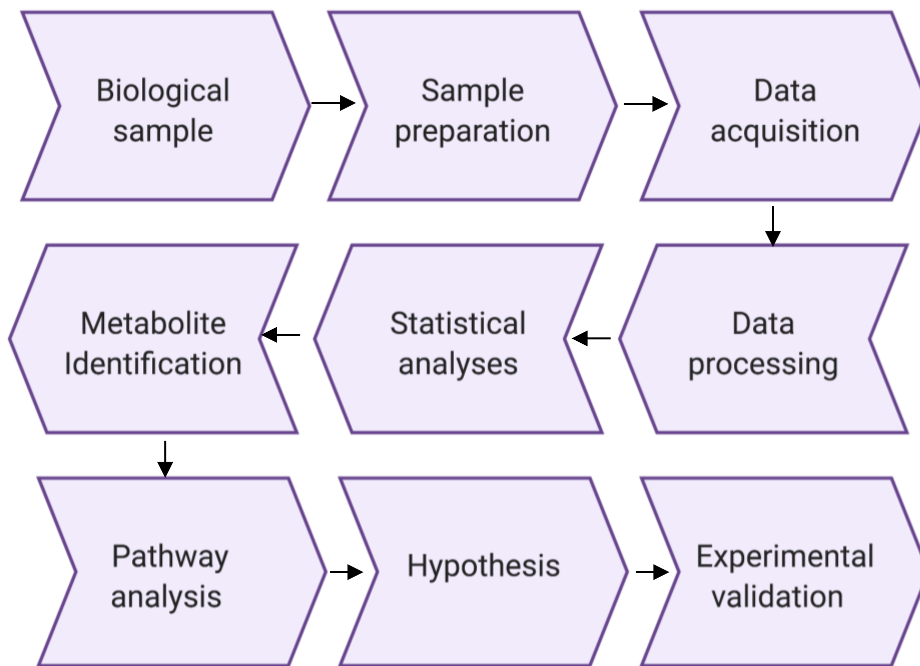



**Figure 6 Untargeted metabolomics workflow for hypothesis generation**

## Experimental design

A well-established experimental design is essential to obtain objective and valid results. Furthermore, it is important to evaluate the number of individuals, samples and matched controls to be included in the study (47). Standardization and control of variables influencing the metabolome must be addressed, e.g. sex, age, ethnicity, diet, physical activity, medication and diseases, which all contribute to biological variation. The design of the LCHF study is shown in **figure 7**. The LCHF had a cross over design, meaning that both groups performed the diet intervention but at different times.

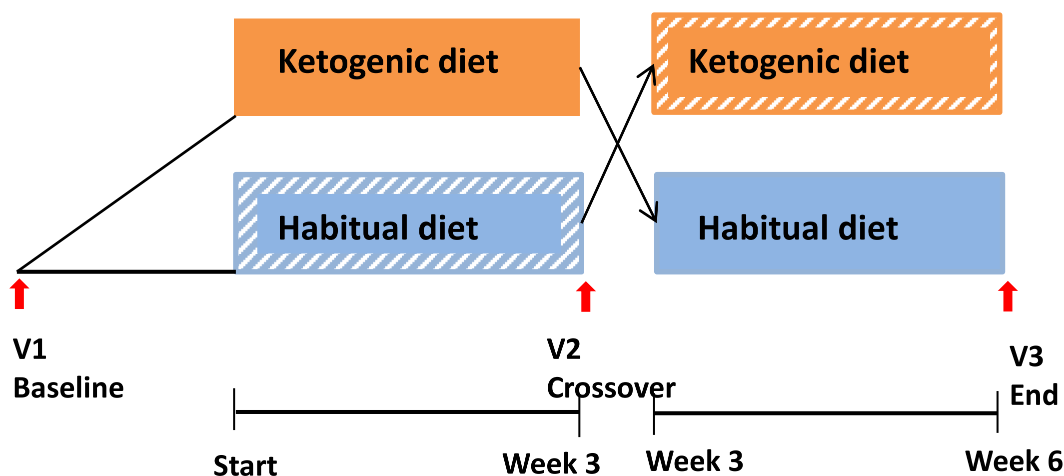



**Figure 7** The LCHF study design with three visits (V1-V3) when samples were obtained.

### Biological material

The composition of metabolites differs in the different sample materials. Plasma, serum, tissue, urine, tear fluid, spinal fluid, whole blood and dried blood spots are different biological materials that can be used in metabolomics (54-58). Choice of material depends on the metabolites of interest and availability of samples.

### Sample preparation

Intervention and control samples must be treated equally prior to analysis. Information about the preanalytical factors e.g. sampling, sample handling, preparation, transport and storage is important (45, 47). Sample preparation in metabolomics is usually non-specific to ensure coverage of as many metabolites as possible in the biological sample .

### Data acquisition

In LC-MS global metabolomics a process blank sample and quality control samples are usually included in addition to the biological samples of interest (49). A process blank sample, e.g. water, is prepared using the same procedure as for the biological samples, and analyzed to remove the background signal which do not reflect the biological effects being studied (49). Quality control samples are used to correct for changes in instrument performance over time (49). A pooled quality control (PQC) sample is often used in global metabolomics studies. A small volume of each biological sample is mixed together to generate a PQC sample, followed by aliquotation to a set of identical PQC samples (49). These PQC samples should be analyzed at designated time points through the analytical sequence (47).

### Full scan and tandem mass spectrometry data acquired

Data acquisition by LC-MS can be done in full MS and/or MS/MS scan mode (59). In full MS scan mode, intact molecular ions in a given *m/z* range are detected (51). In MS/MS scan mode, molecular (precursor) ions are fragmented to produce product ions(51). The fragmentation pattern of a compound can be used for identification purposes by determination of the molecular structure. Data acquired using full MS (molecular mass and isotope pattern) and MS/MS (fragmentation spectra) scan mode can be compared to experimental and established MS libraries and databases for compound identification(51, 60).

### Data preprocessing

Mass and retention time shifts are corrected for using the PQC samples (61). The ionization process may produce ions (fragments and adducts) referred to as features that are not necessarily unique metabolites (45). Features are ideally grouped together to the single metabolite they originate from based on retention times using comprehensive software (47, 61).

### Data analysis

Several bioinformatics statistical tools can be used to determine whether there are differences in the metabolome of the KD compared to the habitual diet samples, and identify significantly altered metabolites (45, 47). Univariate statistical methods are used to analyze metabolomics features independently, while multivariate methods are used to analyze all the metabolomics features, revealing possible patterns (47). Principal component analysis (PCA) is a method that summarizes the complex metabolomics data to visualize the difference between two or more groups (62). PCA is not a statistical test but an ordination analysis to simplify the complex data and aims to uncover factors that account for the major patterns across the original variables and explain most of the variability in the data (63). PCA forms a new coordinate system for separation of samples, and the largest variance of the dataset is illustrated by the first principal component (64). In untargeted metabolomics one point in a PCA plot represents all the features detected in a specific sample.

Differential analysis using volcano plot detect specific compounds differing in amount between two groups based on defined sample ratios (fold change) and calculated p-values. Volcano plot shows which compounds that are significantly up and down-regulated (65). **Figure 8** shows a box and whisker plot and how the data is distributed. The height of the rectangle represents the peak areas in the interquartile range. The first quartile represents 25 % of the distribution, the third quartile represents 75 % of the distribution. In small number of data points, the whiskers end at the highest and lowest data points (65).

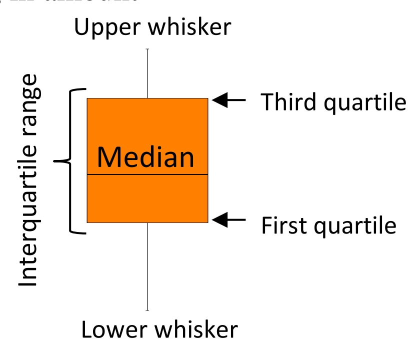



**Figure 8** Box and whisker plot distribution of data

### Metabolite identification

Metabolite identification is partially performed manually, thus being a time consuming step (45). To identify a feature of interest, metabolite databases, e.g. the Human Metabolome Database (http://www.hmdb.ca/ ) are used by searching the accurate mass of the specific compound (45). Chemspider searches databases of MS1 scans by using the mass or predicted formulas and mzCloud database for matching and finding similar fragmentation spectra (65). The metabolite identification is a comprehensive process, where a considerable number of the features detected from biological samples cannot be identified without extensive follow up investigations (60).

## 1.2.5 Metabolomics in nutrition research

There has been an increased interest in using metabolomics for nutrition research (66). The complexity of different diets and how they affect our health has for a long time been of interest among researchers. Untargeted metabolomics has been used to identify biomarkers associated with food intake (67, 68) and as a screening tool for revealing patterns of different dietary interventions (69). However, the associations between complex diets and their desired results as well as adverse outcomes are difficult to address. Untargeted metabolomics can contribute to a wider understanding of how diets effect the health and identify individual metabolites and complex profiles that change in response to the dietary intervention (66).

As earlier described, Retterstøl et al investigated the effect of following a KD for three weeks with targeted analyses to identify and quantify metabolites of interest coupled to cholesterol pathways, lipid profile and peripheral blood mononuclear cells gene expression (70). A global metabolomics analysis approach was not performed at that time.

# 2 Aim of thesis

Studies investigating the impact of KD in human health are sparse. The aim of this thesis was to apply an untargeted metabolomics profiling approach on plasma samples from healthy, young adults on a habitual and ketogenic diet, respectively. In addition to analyzing samples from the first three weeks of the six week cross over intervention study published so far (70), this thesis will also study the effect after cross over from habitual to ketogenic diet.

Specific objectives of this thesis were:

- Compare the metabolomics profile before and after a three weeks long KD intervention. Is there a separation between the metabolomes of habitual diet and KD?
- Investigate which metabolites that significantly change in concentration between the metabolomes of habitual and KD
- How does a KD affect the amino acid profiles among the participants?
- How does a KD affect metabolites involved in energy metabolism?
- Compare the metabolomic profiles between those with the lowest and highest relative LDL-C increase in response to the KD
- Investigate which metabolites that are significantly changed in concentration between those with lowest and highest relative LDL-C increase

# 3 Materials and methods

## 3.1 Low carbohydrate high fat diet study

### 3.1.1 Participants and design of the LCHF diet study

Participants were mainly students and employees at the Department of Nutrition research at the University of Oslo in September 2011 to December 2012 that were invited to participate in a ketogenic (low carbohydrate high fat) diet study after information meetings. Inclusion and exclusion criteria have been described elsewhere in detail (70). **Figure 9** illustrates the six weeks randomized controlled parallel crossover intervention study.

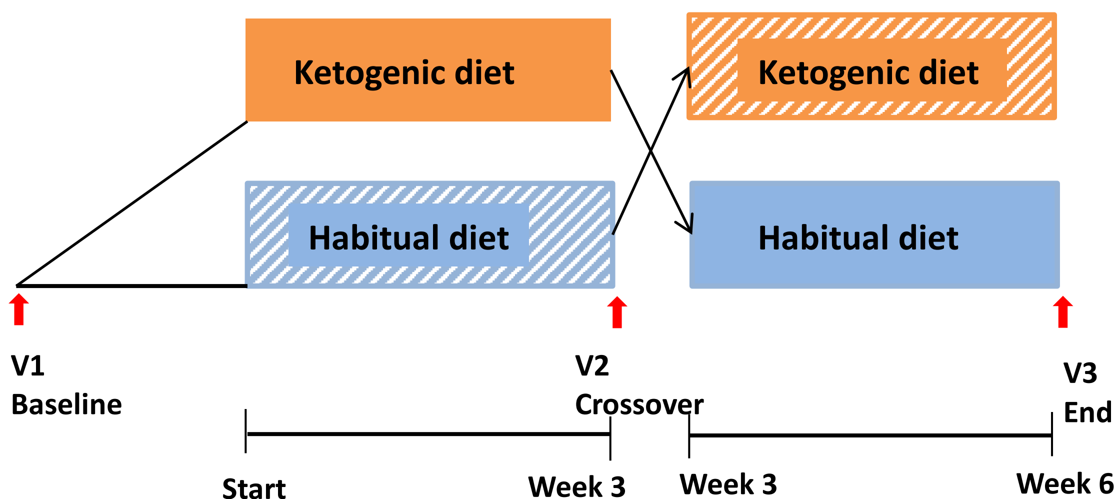



**Figure 9** Design of the six weeks randomized controlled parallel crossover intervention study. Participants were allocated to either a three weeks ketogenic diet or to continue their habitual diet for three weeks, followed by crossover and another three weeks on the other type of diet. Data were collected at baseline (V1), crossover (V2) and end of intervention (V3)

The participants were divided randomly into two groups. One group, called the LK (low carbohydrate) group, started with a KD and ended the dietary intervention after 3 weeks. The LK group then resumed their habitual diet for the next three 3 weeks. The other group continued with their habitual diet for the first three weeks, followed by the KD for the last 3 weeks. The dietary specifications were as reported in Retterstøl et al (70). Specifically, the participants were advised to follow the guidelines in Dr.Atkins´new Diet Revolution (71).

Carbohydrate intake was limited to 20 g per day or were not to exceed 5 E% for the calorie intake. They had no restrictions on type of fat during the dietary intervention.

### 3.1.2 Dietary assessment

21 of the participants performed a four days weighed food record assessment prior study start to evaluate their habitual diet and 19 of the participants performed a three days weighed food record during the LCHF diet period (70). KostBeregningsSystem Version 7.1, University of Oslo, Norway and "MAT på DATA" version 5.2 were used for energy and nutrients calculations (70). This was to check for compliance among the participants on the KD.

## 3.2 Materials for metabolomics and LCHF diet

### 3.2.1 Sample material and storage

All the sample materials were from the LCHF diet study (70). Anthropometric measurements, blood pressure and fasting blood samples were drawn at baseline, after three weeks (crossover) and after six weeks (end of study). Plasma was obtained using EDTA tubes (Becton Dickinson) and serum was collected from silica gel tubes (Becton Dickinson) as described in detail elsewhere (70). Plasma and serum were stored at -80 °C until further analysis.

The frozen samples were placed on blocks of dry ice from the biobank at the Department of Nutrition Research at the University of Oslo and shipped to Department of Medical Biochemistry, Oslo University Hospital, Rikshospitalet, Norway for global metabolomics analysis.

The samples were numerated from p1, p2…p30, where p1 to p15 represented one group (LK group) and p16 to p30 constituted another group (KL group).

### 3.2.2 Preparations for LC-ESI-MS analyses (chemicals)

#### Solvents

Methanol (MeOH) ≥99.9%, formic acid (FA) 98-100 % and ammonium acetate ≥ 99.0% purity were obtained from Merck (Dramstadt, Germany). Type 1 water was used from a

Millipore Milli-Q purification system with Q-guard and Quantum purification cartridge to reach a resistivity of 18,2 MΩ.cm at 25°C (Merck) (72).

### Mobile phases

Mobile phases were prepared prior to each global metabolomics analysis.
Mobile phases A and B used for positive ionization method consisted of water with 0.1 % formic acid (FA) and MeOH with 0.1 % FA, respectively. Mobile phases A and B used for negative ionization method consisted of water with 10 mM ammonium acetate and MeOH with 10mM ammonium acetate, respectively. Mobile phases were stored at room temperature after preparation.

### Extraction solution

The extraction solution consisted of cold MeOH stored at 4 °C.

### Calibration solutions

Pierce LTQ Velos ESI positive Ion Calibration solution and Pierce ESI Negative Ion Calibration solution were obtained from Thermo Fisher Scientific (Waltham, MA, USA) stored at -18 °C.

## 3.3 Sample preparation

Protein precipitation with organic solvent was performed. The sample preparation method was conducted for both plasma and serum samples. The sample preparations were distributed over four and three days for serum and plasma, respectively (See **appendix**, section 7.1.1) However, only plasma is described further due to limited time and that the plasma not the serum is the entity circulating in the blood vessels. Plasma constitutes the liquid portion of blood when blood cells have been removed. For the majority of analyses the measured concentrations will be similar in plasma and serum. The plasma samples were thawed for 50 minutes at room temperature, followed by vortexing for 5 seconds (Genie2, Scientific Industries, Bohemia, NY, USA) and then centrifuged at 3600 rpm at 20°C for 10 minutes (Megafuge 1.0 R, Heraeus instruments).

30 µl blood plasma was transferred to filter paper cards from Whatman 903 Protein Saver Cards from GE Healthcare Life Science (Chicago, IL, USA), and stored at -80°C for possible

future analysis.

90 µl of cold methanol was added to 30 µl blood plasma in a 1.5 ml Eppendorf tube, and then mixed on a table vortex for 5seconds. The samples were centrifuged at 14800 rpm (14.8 xg rpm) at 4°C for 10 minutes with Heraeus Fresco 21 centrifuge from Thermo scientific. 90 µl of the clear supernatant were transferred to an HPLC vial with insert and cap, all from Matriks AS, Oslo, Norway. **Figure 10** shows the sample preparation method (my own photos)

**Figure 10 Sample preparation (private photos)**

To obtain a pooled quality control (PQC) to be used throughout the whole run (analysis), a representative subset consisted of 30 µl of each sample from day1 were collected as shown in **figure 11**. See **appendix section 7.1.2** for detailed description of the preparations of the different pooled quality controls used in the study.

**Pooled quality control preparation**

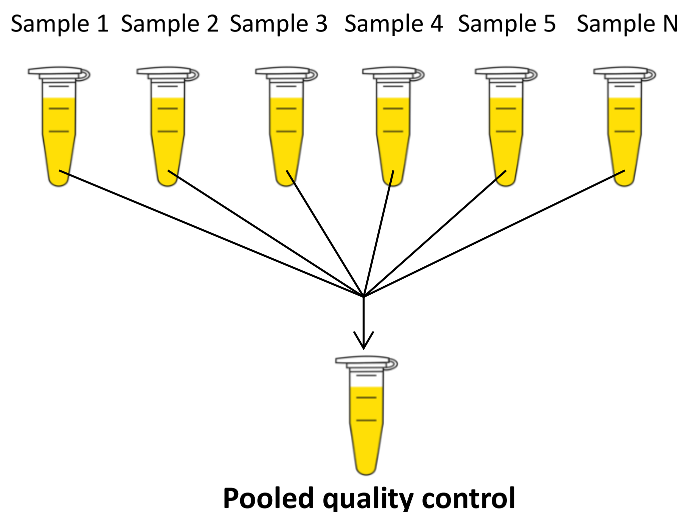



**Figure 11** Preparation of pooled quality control that contains the same metabolites as all the samples to control for possible analytical retention time drift and peak area drift throughout the sequence.

