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Metabolomics of Weight Loss and Weight Maintenance in Obese Humans

Metabolomics of Weight Loss and Weight Maintenance in Obese Humans

Nina Geidenstam



LUND
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DOCTORAL DISSERTATION

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To be defended in “Jubileumsaulan”, Jan Waldenströms gata 5,

Skånes Universitets Sjukhus, Malmö.

Wednesday 4th of May 2016, at 9.00.

Faculty opponent

Professor Henrik Antti

Department of Chemistry, Umeå University, Sweden

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Title and subtitle Metabolomics of weight loss and weight maintenance in obese humans		
<p>Abstract</p> <p>The prevalence of obesity and obesity-related complications are increasing worldwide. Weight loss has shown to improve insulin sensitivity and decrease the risk of developing type 2 diabetes (T2D). Even so, little is known about how metabolites, other than glucose, are affected after weight loss and weight maintenance treatment.</p> <p>Impaired glucose tolerance (IGT) is a common trait of obesity and is studied during an oral glucose tolerance test (OGTT). In this thesis, we identified 16 distinct metabolite OGTT profiles (change from fasting, 30 and 120 min.) that deviated from the glucose tolerant lean group. These deviations were grouped as; a delayed reduction in the levels of five fatty acids, increased levels at 30 min. of five amino acids (incl. isoleucine and leucine), and a blunted increase at 30 min. of six metabolites. When we followed up these obese individuals after weight loss and weight maintenance, roughly half of these metabolites improved towards the expected healthy profile. Specifically, enhanced suppression of aromatic amino acids (tyrosine and phenylalanine) was associated with decreased insulinogenic index after weight loss. On the contrary, the glucose-elicited suppression of four amino acids and three fatty acids improved after weight maintenance, paralleling an improved glucose tolerance. This suggests that diet-induced weight loss followed by weight maintenance results in changes in metabolite OGTT profiles associated with either hepatic insulin sensitivity or peripheral glucose tolerance.</p> <p>Obesity is associated with altered levels of fasting circulating amino acids. We found that eight out of the 18 detected amino acids were associated with obesity, independently of age, sex, T2D and blood pressure. Six of these amino acids were improved after weight maintenance. From this, we created scores based on amino acids and risk factors at baseline that are either informative of the level of association to obesity or the potential benefit, or lack of benefit, by reducing the included amino acids to those that are expected to normalize with a weight loss and weight maintenance program.</p> <p>Finally, we validated previous findings and further explored the data in a larger cohort at baseline and one year after participating in a non-surgical weight loss program. Changed levels in 30 metabolites were unique in those with a $\geq 10\%$ weight loss compared to those with $< 10\%$ weight loss. In addition, we found weight loss to be associated with 13 baseline metabolite levels, and interestingly, many of these are common food additives. We also saw that the obesity predictive scores were modifiable with weight loss.</p> <p>The thesis adds to the understanding of metabolite alterations in obesity-driven IGT and the benefits of weight loss followed by weight maintenance. Our findings suggest several metabolites that may be valuable to consider when evaluating who will benefit from weight loss treatments.</p>		
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Till minne av min pappa

*“The important thing is not to stop questioning.
Curiosity has its own reason for existing”*

Albert Einstein

“Do. Or do not. There is no try.”

Yoda

Content

List of papers	11
Abbreviations	13
Introduction	15
Obesity	15
Metabolic regulation	15
Fasting conditions.....	15
Prandial and postprandial conditions.....	16
Circulating metabolite levels and obesity.....	17
Impaired glucose response	18
Metabolite profiles of impaired glucose tolerance	19
Weight loss and weight maintenance	20
Metabolite changes with weight loss and weight maintenance	20
Aims	23
General methodology	25
Study participants.....	25
Obesity cohort	25
Normal weight glucose tolerant cohort	25
Malmö Diet and Cancer cohort	26
Glucose and insulin analysis	26
Metabolomics analysis	27
Gas chromatography mass spectrometry	28
Liquid chromatography mass spectrometry	29
Sample preparation.....	29
Statistical analysis	29
Obesity and diabetes scores.....	31
Results and Discussion	33
Metabolite responses during an OGTT	33
Differences in metabolite profiles during an OGTT between obese and normal weight individuals (paper I)	33

Change in metabolite profiles during an OGTT after weight loss and weight maintenance (paper II)	35
Heterogeneity within the obese group (paper I-II)	37
Altered fasting metabolite levels improved with weight loss and weight maintenance treatment.....	38
Change in fasting amino acid levels with weight loss and weight maintenance (paper III)	38
Further evaluation of change in amino acid levels after participating in a weight loss program (paper IV)	39
Amino acid levels predictive of weight loss treatment	40
BMI-associated amino acid levels (paper III)	40
Obesity and treatment-modifiable scores (paper III)	41
Evaluation of the obesity scores in a different weight loss cohort (paper IV)	43
Weight loss-associated metabolite levels	44
Baseline metabolites associated with change in BMI (paper IV)	44
Weight-loss induced change in metabolite levels associated with decrease in BMI (paper IV)	45
Major conclusions	47
Summary in Swedish – Populärvetenskaplig sammanfattning	49
Fetma – ett (över)viktigt problem	49
Metabolomik.....	49
Bristfällig ämnesomsättning och glukosbelastning	49
Vikttnedgångsbehandling.....	50
Glukosbelastning efter vikttnedgång	50
Aminosyror relaterade till BMI och förändring vid vikttnedgång.....	51
Sammanfattning.....	51
Acknowledgements	53
References	57

List of papers

This thesis is based on the following manuscripts

- I** **Geidenstam N**, Spégel P, Mulder H, Filipsson K, Ridderståle M, Danielsson A. Metabolite profile deviations in an oral glucose tolerance test – a comparison between lean and obese individuals. *Obesity*. 2014; 22(11):2388-95.
- II** **Geidenstam N**, Danielsson A, Spégel P, Ridderståle M. Changes in glucose-elicited blood metabolite responses following weight loss and long term weight maintenance in obese individuals with impaired glucose tolerance. *Diabetes research and clinical practice*. 2016; 113: 187-97.
- III** **Geidenstam N**, Magnusson M, Danielsson A, Gerszten R, Wang T, Reinius L, Mulder H, Melander O, Ridderståle M. Amino acid signatures predictive of beneficial effects of weight loss. Manuscript submitted.
- IV** **Geidenstam N**, Al-Majdoub, Ekman M, Spégel P, Ridderståle M. Metabolite profiling of obese individuals before and after a one year weight loss program. Manuscript.

Abbreviations

AHT	Anti-hypertensive treatment
BCAA	Branched-chain amino acids
BMI	Body mass index
CIR	Corrected insulin response
CVD	Cardiovascular disease
DI	Disposition index
DM-AA	Diabetes-predictive amino acid score
FFA	Free fatty acids
IFG	Impaired fasting glucose
IGI	Insulinogenic index
IGT	Impaired glucose tolerance
ISI	Insulin sensitivity index
GC/MS	Gas chromatography/mass spectrometer
HOMA-IR	Homeostasis model assessment-estimated insulin resistance
HOMA- β	HOMA for β -cell function
MDC	Malmö diet and cancer
MDC-CC	MDC cardiovascular cohort
OB-BMI score	Score based amino acids associated with obesity (defined by BMI)
OGTT	Oral glucose tolerance test
OPLS-DA	Orthogonal projections to latent structures discriminant analysis
PCA	Principal component analysis
SBP	Systolic blood pressure

TCA cycle	Tricarboxylic acid cycle
T2D	Type 2 diabetes
WLWM program	Weight loss and weight maintenance program
WLWM-BMI score	Score based on amino acids associated with obesity by BMI that are modified after a weight loss and weight maintenance program
WC	Waist circumference

Introduction

Obesity

Obesity is a global public-health problem which started to increase in the mid-20th century¹. The worldwide prevalence of overweight adults was over 1.9 billion (39%) in 2014, and out of these, 600 million (13%) were obese². The obesity epidemic is predicted to continue to such degree that by 2030, 40-50% of the adult population will be obese³. The obesity prevalence in children and adolescents has also become more common, and this increases the risk of developing obesity-related complications earlier than otherwise⁴⁻⁶. Obesity is defined as having a body mass index greater than 30 kg per square meter (kg/m^2) and is most commonly the result of energy surplus⁷. An extended period of over-nutrition, in combination with decreased physical activity, is linked to development of several complications. The etiology of obesity-related complications is not yet fully understood but glucose intolerance as a result of obesity has been linked to conditions such as insulin resistance, type 2 diabetes (T2D), cardiovascular disease, cancer, and osteoporosis⁸⁻¹⁰.

Metabolic regulation

Fasting conditions

All cells need energy to perform various biological processes, and different tissues have different means to maintain a constant energy supply. For instance, red blood cells and the brain use glucose as their energy source (ketone bodies can be used during starvation), whereas most peripheral tissues use both glucose and fatty acids as energy source, depending on blood concentrations of these nutrients¹¹. The human body can efficiently regulate the glucose levels by secreting hormones and through different tissue-specific glucose transporters¹². Through rigorous glucose regulation, circulating glucose levels are present at a relatively constant concentration. Due to that, the red blood cells and brain almost exclusively use glucose as their energy source; the body can store glucose as glycogen in the liver

and skeletal muscles^{13, 14}. When glucose levels decrease (e.g. during the night), release of glucagon from pancreatic α -cells stimulates glycogen breakdown in the liver as well as glucose synthesis via gluconeogenesis. In addition, fatty acids, stored as triglycerides in the adipose tissue, are the main source of energy for peripheral tissues during fasting¹⁵. Triglycerides are degraded to fatty acids and glycerol by lipases¹⁶. They are then released to the bloodstream to become available as energy source for other tissues. Once fatty acids have entered the cell, β -oxidation of fatty acids produces acetyl coenzyme A (acetyl CoA), a substrate for the tricarboxylic acid (TCA) cycle. Glycerol, which is also released, can be converted both to pyruvate and glucose in the liver^{15, 16}.

Amino acids are another source of energy, although the main use of amino acids is in protein metabolism. There are 20 amino acids that are used in proteins. Of these 20 amino acids, nine (histidine, lysine, methionine, phenylalanine, threonine, tryptophan, isoleucine, leucine and valine) are essential (or semi-essential) for humans, and cannot be synthesized *de novo*. The first step of amino acid degradation is removal of the amino group via transamination, and the remaining carbon molecule is metabolized into glucose, acetyl CoA, or one of several TCA cycle intermediates. Most amino acids are mainly degraded in the liver except for branched-chain amino acids (BCAAs): isoleucine, leucine and valine, which are mainly degraded in skeletal muscles, kidneys and heart^{17, 18}. When measuring metabolite levels during an extended fast, ketone bodies and non-esterified fatty acids have shown to increase in the blood already after a few hours. On the contrary, a noticeable increase in BCAAs was first noticed after 24 hours, which is in accordance with amino acids being used primarily for protein metabolism and not as an energy source¹⁹.

