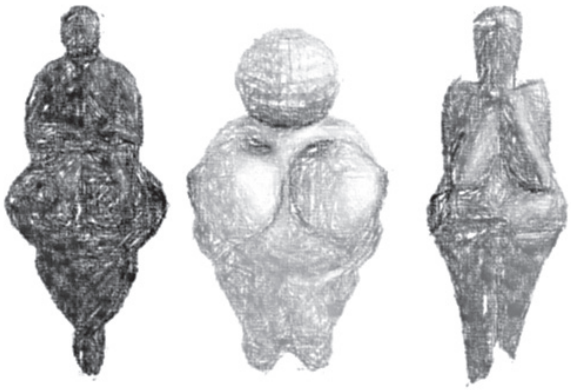
STUDIES IN SPORT, PHYSICAL EDUCATION AND HEALTH
244

Petri Wiklund

Body Composition and Molecular Reflections of Obesity-Related Cardio-Metabolic Disorders

A Cross-Sectional and Longitudinal Study in Women



JYVÄSKYLÄN YLIOPISTO

STUDIES IN SPORT, PHYSICAL EDUCATION AND HEALTH 244

Petri Wiklund

Body Composition and Molecular Reflections of Obesity-Related Cardio-Metabolic Disorders

A Cross-Sectional and Longitudinal Study in Women

Esitetään Jyväskylän yliopiston liikuntatieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston vanhassa juhlasalissa S212
elokuun 27. päivänä 2016 kello 12.

Academic dissertation to be publicly discussed, by permission of
the Faculty of Sport and Health Sciences of the University of Jyväskylä,
in building Seminarium, auditorium S212, on August 27, 2016 at 12 o'clock noon.

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2016

Body Composition and Molecular Reflections of Obesity-Related Cardio-Metabolic Disorders

A Cross-Sectional and Longitudinal Study in Women

STUDIES IN SPORT, PHYSICAL EDUCATION AND HEALTH 244

Petri Wiklund

Body Composition and Molecular Reflections of Obesity-Related Cardio-Metabolic Disorders

A Cross-Sectional and Longitudinal Study in Women

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2016

Editors
Ina Tarkka
Faculty of Sport and Health Sciences, University of Jyväskylä
Pekka Olsbo, Timo Hautala
Publishing Unit, University Library of Jyväskylä

URN:ISBN:978-951-39-6726-0
ISBN 978-951-39-6726-0 (PDF)

ISBN 978-951-39-6725-3 (nid.)
ISSN 0356-1070

Jyväskylä University Printing House, Jyväskylä 2016

"Seek simplicity and distrust it"
-Alfred North Whitehead

To my family and mentors for their love and support

ABSTRACT

Wiklund, Petri
Body composition and molecular reflections of obesity related cardio-metabolic disorders: A cross-sectional and longitudinal study in women
Jyväskylä: University of Jyväskylä, 2016, 110 p.
(Studies in Sport, Physical Education, and Health
ISSN 0356-1070, 244)
ISBN 978-951-39-6725-3 (nid.)
ISBN 978-951-39-6726-0 (PDF)
Finnish summary.
Diss.

Obesity is a major risk factor for the development of cardio-metabolic disease. Unfortunately, our understanding of the role of adiposity and the molecular mechanisms underlying this relationship is still limited. The purpose of this thesis was to investigate and to identify biomarkers that associate with cardio-metabolic risk in young and middle-aged women and peripubertal girls. The thesis was based on two separate studies: EWI study and Calex family study. The study subjects were 100 overweight and obese women (mean age 41.7 years) from the EWI study, 110 women (mean age 36.1) and 396 peripubertal girls (mean age 11.2 years at baseline) from the Calex family study. Body composition, visceral and ectopic fat, serum metabolomics, and adipose and skeletal muscle transcriptomics were assessed. We *first* assessed metabolic profiles of adults with metabolic syndrome, non-alcoholic fatty liver disease and insulin resistance using a cross-sectional study design. The study revealed a key discriminatory role of circulating branched amino acids for individuals with metabolic disorders. Not only was elevated serum branched-chain amino acid level associated with poor metabolic health, but this was also reflected in subcutaneous adipose tissue gene expression profiles. We *then* explored whether increased cardio-metabolic risk in adulthood associated with adiposity originates from childhood, and whether the key discriminatory role of serum branched amino acids found in adults exists already in children. We found that children who were of normal weight but had high body fat percent were susceptible to increased cardio-metabolic risk in adulthood. Furthermore, high levels of branched chain amino acids in childhood were predictive of increases in triglycerides and cardio-metabolic risk later in life. Overall, these results suggest that branched-chain amino acids are viable biomarkers to assess cardio-metabolic health, and thus provide a rationale for continued investigation of the relationship between branched-chain amino acid metabolism, adipose tissue function, and metabolic health.