See **appendix 7.1.3** for the sample sequence used for global metabolomics analysis, including PQC samples injected at regular intervals throughout the analysis.

## 3.4 Liquid chromatography – electrospray ionization– mass spectrometry settings

Metabolites were measured with LC-ESI-MS instrumentation. The LC-ESI-MS used was Dinoex Ultimate 3000 UHPLC system coupled to a Q Exactive Orbitrap MS (Thermo Scientific).The analytical column used was Pursuit XRs (250 x 2.0 mm, particle size 3μm purchased from Agilent Technologies (Santa Clara, CA, USA). The LC-ESI-MS settings used are shown in **tables 1-4** and **figure 12**.

**Table 1** Liquid chromatography settings used for all the analyses

| Parameter | Setting |
|---|---|
| Mobile Phase A | Positive ionization: Water with 0.1 % formic acid<br>Negative ionization: Water with 10 mM ammonium acetate |
| Mobile Phase B | Positive ionization: Methanol with 0.1% formic acid<br>Negative ionization: Methanol with 10 mM ammonium acetate |
| Gradient | See Table 2 and Figure 12 |
| Injection Volume | 2 µl |
| Column temperature | 30 °C |
| Flow rate | 300 µl/min |
| Analysis time | 32.5 minutes |
| Re-equilibration time | 10 minutes |

**Table 2** Flow gradient

Mobil phase A: Water with 0.1% formic acid (positive ionization)

Water with 10 mM ammonium acetate (negative ionization)

Mobil phase B: Methanol with 0.1 % formic acid (positive ionization)

Methanol with 10 mM ammonium acetate(negative ionization)

| Time | Flow | %A | %B |
|---|---|---|---|
| 0 | 0.3 | 98 | 2 |
| 6 | 0.3 | 90 | 10 |
| 8.5 | 0.3 | 25 | 75 |
| 25 | 0.3 | 0 | 100 |
| 32.5 | 0.3 | 0 | 100 |
| 37.51 | 0.3 | 98 | 2 |
| 47.51 | 0.3 | 98 | 2 |

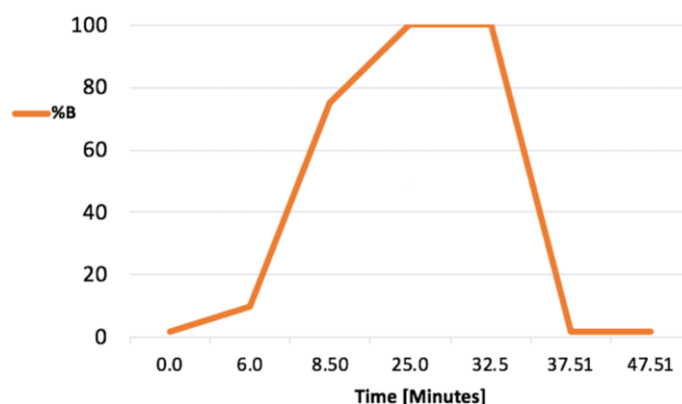



**Figure 12** flow gradient
Mobil phase A: Water with 0.1% formic acid (positive ionization)
Water with 10 mM ammonium acetate (negative ionization)
Mobil phase B: Methanol with 0.1 % formic acid (positive ionization)
Methanol with 10 mM ammonium acetate (negative ionization)

**Table 3** Mass spectrometric settings

| Parameter | Setting |
|---|---|
| Scan ranges *(m/z)* | 70-1050 |
| Fragmentation | Yes |
| Resolution | Full MS: 70 000<br>MS/MS: 17 500 |
| Polarity | Positive and negative |
| Automatic gain control target value | Full MS: 1.00E+06 ion counts<br>MS/MS: 5.00E+05 |
| Maximum injection time | Full MS: 250 ms<br>MS/MS: 100 ms |
| Analysis time | 32.5 minutes |
| Re-equilibration time | 10 minutes |
| MS/MS parameter | Setting |
| Scan type | Data dependent acquisition, top 5 |
| Dynamic exclusion time | 10 s |
| Intensity threshold | 5.00E+04 |
| Stepped normalized collision energy | 20, 50, 80 |

**Table 4** Electrospray settings

| Electrospray parameter | Setting |
|---|---|
| Sheath gas (N2) flow rate | 40 a.u. |
| Auxiliary gas (N2) flow rate | 10 a.u. |
| Sweep gas (N2) flow rate | 2 a.u. |
| Capillary temperature | 250 ℃ |
| S-lens RF level | 50.0 |
| Auxiliary gas heater temperature | 300 ℃ |
| Electrospray voltage | 3.5 kV |
| Electrospray needle position | C |

### 3.4.1 Ethics

All of the participants signed a written consent form, agreeing to the donation of blood, anthropometric measures and terms of the study. The study was approved by Regional Committee for Medical and Health Research Ethics (REC) (protocol approval 2018/2019 – 2011/1365-26). There were no conflicts of interest declared in this thesis.

## 3.5 Data analysis

Xcalibur (Version 4.0) was used to make the global metabolomics analysis sequence and to control the liquid chromatography. Tune (version 2.5 Build 2042) was used to calibrate and to control the mass spectrometric parameters. Compound discover software (version 2.1 SP1) from Thermo Scientific was used for processing raw data files from the global metabolomics analyses using LC-ESI-MS, perform statistics and use the processed data to search in online databases (Chemical formula in ChemSpider and fragmentation spectra (MS/MS) in mzCloud). **Figure 13** shows the workflow tree used for preprocessing the data in compound discover. The workflow template for the pre-processing used in this project is "Metabolomics/untargeted Metabolomics with Statistics Detect Unknowns with ID using Online Databases" created by compound discoverer.

Statistical tools used for differential analysis were mainly principal component analysis (PCA), volcano plot and box plot. Metabolic peak intensities were log transformed and scaled in CD to normalize skewed distribution.

IBM SPSS Statistics 24 for Windows software was used for performing data analysis regarding baseline description and blood sample parameters. Paired t-test was used on normally distributed data. Data are expressed with mean values and standard deviation. The nonparametric test, wilcoxon signed ranks, test was used if the data were skewed to compare two related samples. "R" software was used for performing correlations analysis, both Pearson and Spearman were used. Statistical significance levels were determined to $p<0.05$

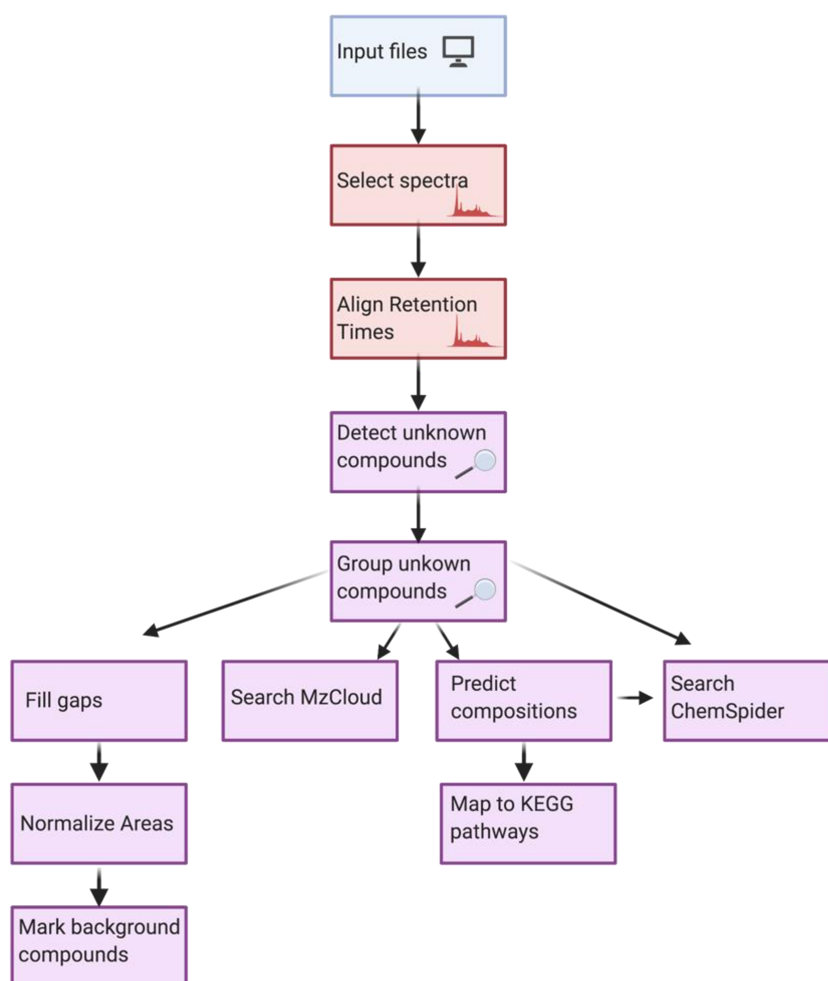



**Figure 13** Workflow tree used for preprocessing the data in compound discoverer(CD). Figure modified from CD software.

# 4 Results

Metabolomic profiling was performed on plasma samples using high-performance liquid chromatography coupled to mass spectrometry. Analysis results were based on retention time (rt), mass to charge ratio *(m/z)*, and peak area of each feature.

## 4.1 Participant characteristics

30 participants were included. Four participants were excluded after metabolomics analysis due to results and further investigations revealing non-compliance with respect to the diet, resulting in a total of 26 participants. See **appendix** section 7.2.1 for details regarding the exclusion. Baseline characteristics of the 26 participants and the changes in anthropometrics measurements and blood parameters following dietary intervention are shown in **table 5**. The mean BMI in the population corresponded to "normal weight", however both men and women lost weight during the study period. The level of atherogenic lipoprotein particles increased on KD, with the exception of triglyceride and triglyceride-rich lipoprotein (TRL) cholesterol which remained unchanged. HDL-C increased significantly.

**Table 5** Characteristics of participants in the project

| | Baseline (n=26) | End-of-intervention (n=26) | P-value[1] |
|---|---|---|---|
| Female, n (%) | 21(80.8) | | 1.000 |
| Age (years) | 25.1±4.7 | | 1.000 |
| Weight (kg) | 63.2±7.9 | 61.6±7.9 | <0.001 |
| BMI (kg/m2) | 21.8±1.3 | 21.2±1.4 | <0.001 |
| Total cholesterol (mmol/l) | 4.3±0.6 | 5.8±1.2 | <0.001 |
| HDL cholesterol (mmol/l) | 1.7±0.4 | 2.0±0.4 | <0.001 |
| LDL cholesterol (mmol/l) | 2.4±0.6 | 3.4±1.11 | <0.001 |
| Triglyceride (mmol/l) | 0.8(0.4-1.5) | 0.8(0.4-1.5) | 0.380[a] |
| Non-HDL-C (mmol/l) | 2.7±0.7 | 3.8±1.2 | <0.001 |
| TRL cholesterol (mmol/l) | 0.3±0.1 | 0.4±0.2 | 0.090 |
| ApoA1 (g/L) | 1.6±0.2 | 1.9±0.3 | <0.001 |
| ApoB (g/L) | 0.7±0.2 | 1.0±0.3 | <0.001 |
| FFA (mmol/l) | 0.4(0.1-0.9) | 0.8(0.3-1.3) | <0.001[a] |

[1]*Data presented as n, number (%) or mean ± SD. Wilcoxon or paired t-test.*
*ApoB; apolipoprotein B; ApoA1; apolipoprotein A1; HDL, high density lipoprotein; LDL, low density lipoprotein; FFA, serum free fatty acid; non-HDL-C (TC minus HDL-C); TRL cholesterol (triglyceride-rich lipoprotein cholesterol; TC minus LDL-C minus HDL-C)*
[a]*Wilcoxon signed ranks test*

**Compliance to the ketogenic diet**

Compliance to the KD among the participants is shown in **table 6**. The mean intake of reported carbohydrate was 17 ± 3 (9-20) g. The energy intake varied a lot between the participants, but there was no difference between the energy intake between habitual diet and following the KD. The reported calorie intake on the habitual diet ranged from 1223 to 4152 kcal and 1326 to 3013 kcal on the KD.

**Table 6** Dietary intake on habitual diet and on ketogenic diet

| | Habitual diet | Ketogenic diet | P-value[1] |
|---|---|---|---|
| Energy, kcal | 2109±637[a] (21)[b*] | 2043±432 (19) | 0.797 |
| Carbohydrate, E% | 46.4±4.5 (21) | 3.2±0.6 (19) | <0.001* |
| Protein, E % | 17.3±2.8 (21) | 26.3±4.6 (19) | <0.001* |
| Fat, E% | 30.3±4.8 (21) | 69.5±5.2 (19) | <0.001* |
| Saturated fat, E% | 10.9±1.8 (20) | 29.5±7.5 (19) | <0.001[2] |
| Monounsaturated fat, E% | 10.6±2.5 (20) | 23.9±3.7 (19) | <0.001[2] |
| Polyunsaturated fat, E% | 5.6±1.2 (20) | 12.6±6.3 (19) | 0.001[2] |
| Fiber | 2.8±0.7 (20) | 2.9±1.0 (19) | 0.430[2] |
| Cholesterol, mg | 266±76 (18) | 1007±370 (18) | <0.001 |

[a]Mean ± SD
[b] number of weighed dietary record for the specific nutrient included in calculations of mean
[1]Paired t-test
*Missing two values on KD, 19 related samples included in paired t-test
[2]Missing one value on KD, 19 related samples included in paired t-test

### 4.1.1 Metabolome at baseline shows random metabolite profiles

Visit 1 was used as baseline for both groups. For the group that continued with their usual diet from week 1 to week 3, visit 1(V1) and visit 2(V2) were compared to investigate whether the participants changed their habitual diet as an unintended response to participation in the study. A principal component analysis plot (PCA) was used to explore and visualize the variation between the samples. The PCA plot showed no substantial difference between the participants´metabolomes at these two visits in the habitual diet group (see **appendix** section 7.2.2), and thus the effect of study inclusion alone had not altered their behaviours in a way that markedly affected their plasma metabolomes.

To explore the potential baseline metabolome difference between the two groups, a PCA plot with all the baseline samples were performed. As presented in **Figure 14,** there were no obvious differences between the two groups at baseline according to the PCA plot.

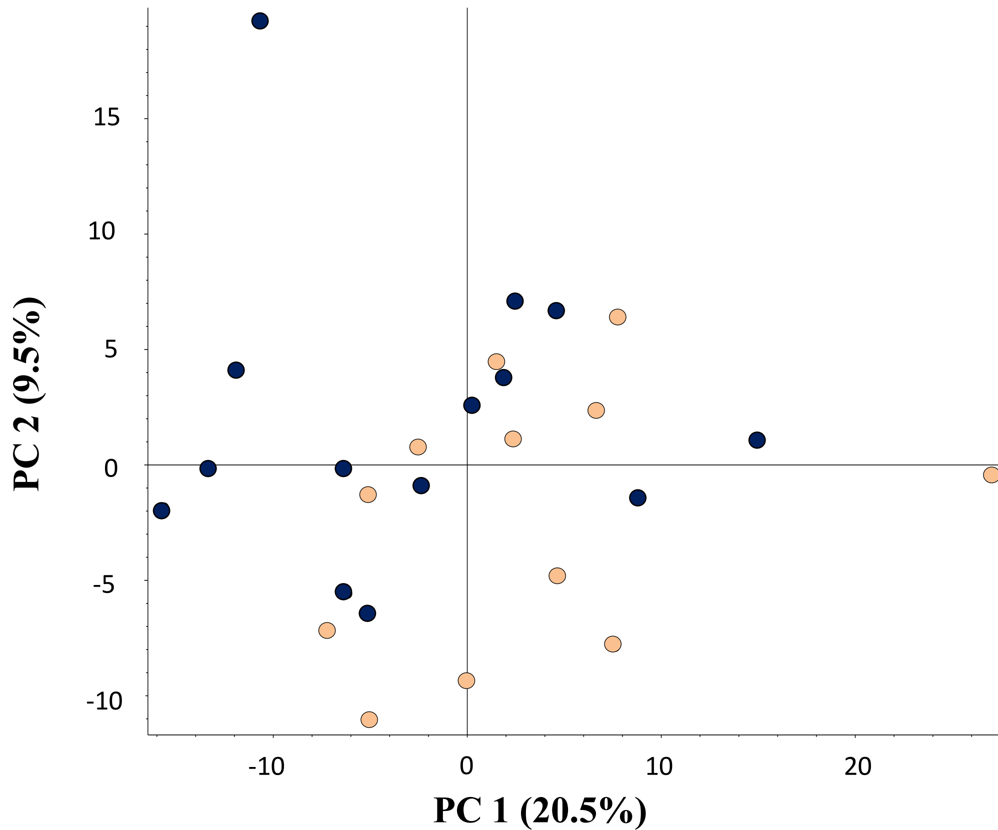



**Figure 14** Principal component analysis (PCA) plot of all detected features from baseline samples prior to intervention. Each point represents all the metabolites detected in that sample. Blue and beige represent the LK (low carbohydrate) and KL (habitual diet) group respectively. The plot shows no clustering between the two groups.

The PCA plot confirmed a randomized population, thus further metabolomics analysis that compared the KD and control diet were conducted. The PQC samples were centered in the PCA plot, meaning that the variation observed for the individual participant samples represents the biological variation in the population and possible preanalytical differences (sample preparation, storage etc).

Because the PCA plot shown in **figure 14** displayed no obvious differences between the two groups, the population was collectively grouped as one with baseline V1-samples representing the habitual diet metabolomes for all participants in the following analyses.