Prandial and postprandial conditions

In food, fatty acids, amino acids and glucose, are found as lipids, proteins and carbohydrates, such as starch. These first need to be degraded into smaller components so nutrients can be absorbed from the small intestine, transported through the hepatic portal vein into the liver to then enter the blood circulation. Larger fatty acids and lipids are hydrophobic and therefore absorbed through the lymphatic vessels before entering the circulation (*i.e.* they bypass the liver). Postprandial increased blood concentrations of glucose, amino acids and fatty acids stimulate and potentiate insulin secretion from the pancreatic β -cells²⁰⁻²². This stimulates the peripheral tissue, like skeletal muscles and adipose tissue, to take up these nutrients from the circulation (Figure 1). These adaptations take place to achieve glucose homeostasis. Also, lipolysis and proteolysis in skeletal muscle, liver and adipose tissue are inhibited, which reduces the release of these

metabolites. This process thereby induces the switch from catabolism to anabolism.

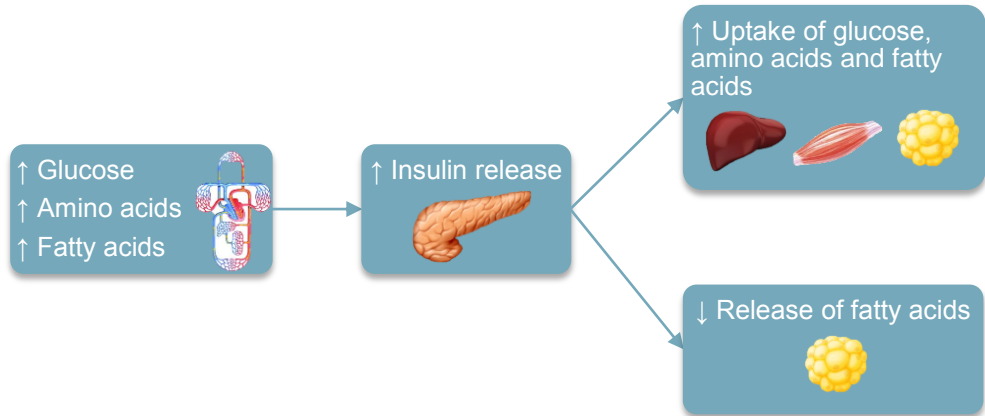


Figure 1 Postprandial regulation

Postprandial circulatory increase of glucose, amino acids and fatty acids leads to increased insulin secretion from the pancreatic β -cells. This further leads to increased uptake of metabolites into peripheral tissues (such as liver, skeletal muscle and adipose tissue) and decreased release of fatty acids from the adipose tissue.

Circulating metabolite levels and obesity

Failure in metabolic regulation is a common trait of obesity which may lead to altered circulating metabolite levels, such as glucose. Pathophysiological changes in people with obesity are often present long before the onset of chronic hyperglycemia and T2D²³. Elevated plasma fatty acids and increased intracellular lipids in obese individuals inhibit insulin sensitivity in muscle, thus dysregulation of fatty acids have been linked to insulin resistance²⁴. It is also believed that chronically high fatty acid concentrations have a “lipotoxic” effect on the pancreas²⁵. Also, increased levels of valine, leucine, isoleucine, tyrosine and phenylalanine, and decreased levels of glycine in non-diabetic obese subjects was reported by Felig *et al* already in 1969²⁶. Others have confirmed these observations through recent advancement in the use of metabolomics techniques, which can efficiently analyze large sets of metabolites²⁷⁻³⁰. In addition, several altered metabolite levels identified in obese, when compared to lean subjects, may be suitable as biomarkers for metabolic complications related to obesity²⁸⁻³⁵. Rauschert *et al* summarized a list of recent reports including potential metabolite biomarkers for obesity³³. For instance amino acids, carnitines and glycerol have

been associated with BMI^{30, 33}. Recent reports have specifically shown elevated levels BCAAs in individuals who are at risk of developing diabetes³⁶, in those with established diabetes^{27, 37}, and in overweight and obese humans²⁷. Also, BCAAs together with aromatic amino acids (tyrosine and phenylalanine), have been associated with, and shown to predict, the development of insulin resistance^{38, 39}, as well as the risk of developing T2D^{36, 40}. Specifically, a set of isoleucine, tyrosine and phenylalanine showed strong predictive potential for incident T2D³⁶. A score calculated from these three amino acids, called diabetes-predictive amino acid score (DM-AA score), has also been associated with risk of future cardiovascular disease (CVD)⁴¹. If there is causation between the BCAA and aromatic amino acids is not known, but they share the same transporter across cell membranes in a competitive manner, *i.e.* uptake is both regulated by the concentration of its own amino acid, but also that of its competitors⁴². Several hypotheses have been presenting trying to link dysregulation of lipids and amino acids, specifically the BCAAs. For instance, the activity of the branched-chain α -ketoacid dehydrogenase (BCKD), which is one of the enzymes in BCAA degradation, may also be inhibited by increased β -oxidation of fatty acids. This would then lead to increased BCAA levels³⁵. In addition, another theory is that over-nutrition leads to preferential use of glucose and lipid substrates, which may lead to a reduced need for amino acid catabolism³⁴. Albeit, one theory is not excluding the other and it is therefore likely that it may be a mixture of several metabolic processes causing the increased levels of BCAAs.

Impaired glucose response

A well-established approach to study the postprandial response is to perform an oral glucose tolerance test (OGTT). Glucose clearance during an OGTT is also used in the clinic to evaluate glucose tolerance⁴³. Healthy glucose tolerant subjects have fasting plasma glucose levels of 5.5 mmol/l or lower and a 2-hour glucose level of <7.8 mmol/l after the OGTT (Figure 2). Insufficient insulin action, either due to decreased insulin sensitivity or insufficient insulin release, results in impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT). People with IFG have elevated fasting plasma glucose (between 5.6-6.9 mmol/l) but normal response during OGTT. IGT is defined as 2-hour glucose levels between 7.8-11.0 mmol/l⁴⁴. IFG and IGT are prediabetic states, and common traits of obesity⁴⁵, that may proceed to develop T2D. T2D is defined as fasting levels of ≥ 7.0 mmol/l and 2-hour plasma glucose levels of ≥ 11.1 mmol/l^{44, 46}.

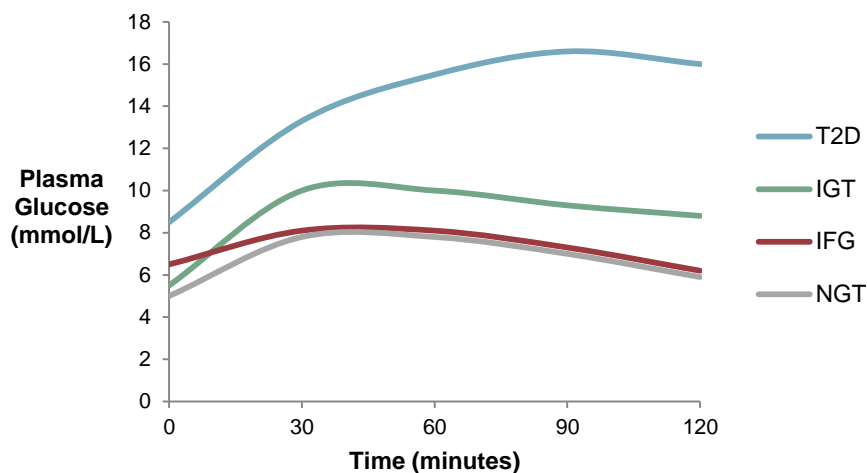


Figure 2 Oral Glucose Tolerance Test

Plasma glucose levels during an OGTT. Graph illustrates example of average glucose response in people with normal glucose tolerance (NGT), the prediabetes states; impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) and type 2 diabetes (T2D). Some individuals may have both IFG and IGT (not illustrated)^{47, 48}.

Metabolite profiles of impaired glucose tolerance

Since obesity is a metabolically complex disorder, it is relevant to study the flux of metabolites that occurs during energy regulation and is thus present in the pathology of obesity and its complications. Metabolomics, the systematic study of small molecules in cells and biological systems, has been performed on blood samples acquired during an oral glucose load in healthy subjects with normal weight^{19, 49-52} and overweight^{52, 53}. In healthy individuals with a normal glucose tolerance, the expected glucose- and insulin-provoked decrease of free fatty acids and glycerol was observed^{49, 50, 52}. This is due to the fact that fatty acid release from adipose tissue is efficiently switched off by insulin, reducing the blood concentration of fatty acids and glycerol. The oral glucose load has also shown decreased circulating levels of amino acids and β -hydroxybutyrate, which reflect the reduced proteolysis and ketogenesis, respectively⁵². Shaham *et al*⁵², and the extended study by Ho *et al*⁵³, observed a blunted decrease in valine, isoleucine/leucine, methionine, β -hydroxybutyrate, pyridoxate, and a blunted increase of lactate during an OGTT in obese insulin resistant subjects. Another study in young obese individuals (20 years), reported 25 metabolite response differences between obese and lean including amino acids (e.g. alanine, glycine, phenylalanine and BCAAs) and fatty acids (C16:0, C16:1, C18:2 and C18:3)⁵⁴.

Weight loss and weight maintenance

Weight loss achieved by a combination of restricted calorie intake, increased physical activity and behavioral support has been reported to reduce the incidence of IGT and the risk of developing T2D in obese individuals⁵⁵⁻⁵⁸. Weight loss can be achieved by restricting caloric intake (e.g. initially by low-calorie diet), bariatric surgery or with drugs (such as orlistat and liraglutide)⁵⁹⁻⁶¹. Bariatric surgery is the most effective way of weight reduction but not all are eligible for surgery, and it would be economically unfeasible to operate everyone who is obese globally. Thus, diet-induced or drug-mediated weight reduction would be preferable. Nevertheless, the drugs that are currently on the market typically report only about 5-10% body weight loss while the corresponding weight loss following bariatric surgery is in the range of 25-30%^{59, 60, 62}. Another dilemma is that weight regain is common, about half of the participants return to baseline weight within five years of weight loss, since it is easy to fall back to previous eating and living habits⁶³. Lifestyle intervention combining low-calorie diet, behavioral therapy, and if possible, physical activity, have showed best success in non-surgical weight loss and also in weight maintenance⁵⁵.