Keywords: body composition, biomarkers, cardio-metabolic disorders, metabolomics, obesity

Author´s address

Petri Wiklund, pt, MSc
Department of Health Sciences
University of Jyväskylä
40014 Jyväskylä
Finland
petri.wiklund@jyu.fi

Supervisors

Professor Sulin Cheng, PhD
Department of Health Sciences
University of Jyväskylä
Jyväskylä, Finland

Professor Markku Alen, MD, PhD
Department of Medical Rehabilitation,
Oulu University Hospital and
Center for Life Course Health Research,
University of Oulu,
Oulu, Finland

Professor Urho Kujala, MD, PhD
Department of Health Sciences
University of Jyväskylä
Jyväskylä, Finland

Reviewers

Associate Professor Tuomo Rankinen, PhD,
Human Genomics Laboratory,
Pennington Biomedical Research Center,
Baton Rouge, LA 70808, USA

Associate Professor David M. Mutch PhD,
Department of Human Health and
Nutritional Sciences, University of Guelph,
Guelph, ON, Canada N1G 2W1

Opponent

Professor Marjo-Riitta Järvelin, MD, PhD,
Faculty of Medicine, School of Public Health,
Imperial College, London, United Kingdom

ACKNOWLEDGEMENTS

This thesis has been written on the basis of studies conducted in the scientific group of Sports and Exercise Medicine at the Department of Health Sciences, University of Jyväskylä. This work began in 2009 as a simple resting energy expenditure study in overweight and obese individuals, but it turned out to be a 7-year endeavor to explore and understand how obesity develops and why obesity causes specific diseases in some people but not in others. Many research articles were published during these years but only few of them are presented herein. These articles are the result of many people, who I wish to thank.

First and foremost I wish to thank my main supervisor, Professor Sulin Cheng. The immeasurable amount of support and guidance you have provided me throughout these years has been truly invaluable. You were always willing to share your wisdom, knowledge, and skills and you have always been genuinely concerned about me and my success. You helped me through tough times and showed me how to find the opportunity in the difficulties I was facing. You held me to high standards, congratulated me when I met your expectations and corrected me when I failed to do so – but in a way where I learned what not to do the next time. Your perseverance, integrity and the passion you have for science continue to inspire me.

I am also deeply grateful to my other supervisor Professor Markku Alén who has taken a great effort to improve my scientific skills. Your professional expertise, insightful opinions and inspiring criticism have truly improved my scientific thinking. With your guidance I have become a better writer and a better researcher. The thoughtful discussions we have had about science and life have inspired me to pursue my goals with hard work and dedication. Everything I have learned from you will forever remain a major contributor behind my success and achievements. I am also indebted to my third supervisor Professor Urho Kujala for his support and professional expertise throughout my studies. Your door was always open for me when I needed advice in scientific issues. Your affluent encouragements have made me more confident, and your constructive criticism push me to think more.

I gratefully acknowledge Professor David Mutch and Tuomo Rankinen who reviewed the manuscript of the thesis and gave me valuable criticism and suggestions for the completion of the work. It is also an honor for me to have Professor Marjo-Riitta Järvelin as the opponent in the public defense of this thesis.

My colleagues Eveliina Munukka, Satu Pekkala, Xiao Tan and Leiting Xu deserve my sincere thanks for their helpful suggestions, hard work on data collection, thoughtful discussions and friendship. I owe special thanks to Ms. Shumei Cheng for her hard work on data collection and whose delicious Chinese food I have had the privilege to enjoy during all these years. I also wish to thank Timo Törmäkangas, Reija Autio and Lingjia Kong for their valuable expertise in statistics.

My deepest appreciation goes to my parents for all their unconditional love and support they have given me. And to my mother in particular, who, when I was a school boy, always encouraged me to ask good questions. Finally, I would like to extend my heartfelt gratitude and appreciation for the overwhelming display of love and support my wife Tiina and our sons Viska and Vili have given me. This thesis is dedicated to you.

I want to acknowledge the Academy of Finland, Finnish Ministry of Education and EVO research grants from Oulu University Hospital for their financial support for this work.

Jyväskylä, July 2016
Petri Wiklund

FIGURES

TABLES

LIST OF ORIGINAL ARTICLES

This dissertation is based on the following original articles, which will be referred to in the text by their roman numerals (I-V).

I Wiklund PK, Pekkala S, Autio R, Munukka E, Xu L, Saltevo J, Cheng S, Kujala UM, Alen M, Cheng S. Serum metabolic profiles in overweight and obese women with and without metabolic syndrome. Diabetology and Metabolic Syndrome 2014 Mar 20; 6(1):40.

II Cheng S, Wiklund P, Autio R, Borra R, Ojanen X, Xu L, Törmäkangas T, Alen M. Adipose tissue dysfunction and altered aystemic amino acid metabolism are associated with non-alcoholic fatty liver disease. PLoS One 2015 Oct 6; 10(10):e0138889.