## 4.2 Comparison of ketogenic and habitual diet

### 4.2.1 PCA plot shows a separation between ketogenic and habitual diet

**Figure 15** shows the PCA plot from the analysis run in negative ionization mode of all samples taken at baseline (habitual diet) and immediately following intervention with KD among all the participants. The PCA plot shows distinct clustering discriminating the ketogenic and the habitual diet groups with a few exceptions (p18, p22 and p1). Principal component 1 (PC1) represented 19.4 % of the variability among the samples, while principal component 2 (PC2) explained 8.6 %. The distance in the PC1 direction represented a larger difference than in the PC2 direction. One point represents all the features detected in one sample, which implies that the closer the samples are located in the plot, the more similar their metabolomes are. The features that contribute to the largest variation were therefore relatively similar within each diet group, and differs between the groups.

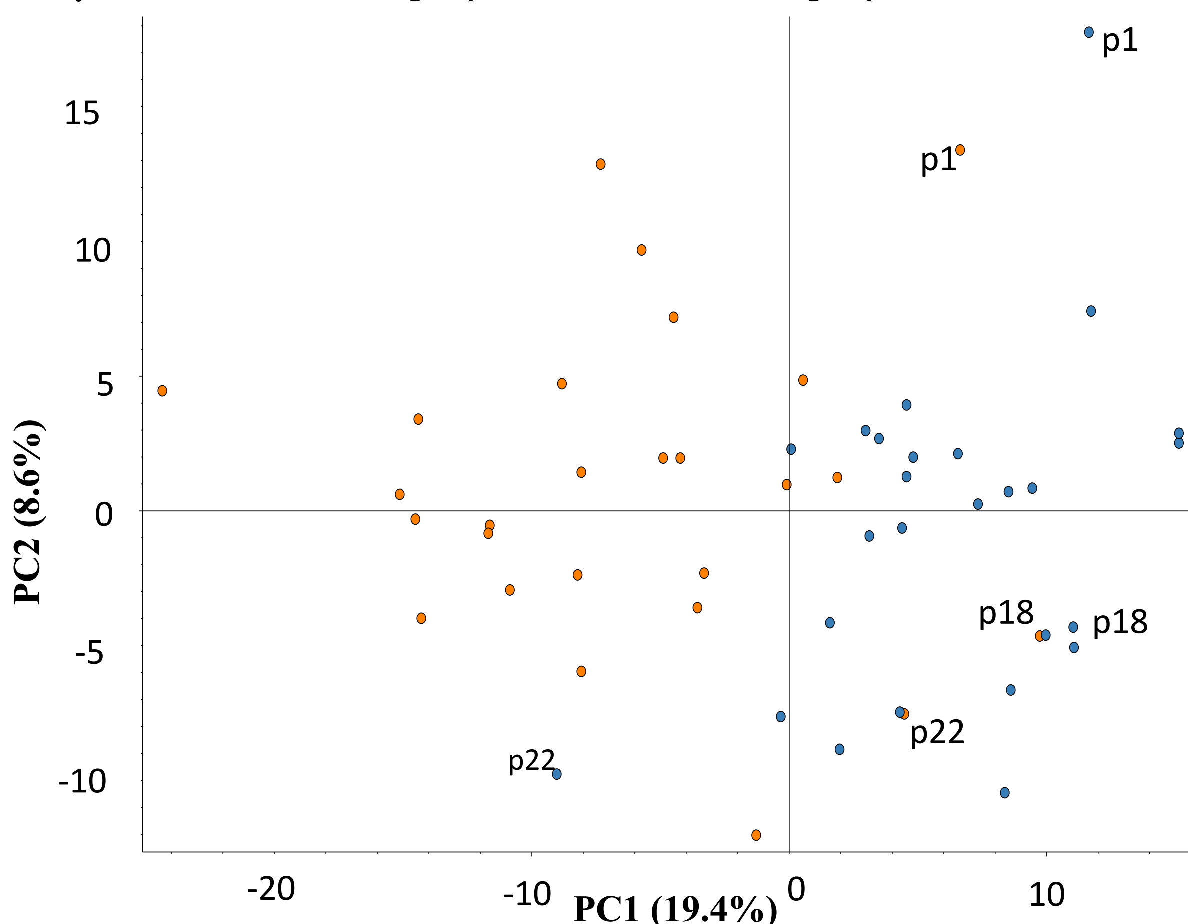



**Figure 15** PCA plot from the analysis run in negative mode that display a separation between the habitual diet and the ketogenic diet, except from some individuals (p18,p22, p1). A point represents all the metabolites detected in that sample. An orange colored point represents the metabolome with a ketogenic diet and a blue colored point represent the metabolome at baseline (habitual diet).

See **appendix section 7.2.3** for the PCA plot in positive ionization mode with PQC samples.

To further investigate the separation seen in **figure 15**, volcano plot was used to look at the individual metabolites that were significantly increased or decreased following the KD intervention.

### 4.2.2 Volcano plot revealed numerous features that were significantly quantitatively changed following a ketogenic diet

From 26 young adults on KD, an average of 1085 of 2637 (1552 filtered out in the preprocessing) features in positive mode and 419 of 1116 (697 filtered out) features in negative ionization mode were detected.

Statistical analysis using volcano plot, see **Figures 16 and 17**, revealed that the quantity of numerous features was significantly changed in the participants at the end of the three week period on KD. The x-axis represents the generated ratio between the ketogenic and habitual diet in log2 scale. The y-axis represents the significance of the generated ratio in negative log10 scale. $P<0.05$ was used as the cut off value for significance and log2 fold change values was set to $>1$ and $<1$, which represents a hundred percent increase or a fifty percent reduction in the ratio, respectively. In positive ionization mode 48 features were significantly increased and had a log2 fold change greater than or equal to 1 in the KD (red in the volcano plot), with seven metabolites successfully identified: hydroxybutyrate, trihomomethionine, acetoacetate, stearoylcarnitine, acetyl-l-carnitine, hexanoylcarnitine and cis-5-tetradecanoylcarnitine. See **appendix** section 7.2.4 **table 15 and table 16** for *m/z* and rt for the remaining, unidentified metabolites. 277 additional features were significantly increased ($p < 0.05$), but the increase was less than 100 %.

Nine features were significantly decreased (green in the volcano plot), with four successful metabolites identifications: stachydrine, methyl indole 3-acetate, caffeine and N-acetylvaline. 130 additional features were significantly decreased but the reduction was less than 50 %. 11 had a reduction of more than 50 %, but the change was not significant.

Measured peak areas of identified metabolites that changed significantly in the KD samples and habitual samples are shown in section 4.2.3 in **table 7.**

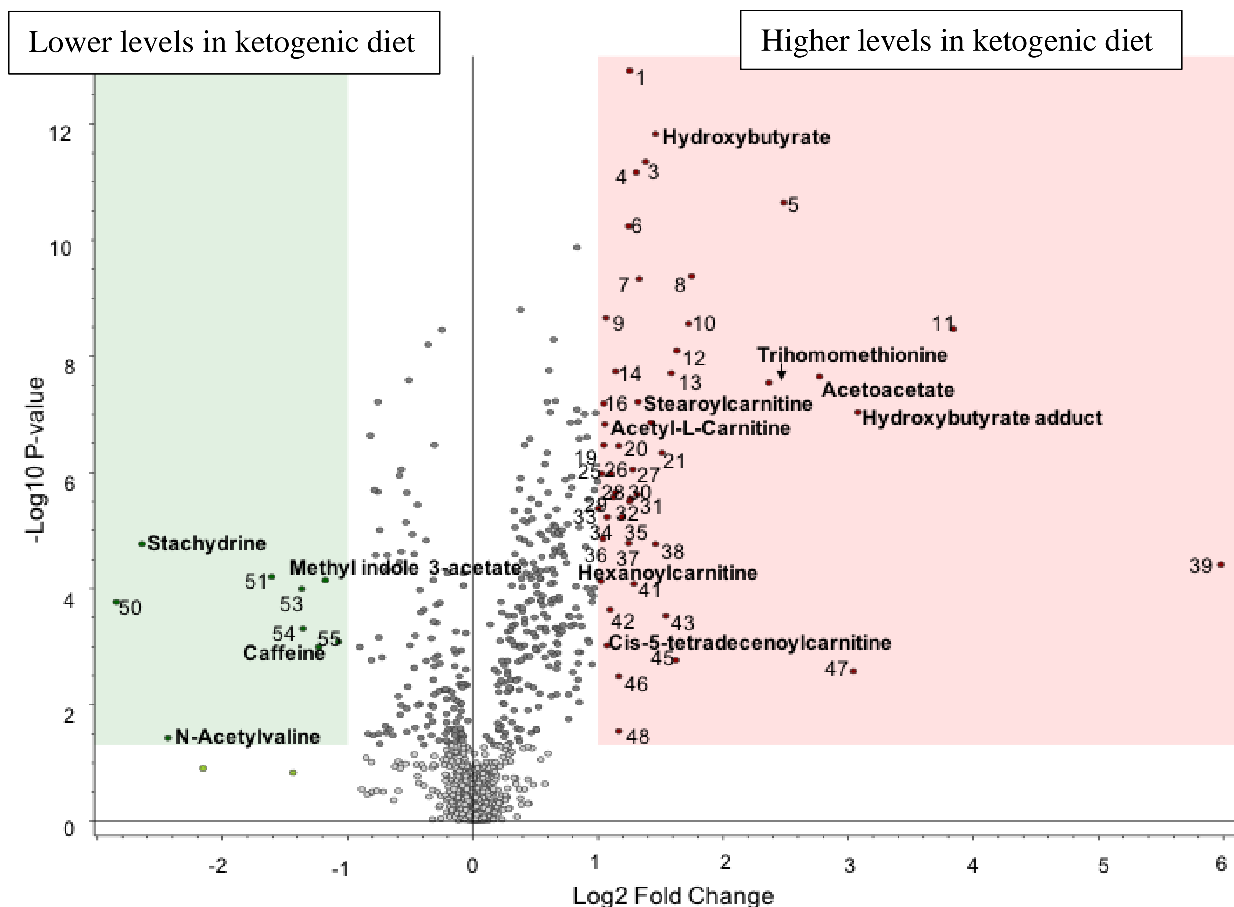



**Figure 16** Differential analysis using volcano plot of data obtained from the positive ionization mode analysis of samples taken following ketogenic diet intervention. Metabolites identified are presented in bold text. The points in the green and red areas display metabolites with both fold changes values as well as statistical significance.

The volcano plot with negative ionization mode is shown in **figure 17**. 32 compounds were detected in significantly higher levels in samples taken after KD versus habitual diet and had a log2 fold change greater than or equal to 1, with five successful metabolite identifications: hydroxybutyrate, methyl octadecenoate, arachidic acid, hydroxyvaleric acid and acetylglycine. 129 features were significantly increased, but the fold change was less than 1. One feature was more than doubled, but the increase was not significant. Four compounds had significantly lower levels in samples taken after KD versus habitual diet, but without any successful metabolite identifications. 57 features were significantly decreased, but the reduction was less than 50 %. Two features were reduced more than 50 %, but the change was not statistically significant.

Measured peak area of identified compounds that changed significantly in the KD samples and habitual diet samples are shown in section 4.2.3 in **table 7.**

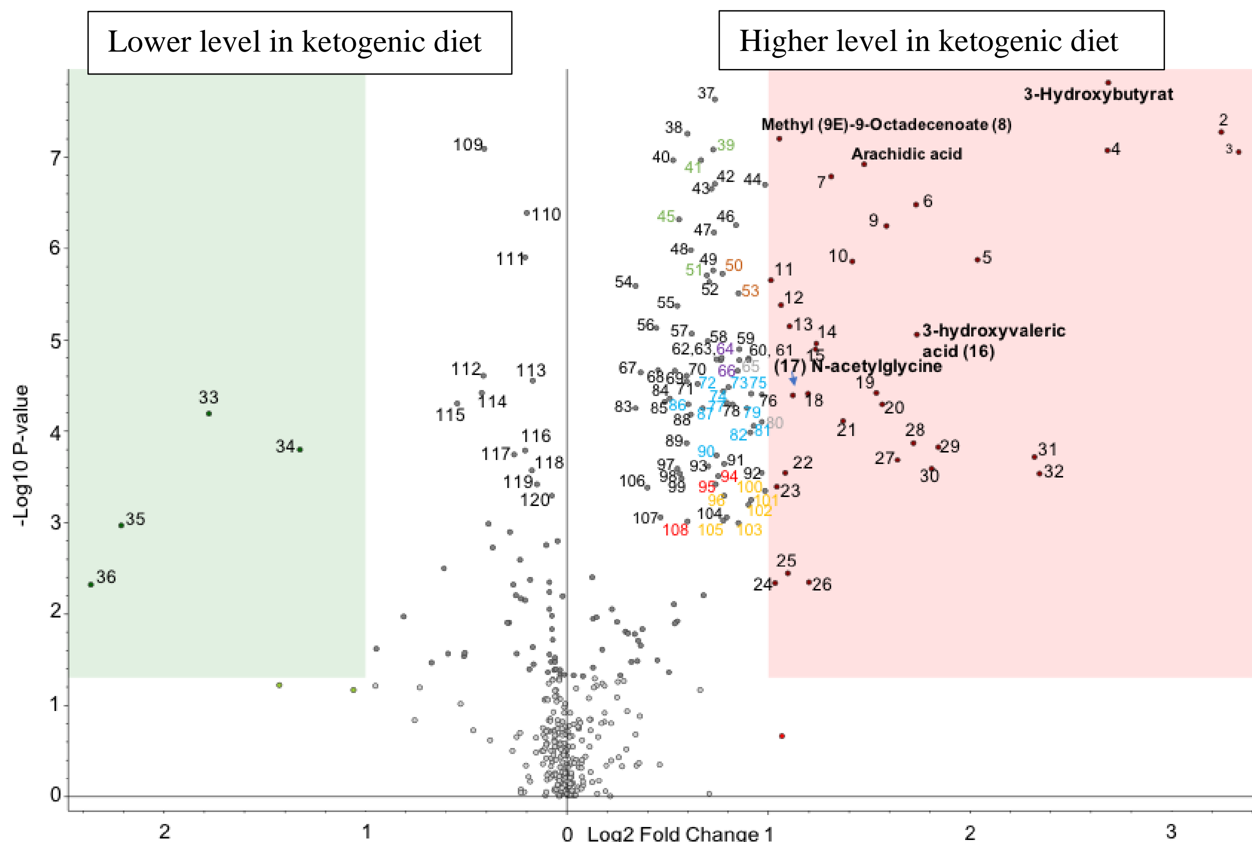



**Figure 17**
Differential analysis using volcano plot of data obtained from the negative ionization mode analysis of samples following ketogenic diet. Metabolites identified are marked in bold text. Numbers with the same colors had the same pattern which means they can be fragments or adducts from the same compound.

### 4.2.3 Peak area quantification for identification of significantly altered metabolites

**Table 7** Metabolites identified that were markedly and significantly altered in quantity following 3 weeks on ketogenic diet

| Compound | Group Areas | | Ratio | P-value |
|---|---|---|---|---|
| | Baseline | Ketogenic diet | Ketogenic diet/baseline | |
| Positive mode | | | | |
| Hydroxybutyrate | 5.01E+07 | 5.59E+08 | 11.16 | 9.30E-08 |
| Acetoacetic acid | 2.32E+07 | 1.53E+08 | 6.59 | 2.32E-08 |
| Trihomomethionine | 2.43E+05 | 1.11E+06 | 4.58 | 2.88E-08 |
| 3-Hydroxyisobutyric acid | 2.04E+07 | 6.34E+07 | 3.11 | 1.53E-12 |
| Stearoylcarnitine | 9.90E+05 | 2.91E+06 | 2.94 | 6.14E-08 |
| Acetyl-L-carnitine | 1.18E+08 | 2.43E+08 | 2.06 | 1.52E-07 |
| Hexanoylcarnitine | 2.99E+06 | 5.86E+06 | 1.96 | 7.49E-05 |
| Caffeine | 1.27E+08 | 5.74E+07 | 0.45 | 1.02E-03 |
| Methyl indole-3-acetate | 4.08E+07 | 1.63E+07 | 0.40 | 7.44E-05 |
| N-Acetylvaline | 2.47E+06 | 4.15E+05 | 0.17 | 3.75E-02 |
| Stachydrine | 2.20E+08 | 2.58E+07 | 0.12 | 1.75E-05 |

| Compound | Group Areas | | Ratio | P-value |
| --- | --- | --- | --- | --- |
| | Baseline | Ketogenic diet | Ketogenic diet/baseline | |
| Negative mode | | | | |
| Hydroxybutyric acid | 4.32E+05 | 2.70E+06 | 6.26 | 8.74E-05 |
| Acetoacetate | 1.11E+04 | 6.52E+04 | 5.85 | 6.84E-04 |
| Arachidic acid | 5.40E+04 | 1.42E+05 | 2.63 | 3.56E-05 |
| Hydroxyvaleric acid | 2.00E+05 | 5.08E+05 | 2.54 | 6.95E-04 |
| Adrenic Acid | 4.56E+04 | 1.04E+05 | 2.28 | 5.27E-06 |
| N-Acetylglycine | 4.88E+04 | 1.07E+05 | 2.20 | 4.31E-04 |
| Octadecanedioic acid | 1.37E+05 | 2.79E+05 | 2.04 | 6.58E-04 |

**Figure 18** shows a box and whiskers plot of the measured peak area of hydroxybutyrate and n-acetylvaline, two compounds that were significantly changed after a KD.