Metabolite changes with weight loss and weight maintenance

Altered fasting metabolite levels observed in insulin resistant obese subjects have revealed several metabolic changes that are related to weight reduction⁶⁴⁻⁶⁷. For instance, elevated saturated fatty acids (myristic-, palmitic- and stearic acid), monounsaturated fatty acids (oleic- and eicosenoic acid) and several polyunsaturated fatty acids (including linoleic- and arachidonic acid) decreased after an 8-week weight loss⁶⁴. These authors also observed a positive association between palmitoleic acid at baseline and change of body fat, but the decrease of fatty acid was not significant. Lien *et al* performed metabolite profiling in 27 obese individuals after a behavioral weight loss intervention, after weight maintenance and after weight regain⁶⁵. They observed change in levels of fasting metabolites, hormones and clinically measured variables, and thereby showing large metabolic change depending on if they were catabolic, anabolic or when achieving a new steady state, at different stages (*i.e.* weight loss, weight gain and weight maintenance). This illustrates that levels of many metabolites, in addition to glucose, were affected by weight change. Another study found BCAAs to predict improvement in insulin resistance with moderate weight loss⁶⁶. However, the potential weight loss associated improvements of the BCAAs are controversial, and further analysis is required⁶⁴⁻⁶⁸. Furthermore, metabolite analysis after weight loss have also been performed in obese children⁶⁹. This study observed significant

increase of glutamine, methionine, the acyl-alkyl phosphatidylcholine PCaeC36:2 and the three measured lysophosphatidylcholines (LPCaC18:1, LPCaC18:2 and LPCaC20:4) one year after weight loss, compared to the group without weight reduction. Thus, intriguing findings regarding metabolite changes due to weight loss have been presented, but further research is necessary to provide clarity in which metabolites are linked to a healthier metabolic profile.

Aims

The overall aim of this thesis was to elucidate the metabolite changes present in obese humans after diet-induced weight loss followed by weight maintenance. I investigated this by including both fasting levels and the metabolite response during an oral glucose load.

Specific aims for the respective papers:

- I** Identify differences in metabolite profiles during an OGTT in insulin resistant obese individuals and insulin sensitive individuals with normal weight.
- II** Investigate if the altered metabolite profiles during an OGTT in obese individuals (from Paper I) are modified with a weight loss and weight maintenance intervention.
- III** Identify fasting amino acid levels that are associated with obesity and investigate if weight loss and weight maintenance can improve levels of these amino acids. We also aim to test if amino acids can predict the potential benefit of a combined weight loss and weight maintenance program.
- IV** Validate previous findings and further explore metabolite data using a larger cohort. Specifically, analyze if weight reduction is associated with baseline metabolite levels and weight loss-induced change in metabolites levels.

General methodology

This section describes an overview of the methodology used in this thesis. For a detailed description, please review the respective paper.

Study participants

Obesity cohort

In all studies, some or all included participants attended the obesity outpatient unit at the Department of Endocrinology, Skåne University Hospital, Malmö, Sweden. Paper I and II included 14 obese participants with IGT, who were subjected to an OGTT. The intervention study (paper II-III) included diet-induced weight loss (per protocol $\geq 10\%$, low-calorie diet of $< 1,200$ kcal/day) and weight maintenance (per protocol $\pm 5\%$ weight change to define weight stability). The intervention also included group-based therapy lead by a dietitian. OGTT was conducted at baseline (paper I), after weight loss and weight maintenance (paper II). In paper III, 12 obese individuals participating in the intervention study were included to evaluate change in fasting levels of glucose, insulin and metabolite levels. None of the participants in paper I-III were diagnosed with T2D, cardiovascular disease or taking any medications related to metabolic disease. In paper IV, a total of 84 individuals visited the outpatient unit and participated in a program with the aim to lose weight by non-surgical means. This weight loss program consisted of participation in behavioral therapy (individually or in group) and a prolonged period of low-calorie diet. Overnight fasted blood samples were collected before and after treatment. The study outline of the obese cohort is illustrated in Figure 3. All participants gave their written informed consent and the ethics committee at Lund University, Sweden, approved the study.

Normal weight glucose tolerant cohort

A small glucose tolerant group with normal weight ($n=6$) was included for comparison in paper I and II. This group has been reported in detail previously⁴⁹.

Metabolite profiling was performed during an OGTT after an overnight fasting, and samples were collected at 15, 30, 45, 60, 75, 90, 105 and 120 minutes. In paper I, data was reanalyzed using only time points 0, 30 and 120 minutes.

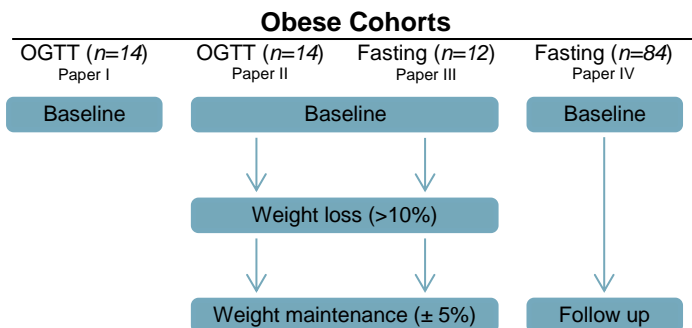


Figure 3 Study outline

Study outline for the obese cohort. Paper I included only OGTT at baseline, and was compared to metabolite response during an OGTT from a lean cohort. Paper II included OGTT at baseline, weight loss and weight maintenance. Paper III included fasting samples of obese participating in the weight loss intervention (pilot) and Paper IV included subjects participating in a weight loss program (validation).

Malmö Diet and Cancer cohort

In paper III, a subset of the population-based Malmö Diet and Cancer (MDC) cohort of 28,449 people, enrolled between 1991 and 1996 in Southern Sweden, was included. A subset of 6,103 individuals was randomly selected to participate in the MDC cardiovascular cohort (MDC-CC)⁷⁰. Metabolomics analysis was performed in subjects from a nested incident CVD case-control study (n=506) with subjects matched for gender, age, Framingham risk score, and from a nested incident diabetes case-control study (n=326) in the MDC-CC⁴¹. A total of 804 subjects were included in the analysis, after exclusion of subjects who participated in both studies and with incomplete data.

Glucose and insulin analysis

Oral glucose tolerance test (OGTT) was performed according to standard procedures of 75g glucose mixed in water and consumed after an overnight fast. Blood samples were collected at fasting (0 minutes) and after 30 and 120 minutes (paper I-II). Plasma glucose and serum insulin levels were measured at fasting (paper I-IV) and during an OGTT (at 30 and 120 minutes, paper I-II). Indices were calculated to evaluate insulin sensitivity and response including; insulin sensitivity

index (ISI), corrected insulin response (CIR), disposition index (DI) and insulinogenic index (IGI)^{47, 71, 72}. Insulin resistance and β -cell function were estimated by the homeostasis model assessment-estimated insulin resistance (HOMA-IR) and HOMA- β , respectively⁷³. How each index was calculated is presented as follows.

$$ISI = \frac{10,000}{\sqrt{([Glucose_{0 \min} \times Insulin_{0 \min}] \times [mean OGTT_{glucose} \times mean OGTT_{insulin}])}}$$

$$CIR = \frac{100 \times Insulin_{30 \min}}{Glucose_{30 \min} \times (Glucose_{30 \min} - 3.89)}$$

$$DI = CIR \times ISI$$

$$IGI = \frac{(insulin_{30 \min} - insulin_{0 \min})}{(glucose_{30 \min} - glucose_{0 \min})}$$

$$HOMA - IR = \frac{(fasting \ glucose \ [mmol/l] \times \ fasting \ insulin \ [\mu U/ml])}{22.5}$$

$$HOMA - \beta = \frac{fasting \ insulin \ [\mu U/ml] \times 20}{fasting \ glucose \ [mmol/l] - 3.5}$$

Metabolomics analysis

The collectively named “omics”-fields include genomics (the study of the genome, *i.e.* “what can happen”), transcriptomics (the study of the transcriptome, *i.e.* “what appear to be happening”), proteomics (the study of the proteome, *i.e.* “what makes it happen”) and metabolomics (the study of the metabolome, *i.e.* “what is happening and has happened”). Hence, metabolomics links the genotype with the phenotype⁷⁴. Metabolomics is the systematic study of metabolites, *i.e.* small molecules (<1500 Da) from cells, tissues or biofluids that are substrates, intermediates or end products of metabolic reactions⁷⁵. Several metabolomics techniques are available that efficiently detect and quantifies compounds, for example gas chromatography mass spectrometry (GC/MS)⁷⁶, liquid chromatography mass spectrometry (LC/MS)⁷⁷ and nuclear magnetic resonance (NMR) spectroscopy⁷⁸. The advantage of NMR is that it is a quantitative method; however, the disadvantages, compared to MS-based methods, are lower sensitivity, dynamic range and resolution. In this thesis, I have not used NMR so it will not be addressed further. Advantages and disadvantages for the use of GC/MS and LC/MS, will be addressed in following paragraphs. Overall, it is possible to

detect up to thousands of metabolites using MS-based techniques, although this necessitates the use of several methods with different characteristics.

Gas chromatography mass spectrometry

In my thesis, I have used GC/MS to analyze metabolites in the obese and normal weight cohorts. The advantages with this method are that it has a high separation efficiency, robustness and throughput, while the main disadvantage is that it only measures volatile analytes⁷⁹. Metabolites are generally non-volatile, but can be made volatile by derivatization⁸⁰. The retention is based on partitioning between the mobile phase (consisting of a carrier gas) and a stationary phase (consisting of a liquid residing on the inside the capillary wall). The GC is coupled to a time-of-flight (TOF) mass analyzer, *via* electron ionization (EI) ion source. In the EI source, an electron is extracted from the analyte, resulting in the formation of an unstable radical cation. This ion then fragments, yielding a set of fragments characteristic for a specific analyte. The hereby formed ions are accelerated into the TOF mass analyzer, where they are separated based on their mass-to-charge ratio (m/z). The analytes are identified based on the time when they elute from the GC column, normally expressed as a retention index, and by matching their fragmentation pattern, *i.e.* their mass spectra, to mass spectra found in databases. The general procedure is illustrated in Figure 4.

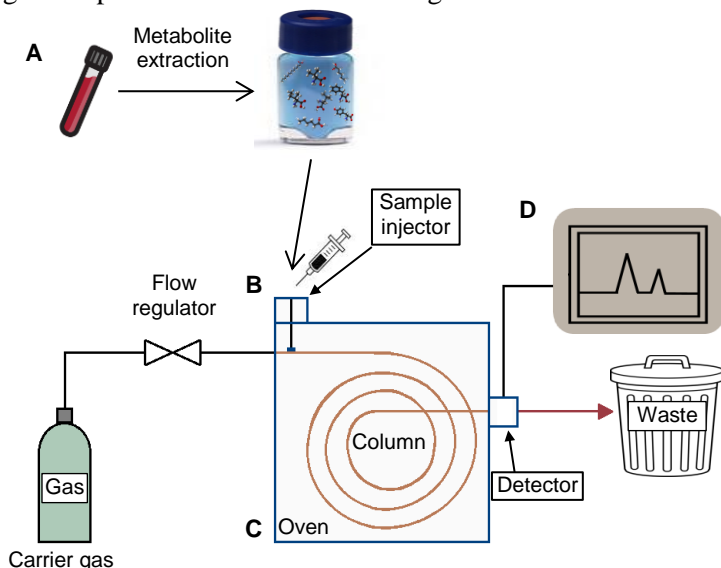


Figure 4 Schematic protocol for metabolite profiling by gas chromatography mass spectrometry

Metabolites are extracted from blood (serum or plasma), derivatized (A) and injected into the gas chromatograph (B). The metabolites are separated based on their partitioning between the gas phase and a thin layer on the inner surface of a capillary column (C). The metabolites are ionized. In this process, several fragments are formed from the same metabolite. These fragments are then separated in the TOF analyzer, and their flight time (*i.e.* the time it takes for them to reach the detector), which is proportional to the m/z of the metabolite, determine (D).

Liquid chromatography mass spectrometry

Amino acids in the MDC-CC cohort was profiled using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and has been described in detailed^{36, 41}. The difference between GC and LC is that in an LC the separation is based on partitioning of analytes between a liquid phase (instead of gas) and a stationary phase that usually is covalently bound to solid particles. An advantage with LC is that it is applicable also to non-volatile metabolites, without any prior derivatization. Hence, LC can be used to analyze large polar metabolites as well as thermolabile metabolites. The disadvantages with LC/MS compared to GC/MS are lower reproducibility, lower robustness, longer analysis times and lower separation efficiency^{74, 79}.

Sample preparation

Sample preparation prior to metabolomics analysis is very important to reduce biases in the determination of metabolite concentrations. In my thesis, I have analyzed both serum (paper I-II and IV, obese cohort) and plasma samples (paper I, lean cohort and paper III, both obese and MDC-CC cohort). Absolute concentration of metabolites may vary between plasma and serum samples⁸¹. However, in my work I have focused on variation, rather than absolute levels.

Analysis by GC/MS is associated with some variation in the extraction and derivatization yield, as well as in the performance of the GC/MS. I have used a cocktail of isotope labeled standards, added prior to extraction, to correct for this variation.

Statistical analysis

Metabolomics techniques generate datasets with a large number of variables (metabolites) and many metabolites are also, since they are often strongly biologically linked, highly correlated. This increases the complexity of analyzing metabolomics data. In addition, analyses by GC and LC are associated with drift, due to e.g. a continuous contamination of the equipment which affects the sensitivity. Because of this we have restricted the number of samples analyzed in a batch to approximately a hundred. Variation within a batch can be corrected for using internal standards⁸², whereas variation between batches is corrected for using other methods. In paper IV we used ComBat to adjust for batch effects^{83, 84}. Samples were scaled to unit variance to reduce inter-individual variations within an analysis and double-centered (paper I-III), *i.e.* normalized to the mean of the

three measurements for each individual (either the three OGTT time points or baseline, weight loss and weight maintenance) to reduce intra-individual variation⁸⁵. Most statistics assumes the data to be normally distributed however sometimes this requirement is not fulfilled. Metabolites with skewed distribution were therefore transformed to resemble a normal distribution prior to analysis.

Multivariate data analysis is a useful tool for examination and visualization of large datasets. Many of these methods aim to find latent variables and structures that more efficiently describe the data (*i.e.* with fewer dimensions), as compared to traditional uni-, bi-, and few-variable methods. For these calculations we used SIMCA 13 (Umetrics, Umeå, Sweden). Principal component analysis (PCA) is an unsupervised method, which aims at describing the variation in the data. PCA was used to examine the datasets for potential outliers and to generate a first overview of the data⁸⁶. Orthogonal projections to latent structures discriminant analysis (OPLS-DA) is a supervised classification method which focuses the analysis on variation in metabolite levels responsible for the class discrimination⁸⁷. OPLS-DA was used to find metabolite patterns discriminating between OGTT time points (paper I-II) or between weight loss phases (paper III). All multivariate models were evaluated by a 7-fold cross-validation⁸⁸. In cross-validation, the analysis is performed on a subset of the samples (*i.e.* training set) and the analysis is then validated in another subset of the data (*i.e.* testing set), this was then performed seven times with different subsets to reduce variability. Significant changes were estimated from jack-knifed confidence intervals⁸⁹, which is based on “leave one out” procedure which means starting from the whole sample, then leaving one sample out and the parameter of interest is estimated from this smaller sample set.

For descriptive analysis of clinical variables, paired Student’s t-test (for normally distributed data), Wilcoxon Signed Rank Test (for skewed data) and χ^2 -test for categorical variables, was used to assess difference in anthropometric data among the obese individuals. Non-parametric Mann-Whitney’s U test was used when comparing measures between obese and lean individuals. Spearman’s rank correlation coefficient was used to test for correlations between different variables or small sets of metabolites. Multiple testing was performed using Benjamini-Hochberg false discovery rate (FDR) correction (paper IV)⁹⁰. Linear regression was performed to associate BMI, or change in BMI with metabolite levels, adjusting for age, gender and T2D status (paper III-IV). Statistics were calculated using IBM SPSS statistics v.20 (IBM Corp. 2011, Armonk, NY, USA) or in R⁸⁴.

Obesity and diabetes scores

OB-BMI score & OB-WC score

In paper III, we aimed to create amino acids profile scores for obesity (OB): OB-BMI and OB-WC, which included amino acids associated with obesity and known risk factors for CVD and T2D status. Noticeably, amino acids were chosen over other known altered metabolites, such as fatty acids, due to recent findings that amino acids alone are associated with metabolic diseases^{36, 39, 41, 91}. This encouraged us to test this for obese subjects as well. In order to construct these scores, backward elimination regression was performed for all amino acids associated with BMI or waist circumference (WC), respectively, in the MDC-CC cohort adjusted for age, sex, diabetes status, anti-hypertension treatment and systolic blood pressure. The resulted amino acids were tested for their association to categorical measures of obesity database (BMI and WC, general obesity [BMI>30 kg/m²], and abdominal obesity [WC>108 cm for men; >88 cm for woman³²]). The OB-BMI and OB-WC scores were constructed from the amino acid levels weighted by the β -coefficients. By constructing a score that includes both amino acids and known risk factors such as blood pressure and age, we may access a metabolic risk score more reflective of metabolic health than solely looking at BMI.

WLWM-BMI score & WLWM-WC score

From the OB-scores we constructed a second set of scores for BMI and WC that aimed to reflect the treatment-modifiable part of the amino acid constitution by including only if they are modifiable in the obesity cohort. Thus, these scores were created using the same variables as for the OB-scores but without those amino acids that did not show improvement as a result of the full weight loss and weight maintenance (WLWM) intervention, *i.e.* WLWM-BMI and WLWM-WC (in Paper III). Hence, the OB-scores assess the total amino acid-associated burden of obesity, whereas the WLWM-scores assess the portion of this burden which may be improved by weight loss and weight maintenance programs. The OB and WLWM-scores were constructed in paper III and further tested in the larger cohort in paper IV.

DM-AA score

The diabetes-predictive amino acid score (DM-AA score) consists of isoleucine, tyrosine and phenylalanine and has shown to predict the risk of developing both T2D and cardiovascular disease^{36, 41}. The DM-AA score is equal to the standardized score of z-score of log isoleucine + z-score of log tyrosine + z-score of log phenylalanine. This score was evaluated both in paper III and IV.

Results and Discussion

Metabolite responses during an OGTT

Differences in metabolite profiles during an OGTT between obese and normal weight individuals (paper I)

In order to access the postprandial alterations present in obesity, an oral glucose challenge was performed in 14 insulin resistant obese individuals (BMI 43.6 ± 1.5 kg/m² [mean \pm SEM]) at three time-points (0, 30 and 120 minutes). These OGTT-profiles were then compared to the response found in an insulin sensitive group with normal weight (BMI 22.4 ± 2.4 kg/m²). Fasting and 30 minute concentrations of glucose in the obese group were the same as in the lean group, whereas 2-hour glucose concentrations were elevated in the obese group ($p < 0.01$). Both fasting and 2-hour insulin concentrations were elevated in the obese group ($p < 0.0001$ and $p < 0.01$, respectively). This together with higher HOMA-IR ($p < 0.0001$), HOMA- β ($p < 0.001$), insulin sensitivity index (ISI; $p < 0.0001$), corrected insulin response (CIR; $p = 0.017$) but not the disposition index (DI) indicated that the peripheral insulin sensitivity was decreased but β -cell activity appeared to be adequate.

Even though IGT is often observed prior to established T2D, not all people with IGT develop T2D. Thus, additional metabolic markers found during a conventional OGTT may improve the understanding of insulin resistance^{45, 46}. In the obese group, 59 metabolite profiles were identified and OPLS-DA analysis showed clear metabolite level separation between the OGTT time points. Figure 5 show an onset-decay-plot of the metabolite response during an OGTT in the obese group. Metabolite levels that are responsible for the classification of OPLS-DA in the obese group reveal several alterations compared to the expected response seen in lean individuals^{49, 52, 53}. Under healthy conditions, circulating fatty acids decrease rapidly upon glucose-provoked insulin release⁴⁹. More specifically, a faster decline has been observed for the monounsaturated fatty acids compared to the saturated counterparts⁵⁰. Although, a delayed response of fatty acids was observed in the obese group (Figure 5). Additionally, the OGTT response of amino acids and their derivatives has been studied and the BCAAs, among others, are also expected to decrease upon insulin release in healthy individuals^{49, 52, 53}.

However, a delayed and/or lack of decrease was observed in the insulin resistant obese group (Figure 5 and paper I).

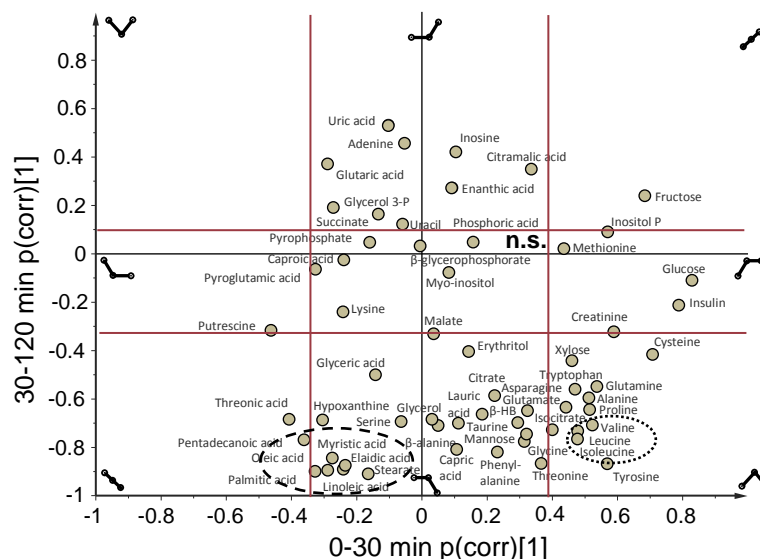


Figure 5 Onset-decay-plots of metabolite levels during an OGTT in the obese group

Figure 3. Cluster study plots of metabolite levels during an OGTT in the obese group. Alterations in metabolite profiles during an OGTT in the obese group. Two horizontal and two vertical lines (red) in the plot represent the relative significance border, i.e. metabolites in the center square have non-significant (n.s.) change during the OGTT. Fatty acid and amino acid clusters are highlighted by dashed and dotted circles, respectively. P(corr)[1]: loadings f0or the predictive component of assigned model scaled as correlations. α -KG; α -ketoglutarate, β -HB; β -hydroxybutyrate, P: phosphate.