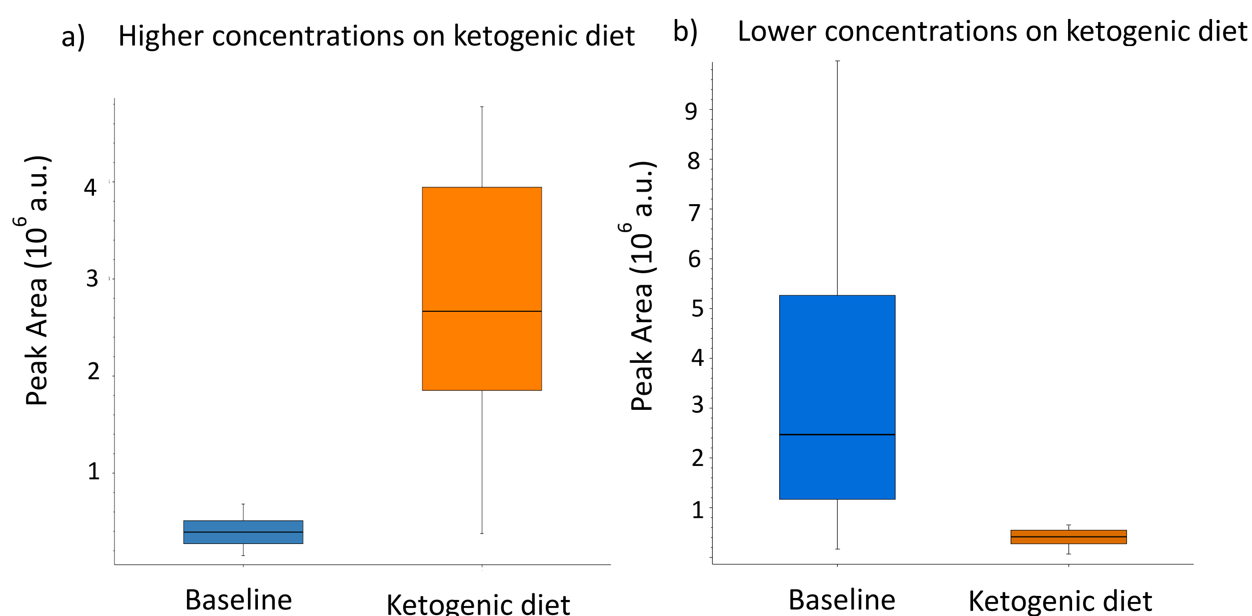



**Figure 18** Box plot of changes in a) hydroxybutyrate and b) N-acetylvaline following three weeks on a ketogenic diet.

See appendix section 7.2.4 for box and whisker plots of acetoacetate, hydroxyvaleric acid, acetylglycine, arachidic acid, n-acetylvaline and caffeine.

To further characterize the human metabolism after a KD, a more targeted approach was conducted to look at specific metabolite abundances, such as fatty acids related to LDL-C , carnitine-bound metabolites necessary for lipid transport (10) and amino acids (73).

### 4.2.4 Fatty acids

Levels of fatty acids related to possible increase or decrease in LDL-C are shown in **table 8**.

**Table 8** Saturated fatty acids that were markedly and significantly altered in quantity following 3 weeks on ketogenic diet

| Compound | Group Areas | | Ratio | P-value |
|---|---|---|---|---|
| | Baseline | Ketogenic | | |
| Oleic acid | 2.64E+07 | 5.45E+07 | 2.1 | 1.20E-04 |
| Arachidonic acid | 4.68E+05 | 7.92E+05 | 1.7 | 1.80E-05 |
| Myristic acid | 2.58E+06 | 4.43E+06 | 1.7 | 0.01 |
| Palmitic acid | 2.51E+07 | 4.09E+07 | 1.6 | 0.01 |
| Lauric acid | 3.29E+06 | 3.88E+06 | 1.2 | 0.05 |

### 4.2.5 Carnitine-bound metabolites were elevated in plasma on ketogenic diet

The identification of the carnitine-bound metabolites was not completed due to lack of time, and the carnitine conjugates listed in **table 9** are only the most likely compounds based on software searches in available databases. Higher levels of carnitine conjugates from baseline to 3 weeks on the KD are shown. Acylcarnitines linked to fatty acid metabolism showed higher levels, whereas acylcarnitines linked to amino acid metabolism were not detected, except for propionylcarnitine.

**Table 9** Alterations in carnitine and carnitine conjugates from baseline to after ketogenic diet

| | Compound | Group Areas | | Ratio | P-value |
|---|---|---|---|---|---|
| | | Baseline | Ketogenic | | |
| C0 | **L(-)-Carnitine** | 2.72E+06 | 2.77E+06 | 1.0 | 0.153 |
| C2 | **Acetyl-L-carnitine** | 2.01E+06 | 3.88E+06 | 1.9 | 0.004 |
| C3 | **Propionylcarnitine** | 1.83E+05 | 1.94E+05 | 1.1 | 0.849 |
| C6 | Hexanoylcarnitine [1] | 4.56E+04 | 9.13E+04 | 2.0 | 0.015 |
| C8 | Octanoylcarnitine [2] | 1.76E+05 | 3.55E+05 | 2.0 | 0.063 |
| C14 | Myristoylcarnitine* | 2.14E+04 | 5.91E+04 | 2.8 | <0.001 |
| C16 | L-Palmitoylcarnitine* | 7.63E+04 | 1.24E+05 | 1.6 | <0.001 |
| C18 | Stearoylcarnitine | 1.58E+04 | 5.76E+04 | 3.6 | <0.001 |
| | 3-hydroxyoctanoylcarnitine* | 3.85E+04 | 5.87E+04 | 1.5 | 0.019 |

Bold text; full match, *no mzcloud, [1]no chemspider [2]invalide mass

### 4.2.6 Markers of energy metabolism

**Table 10** shows detected and identified metabolites involved in energy metabolism. Citric, malic and alpha-ketoglutaric acid, as intermediate in citric acid cycle, were significant upregulated. Creatine were significantly elevated.

**Table 10** Energy intermediates Alterations in energy intermediates from baseline to after ketogenic diet

| Compound | Group Areas | | Ratio | P-value |
|---|---|---|---|---|
| | Baseline | Ketogenic | | |
| Citric acid | 5.65E+07 | 8.60E07 | 1.5 | 6.19E-08 |
| Malic acid | 2.36E+07 | 3.13E+07 | 1.3 | 3.33E-04 |
| Alpha-ketoglutaric acid | 3.03E+07 | 4.61E+07 | 1.4 | 1.71E-05 |
| Creatine | 2.82E+06 | 4.09E+06 | 1.45 | 0.001 |
| Lactic acid | 7.38E+07 | 8.34E+07 | 1.13 | 0.972 |
| Creatinine | 6.60E+08 | 6.82E+08 | 1.03 | 0.585 |

### 4.2.7 Alterations in amino acid profiles on ketogenic diet

Examination of the amino acid profiles from baseline to the end of three weeks on a KD revealed significantly decreased levels of threonine, asparagine, tryptophan, proline, β-alanine, leucine, arginine, methionine, glutamine and alanine. However, the average level of valine was elevated 43 % compared to baseline levels. The average peak areas and ratios of amino acids after 3 weeks on a KD versus habitual diet are presented in **table 11.**

**Table 11** Average peak areas and ratios of amino acids from baseline and after a ketogenic diet

| Name | Group Areas | | Ratio | P-value |
|---|---|---|---|---|
| | Baseline | Ketogenic diet | Ketogenic diet/Baseline | |
| y-aminobutyric acid (GABA) | 2.40E+06 | 4.09E+06 | 1.65 | 1.75E-06 |
| Valine | 8.86E+06 | 1.26E+07 | 1.43 | 4.41E-07 |
| Isoleucine | 4.20E+07 | 5.06E+07 | 1.21 | 1.27E-05 |
| Citrulline | 4.62E+06 | 4.79E+06 | 1.04 | 4.23E-01 |
| Phenylalanine | 2.10E+07 | 2.05E+07 | 0.98 | 2.06E-01 |
| Lysine | 4.03E+06 | 3.92E+06 | 0.97 | 5.64E-01 |
| Cystine | 1.89E+05 | 1.79E+05 | 0.95 | 1.93E-01 |
| Tyrosine | 1.06E+07 | 1.00E+07 | 0.95 | 1.05E-01 |
| Glutamic acid | 2.34E+06 | 2.21E+06 | 0.95 | 8.65E-01 |
| Histidine | 1.00E+06 | 9.30E+05 | 0.93 | 3.43E-01 |
| Serine | 3.74E+06 | 3.47E+06 | 0.93 | 1.61E-01 |
| Arginine | 1.96E+06 | 1.79E+06 | 0.92 | 7.95E-03 |
| Glycine | 8.27E+04 | 7.55E+04 | 0.91 | 1.49E-01 |
| Asparagine | 3.72E+06 | 3.36E+06 | 0.90 | 1.74E-05 |
| Proline | 6.35E+06 | 5.58E+06 | 0.88 | 1.54E-03 |
| Alanine | 3.30E+05 | 2.88E+05 | 0.87 | 7.09E-03 |
| Leucine | 2.52E+04 | 2.16E+04 | 0.86 | 3.15E-03 |
| β-Alanine | 1.02E+07 | 8.67E+06 | 0.85 | 2.66E-03 |
| Methionine | 6.52E+06 | 5.45E+06 | 0.84 | 8.11E-03 |
| Glutamine | 3.32E+06 | 2.78E+06 | 0.84 | 2.73E-04 |
| Tryptophan | 2.21E+07 | 1.77E+07 | 0.80 | 3.88E-04 |
| Threonine | 1.02E+07 | 7.76E+06 | 0.76 | 7.70E-06 |

### 4.2.8 Summary of metabolic data from the comparison of ketogenic and habitual diet

**Table 12** summarizes the quantitative changes in metabolite groups and single metabolites following a three week period on a KD based on an untargeted and targeted approach.

**Table 12** Biochemical effects of a ketogenic diet for three weeks

| Elevated after ketogenic diet | Reduced after ketogenic diet |
|---|---|
| Ketone bodies, lipids, trihomomethionine, 3-hydroxyvaleric acid, acetylglycine,acylcarnitines, amino acids; valine, isoleucine, citric acid, malic acid, alpha-ketoglutaric acid, creatine | Acetylvaline, Caffeine, Methyl Indole 3-acetate, Stachydrine, amino acids; threonine, asparagine, tryptophan, proline, b-alaine, leucine, arginine, methionine, glutamine, alanine |

Because the individual increase in LDL-C from baseline varied between 5 % to 128% following a KD for three weeks, the last analysis conducted by LC-ESI-MS global metabolomics in this thesis was based on the relative change of LDL levels.

## 4.1 Comparison of ketogenic and habitual diet metabolomes among those with lowest and highest relative LDL-C increase

Changes in plasma levels of LDL-C from baseline to the end of three weeks on a KD were determined for each participant based on data collected in 2012, see **appendix** section 7.3 **table 17** for further details. The participants were divided into either a low or a high LDL increase group. **Figure 19** displays the PCA plot in negative ionization mode with the participants that had the highest and the lowest relative LDL-C increase from the habitual diet baseline to the end of intervention with three weeks on a KD. The two groups clustered in the PC3 direction, which describes 10.1 % of the variability in the sample metabolomes. Two of the individuals in the high LDL increase group were clustered with samples from the low LDL increase group, which implied that these metabolomes were more similar to each other.

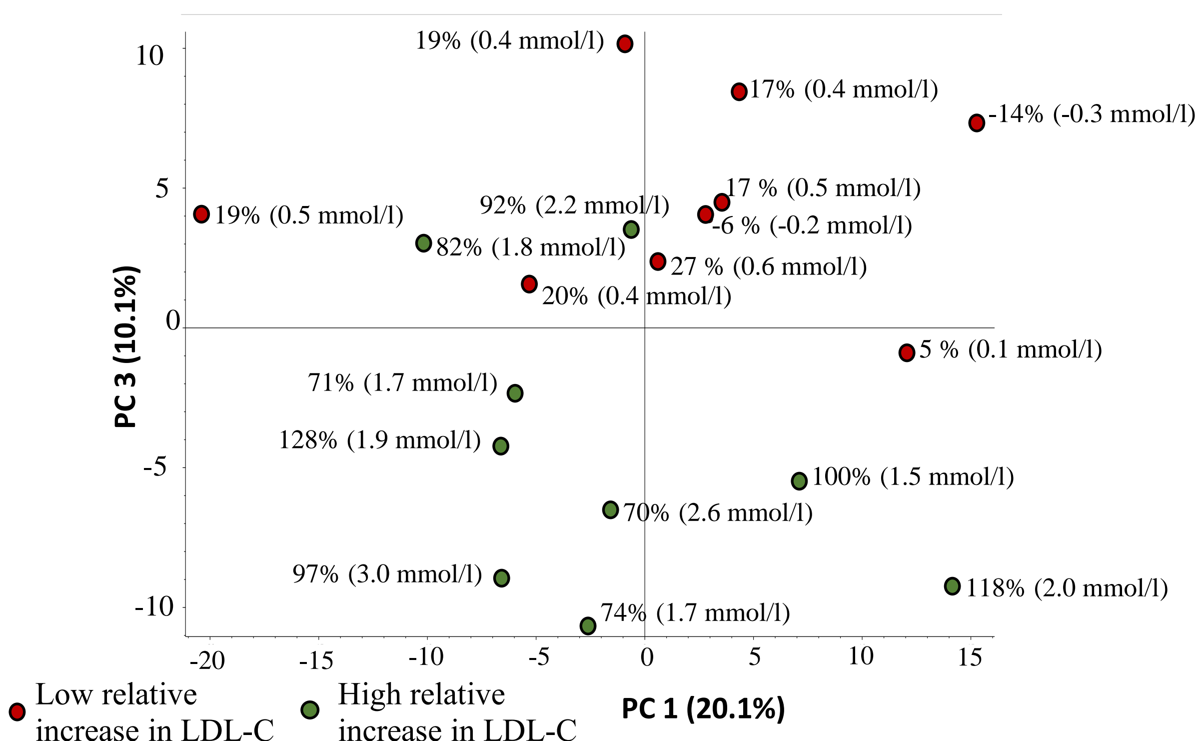



**Figure 19** Principal component analysis plot of all detected compounds among those with high relative LDL-C increase (green) and low relative LDL-C increase (red) after three weeks on a ketogenic diet. The numbers represent percentage increase from baseline to end of ketogenic diet, and LDL-C increase levels in mmol/l. PC1 describes 20.1 % of the variability of the samples, and PC3 explains 10.1 %.

Since the PCA plot gave a pattern compatible with a high relative LDL-C increase group and a low relative LDL-C increase group, differential analysis was conducted to determine which components that changed the most between these two groups.

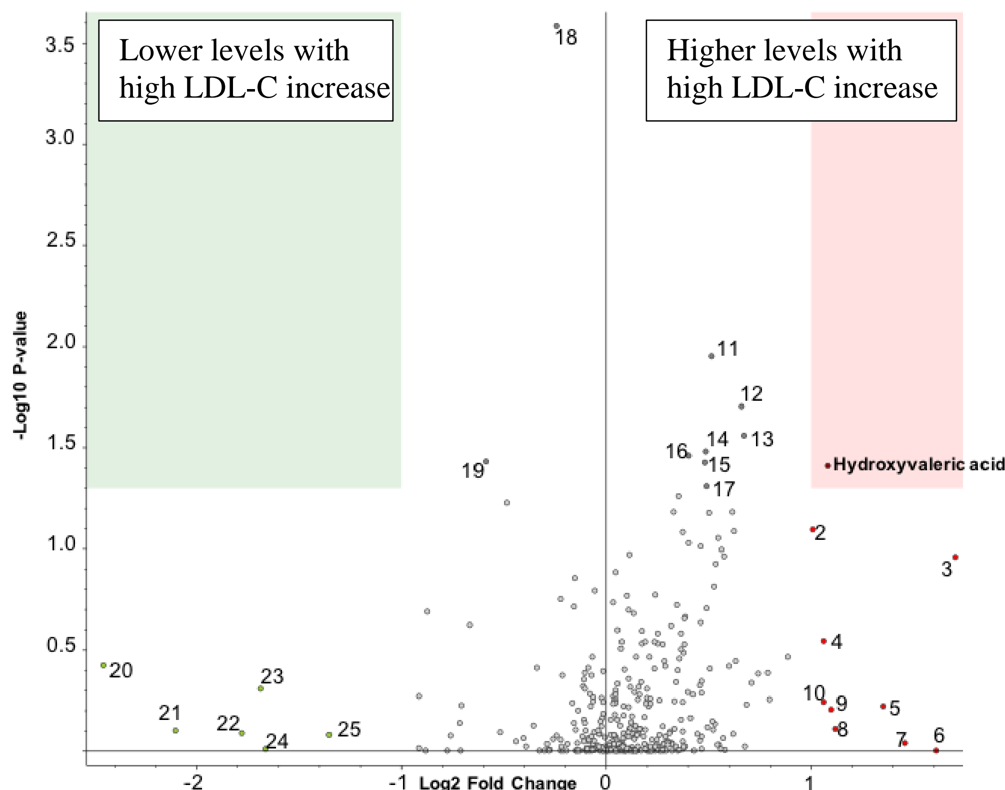



**Figure 20** Differential analysis using volcano plot of data obtained from the negative ionization mode analysis of samples following ketogenic diet intervention among the participants with highest and lowest percent increase in LDL-C levels. The points in the green and red areas display metabolites with both fold change values as well as statistical significance. Metabolites identified are presented in bold text. Numbers with the same colors can be fragments or adducts of the same compound.

As shown in **figure 20,** the differential analysis revealed one features detected in significantly higher levels with a log2 fold change >1 among the high LDL-C increase group compared to the low LDL-C increase group, successfully identified as hydroxyvaleric acid. See **table 18** in **appendix** section 7.3.2 for molecular weight and retention time for the non-identified compounds. **Figure 21** display box and whiskers plot for hydroxyvaleric acid.

See **appendix** section 7.3.3 for volcano plot in positive ionization mode.

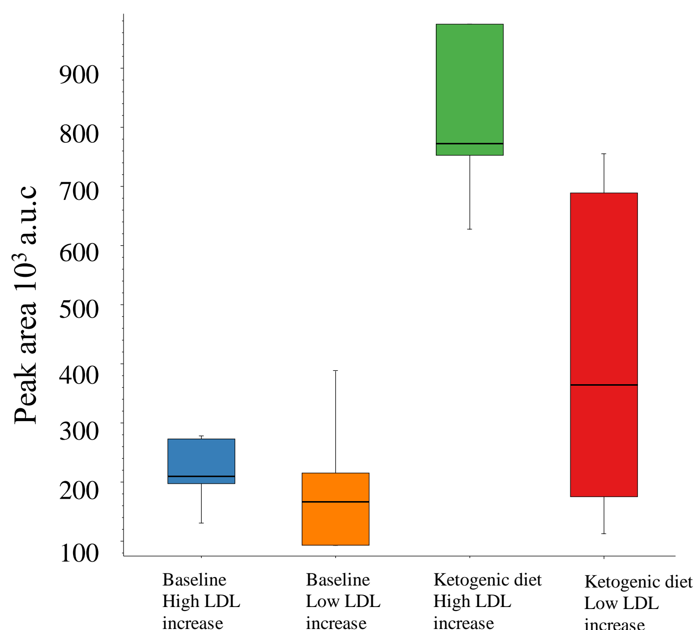



**Figure 21** box and whiskers plot for hydroxyvaleric acid. There were no significant differences between the groups at baseline. The group with highest LDL increase had a greatest elevation of hydroxyvaleric acid following the ketogenic diet

## Association between amino acids levels and LDL-C

Due to the interesting finding of altered amino acids levels following three weeks on a KD, shown in 4.2.7 **table 11**, correlation analyses were performed to check the association between LDL-C and amino acid levels in blood.