When comparing the metabolite OGTT-response in the insulin resistant obese to the glucose tolerant lean group, 16 deviating metabolite profiles (out of the 32 metabolites which were common in both datasets). These deviations were categorized into three groups. 1) Delayed reduction in levels of five fatty acids (including palmitic acid, lauric acid, oleic acid, pentadecanoic acid and stearic acid). 2) Increased levels at 30 minutes of five amino acids (including asparagine, glutamate, taurine, tyrosine, isoleucine and leucine). 3) A blunted increase at 30 minutes of six metabolites (including pyrophosphate, threonic acid, phenylalanine, serine, glyceric acid and aspartate). The delayed responses indicate that insulin resistance in peripheral tissues affects many metabolites, either directly or indirectly. In addition, a delayed decrease in levels of β -hydroxybutyrate, glycerol, hypoxanthine and several amino acids may reflect a dysregulation of ketogenesis, lipolysis, nucleotide degradation and proteolysis^{52, 53}.

Change in metabolite profiles during an OGTT after weight loss and weight maintenance (paper II)

All 14 non-diabetic obese individuals ($\text{BMI}=43.7\pm1.5 \text{ kg/m}^2$) completed the weight loss and weight maintenance program, with a mean weight loss of 17% (BMI change from weight loss $36.2\pm1.7 \text{ kg/m}^2$ to weight maintenance $34.9\pm1.8 \text{ kg/m}^2$). Insulin resistance and sensitivity (judged by fasting insulin, HOMA-IR, ISI) and β -cell function (judged by HOMA- β , CIR, IGI) improved during weight loss, reflecting improved hepatic insulin response. Furthermore, improvement of glucose tolerance (judged by IGT-status or 2-hour glucose levels, $\text{AUC}_{\text{Glucose}}$ and $\text{AUC}_{\text{Insulin}}$), was first noticed after weight maintenance, thus reflecting improved peripheral insulin sensitivity.

A total of 58 metabolite profiles during an OGTT (at fasting, 30 and 120 minutes) at baseline, weight loss and weight maintenance were analyzed in the obese group. In addition, the changes in the OGTT-elicited metabolite patterns occurred differentially during weight loss and weight maintenance. Metabolite OGTT-response improvement was determined from the OGTT response reported in lean healthy individuals^{49, 52, 53}. Out of the 16 altered metabolite profiles identified in paper I, roughly half of these improved towards a lean profile after the weight loss program. Table 1 shows groups of improved metabolite profiles, either after weight loss or weight maintenance, compared to the expected healthy profile. Surprisingly, only three metabolites (tyrosine, malate and pyrophosphate) were identified to have a statistically significant improved profile after weight loss. It is probable though, that more early improvements would be significant in a larger cohort. In addition, phenylalanine was not grouped with weight loss improvement since it did not share the same profile as the primary lean reference group⁴⁹, but it shares the same profile reported by Shaham *et al*⁵². Therefore, further investigation may clarify whether phenylalanine should be included in the weight loss improvement group. In addition, both phenylalanine and tyrosine, which are closely biologically linked, showed alteration during the first 30 minutes. IGI reflects the initial insulin response (during the initial 30 minutes). This motivated the analysis to determine if IGI was associated with change in the aromatic amino acids. Glucose-elicited suppression of the aromatic amino acids tyrosine and phenylalanine was enhanced after weight loss. The enhanced suppression (analyzed as AUC) of tyrosine and phenylalanine, respectively, was associated with improved IGI after weight loss (tyrosine: $r=0.72$, $p=0.013$; phenylalanine: $r=0.63$, $p=0.039$).

The OGTT-elicited suppression and/or lack of increase in levels of nine metabolites, together with glucose and insulin, improved towards the lean profile after weight maintenance, paralleling an improvement in glucose tolerance (Table 1). All identified fatty acids improved towards the lean profile first after weight

maintenance, except stearate, which did not improve during the course of weight loss treatment. Stearate, which is a saturated fatty acid, has shown a slower decrease during OGTT than its monounsaturated counterpart oleate⁵⁰.

Table 1 Metabolite OGTT-profiles at baseline, weight loss and weight maintenance

		OBESE		OBESE		OBESE		LEAN	
	Metabolite (Group)	Baseline 0, 30, 120 min	0-120 p-value	Weight loss 0, 30, 120 min	0-120 p-value	Weight maintenance 0, 30, 120 min	0-120 p-value	Reference profile 0, 30, 120 min	Ref.
Weight loss improvement	Tyrosine (Amino acid)		0.019		0.011		0.004		49, 52
	Malate (TCA-cycle)								49
	Pyrophosphate* (Inorganic ion)								49
Weight maintenance improvement	Glutamate (Amino acid)								49
	Glutamine (Amino acid)				0.023				49
	Isoleucine (Amino acid)		0.035				0.004		49, 52
	Leucine (Amino acid)		0.048				0.005		49, 52
	Laurate (Fatty acid)		<0.001		<0.001		<0.001		49
	Oleate (Fatty acid)		<0.001		<0.001		<0.001		49
	Palmitate (Fatty acid)		<0.001		<0.001		<0.001		49
	Glucose (Carbohydrate)		<0.001		<0.001		0.019		49
	Insulin (Hormone)		0.002		<0.001		<0.001		49
	Hypoxanthine (Purine)		0.019						52, 53
	Glycerol (Sugar alcohol)		0.001		0.055		<0.001		52

Grey shadow represent metabolite profiles at baseline for obese individuals which was presented and published in paper I. *Pyrophosphate improves during weight loss, although return to baseline profile after weight maintenance.

Recent reports have shown that a cluster of BCAAs, together with the aromatic amino acids (tyrosine and phenylalanine), were associated with HOMA-IR in overweight and obese humans³⁸. Importantly, weight loss-mediated improvement of this cluster paralleled an improvement in HOMA-IR⁶⁶. Furthermore, HOMA-IR has been reported to associate with hepatic insulin resistance regardless of glucose tolerance capacity⁹², and therefore, our results may indicate a connection between aromatic amino acids and hepatic insulin sensitivity. Likewise, the improved insulin-mediated deterioration of BCAAs and fatty acids may indicate increased insulin sensitivity in peripheral tissues after weight maintenance. This points towards careful consideration of metabolite markers during a weight loss program to determine when a metabolic improvement is expected to show. Moreover, in paper I and II, we identified several altered metabolite responses during an OGTT to improve towards a healthier profile after participating in the weight loss program. A summary of the metabolic pathways that improved in the obese group after weight loss and weight maintenance, along with markers that were identified during the OGTT, are presented in Figure 6.

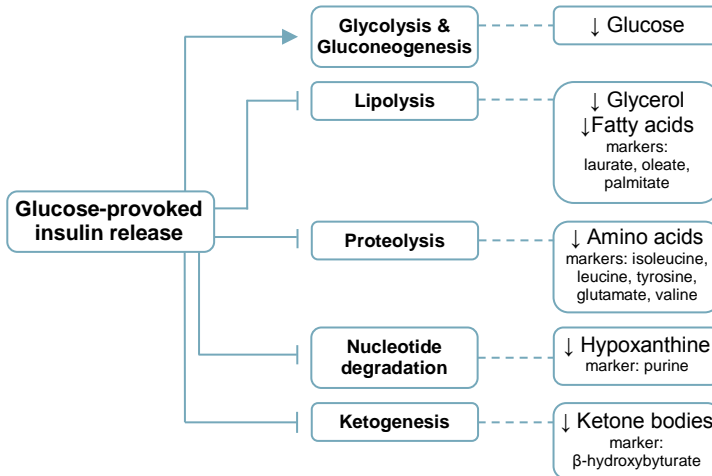


Figure 6 Summary of metabolites during an OGTT

These metabolites (with specific markers pointed out) are expected to decrease in insulin sensitivity situation, but show altered response in obese insulin resistant humans and, more importantly, improve with weight loss treatment. These markers may therefore be used to evaluate improved energy metabolism after weight loss and weight maintenance. Tyrosine improved already after weight loss, whereas the others have a noticeable improvement after weight maintenance, when peripheral insulin sensitivity improved.

Heterogeneity within the obese group (paper I-II)

Metabolite analysis also revealed a larger heterogeneity in metabolite response during the OGTT in the obese group compared to lean (paper I). To illustrate this, we focused on the 2-hour decline of the BCAAs isoleucine and leucine, as well as FFAs (palmitic-, lauric-, oleic-, pentadecanoic- and stearic acid). Concerning isoleucine and leucine, we observed a consistent decrease in lean of $51 \pm 2\%$, whereas in obese the response was scattered with an average of $19 \pm 13\%$. Considering the fatty acids, a strong decrease of $79 \pm 2\%$ was noticed in the lean group and a weaker decrease of $55 \pm 3\%$ in the obese group. A potential explanation may be that the β -cell function (assessed by HOMA- β) associated with levels of isoleucine and leucine at each time point (fasting: $p < 0.0001$, 30 minutes: $p = 0.016$ and 120 minutes: $p = 0.02$). This may indicate the ability to secrete compensatory insulin, is affecting the isoleucine and leucine levels. In addition, the 2-hour fatty acid level or the decrease of fatty acids were associated with HOMA- β ($p = 0.008$), 2-hour glucose ($p < 0.0001$), 2-hour insulin ($p < 0.001$), HOMA-IR ($p = 0.001$) and ISI ($p < 0.0001$). This suggests that fatty acids, which are normally very responsive to insulin, are also strongly affected by insulin resistance. Recent studies have observed elevated BCAA levels together with a high-fat diet to be associated with development of obesity-related insulin resistance^{26, 28}.

Consequently, we also investigated the heterogeneous response in the obese group after weight loss and weight maintenance in paper II. Interestingly, we observed a greater heterogeneity in the response after weight loss than at baseline in the obese, compared to lean subjects (from paper I). Despite this, the heterogeneous response was markedly reduced after weight maintenance (Figure 7).

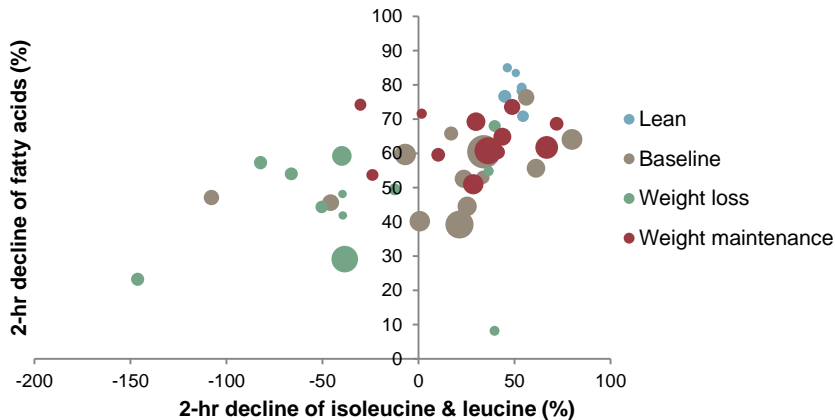


Figure 7 Two-hour decline of fatty acids, isoleucine and leucine during an OGTT

Weight loss decrease of fatty acids (laurate, oleate and palmitate: $-56.0 \pm 4.1\%$) and scattered response of isoleucine and isoleucine ($3.8 \pm 14.2\%$) is more variable than at baseline (reported in paper I). A larger decline of both fatty acids and the BCAAs isoleucine and leucine was observed after weight maintenance (fatty acids: $-73.0 \pm 1.5\%$, isoleucine and leucine: $-28.5 \pm 7.9\%$).

Altered fasting metabolite levels improved with weight loss and weight maintenance treatment

Change in fasting amino acid levels with weight loss and weight maintenance (paper III)

Fasting amino acid levels were studied after the obese individuals participated in the weight loss and weight maintenance program. The obese participants lost on average 20% of their initial weight and sustained this weight during a six month weight maintenance phase ($\pm 3.9\%$). An improved amino acid profile was determined as change toward levels found in normal weight subjects. Out of the 18 amino acids analyzed, improved levels of ten amino acids were observed after weight loss, and a total of eleven amino acids improved after weight maintenance. Lysine and valine improved after weight loss but then returned to baseline levels after weight maintenance.

Further evaluation of change in amino acid levels after participating in a weight loss program (paper IV)

To validate our findings from paper III, fasting levels were analyzed in a larger obese cohort (n=84) before (BMI 42.6 ± 5.6 , mean \pm SD) and after participating in a weight loss program (BMI 36.1 ± 6.5 , duration 0.9 years [range: 0.3-3.1]). Over 70 serum metabolites were identified in all subjects at baseline and follow-up, and 58% metabolites changed with the average weight loss of 18.8 ± 14 kg. Metabolite change from baseline to follow-up was observed for 42 metabolites (58%) and 30 of these metabolites were unique for those with a weight loss greater than 10%. Due to the timespan and structure of the weight loss program, baseline and follow-up in this paper are comparable with baseline and weight maintenance in paper III. Weight loss-induced change in levels of asparagine, alanine, aspartate, tyrosine, phenylalanine, glutamate, isoleucine and leucine were confirmed in this paper and consistent with other reports^{65, 66, 68}. Even so, the decrease in levels of the BCAA valine, could not be confirmed in this report, compared to paper III, and has shown inconsistent change in other reports as well^{64, 66-68}. A summary of metabolites that are modifiable with weight loss and weight maintenance is presented in Figure 8.

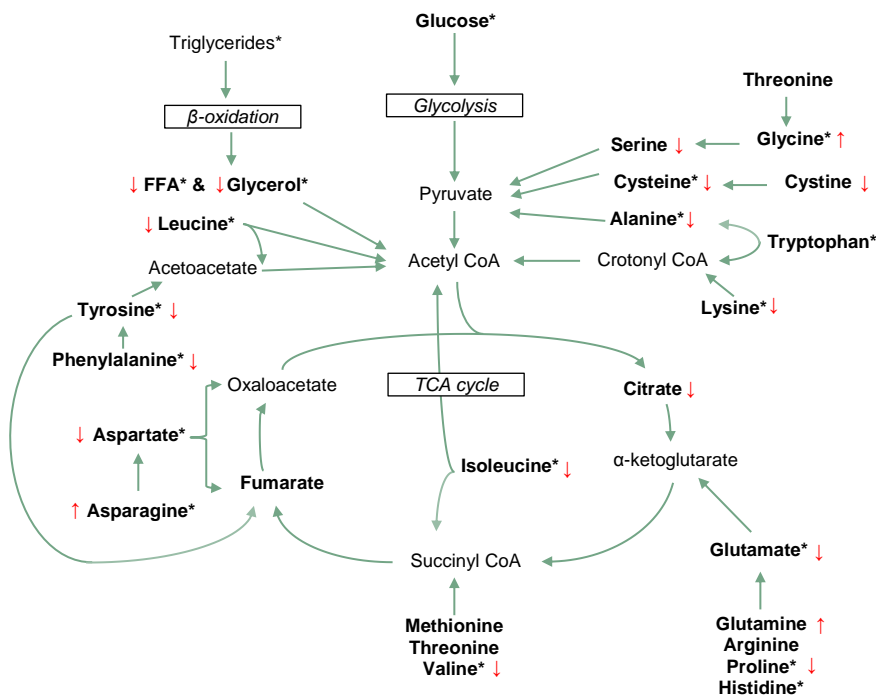


Figure 8 Metabolite changes with weight loss and weight maintenance in obese humans

A schematic overview of some amino acids and general fatty acid involvement in energy metabolism. Bold font indicates that the metabolite was analyzed in this thesis. Red arrow indicates if we observed change during the weight loss treatment. *indicates if the altered metabolite levels have been reported in obese individuals, all except glycine and asparagine are elevated. The potential decrease of valine was not conclusive and needs to be further validated.

Amino acid levels predictive of weight loss treatment

BMI-associated amino acid levels (paper III)

Many studies have observed altered fasting metabolite levels in people with obesity and/or T2D, compared to healthy glucose tolerant individuals^{27-29, 93}. Particularly the amino acids have been associated with metabolic disorders and, due to their diverse and important metabolic role, have recently received more focus^{34-36, 94, 95}. In paper III, fasting amino acid levels were tested against obesity traits (BMI and waist circumference [WC]) in the MDC-CC cohort, an independent prospective study and separated from the obese cohort. After backward elimination, eight of 18 detected amino acids were associated with obesity (positive association: alanine, glutamate, isoleucine, phenylalanine, tyrosine and valine, and negative association: asparagine and glycine), and adjusted for age, gender, T2D, systolic blood pressure and anti-hypertensive treatment. The amino acids were also tested against categorical measures of obesity (BMI and WC, Figure 9).

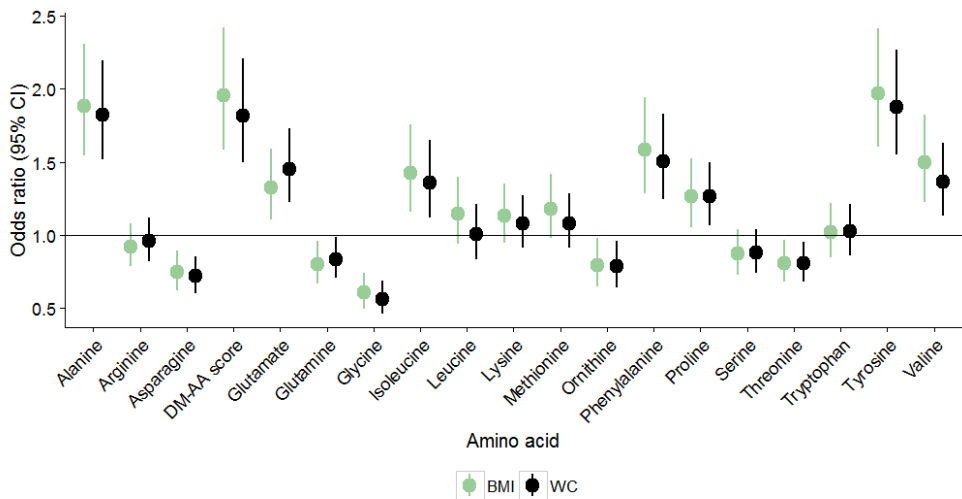


Figure 9 Odds ratio for amino acids associated with obesity

Amino acid levels were analyzed against categorical measures of obesity as either waist circumference (WC) or BMI (WC>88 cm for women and WC>102 cm for men, or BMI≥30 kg/m²). Analysis was adjusted for age, gender, systolic blood pressure, anti-hypertensive treatment and type 2 diabetes status. DM-AA; diabetes-predictive amino acids score (including isoleucine, tyrosine and phenylalanine).

The same amino acids were significant in both the BMI and WC models, which included alanine, asparagine, glutamine, glutamate, glycine, isoleucine, ornithine,

phenylalanine, proline, threonine, tyrosine, valine and the DM-AA score. Since DM-AA score consists of isoleucine, tyrosine and phenylalanine, it is not surprising that this score showed strong significance. However, considering that this score was originally found to predict the risk of developing T2D^{36, 41}, it may be that this score, and thereby also the individual amino acids, are linked to the insulin resistant part of the obese individuals. Others have previously reported association of these amino acids with BMI, however, some studies have also shown contradicting results^{28-30, 96-100}. The most consistent findings have been regarding the BCAAs isoleucine and valine and their elevated levels are associated with increased BMI, as it was also observed in this report^{28-30, 97}.

Obesity and treatment-modifiable scores (paper III)

By identifying BMI-associated amino acids, we could utilize these data to create scores informative of the potential benefit, or lack of benefit, of a weight loss and weight maintenance program based on the amino acid changes observed. Thus, the OB-BMI score represents the overall risk, whereas the WLWM-BMI score represents the treatment-modifiable risk. Both scores were adjusted for gender, age, systolic blood pressure (SBP), anti-hypertensive treatment (AHT) and T2D status. We tested the scores both for BMI and WC but since the association model for amino acids and BMI was higher ($r^2=0.54$) than for WC ($r^2=0.31$), further results are limited to the association with BMI. The amino acids included in the scores are alanine (Ala), asparagine (Asn), glycine (Gly), isoleucine (Ile), tyrosine (Tyr) and valine (Val).

$$OB - BMI = 26.8 + 0.92[Ala] - 1.40[Asn] - 0.58[Gly] - 0.61[Ile] + 1.03[Tyr] + 1.01[Val] \\ + 0.12[sex] - 0.061[age] + 0.02[SBP] + 0.98[AHT] + 1.50[T2D]$$

$$WLWM - BMI = 27.9 + 0.94[Ala] - 1.62[Asn] + 1.33[Tyr] - 0.22[sex] - 0.07[age] \\ + 0.02[SBP] + 1.04[AHT] + 1.84[T2D]$$

Whether or not these scores are applicable for clinical evaluation needs to be tested in an independent study. However, to exemplify and compare the performance of the OB-BMI and WLWM-BMI scores before and after weight loss, we simulated different scenarios using realistic amino acid concentrations. We created two non-diabetic fictional persons, with same sex, age, systolic blood pressure and no hypertensive treatment. They were constructed to have equal pre-

treatment OB-BMI score but containing different amino acid concentration compositions of the amino acids associated with BMI. Even though the amino acid levels are realistic, they were intentionally chosen to be highly modifiable by weight loss for one subject while only partly for the other. The post-treatment OB score differs depending on how these amino acid profiles are affected by treatment illustrating the differential benefit of weight loss over the other (Figure 10). When instead using the treatment-modifiable score, WLWM-BMI, this relative benefit is apparent already when evaluating the obese individuals prior to weight loss.