Scatter plots of change in LDL-C levels (mmol/l) on the x-axis vs. change in the amino acid peak area on the y-axis (ratio) following a three week KD are shown in **figure 22**.

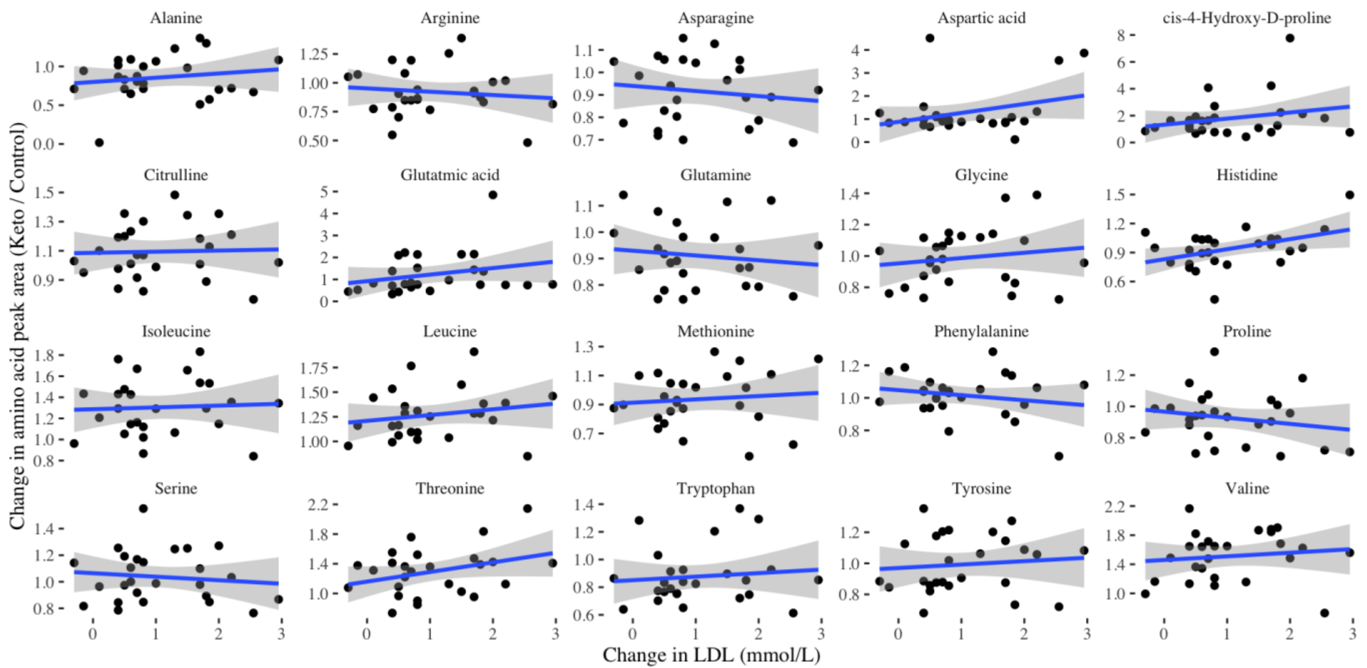



**Figure 22** shows scatter plots of the change in LDL(mmol/L) on the x-axis and change in amino acid peak area (keto/habitual diet(control)) on the y-axis.

Univariate correlation matrix for change in LDL-C with change in amino acids showed positive correlations between change in LDL-C and change in histidine, threonine and aspartic acid. Histidine was the only amino acid that had a significantly (p-value: 0.03) positive correlation with change in LDL-C with a correlation coefficient of 0.44 (CI 0.06, 0.71). **Table 13** displays correlation coefficients between change in LDL-C and histidine, threonine and aspartic acid.

**Table 13** Correlation coefficients between change in LDL-C and change in the three amino acids histidine, threonine and aspartic acid

| | Correlation coefficient LDL-C | CI | P-value[1] |
|---|---|---|---|
| Histidine | 0.44 | 0.06, 0.71 | 0.03 |
| Threonine | 0.34 | -0.06, 0.64 | 0.09 |
| Aspartic acid | 0.31 | -0.09, 0.62 | 0.12 |

[1]Pearson´s correlation

# 5 Discussion

This thesis is an extension of the low carbohydrate high fat study from 2011/2012 (70) and investigates the effect on the metabolome of following KD for three weeks using a global metabolomics platform. To the author`s knowledge, this is the first study studying the KD effect with a global metabolomics approach in normal weighed humans. Previous studies have investigated the effect of KD as a weight loss method or as a treatment for different diseases; whereas we have looked at the global effect of three weeks on KD on healthy individuals in a study with no specific goals with respect to health or weight reduction.

## 5.1 Discussion of subjects and methods

### 5.1.1 Subject and study design

A great strength of this thesis is the randomized, cross over design in the study of Retterstøl et al. (70). Each participant acted as his or her own control; thus, the KD intervention was evaluated within the same subject and accordingly between subject variability was removed. Furthermore, both groups were combined into one group for evaluating the changes in the metabolomes from the baseline on habitual diet to the end of KD intervention. The two groups completed the study at different time points; however, we showed that this did not influence the results.

The population in the study was almost exclusively clinical nutrition students and employees from The University of Oslo, which makes it a homogenous group. The sample size of 26 participants was relatively small due to exclusion and withdrawal rates. Despite this fact, a highly statistical major change in the metabolomes after following a KD was seen. There was a relatively large variation in abundance of the different metabolites between the individuals, and it is difficult to determine if changes are due to random variation or real biological differences. Furthermore, since global metabolomics gives an enormous amount of complex data, a smaller sample size has the advantage that it takes less data processing time and is more manageable within the time limit of this thesis.

**Compliance to the diet regimen**

The risk of recall bias was minimized since the food record diet was collected prospectively. However, bias may have been induced by under- or overreporting of participants´ dietary

intakes since the data was self-reported. This is a major challenge in collecting dietary data in nutrition research (74). Weighed food record requires time and effort by the subjects, but since the subjects were primarily students and employees at the University of Oslo, it is likely that these weighed food records were performed meticulously. Due to the limited amount of time a comprehensive comparison of the dietary data and metabolomics data was not conducted. Long-term compliance is typically low and can be a big issue with KD as well as other interventions that require major lifestyle changes. Nine of the participants had adverse effects on the KD, whereas two were serious adverse events and seven experienced common side effects like headache and fatigue. We speculate whether the significantly lower levels of caffeine after KD compared to habitual diet may have caused these reported side effects.

Despite no significantly reported changes in energy intake between habitual and KD, all the participants lost weight during the ketogenic diet intervention. The participants were encouraged to eat according to their energy needs and the same level of physical activity (70). Some of the weight reduction can be related to water loss as a consequence of rapid glycogen depletion (21). In addition, the participants may have taken healthier choices in general or unintentionally reduced their energy intake when following an intervention study. Furthermore, ketosis is suggested to suppress the increase in appetite (75).

### 5.1.2 Laboratory and LC-MS method

Factors that may have affected the outcome includes variations both in the manual laboratory work and other technical challenges related to sample handling and storage, instrumentation, software etc. The blood samples had been stored in a – 80°C freezer since 2012. This results in uncertainty regarding how the long-term storage period may have influenced the samples and potentially reduced or elevated the amount of different analytes in the samples (76).

Dried blood spot (DBS), is blood drops spotted onto a filter paper and dried. We could have used DBS as sample preparation material instead of plasma, since the LC-MS method is optimized for DBS and is used in the routine for the newborn screening because the paper makes the analytes less reactive and more stable. However, plasma was chosen because it was readily available and because there is higher concentrations in undiluted plasma samples than

in plasma applied to and diluted from DBS filter paper. Furthermore, there is a risk that some components are retained on the filter paper and thereby lost for metabolomics analyses.

A strength of the laboratory preparation design was the implementation of a pilot with a limited number of samples to evaluate how the samples were handled and how the whole sample handling process worked. The samples were partitioned in batches over several days, thus theoretically increasing the probability of day to day variation in the sample preparation. However, the only real possible error is pipetting errors, and the platform uses a semi-quantitative method that is not markedly affected by such small pipetting errors.

The LC-MS/MS analysis used is an optimized method to analyze as many compounds as possible, and is a well-established method at the Department of Medical Biochemistry, Oslo University Hospital, Rikshospitalet, Norway.
Since the consumption of fat changed enormously on a KD, the mobile phase for negative ionization was adjusted to increase lipidomic coverage (77, 78). Positive ionization mode detected notable more features than negative ionization. Some features are being ionized only in positive or negative modes, while some features are detected in both positive and negative ionization mode. Consequently, the two ionization modes are complementary.

For data processing, the different settings in the compound discover metabolomics processing workflow affect the results of the analysis and are thus chosen based on well-founded and well-documented criteria to detect and determine the possible identity of the metabolites. Despite this, the identification of metabolites is a comprehensive, complicated, and very time-consuming process that requires analytical chemistry expertise. Features could be generated from the same compounds(fragments and adducts) or related by other means, such as being a part of a shared metabolic pathway. Many of the detected features from the analyses have not been successfully identified. However, these compounds could be of high importance regarding the KD effects, and ideally this should be followed up in the future.

## 5.2 Discussion of the results

### 5.2.1 Comparison of ketogenic and habitual diet

An untargeted metabolomics approach reveals changes in a diverse set of compounds, some of which are anticipated and some are unexpected. The expected changes included elevated levels of ketones and lipids. Some unexpected findings includes decreased amino acid levels and correlation between histidine and LDL-C.

First, PCA plots visualized a difference in the metabolomes after following the KD for three weeks. However, the response to the KD varied considerably between the participants. Participant p1, p18 and p22 deviated from the separation seen in the other study participants in the PCA plot based on their diets. Both p1 and p18 moved in the same direction as the other participant`s metabolomes but their KD metabolome did not differ significantly from their habitual diet. Surprisingly, p22 on habitual diet was positioned with the KD metabolomes. There are many possible explanations to this, and this will be discussed later.

### 5.2.2 Elevated ketone bodies

As expected, levels of ketone bodies (hydroxybutyrate and acetoacetate) were significantly elevated after three weeks on a KD. These findings are in line with well-established knowledge on how the diet alters the energy metabolism from primarily glucose utilization to creating ketone bodies for energy (1, 10, 79). A study conducted by Ulven et al, the Noma study, showed significantly elevated levels of acetoacetate and acetate when saturated fat was replaced with polyunsaturated fat, and accordingly significant reduced LDL-C levels. However no change in hydroxybutyrate was observed. Three of the participants, p1, p18, and p22 (the participants mentioned in 5.2.1) did not have a high increase in ketone bodies (data not shown). The levels of ketone bodies may be influenced by many different factors. Higher consumption of carbohydrates than allowed can interrupt the physiological adaption to fat oxidation. However, food records of these participants showed a carbohydrate intake lower than 20 g per day. Errors in self reporting occurs and cannot be excluded. Another possible explanation is lowered ketone levels after exercise. Unfortunately, no registration of physical activity during the intervention was performed. Furthermore, body composition and variations in basal metabolic rate can also contribute to the metabolite levels observed (20).

Ketone bodies have been used as biomarkers for KD to identify noncompliance to the diet (1). Despite the lower levels of ketone bodies, these participants were not excluded due to the food records that were in agreement with the dietary regimen. In addition, p1 and p18 had a 5 % and -14 % change in LDL-C concentration, respectively, following KD. In contrast, p22 had a 118% increase in LDL-C concentration, thus being one of the participants with the highest LDL-C increase among all of the subjects. A relationship between the concentration of ketone bodies and LDL-C was not seen (data not shown).
More investigation revealed higher ketone bodies in subject p18 prior KD intervention. This finding may indicate that the participant started the KD intervention earlier than supposed. However, this was only seen in plasma and not serum samples and mislabeling or sample mix up prior to this project is therefore probable. This exemplifies that every extra step of manual handling increases the likelihood of mislabeling and sample mix up. Thus, p18 was mistakenly included in the analysis, and should have been excluded. This may have affected the results. Although, excluding one subject will not necessary affect the statistical results due to the sample size, it may weaken the precision of the estimates of the effect (80). At present, since there is not time enough to perform metabolic reanalyses, we can only speculate whether exclusion of this participant would have shown an even greater effect of the ketogenic diet, since both of the included samples from this participant most likely reflected the habitual diet.

The ketogenic diet itself may cause epigenetic modifications, and the effect of the ketone bodies produced have been linked to epigenetic effects (81). Epigenetic changes of KD, such as DNA methylation and histone modifications, contribute to the changes in gene transcription profiles that again lead to metabolic changes and altered metabolomes. The postulated anticonvulsant effect of ketone bodies themselves has been questioned (82), and this remains to be resolved. An animal study conducted by Kennedy et al evaluated the physiological and metabolic effect of a ketogenic diet in mice. They showed a unique gene expression pattern in KD, where expression of genes in fatty oxidation and ketogenic pathways were increased and expression of genes in lipid synthesis pathways were reduced (83). In this present study, Retterstøl et al found that gene expression of transcription factor SREBP-1 was significantly different between the KD and habitual diet, but no differences between the diets in the change of expression of the other lipid-related genes investigated were observed. However, several within-group changes in gene expression in both the

habitual and KD were shown. In addition, borderline reduction in expression of LDL receptor was observed in the KD (70). SREBP-1 is a key regulator of the lipogenic genes in the liver, and represents a possible mechanism by which dietary saturated fats can lead to hyperlipidemia and atherogenesis (84).

### 5.2.3 Raised fatty acids

Not surprisingly, plasma fatty acids were higher in KD, particularly hydroxyvaleric acid, saturated fatty acids; arachidic acid, medium-chain fatty acids; octadecanedioic acid, polyunsaturated fatty acids; arachidonic acid and adrenic acid and monounsaturated fatty acid; oleic acid. The elevation of free fatty acids as a consequence of lipolysis due to lower plasma glucose and insulin are characteristic of reduced carbohydrate availability. No restriction on the type of fats was given to the participants. Thus, fatty acid content and composition most likely differed between the participants explaining part of the differences observed.

Participants reported a higher intake of saturated fat while on KD. The saturated fatty acids that significantly increase total LDL-C, namely lauric, myristic and palmitic were all elevated at the end of the intervention groups. No correlations between relative LDL-C increase and peak area of the respectively saturated fatty acids were found (data not shown). Endogenously produced fat makes it challenging to use the saturated fatty acids as biomarkers of fat and fatty acid intake (85). Thus, the large variation in LDL-C response between the participants cannot be studied by using plasma levels of saturated fat. As earlier mentioned, cholesterol levels in some participants were almost unaffected by the KD with self-reported high intake of saturate fat, whereas other participants experienced a dramatic increase. This variation in response to a KD suggests that the diet needs to be implemented with caution if one wished to avoid large increases in LDL-C.

A higher intake of mono- and polyunsaturated fat than of saturated fat is preferable to decrease cardiovascular risk. Compared to the recommended guidelines of saturated fat intake < 10 E% (36), the participants had a mean intake of 29.5 E%. The saturated fatty acid arachidic acid (C20:0) had a 164% increase from habitual diet to ketogenic diet, thus being the fatty acid with the largest change. It is probable that the lipid profiles of the subjects in this study would have been improved by a higher proportional intake of foods with polyunsaturated fat. The monounsaturated fatty acid oleic acid, had a 110% increase from

habitual diet to KD. A study conducted by Ulven et al found a reduction in total cholesterol when exchanging a few regularly consumed food items with others with improved fat quality. This may have a potentially impact on future CVD risk (86).

### 5.2.4 Carnitine levels increased

Carnitine is involved in transport of long chain fatty acids into mitochondria for oxidation. Consistent with prior findings (23), we observed that those coupled to lipid metabolism were elevated, particularly myrisotyl-, palmitoyl-, stearonyl- and hexanoylcarnitine. Unfortunately, those coupled to amino acid metabolism were not detected due to the properties of the analytical technique chosen. Requirements of carnitine may increase when ingesting a high fat diet (1). However, no change in free carnitine levels was seen. Carnitine is a reservoir for excess acyl residues, generated by high rates of beta-oxidation in mitochondria (10). Humans are able to synthesize carnitine from the essential amino acids lysine and methionine (10) and also gets it from dietary sources, mainly animal sources.

The Noma study showed significantly reduced levels of myristoyl-, and palmitoylcarnitine when replacing saturated fat with polyunsaturated fat (37). Accordingly, in this thesis, a higher intake of saturated fat significantly increased the levels of myriostoyl-, and palmitoylcarnitine. Studies have suggested acylcarnitines to be biomarkers of CVD risk in obese and diabetes patients (87) and to be associated with cardiovascular events in the elderly (88).

### 5.2.5 Changes in markers of energy metabolism

Some metabolites involved in the energy metabolism were investigated to see if they were up- or down-regulated. Only citric acid, malic acid, alpha-ketoglutaric acid as intermediates in the citric acid cycle were successfully identified, all of which were significantly upregulated. These changes suggest an increased flux through the citric acid cycle. In addition, no significant differences in citrulline and ornithine were observed, indicating that there is no obvious elevation in amino acid degradation. However, degradation products were not investigated. Thus, these findings should be interpreted with care.

### 5.2.6 Alterations in amino acid levels

In this study, ten amino acids were significantly decreased in connection with the KD. Since amino acids levels were reduced, a possible explanation could be that these amino acids had been used in ketogenesis or glucogenesis. In contrast to these finding, the participants reported protein intake that increased significantly from 17.3 E% on habitual diet to 26.3 E% on KD.