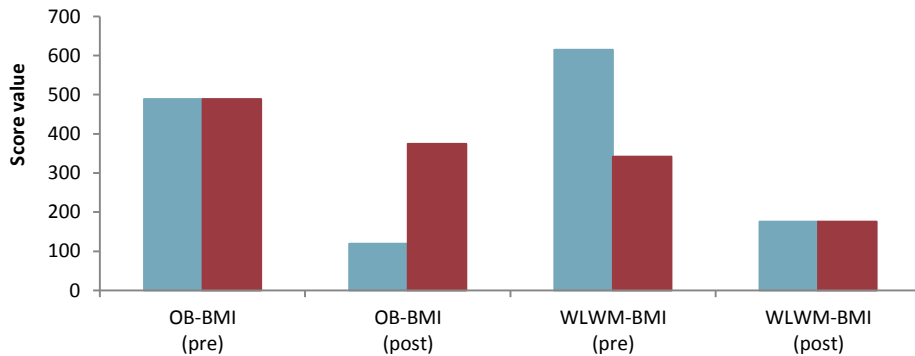


Figure 10 OB-BMI and WLWM-BMI before and after weight loss

Hypothetical, but realistic, amino acid compositions for two individuals and their OB-BMI and WLWM-BMI scores before (pre) and after (post) a weight loss intervention. The OB-BMI score at baseline was set to be equal, although the specific amino acid concentrations were different.

As this example illustrated, two individuals with the same OB-BMI score at baseline, but consisting of different amino acid concentrations, show an expected large score difference after weight loss. Due to this difference in the outcome of OB-BMI score (post), it is noticeable that the score is sensitive to difference in amino acid composition. Thus, due to different concentrations of the same amino acids, one individual (blue) is shown to benefit more over the other (red). When focusing on the amino acids that are expected to improve, *i.e.* WLWM score, then it is possible to see already before treatment which one who would benefit more from the weight loss program. The WLWM-score was different from the OB-score depending on the amount of modifiable burden. Hypothetically, the WLWM-scores could be used in evaluating the treatment-specific likelihood of benefit for an individual, although this needs to be tested in a clinical trial. Previous prediction models of weight loss and weight maintenance have included fasting glucose, HOMA-IR, initial weight trajectories, circulating angiotensin-converting enzyme, inflammatory markers, psychosocial factors, leptin concentrations or first evaluating initial weight loss trajectories to predict the outcome¹⁰¹⁻¹⁰⁷. However, no model has been successfully implemented in clinical praxis so far. There are

several factors that need to be improved for application of such model, like easier analysis tools, better prediction and individualized indicator¹⁰⁸.

Evaluation of the obesity scores in a different weight loss cohort (paper IV)

In paper IV, we used this larger cohort to also evaluate the obesity scores, OB-BMI and WLWM-BMI, from paper III. Neither the OB-BMI nor the WLWM-BMI scores differed at baseline and follow-up when analyzing the complete cohort or when divided into the weight loss groups ($\geq 10\%$ and $<10\%$ weight loss). However, when looking at the change from baseline to follow-up in score level, then both scores showed a significant change ($p < 0.0001$). There was no significant difference when comparing the OB-BMI and WLWM-BMI scores against each other, which may be due to that the scores only differ in a few amino acids, but it may also be due to lack of power. Nevertheless, when comparing the change in scores of an individual from 5 to 10 units decrease in BMI, a larger change was observed in the WLWM-BMI score, compared to the OB-BMI score (Figure 11).

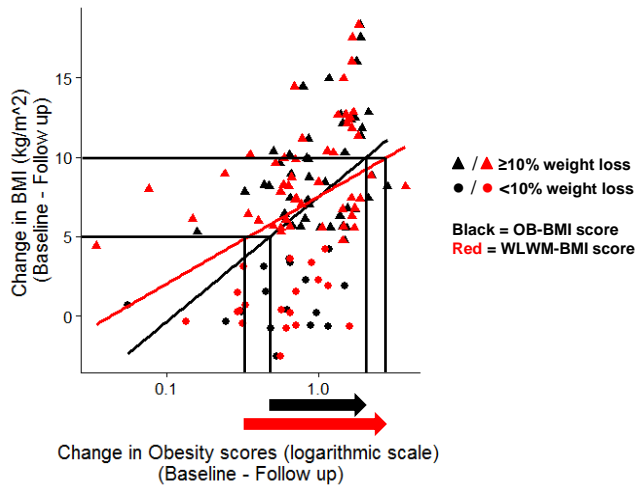


Figure 11 The change in BMI against change in obesity score

Scatter plot illustrating the change in obesity score *i.e.* OB-BMI (black) and WLWM-BMI (red), from baseline to follow-up and the relative change in BMI. Scores were presented in a logarithmic scale. Shape is depending on weight loss: $\geq 10\%$ (triangles) and weight loss $<10\%$ (circles). Correlation coefficient was calculated for each analysis using Spearman's rank correlation test. Correlation for OB-BMI score: $r = 0.58$, $p < 0.0001$ and correlation for WLWM-BMI score: $r = 0.55$, $p < 0.0001$.

The DM-AA score was also analyzed, but excluding participants with T2D ($n=27$). In paper III, we observed a decreased DM-AA score with weight loss treatment. In the current study, we saw a borderline significance ($p=0.07$) in DM-AA decrease in the $\geq 10\%$ weight loss group. On the contrary, a significant increased risk was observed in the $<10\%$ weight loss group. When including all participants, a correlation between the DM-AA score with decreased BMI ($r = 0.39$, $p<0.001$) and with HOMA-IR ($r \geq 0.3$, $p \geq 0.01$) was observed. The DM-AA score, which consists of isoleucine, tyrosine and phenylalanine, has been associated with insulin resistance, both in this report (paper IV) and by others^{39, 66}.

Weight loss-associated metabolite levels

Baseline metabolites associated with change in BMI (paper IV)

Disturbed energy metabolism is a common trait of pre-diabetic obese individuals, although the mechanisms behind it are not yet fully understood. Fortunately, weight loss has shown to improve several obesity-related complications^{9, 45, 56, 57}. It would therefore be profitable to identify metabolites alterations that are already at baseline associated with weight loss success, and thereby provide important information about how to guide treatment options on an individual level. In paper IV, we found decreased BMI to be associated with 13 baseline metabolite levels (Figure 12). Several of these, including glucose, myo-inositol, malate, fumarate, xylitol and heptanoic acid are involved in energy metabolism. Out of these metabolites, 2-aminobutyric acid, glyceric acid and xylitol were significantly associated with categorical separation of weight loss (defined as limit of 10% weight change). We further looked only at xylitol and found that those subjects that had lower levels of xylitol (split by half) had a 5.5-fold larger chance of belonging to the group with $\geq 10\%$ weight loss. How xylitol is absorbed from food and metabolized in humans is not well known, but due to their low calorie count and weak insulin stimulation, it is used as a sweetener. In other words, subjects that eat these food additives were less likely to benefit from this type of weight loss program in this report. However, more research needs to be further conducted to learn more about the metabolic route of xylitol. Since the metabolic regulation and implication of xylitol is largely unknown, it is not possible at this point to determine if xylitol is mainly reflecting dietary habits or metabolic processes. For this reason, it is too preliminary to conclude whether xylitol is involved in a complex phenotype where sweetened food, other than carbohydrates, has a negative influence on energy metabolism, or if it rather reflects at a dietary pattern.

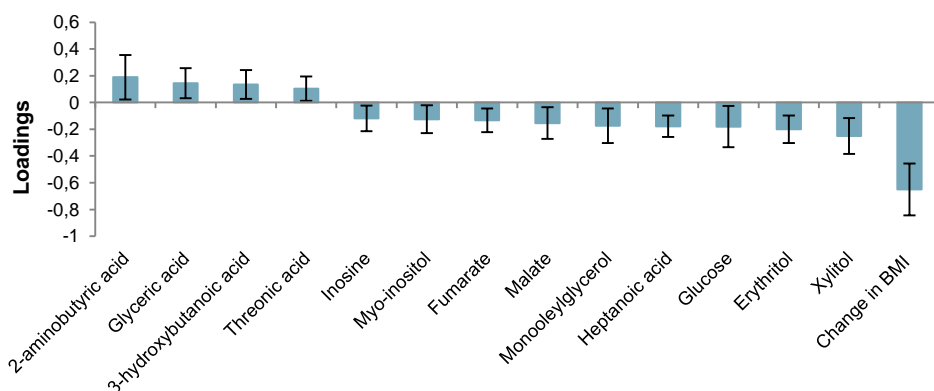


Figure 12 Baseline metabolites associated with change in BMI

Significant metabolites at baseline that was associated with change in BMI. Loadings from the first principal component obtained from multivariate data analysis. Positive loadings reflect higher baseline levels and negative loadings reflect lower baseline levels. Negative change in BMI reflect a decrease from baseline for follow-up.

Weight-loss induced change in metabolite levels associated with decrease in BMI (paper IV)

We also analyzed the association between change in metabolite levels and weight loss (Figure 13). In this model, we identified 15 metabolite levels that changed with weight loss and were associated with a decrease in BMI. These metabolites included the three BCAAs and tyrosine, all of which were also associated with improved insulin resistance (data not shown), and are in accordance with a report by Shah *et al*⁶⁶.