Two clinical studies have reported significant increases in GABA levels in the brain following KD treatment of children with refractory epilepsy (89, 90). GABA is synthesized from glutamate, and is the main inhibitory neurotransmitter in the body. Consistent with these findings, plasma levels of GABA were elevated after a KD in our healthy population. However, these results might not be directly comparable due to different biological sample materials and different study populations.

Dietary amino acids have been related to serum lipid levels (73). On comparing the amino acid levels with LDL-C change levels, a significant correlation between histidine and LDL-C was observed. To the best of the author`s knowledge, this is one of only a few previous studies that have investigated and compared change in plasma amino acids peak areas with change in LDL-C levels (mmol/L) (37). Similar to our findings, The Noma study with significant reduced levels of LDL-C when replacing saturated fat with polyunsaturated fat, found borderline elevated levels of histidine and glycine, and significantly higher levels of the amino acids serine, asparagine, proline and cystathionine. The clinical significance of this finding remains unanswered and should be further investigated. A study conducted by Teymoori et al investigated the association between dietary amino acids and prospective changes in the serum lipid profile in adults. In contrast to our study, they found no significant correlation with histidine. Despite weak correlations, they found that alanine and tryptophan were associated with higher and lower LDL-C changes respectively. Lysine, alanine, methionine, aspartic acid and alkaline amino acids showed positive association with changes in total cholesterol (91).

As in Cappuccio et al, the acetylated amino acid, acetylglycine, were significantly raised after KD. In this study, a 120 % increase was shown. Acetylglycine has been significantly associated with dietary fiber intake (92). In contrast to these findings, we found no correlation

between acetylglycine and reported dietary fiber intake (data not shown). The relevance of acetylglycine and the effect of ketogenic diet remain unclear.

#### 5.2.7 Comparison of ketogenic and habitual diet metabolomes among those with lowest and highest relative LDL-C increase

Hydroxyvaleric acid was significantly elevated after KD. Hydroxyvaleric acid belongs to the class of fatty acyls known as hydroxy fatty acids. Interestingly, a greater elevation of hydroxyvaleric acid was detected in the group with highest relative LDL-C increase (>70%) compared to the low relative LDL-C increase group (<27%). The box and whiskers plot for hydroxyvaleric acid shows a large variation between the individual peak areas measured, especially in the low relative LDL-C increase group. In literature, too, there are studies suggesting a correlation between 2-hydroxyvaleric acid and LDL-C levels. Trupp et al, studied the effects on intermediary metabolism of reducing LDL-C with statin. 2-hydroxyvaleric acid was significantly decreased after treatment with statin. Furthermore, there was a correlation between low baseline 2-hydroxyvaleric acid levels and decreased LDL-C levels described (93). This finding is consistent with my results, although my study setup demonstrates the correlation between elevated levels of Hydroxyvaleric acid and LDL-C.

Why the change in LDL-C levels following KD varied so much between individuals remains unanswered. However, the metabolites that were found to change markedly and significantly on KD, but that were not identified, could be of importance related to the large inter-individual LDL-C response to KD.

# 6 Conclusion and future perspective

Metabolomic profiling using high-performance liquid chromatography coupled to mass spectrometry showed:

- Distinct clustering discriminating the ketogenic diet and the habitual diet using principal component analysis (PCA) plot. The features that contribute to the largest variation were therefore similar within each diet group and differed between the groups. However only a few of these features were successfully identified.

- Alterations in ketone bodies, lipids, acylcarnitine and amino acid profiles using the differential analysis; volcano plot.

    - Hydroxybutyrate, acetoacetic acid, trihomomethionine, steryoylcarnitine, acetyl-l-carnitine, hexanoylcarnitine, 3-hydroxyisobutyric acid, hydroxyvaleric acid, arachidic acid, octadecanedioic acid, adrenic acid, n-acetylglycine, arachidonic acid were successfully identified and significantly elevated after following KD diet with a log2 fold change >1. GABA was significantly elevated, but with a log2 fold change lesser then 1.

    - Four metabolites; stachydrine, methyl indole 3-acetate, caffeine and N-acetylvaline were successfully identified and significantly decreased after following KD diet with a log2fold change >1 in positive ionization mode

    - The amino acid profiles among the participants after a KD were altered. Plasma levels of the amino acids threonine, asparagine, tryptophan, proline, b-alanine, leucine, arginine, methionine, glutamine and alanine were decreased, whereas levels of valine and isoleucine were significantly higher after KD.

- Intermediates in citric acid cycle were upregulated, in particular citric acid, malic acid and alpha-ketoglutarate. This may suggest an increased citric acid cycle flux.

- The metabolomic profiles among those with the lowest and highest relative LDL-C increase on KD differed from each other and were similar within each group based on the PCA plot.

- Significantly elevated levels of hydroxyvaleric acid among those with highest relative LDL-C increase.

- Histidine was positively associated with LDL-C

In conclusion, applying global metabolomics in this randomized controlled study identified substantial changes in the metabolome as a response to the dietary intervention. We found alterations in a diverse set of compounds, but unfortunately we were unable to explain why the individual LDL-C levels on KD varied so much.

### Future perspective

The global metabolomics platform is implemented as a valuable tool in the diagnostic lab at the Department of Medical Biochemistry at OUS and is used in many different research projects. The analytical method used in this thesis is not optimized for lipids. Further research with a lipidomics platform could be of interest to increase the knowledge of how the ketogenic diet with high fat intake alters the metabolism. Furthermore, a longer intervention time would have been necessary to investigate the more long-term effects of a KD than that of a three week intervention.

In addition, a comprehensive comparison of the dietary data and metabolomics data can address an association between reported food intake and measured peak areas, and find potential biomarkers of a ketogenic diet and ideally also establish reference ranges for a normal metabolome and possibly even the ketogenic metabolome.

Metabolite identification is a difficult and time consuming task, and in this project only a few of the many compounds that changed significantly as a consequence of the dietary intervention were identified due to limited time. Further investigations into metabolite identification and statistical analyses of the results can be done to gain more knowledge of the effect of ketogenic diet on the metabolome.

Why the LDL-C levels varied a lot between the participants deserve further investigations. Ideally, metabolomics can be used to characterize similar metabolomic patterns to be able to identify the mechanism by which a ketogenic diet can cause a marked increase in LDL-C an find underlying susceptibility and resilience factors explaining the large individual variation in response, enabling a personalized way to predict if an individual will likely respond with a marked increase in LDL-C levels on a KD.

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# Appendix

Appendix 7.1 Sample preparation

Appendix 7.2 Comparison of ketogenic and habitual diet

Appendix 7.3 Comparison of ketogenic and habitual diet metabolomes among those with lowest and highest relative LDL-C increase

Appendix 7.4 Approval by the Regional Ethical Committee for Medical Research

# 7 Appendix

## 7.1 Sample preparation

### 7.1.1 Sample preparation set up

The sample preparations were distributed over four and three days for serum and plasma

**Serum**
90 samples - 4 days + PQC + processed blank

Day 1: 8 subjects x 3 visits =>24 samples

Day 2: 7 subjects x 3 visits=>21 samples

Day 3: 8 subjects x 3 visits =>24 samples

Day 4: 7 subjects x 3 visits =>21 samples

*LC-MS analysis time: FullMS Pos + FullMS Neg = 6 days*

**Plasma**
90 samples - 3 days + PQC + processed blank

Day 1:15 subjects x 3 visits => 45 samples

Day 2: 8 subjects x 3 visits => 24 samples

Day 3: 7 subjects x 3 visits => 21 samples

*LC-MS analysis time: FullMS Pos + FullMS Neg = 6 days*

### 7.1.2 PQC preparation

PQCmain (from day 1)
- Check precision in the PCA plot
- Correct for analytical drift for each metabolite using linear regression

PQCday (2,3,4)
- Pooled all samples from day 2-4
- MS/MS spectra to provide information about the chemical structure to identify metabolites

## PQC procedure

**PQCmain (day1)**

- Collected all biological samples from day 1 in a 1,5 ml Eppendorf tube (24 biological samples)
- Mixed on a table vortex for 5 seconds
- Aliquoted 30 µl to five Eppendorf tubes
- Prepared the samples separately (see protein precipitation)
- Collected all the samples to a 1,5 ml Eppendorf tube
- Aliquoted to eight HPLC vials

**PQCday2**

- Collected all biological samples from day 2 in a 1,5 ml Eppendorf tube (21 biological samples)
- Mixed on a table vortex for 5 seconds
- See protein precipitation
- Transferred to a HPLC vial

**PQCday3**

- Collected all biological samples from day 3 in a 1,5 ml Eppendorf tube (24 biological samples)
- Mixed on a table vortex for 5 seconds
- See protein precipitation
- Transferred to a HPLC vial

**PQCday4**

- Collected all biological samples from day 4 in a 1,5 ml Eppendorf tube (21 biological samples)
- Mixed on a table vortex for 5 seconds
- See protein precipitation
- Transferred to a HPLC vial

**PQCtotal**

- Collected 190µl from PQCday1 (main), day2, day3 and day4
- Mixed on a table vortex for 5 seconds
- Aliquoted 30 µl to five Eppendorf tubes
- Prepared the samples separately (protein precipitation)
- Collected 30 µl of all the samples to a 1,5 ml Eppendorf tube
- Transferred to a HPLC vial

### 7.1.3 Analysis sequence

**Table 14** The analysis sequence applied in the thesis. A blank sample was analyzed at the start of the analytical batch, a blank extraction sample was analyzed twice, followed by two system conditioning QC samples. Five biological samples were analyzed between each PQC. A set of PQC samples was applied separately at the end of the run for MS/MS data acquisition.

**Table 14** The analysis sequence

| Injection no. | Sample type | Comments |
|---|---|---|
| 1 | Blank sample | Water |
| 2 | Process blank sample | Extraction of background |
| 3 | System conditioning QC sample 1 | Not included in data processing |
| 4 | System conditioning QC sample 2 | Not included in data processing |
| 5 | Pooled QC main sample 1 | |
| 6 | Pooled QC main sample 2 | |
| 7 | Biological sample | |
| 8 | Biological sample | |
| 9 | Biological sample | |
| 10 | Biological sample | |
| 11 | Biological sample | |
| 12 | Pooled QC main sample 3 | |
| 13 | Biological sample | |
| . | Biological sample | |
| . | Biological sample | |
| . | Biological sample | |
| 35 | Biological sample | |
| 36 | Pooled QC main sample 8 | |
| 37 | Pooled QC day2 MSMS | |
| 38 | Biological sample | |
| . | Biological sample | |
| . | Biological sample | |
| . | Biological sample | |
| 109 | Biological sample | |
| 110 | Pooled QC main sample 19 | |
| 111 | Biological sample | |
| 112 | Biological sample | |
| 113 | Biological sample | |
| 114 | Biological sample | |
| 115 | Biological sample | |
| 116 | Pooled QC main sample 20 | |
| 117 | Pooled QC main sample 21 | |
| 118 | Pooled QC main MSMS | For identification |
| 119 | Pooled QC day4 MSMS | For identification |
| 120 | Pooled QC total MSMS | For identification |

# 7.2 Comparison of ketogenic and habitual diet

## 7.2.1 Exclusion of participants because of non-compliance

All three blood samples were required. Since p25 was missing a blood sample, this participant was excluded from the analysis.

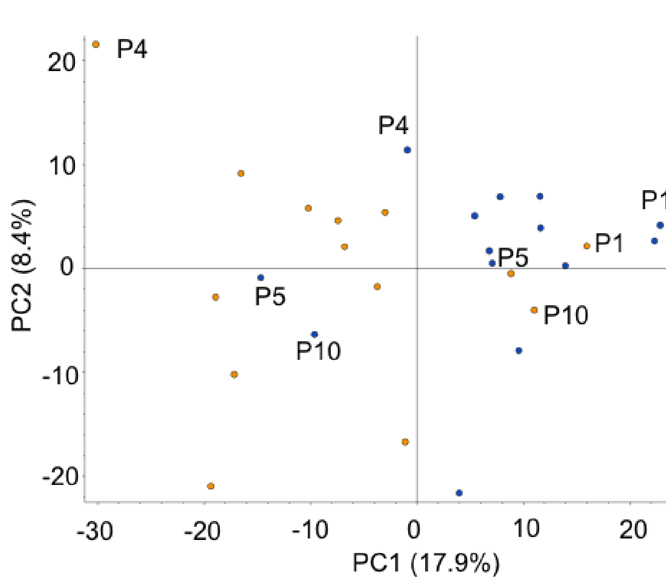


Figure 23 PCA plot of the LK group in positive ionization mode

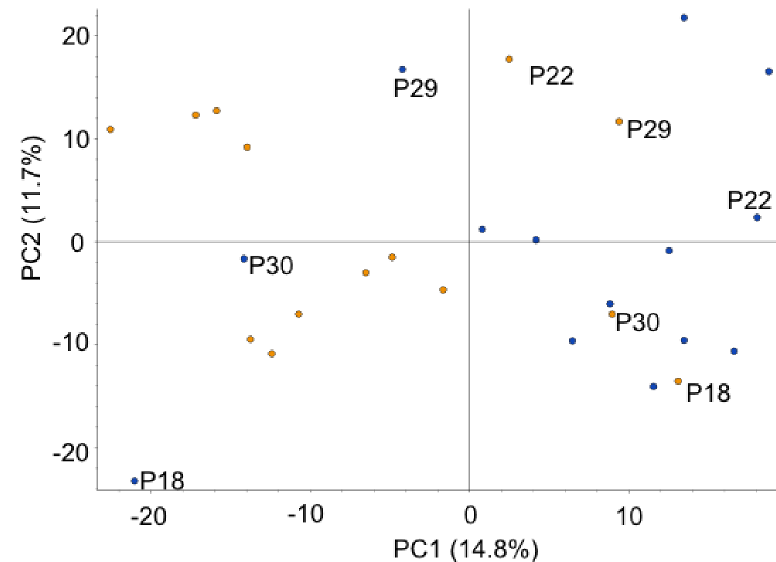


Figure 24 PCA plot of the KL group in positive ionization mode

As shown in **figure 23 and 24**; p5, p10, p29 and p30 deviated from the separation seen in the other study participants in the PCA plot based on their diets both in plasma and serum samples. High levels of b-hydroxybutyrate prior intervention (shown in figure 25 and 26) and low levels of b-hydroxybutyrate during the intervention identified non-compliance, or at least insufficient adherence, to the ketogenic diet (shown in figure 27 and 28). Further investigation revealed that p30 started the ketogenic diet earlier, and the participant was recoded and included in the analysis, while p5,p10 and p29 were excluded for further analysis.

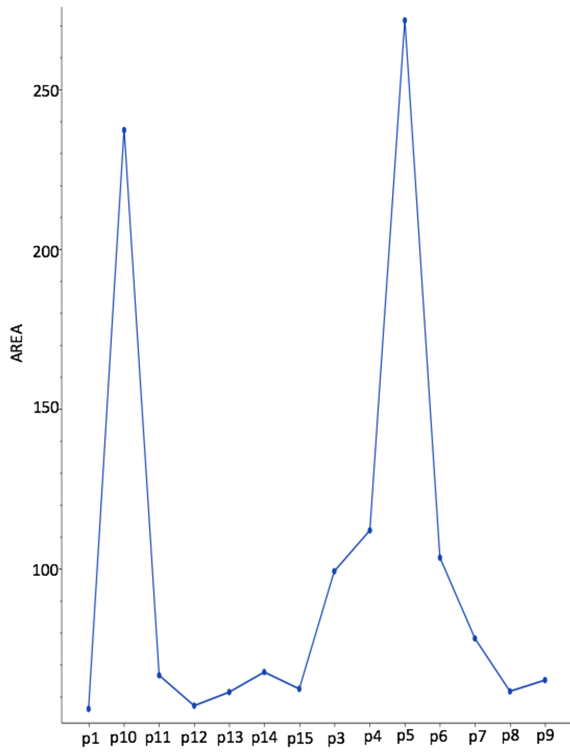


**Figure 25** relative abundance of hydroxybutyrate prior to ketogenic diet in the LK group, where p10 and p5 shows elevated levels of the ketone body

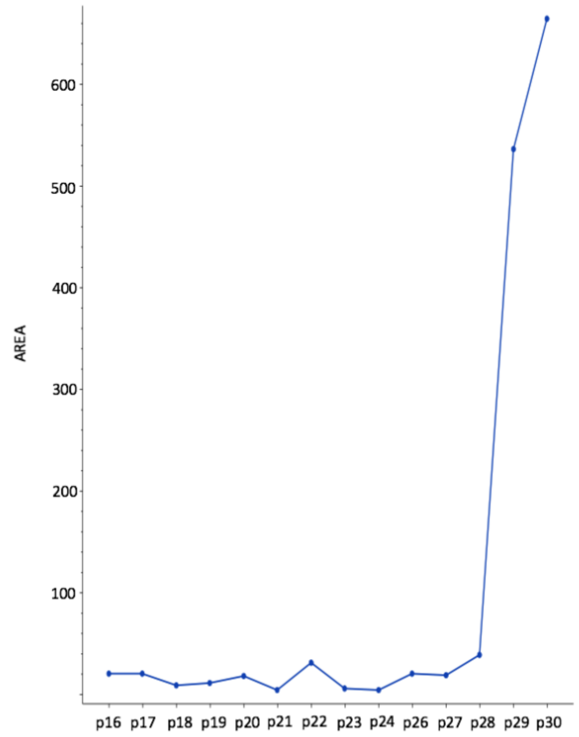


**Figure 26** relative abundance of hydroxybutyrate prior to ketogenic diet in the KL group, where p29 and p30 have elevated levels of the ketone body

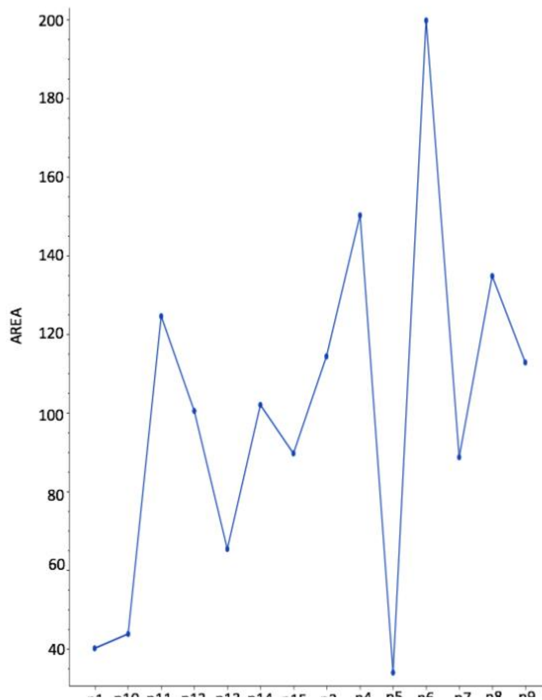


**Figure 27** relative abundance of hydroxybutyrate on ketogenic diet in the LK group, where p1, p5 and p10 shows decreased levels of the ketone body

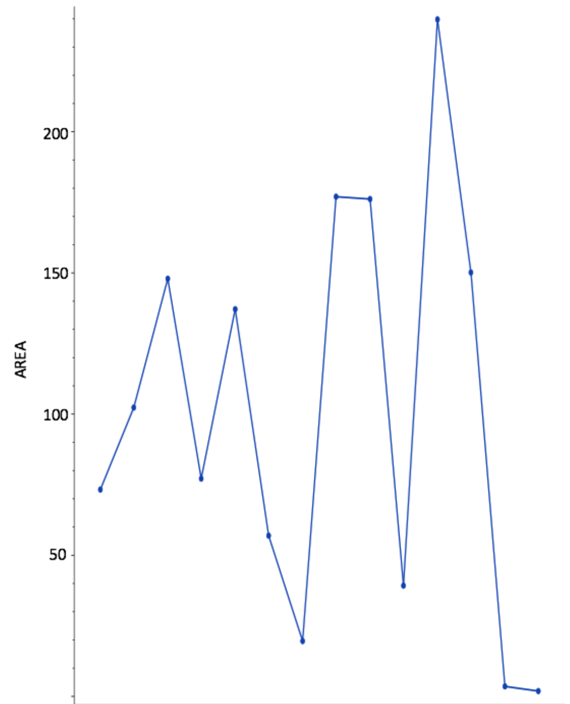


**Figure 28** relative abundance of hydroxybutyrate on ketogenic diet in the KL group, where p29 and p30 shows decreased levels of the ketone body

### 7.2.2 Did study participation affect the habitual diet?

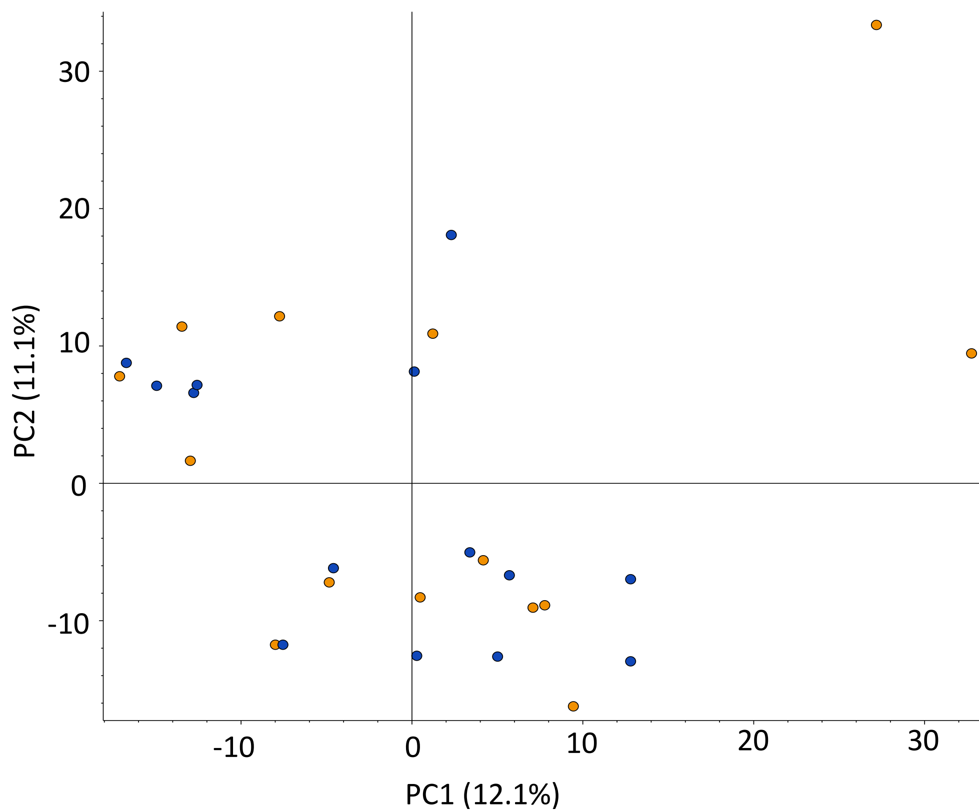


**Figure 29** PCA plot of habitual diet at visit1 and visit2 for the KL group. Blue points represent visit1 and yellow points represents visit2.

The principal component analysis (PCA) plot showed no substantial difference between the metabolomes of the participants at these two visits on the habitual diet as shown in **figure 29**.

### 7.2.3 PCA plot with quality controls

**Figure 30** shows the PCA in positive ionization mode. PCA plot from the analysis run in positive ionization mode of all samples taken at baseline (habitual diet) and immediately following intervention with KD among all the participants. The PCA plot shows the same pattern as negative ionization mode, with a distinct clustering discriminating the ketogenic and the habitual diet groups with a few exceptions (p18, p22 and p1). The pooled quality control (PQC) samples were focused and centered, indicating that the results were not affected by analytical drift.

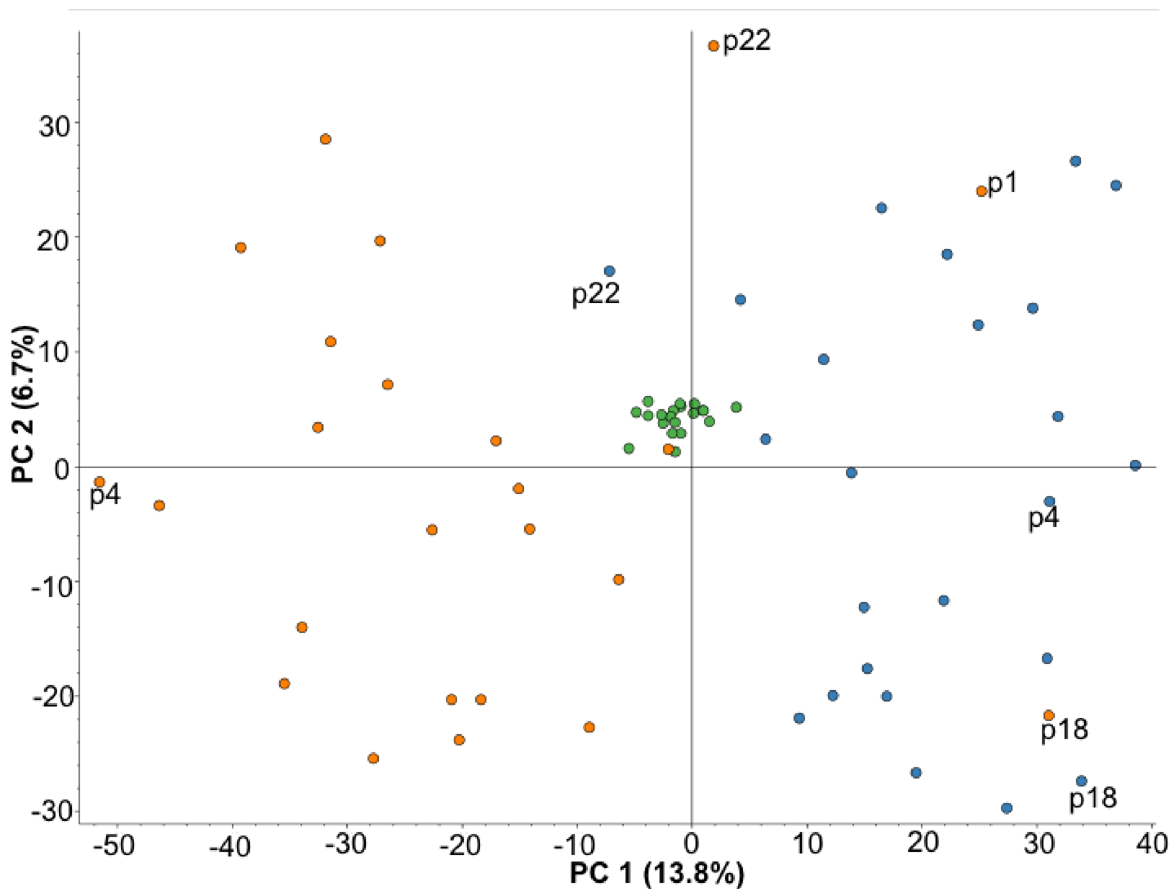


**Figure 30** PCA plot from the analysis in positive mode that display a separation between the habitual diet and the ketogenic diet, except from some individuals (p18,p22, p1) . A point represents all the metabolites detected in that sample. An orange colored point represents the metabolome with a ketogenic diet and a blue colored point represent the metabolome at baseline (habitual diet). The green colored point represents the pooled quality control samples.

### 7.2.4 Molecular weight and retention time to volcano plot

Molecular weight and retention time to detected compounds in positive and negative ionization mode respectively are listed in **table 14** and **table 15**.

**Table 15** Molecular weight and retention time to detected compounds in positive ionization mode

| no | Molecular weight | RT | Suggestions | no | Molecular weight | RT | Suggestions |
|---|---|---|---|---|---|---|---|
| 2 | 186.05040 | 2.161 | hydroxybutyrate adduct | 46 | 270.25597 | 18.794 | margaric acid |
| 3 | 202.02446 | 2.148 | hydroxybutyrate adduct | 47 | 576.33050 | 19.694 | |
| 4 | 200.03534 | 2.155 | hydroxybutyrate adduct | 48 | 117.07901 | 2.701 | 5-aminovaleric acid |
| 5 | 298.12653 | 2.449 | | 49 | 103.06347 | 2.439 | y-aminobutyric acid (GABA) |
| 6 | 568.54353 | 19.687 | | 50 | 792.37102 | 18.466 | similar to 53 |
| 7 | 448.13769 | 12.084 | | 51 | 394.27536 | 19.697 | similar to 41 |
| 8 | 296.27147 | 19.605 | | 52 | 448.27791 | 19.689 | |
| 9 | 749.53691 | 29.397 | | 53 | 304.24036 | 18.464 | arachidonic acid |
| 10 | 590.52547 | 19.685 | similar to Stearic acid | 54 | 131.09461 | 3.393 | beta-leucine |
| 11 | 616.46583 | 17.904 | similar to nr 12 | 55 | 406.27522 | 18.724 | |
| 12 | 534.46245 | 17.921 | | 56 | 836.36305 | 17.915 | |
| 13 | 156.07864 | 12.054 | 2,5- dimethyl-4 ethoxy-3(2H)-furanone | 57 | 354.21707 | 17.925 | |
| 14 | 512.48037 | 17.929 | similar to nr 12/11 | 58 | 574.31497 | 18.724 | |
| 15 | 188.14132 | 12.927 | 5-hydroxydecanoic acid | 59 | 848.36372 | 18.723 | |
| 16 | 118.06305 | 2.475 | | 60 | 342.27720 | 18.726 | |
| 17 | 117.04265 | 2.208 | | 61 | 152.06856 | 2.569 | xylitol |
| 18 | 558.46271 | 17.974 | similar to nr 12/11 | 62 | 308.27166 | 19.699 | |
| 19 | 586.49409 | 18.724 | similar to oleic acid | 63 | 436.30105 | 18.723 | |
| 20 | 564.51207 | 18.722 | | 64 | 364.25917 | 18.723 | similar to 66 |
| 21 | 536.48091 | 17.974 | 5-9Z-9-Hexadecenoyloxy Octadecanoic acid | 65 | 184.14634 | 13.958 | similar to 80 |
| 22 | 462.19001 | 13.523 | | 66 | 282.25588 | 18.724 | Oleic acid |
| 23 | 350.21306 | 17.113 | | 67 | 862.37931 | 18.727 | |
| 24 | 224.17783 | 15.327 | | 68 | 592.30767 | 16.375 | |
| 25 | 581.36952 | 21.947 | similar to sorbitan monooleate | 69 | 770.38643 | 18.725 | |
| 26 | 534.05004 | 12.249 | | 70 | 392.25968 | 18.725 | |
| 27 | 506.01921 | 11.933 | | 71 | 588.33069 | 19.725 | |
| 28 | 582.46259 | 18.018 | | 72 | 768.37082 | 18.024 | |
| 29 | 560.48094 | 18.026 | similar to 9-nitrooleate | 73 | 362.24356 | 18.019 | |
| 30 | 616.11061 | 11.969 | | 74 | 586.31503 | 18.018 | |
| 31 | 184.14622 | 11.893 | | 75 | 340.26143 | 18.017 | |
| 32 | 520.03423 | 12.115 | | 76 | 378.24393 | 18.722 | |
| 33 | 481.13790 | 11.922 | silmar to IpA | 77 | 404.25948 | 18.013 | |
| 34 | 189.07901 | 11.918 | IpA | 78 | 268.24025 | 17.891 | |
| 35 | 154.02670 | 11.164 | | 79 | 280.24024 | 18.015 | linoelaidic acid |
| 36 | 397.04708 | 11.360 | | 80 | 476.20525 | 13.956 | Similar to 65 |
| 37 | 380.25956 | 19.689 | | 81 | 376.22825 | 18.016 | |
| 38 | 772.40210 | 19.696 | | 82 | 390.24403 | 18.020 | |
| 39 | 366.27469 | 19.696 | similar to 41 | 83 | 227.08265 | 3.399 | |
| 40 | 174.01644 | 1.642 | | 84 | 268.13108 | 13.163 | |
| 41 | 284.27156 | 19.696 | stearic acid | 85 | 850.37925 | 19.692 | |
| 42 | 283.12676 | 2.428 | similar to gaba | 86 | 444.24654 | 18.021 | |
| 43 | 408.29087 | 19.686 | | 87 | 572.29923 | 18.020 | |
| 44 | 332.27179 | 20.044 | adrenic acid | 88 | 312.30290 | 21.564 | arachidic acid |
| 45 | 590.34624 | 19.696 | Similar to 41 | 89 | 306.25603 | 19.009 | dihomo-gamma-linolenic acid |

| no | Molecular weight | RT | Suggestions | no | Molecular weight | RT | Suggestions |
|---|---|---|---|---|---|---|---|
| 90 | 846.34819 | 18.020 | | 107 | 131.06951 | 2.710 | creatine |
| 91 | 226.19343 | 15.735 | myristoleic acid | 108 | 766.35511 | 17.492 | |
| 92 | 170.13089 | 13.521 | | 109 | 190.04781 | 2.97 | |
| 93 | 742.35504 | 17.109 | | 110 | 164.06858 | 2.605 | |
| 94 | 278.22458 | 17.491 | alpha Eleostearic acid | 111 | 224.08964 | 2.578 | |
| 95 | 360.22790 | 17.494 | | 112 | 128.05863 | 2.657 | dimethylhydantoin |
| 96 | 254.22464 | 17.109 | hexadecenoic acid | 113 | 228.07249 | 2.371 | |
| 97 | 446.26220 | 18.724 | | 114 | 119.05830 | 2.341 | threonine |
| 98 | 330.25610 | 19.481 | | 115 | 255.12167 | 2.366 | |
| 99 | 134.02157 | 1.696 | malic acid | 116 | 234.08524 | 1.731 | |
| 100 | 314.24593 | 17.114 | | 117 | 132.05350 | 2.305 | asparagine |
| 101 | 820.33228 | 17.116 | | 118 | 146.06917 | 2.366 | glutamine |
| 102 | 336.22787 | 17.109 | | 119 | 326.13263 | 2.379 | |
| 103 | 618.32349 | 17.114 | | 120 | 1596.10110 | 2.858 | |
| 104 | 310.28731 | 20.499 | | | | | |
| 105 | 364.22840 | 17.109 | | | | | |
| 106 | 420.24660 | 17.924 | | | | | |

**Table 16** Molecular weight and retention time to detected compounds in negative ionization mode

| no | Molecular weight | RT | Suggestions | no | Molecular weight | RT | Suggestions |
|---|---|---|---|---|---|---|---|
| 1 | 192.02135 | 4.883 | | 30 | 750.53630 | 26.437 | |
| 2 | 104.04729 | 4.864 | | 31 | 789.49773 | 26.761 | |
| 3 | 126.02920 | 4.900 | | 32 | 723.51922 | 26.032 | |
| 4 | 206.03694 | 4.920 | | 33 | 884.71328 | 20.312 | |
| 5 | 142.00226 | 4.440 | | 34 | 751.54966 | 26.760 | (1Z-octadecenyl)-2-aracgidonoyl-sn-glycero-3-phosphoethanolamine |
| 6 | 206.01018 | 4.925 | | 35 | 749.53469 | 26.421 | |
| 7 | 154.06046 | 11.968 | | 36 | 806.66646 | 19.566 | |
| 8 | 86.03679 | 4.851 | biacetyl/ succinaldehyd/ fragment acetoacetate | 37 | 397.31900 | 13.870 | |
| 9 | 187.99959 | 4.917 | | 38 | 118.06293 | 8.998 | |
| 10 | 204.07617 | 14.246 | | 39 | 208.09446 | 4.443 | |
| 11 | 230.03687 | 4.819 | | 40 | 259.17813 | 11.932 | hexanoylcarnitine |
| 12 | 331.25403 | 13.631 | | 41 | 789.56389 | 28.941 | |
| 13 | 303.22300 | 13.175 | | 42 | 285.19379 | 12.303 | |
| 14 | 273.17591 | 12.723 | | 43 | 604.44382 | 19.686 | |
| 15 | 427.36595 | 15.005 | stearoylcarnitine | 44 | 369.28751 | 13.347 | cis-5-tetradecenoylcarnitine |
| 16 | 256.13936 | 2.708 | | 45 | 546.39584 | 18.784 | |
| 17 | 203.11562 | 3.674 | Acetyl-L-Carnitine | 46 | 145.07377 | 2.468 | |
| 18 | - | - | | 47 | 320.07765 | 8.979 | |
| 19 | 371.30317 | 13.553 | | 48 | 246.15914 | 16.811 | |
| 20 | 178.06043 | 13.845 | Similar to N-Acetyl-L-cysteine | 49 | 143.09453 | 4.163 | stachydrine |
| 21 | 240.03076 | 13.854 | | 50 | 466.26897 | 17.858 | |
| 22 | 191.09766 | 11.388 | trihomomethionine | 51 | 272.03113 | 13.439 | |
| 23 | 102.03163 | 4.756 | acetoacetic acid | 52 | 189.07884 | 13.440 | methyl indole-3-acetate |
| 24 | 104.04729 | 104.04729 | hydroxybutyrate | 53 | 129.05783 | 13.439 | |
| 25 | 201.07891 | 11.762 | 2-amino-2,3-biphenyldiol | 54 | 280.16476 | 14.985 | |
| 26 | 767.58190 | 28.873 | - PC(o-18:2(9Z,12Z)/18:2(9Z,12Z)) | 55 | 210.12291 | 12.931 | |
| 27 | 745.50122 | 26.032 | | 56 | 194.08028 | 12.762 | caffeine |
| 28 | 771.51647 | 26.433 | | 57 | 159.08939 | 2.736 | N-Acetylvaline |
| 29 | 556.44392 | 19.571 | | | | | |