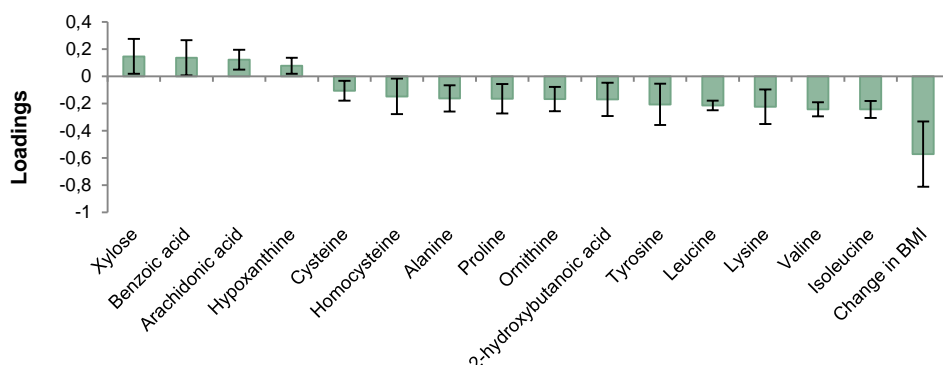


Figure 13 Change in metabolite levels associated with change in BMI

Change in metabolite levels that were significantly associated with change in BMI. Loadings from the first principal component obtained from multivariate data analysis. Positive loadings reflect increased levels and negative loadings reflect decreased levels from baseline to follow-up. Negative change in BMI reflect a decrease from baseline for follow-up.

Major conclusions

In this thesis, I have used a metabolomics technique to explore relative metabolite levels found in glucose intolerant obese individuals after a weight loss regime. With the aim to identify altered levels typical for obesity traits, and those which may improve with non-surgical treatment, might lead to better understanding and evaluation of the heterogeneity normally seen in metabolic disorders. By combining our results, we hope to provide a more comprehensive insight into the metabolite changes in obese humans. Furthermore, we identified over 70 metabolites that are present in the obese state, as well as how it may, or may not, change with weight loss treatment. With our findings, we have been able to glimpse into the metabolic processes occurring after weight loss, which may thus reflect a person's metabolic health.

A summary of the major conclusions:

- ◆ Metabolite profiles during an OGTT in obese individuals compared to lean individuals revealed 16 deviating metabolite profiles. These deviations were categorized into three groups; 1) delayed reduction of five fatty acids, 2) increased levels at 30 minutes for five amino acids, and 3) a blunted increase at 30 minutes of six metabolites (aim I).
- ◆ Roughly half of the 16 deviating metabolite profiles at baseline improved towards the lean profile after the weight loss program (aim II).
- ◆ Differential OGTT improvement of metabolite profile during an OGTT after weight loss compared to weight maintenance. Specifically, aromatic amino acids improved after weight loss when hepatic insulin sensitivity increased. On the other hand, BCAAs and several fatty acids improved first after weight maintenance, concomitant with increased peripheral insulin sensitivity (aim II).
- ◆ We found that the heterogeneity observed in the OGTT response at baseline was surprisingly even more deteriorated after weight loss and distinctly improved after weight maintenance. Specifically, we observed an improved decrease of 34% for isoleucine and leucine, and of 17% for FFA from weight loss to weight maintenance.

- ◆ Ten fasting amino acids were associated with categorical obesity measurements, *i.e.* BMI and WC (aim III).
- ◆ Eleven fasting amino acids improved with weight loss and weight maintenance (aim III), and eight out of these amino acids were validated in a larger cohort, including asparagine, alanine, tyrosine, phenylalanine, glutamate, isoleucine and leucine (aim IV). In total, we observed changed levels of 30 metabolites in the $\geq 10\%$ weight loss group in paper IV.
- ◆ Calculated obesity scores, both for baseline and modifiable with weight loss were constructed in paper III (aim III) and were further analyzed in the larger cohort in paper IV (aim IV). In paper IV, we observed that even though both scores were associated with weight loss, the WLWM-BMI score indicated a higher responsiveness to weight change. Nevertheless, it is possible that a larger cohort and/or additional variables are necessary for a stronger prediction.
- ◆ We identified 13 metabolites at baseline that were associated with weight loss, among which xylitol showed a strong and interesting association (aim IV). Since many of these 13 metabolites are also frequently used sweeteners, these findings may indicate that dietary habits prior to weight loss can influence weight loss success.
- ◆ Finally, weight loss was associated with change in 15 metabolite levels, including amino acids such as BCAAs, tyrosine and lysine (aim IV).

Collectively, an increased understanding of circulating metabolite changes associated with weight loss, as well as sustained weight maintenance, was presented in this thesis. This knowledge is useful to evaluate beneficial weight loss treatment in the future.

Summary in Swedish – Populärvetenskaplig sammanfattning

Fetma – ett (över)viktigt problem

Förr i tiden ansåg man att övervikt och fetma var ett tecken på rikedom och god hälsa då det visade att man hade god tillgång till mat. I takt med människans livsstil har förändrats genom att den är mindre fysiskt krävande och att mat med hög kalorihalt ofta är både lättillgänglig och billigare, har energiförbrukning minskat och energiintaget ökat. År 2014 var 1,9 miljarder (39%) vuxna personer överviktiga i världen, varav 600 miljoner (13%) var diagnostiserade med fetma. Denna fetmaepidemi förutspås att öka så att upp till 50% av jordens befolkning kommer att vara feta år 2030. Normalvikt, övervikt och fetma definieras genom att man räknar ut ett kroppsmasseindex (*eng.* body mass index [BMI], med enheten kg/m^2). Övervikt klassas om man har ett BMI mellan 25,0 – 29,9 kg/m^2 och fetma om man har ett BMI över 30,0 kg/m^2 . Studier har visat att personer med fetma har ökad risk för att utveckla följsjukdomar så som typ 2 diabetes (T2D), hjärt-kärlsjukdomar och sömnapné.

Metabolomik

Metabolomik är en analysmetod då man kan studera mängder med substanser, så kallade *metaboliter* (små molekyler) som finns i vävnader, blod och celler. Detta gör det möjligt att studera ämnen som medverkar i biologiska processer som sker i kroppen. Vi har använt denna metod för att mäta metaboliter i blodet och för att studera metabolismen (ämnasomsättningen) hos individer med fetma.

Bristfällig ämnasomsättning och glukosbelastning

Ett ökat energiintag och otillräcklig energiförbrukning, som kombinerat är den största bidragande faktorn till fetma, resulterar i att fettvävnad lagrar energiöverskottet som fett. Många med fetma får som konsekvens en bristfällig

ämnestsättning då kroppen inte kan ta upp och omsätta överskottet av energi från mat. De näringsämnen som främst används som energi är kolhydrater (som bryts ner till glukos), protein (bryts ner till aminosyror) och fett (fettsyror). Mat tas upp från magsäcken och tarmen till blodet för att förse kroppen med näring och samtidigt frisätter så kallade β -celler i bukspottskörteln insulin. Detta hormon får vävnader att ta upp glukos från blodet och lagra det som glykogen i lever.

Ett sätt att undersöka glukosregleringen efter en måltid är att göra ett s.k. glukosbelastningstest då man efter en natts fasta dricker en sockerlösning och därefter mäter blodsocker- och insulinnivåen i blodet. I normala fall ökar nivån av insulin då sockernivån ökar, vilket får kroppen att ta upp socker från blodet till vävnader, så den ökade blodsockernivån sjunker tillbaka till grundnivån (sker oftast inom två timmar). Det är vanligt att personer med fetma har försämrad förmåga att reglera glukosnivån till en normal nivå och de har då utvecklat insulinresistens. Detta kan med tiden utvecklas till T2D. Vi har under glukosbelastningstestet studerat andra metaboliter än glukos i blodet hos personer med fetma. Det är nämligen känt sedan tidigare att även nivåerna av aminosyror och fettsyror sjunker vid frisättning av insulin hos friska individer, men det är mindre känt hur de beter sig vid fetma. Vi har jämfört resultatet från glukosbelastningen vid fastande, och efter 30 samt 120 minuter mellan individer med eller utan fetma. Vi kunde då se att de personerna med fetma hade en halvtimmes försenad sänkning av fem fettsyror jämfört med friska personer. Hos de överviktiga personerna ökade aminosyranivån de första 30 minuterna innan de började minska. Medan hos de friska personerna inte fanns någon ökning, bara sänkning. Dessa skillnader ger en inblick hur en rubbad metabolism ser ut vid fetma efter en måltid. Vi har härmed identifierat metaboliter med försämrad respons som är relaterat till den metabolt komplexa åkomman fetma.

Viktnerdgångsbehandling

Dagens hjälp för att gå ner i vikt är väldigt generaliserad och inga tydliga riktlinjer finns. Viktuppgång efter avslutad behandling är vanligt. Vår forskargrupp har därför studerat olika metaboliter så som aminosyror, fettsyror och kolhydrater efter viktnerdgång, samt efter cirka sex månaders viktstabilitet. Syftet med att studera detta är att få en ökad förståelse över potentiell förbättring i ämnestsättning.

Glukosbelastning efter viktnerdgång

När vi tittade på metaboliternas respons under en glukosbelastning hos personer med fetma efter att de gått ner mer än 10% av sin initiala vikt såg vi att

aminozyran tyrosin och potentiellt fenylalanin, en relaterad aminosyra till tyrosin, hade en förbättrad profil efter 30 minuter. Även insulinresistensen verkade vara förbättrad i levern efter vikttnedgångsfasen. Något förvånande såg vi att fler aminosyror så som isoleucin och leucin, samt några fettsyror profil förbättrades först efter viktstabilitetsfasen. Då verkade även insulinkänsligheten i övrig vävnad (så som muskler och fettvävnad) förbättras.

Aminosyror relaterade till BMI och förändring vid vikttnedgång

Vi och andra forskare har sett att vissa aminosyror är associerade med BMI. Exempelvis så har ökad blodkoncentration av isoleucin, tyrosin och fenylalanin observerats i personer med fetma. Dessa aminosyror har även associerats till insulinresistens och T2D. I vår studie kunde vi se att viktminskning resulterade i minskade nivåer i dessa aminosyror, och de bibehölls lägre även vid viktstabilitet. Genom att studera aminosyror relaterade till BMI och vilka som förbättrades med vikttnedgång kunde vi konstruera en ekvation med aminosyror som var relaterad till BMI. Denna ekvation inkluderade även status på högt blodtryck och T2D och därmed även fångade andra fetmarelaterade riskfaktorer. I en annan vikttnedgångstudie, kunde vi testa ekvationen och såg att risknivån sänktes med vikttnedgångsbehandling. Dock är det för tidigt att utvärdera om ekvationen är tillräcklig för att kunna användas på individuell nivå.

Sammanfattning

Vår forskning har identifierat en förbättring i flera fetma-relaterade aminosyror och fettsyror hos personer med fetma efter en vikttnedgångsbehandling som även inkluderade en viktsabilitetsfas. Vi såg att förbättringen sker i olika faser, sannolikt relaterat till när insulinkänsligheten förbättras vid vikttnedgång. Slutligen kan vi bekräfta att metabolomik är en användbar metod för att studera metaboliter hos personer med fetma för att få ökad förståelse över vilka metaboliter som kan tänkas användas för utvärdering av försämrade, men även förbättrade, ämnesomsättning.

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May the Fourth Be With You

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