### 7.2.5 Peak areas of significantly altered identified metabolites

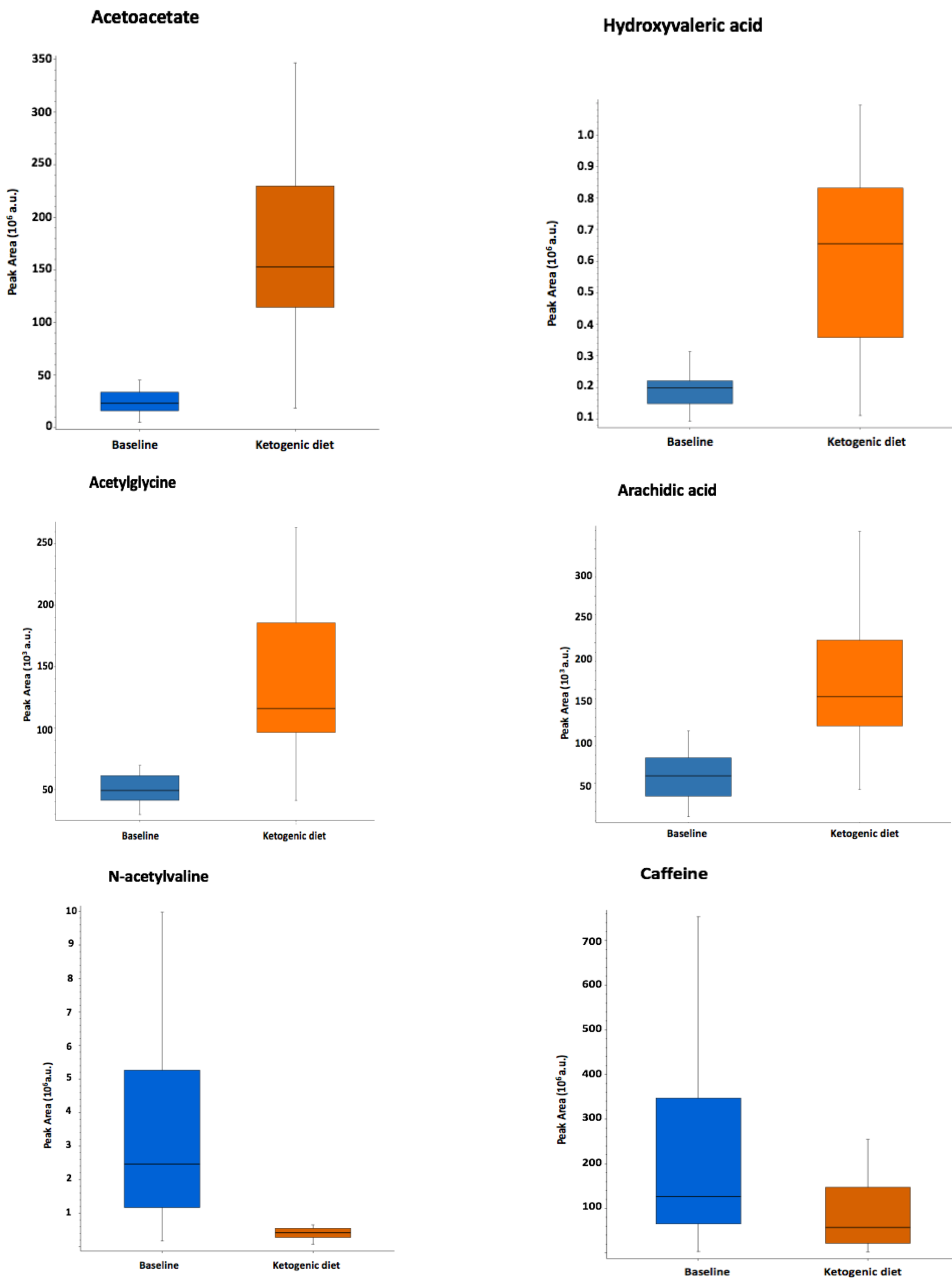


**Figure 31** Peak areas of significantly altered identified metabolites

# 7.3 Comparison of ketogenic and habitual diet metabolomes among those with lowest and highest relative LDL-C increase

## 7.3.1 LDL-C change values for each participant

**Table 17** gives the LDL-C change values estimated for each participant based on data collected in 2012. Changes are given both based on absolute values and LDL-C% change. “H” and “L” represent the group with high LDL-C change and low LDL-C change respectively.

Table 17 LDL-C change levels in mmol/l and percentage change

| ID | LDL-C change mmol/L[1] | LDL-C %Change[2] | Group |
|---|---|---|---|
| P17 | 1.9 | 128 % | H |
| P22 | 2.0 | 118 % | H |
| P21 | 1.5 | 100 % | H |
| P28 | 3.0 | 97 % | H |
| P11 | 2.2 | 92 % | H |
| P12 | 1.8 | 82 % | H |
| P27 | 1.7 | 74 % | H |
| P8 | 1.7 | 71 % | H |
| P26 | 2.6 | 70 % | H |
| P7 | 1.3 | 43 % | |
| P23 | 0.8 | 40 % | |
| P6 | 0.7 | 39 % | |
| P14 | 0.6 | 38 % | |
| P2 | 0.8 | 32 % | |
| P30 | 0.8 | 32 % | |
| P3 | 07 | 32 % | |
| P24 | 1 | 26 % | |
| P15 | 0.6 | 27 % | L |
| P20 | 0.4 | 20 % | L |
| P4 | 0.5 | 19 % | L |
| P13 | 0.4 | 19 % | L |
| P9 | 0.4 | 17 % | L |
| P16 | 0.5 | 17 % | L |
| P1 | 0.1 | 5 % | L |
| P19 | -0.2 | -6 % | L |
| P18 | -0.3 | -14 % | L |

1 Group1:visit2-visit1, Group2: $visit\ 3 - \frac{visit1+visit2}{2}$

2 Group1: $\frac{LDLchange}{Visit1}$, Group2: $LDLchange{:}\frac{visit1+visit2}{2}$

## 7.3.2 Table of features that were significantly changed in amount in volcano plot in negative ionization mode

**Table 18** Features detected from volcano plot in negative ionization mode LDL-C comparison

| no | Molecular weight | RT | Suggestions |
|---|---|---|---|
| 2 | 298.12653 | 2.452 | |
| 3 | 477.28551 | 18.920 | 2-linoleoyl-sn-glycero-3-phosphoethanolamine |
| 4 | 312.23023 | 14.385 | (10E,12Z)-9-Hydroperoxy-10,12-octadecadienoic acid |
| 5 | 568.54390 | 19.693 | Stearic acid |
| 6 | 590.52547 | 19.668 | |
| 7 | 214.03020 | 12.586 | |
| 8 | 179.05827 | 7.002 | Hippuric acid |
| 9 | 513.27633 | 13.919 | |
| 10 | 353.02354 | 6.991 | |
| 11 | 510.04712 | 11.225 | |
| 12 | 698. 07895 | 11.518 | |
| 13 | 130.06307 | 4.589 | (hydroxyethyl) methacrylate – ethyl-acetoacetate |
| 14 | 579.35364 | 20.281 | similar to linoleic acid |
| 15 | 311.12181 | 2.435 | Similar to 16 |
| 16 | 131.05824 | 2.443 | cis-4-hydroxy-D-proline |
| 17 | 298.97981 | 2.319 | |
| 18 | 227.08266 | 3.396 | similar to N-acetyl- D-alloisoleucine |
| 19 | 762.58978 | 28.760 | |
| 20 | 555.35394 | 21.003 | |
| 21 | 198.98060 | 1.990 | |
| 22 | 161.03248 | 1.694 | |
| 23 | 204.00946 | 11.524 | |
| 24 | 202.03007 | 12.346 | |
| 25 | 484.03180 | 11.078 | |
| 26 | 218.02397 | 12.143 | |
| 27 | 246.05604 | 11.922 | |
| 28 | 481.13797 | 11.928 | |

## 7.3.3 Volcano plot in positive ionization mode

**Figure 32** Differential analysis using volcano plot of data obtained from the positive ionization mode analysis of samples following ketogenic diet intervention among the participants with highest and lowest percent change in LDL-C levels. The points in the green and red areas display metabolites with both fold change values as well as statistical significance. Numbers with the same colors can be fragments or adducts of the same compound.

**Table 19** shows molecular weight and retention time to detected compounds in positive ionization mode

## 7.3.1 Table of features that were significantly changed in amount in volcano plot in positive ionization mode

**Table 19** Molecular weight and retention time to detected compounds in positive ionization mode among the participants with highest and lowest percent change in LDL-C levels.

| | Molecular weight | RT | Suggestions | | Molecular weight | RT | Suggestions |
|---|---|---|---|---|---|---|---|
| 1 | 1076.61122 | 19.231 | | 40 | 131.05814 | 2.400 | cis-4-hydroxy-D-proline/ 5-aminolevulinic acid |
| 2 | 1058.62837 | 19.196 | | 41 | 228.11080 | 2.741 | tetraacetylethylenediamine (hydroxyprolylproline) |
| 3 | 557.27894 | 19.235 | | 42 | 845.58119 | 21.137 | |
| 4 | 542.31761 | 18.660 | | 43 | 503.29845 | 18.915 | |
| 5 | 188.04481 | 12.800 | | 44 | 1553.95868 | 19.178 | |
| 6 | 320.07765 | 8.979 | | 45 | 990.66326 | 19.149 | |
| 7 | 192.05764 | 8.972 | (cys-ala) | 46 | 471.29595 | 16.254 | |
| 8 | 519.33230 | 19.226 | (1-linoleoyl-sn-glycerol-3-phosphocholine | 47 | 449.31382 | 16.228 | |
| 9 | 220.02578 | 8.992 | | 48 | 1080.64255 | 20.015 | |
| 10 | 234.03178 | 8.991 | Similar as 9 | 49 | 195.05306 | 11.777 | |
| 11 | 220.05248 | 8.990 | Similar as 9 | 50 | 137.04756 | 3.711 | 4-aminobenzoic acid |
| 12 | 140.04481 | 8.985 | Similar as 7 | 51 | 1084.67383 | 21.080 | |
| 13 | 118.06293 | 8.999 | Similar as 9 | 52 | 518.21256 | 13.719 | |
| 14 | 1012.64578 | 19.151 | | 53 | 496.23036 | 13.704 | |
| 15 | 750.53630 | 26.445 | | 54 | 270.03384 | 12.227 | |
| 16 | 817.59447 | 29.979 | | 55 | 301.22496 | 12.433 | |
| 17 | 427.36595 | 15.003 | stearoylcarnitine | 56 | 367.06624 | 12.361 | |
| 18 | 704.58190 | 26.033 | | 57 | 104.02614 | 12.361 | |
| 19 | 232.14200 | 2.705 | | 58 | 179.05807 | 12.361 | Hippuric acid |
| 20 | 676.55104 | 24.865 | | 59 | 232.11855 | 12.910 | |
| 21 | 204.03977 | 11.845 | | 60 | 263.01035 | 12.363 | |
| 22 | 773.5335 | 26.761 | | 61 | 249.03106 | 12.363 | |
| 23 | 339.33445 | 14.195 | palmiotylcarnitine | 62 | 332.19591 | 16.881 | |
| 24 | 1585.07178 | 25.719 | | 63 | 167.03777 | 10.947 | |
| 25 | 227.92603 | 1.647 | | 64 | 150.13674 | 2.437 | |
| 26 | 792.53577 | 25.743 | | 65 | 199.00676 | 11.275 | |
| 27 | 311.12114 | 2.404 | | 66 | - | - | |
| 28 | 164.08378 | 24.989 | | 67 | 430.26923 | 16.283 | |
| 29 | 767.58190 | 28.906 | | 68 | 155.06925 | 3.151 | L-Histidine |
| 30 | 202.01514 | 8.986 | | 69 | 356.19590 | 14.537 | |
| 31 | 477.28526 | 17.097 | | 70 | 466.26922 | 18.564 | |
| 32 | 725.05014 | 25.712 | | 71 | 316.20356 | 14.537 | |
| 33 | 379.24843 | 16.025 | Sphingosine 1-phosphate | 72 | 444.28715 | 18.564 | |
| 34 | 1449.09763 | 25.723 | | 73 | 173.14152 | 12.644 | |
| 35 | 703.15069 | 2.367 | | 74 | 155.09461 | 12.432 | |
| 36 | 245.16238 | - | 2-methylbutyrylcarnitine | 75 | 232.10686 | 15.124 | Succinyl –L –Ornithine |
| 37 | 85.08908 | 3.514 | | 76 | 700.19416 | 2.465 | |
| 38 | 780.53293 | 28.327 | | 77 | 498.31642 | 16.280 | |
| 39 | 557.28788 | 19.238 | | 78 | 129.05782 | 13.439 | |

| Region: | Saksbehandler: | Telefon: | Vår dato: | Vår referanse: |
|---|---|---|---|---|
| REK sør-øst | Finn Skre Fjordholm | 22845821 | 18.06.2019 | 2011/1365 REK sør-øst D |
| | | | Deres dato: | Deres referanse: |
| | | | 23.05.2019 | |

Vår referanse må oppgis ved alle henvendelser

Kjetil Retterstøl
Oslo universitetssykehus

**2011/1365 Virkningen av høyfettdiett**

**Forskningsansvarlig:** Oslo universitetssykehus HF
**Prosjektleder:** Kjetil Retterstøl

Vi viser til søknad om prosjektendring datert 23.05.2019 for ovennevnte forskningsprosjekt. Søknaden er behandlet av sekretariatet for REK sør-øst D på fullmakt, med hjemmel i helseforskningsloven § 11.

Endringen består i at tre nye medarbeidere skal delta i prosjektet:

-Helge Rootwelt, overlege
-Katja Benedicte Prestø Elgstøen, forsker
-Katrine Pettersen, masterstudent

**Vurdering**
Sekretariatet i REK har vurdert den omsøkte endringen, og har ingen forskningsetiske innvendinger til endringen slik den er beskrevet i skjema for prosjektendring.

Det opplyses at det er Katrine Pettersen som skal skrive masteroppgaven som er nevnt i endringsmeldingen av 23.04.2019. Protokollen for oppgaven er vedlagt søknaden og tas til orientering.

Videre er det lagt ved reviderte utgaver av informasjonsskriv med to samtykkeskjema og følgebrev. REK forutsatte i vedtak den 13.05.2019 at dato for sletting i disse ble korrigert til 31.12.2028.

Dato er endret i korrekt i informasjonsskrivet og REK tar dette til orientering.

I bekreftelse av nektet samtykke og følgebrevet heter det fremdeles at dato for sletting er den 31.12.2023. Det er prosjektperioden som er utvidet til 2023. Sletting av studiematerialet skal ikke skje før i 2028.

REK viser til vedtak den 13.05.2019, og ber igjen om at dato for sletting endres i alt materiell som sendes ut til deltagerne. Korrekte versjoner må sendes REK til orientering.

REK ber om tilbakemelding dersom noe skulle være uklart.

**Vedtak**
Sekretariatet har gjort en forskningsetisk vurdering av endringen i prosjektet og godkjenner prosjektet slik det nå foreligger, jfr. helseforskningsloven § 11, annet ledd.

Tillatelsen er gitt under forutsetning av at prosjektet gjennomføres slik det er beskrevet i søknaden, endringssøknad, oppdatert protokoll og de bestemmelser som følger av helseforskningsloven med forskrifter.

Vi gjør samtidig oppmerksom på at etter ny personopplysningslov må det også foreligge et behandlingsgrunnlag etter personvernforordningen. Det må forankres i egen institusjon.

**Klageadgang**
Vedtaket kan påklages, jf. forvaltningslovens § 28 flg. Eventuell klage sendes til REK sør-øst. Klagefristen er tre uker fra du mottar dette brevet. Dersom vedtaket opprettholdes av REK sør-øst, sendes klagen videre til Den nasjonale forskningsetiske komité for medisin og helsefag for endelig vurdering.

Vennligst oppgi vårt referansenummer i korrespondansen.

Til informasjon bytter REK søknadsportal i sommer. Den nye portalen vil være klar i august. Se våre hjemmesider «Aktuelle meldinger» for oppdatert informasjon.

Henvendelser kan sendes inn på e-post post@helseforskning.etikkom.no.

Med vennlig hilsen

Knut Ruyter
avdelingsdirektør
REK sør-øst sekretariatet

Finn Skre Fjordholm
rådgiver

**Kopi til:** *Lene Kristine Seland: lekrse@ous-hf.no*
*Oslo universitetssykehus HF: oushfdlgodkjenning@ous-hf.no*