III Wiklund P, Zhang X, Pekkala S, Autio R, Kong L, Yang Y, Keinänen-Kiukaanniemi S, Alen M, Cheng S. Insulin resistance is associated with altered amino acid metabolism and adipose tissue dysfunction in normoglycemic women. Scientific Reports 2016 Apr 15; 6:24540

IV Wiklund P, Törmäkangas T, Shi Y, Wu N, Vainionpää A, Alen M, Cheng S. Normal weight obesity and cardio-metabolic risk in females: A 7-year longitudinal study from pre-puberty to early adulthood. (Submitted for publication)

V Wiklund P, Zhang X, Tan X, Keinänen-Kiukaanniemi S, Alen M, Cheng S. Serum amino acid profiles in childhood predicts triglyceride level in adulthood: A 7-year longitudinal study in girls. Journal of Clinical Endocrinology and Metabolism 2016 May; 101(5):2047-55

Additionally, some previously unpublished results are included in the thesis.

ABBREVIATIONS

ANOVA	Analysis of variance
BMI	Body Mass Index
DEG	Differentially expressed genes
DXA	Dual-energy X-ray absorptiometry
EMCL	Extra-myocellular lipid
FDR	False discovery rate
HOMA-IR	Homeostatic model assessment of insulin resistance
IMCL	Intra-myocellular lipid
KEGG	Kyoto Encyclopedia of Genes and Genomes
LTPA	Leisure-time physical activity
MetS	Metabolic syndrome
MHO	Metabolically healthy obese
MRI	Magnetic resonance imaging
NMR	Nuclear magnetic resonance
NWO	Normal weight obesity
PCA	Principal Component analysis
TRM	Time relative for menarche
VO_{2max}	Maximum oxygen uptake

CONTENTS

1 INTRODUCTION

Until the first decades of the 20th century, food shortages and malnutrition were major public health problems and a great socio-economic burden on societies (Caballero 2007). However, with the advent of industrialized agriculture and the forces of globalization, food supplies of today are nothing less than profuse, providing unprecedented quantities of affordable, easily accessible, energy-dense foods (Eknoyan 2006). Simultaneously, mechanization has impinged upon our modes of living in such profound ways that the energy expenditure required for daily living has decreased considerably (Church, Thomas et al. 2011). Together these changes have created an environment that encourages people to eat too much and not do enough physical activity (Swinburn, Egger et al. 1999). This contemporary way of living has spread inexorably across societies in all parts of the world in recent decades. As a result, the number of overweight and obese people in the world today is greater than ever before in the history of human evolution; over 2 billion adults are currently considered to be overweight or obese (Ng, Fleming et al. 2014), and all indications are that these numbers are only going to increase in the foreseeable future (Finkelstein, Khavjou et al. 2012).

The concern surrounding obesity as a public health epidemic lies in the far reaching negative effects it has on health (Pi-Sunyer 2002). The conventional wisdom is that obesity leads to high blood pressure (Kotsis, Nilsson et al. 2015), insulin resistance and glucose intolerance (Kahn, Hull et al. 2006), fattening of the liver (Fabbrini, Sullivan et al. 2010) and lipid disorders such as hypertriglyceridemia (Subramanian and Chait 2012). For these reasons, obesity increases the risk of cardiovascular disease and type II diabetes (Boden and Salehi 2013). As research aims to improve our understanding of the ways in which obesity influences cardio-metabolic health, it has become clear that not all obese individuals develop these metabolic disorders (Badoud, Perreault et al. 2015). Paradoxically, a good number of people with normal body weight have signs of cardiovascular disease or metabolic dysfunction (Ruderman, Chisholm et al. 1998; Conus, Rabasa-Lhoret et al. 2007). This suggests that obesity-related metabolic complications are not attributable to excess body weight alone. Consequently,

the underlying molecular mechanisms of obesity-related metabolic disorders remain incompletely understood.

Cardio-metabolic diseases tend to develop gradually over time; therefore, subclinical signs of their presence can be observed long before clinical manifestations occur. Early and accurate identification of high-risk individuals who are seemingly healthy but have underlying illness would provide an optimum window for preventive treatments (Soininen, Kangas et al. 2015). Thus, biomarkers that accurately predict diseases are of particular importance (Roberts and Gerszten 2013). Metabolomics is nowadays increasingly applied in biomarker discovery because it can provide highly reproducible, high-throughput quantitative data on systemic metabolism in a very cost-effective manner (Ala-Korpela, Kangas et al. 2012). Such comprehensive systemic data is well suited for cardio-metabolic risk research because it can simultaneously explore multiple metabolic pathways. Therefore, serum metabolite profiling is an attractive approach with which to obtain a broad understanding of the molecular perspectives for disease progression

In this thesis I provide a summary of our five original research articles that focus on typical interrelated cardio-metabolic disorders associated with obesity. The purpose is to explore the relationship between adiposity and cardio-metabolic risk, and through metabolomic and transcriptomics approaches to identify biomarkers that associate with common obesity-related metabolic disorders in children and adults.

2 REVIEW OF THE LITERATURE

2.1 Obesity

2.1.1 Definition

Obesity is generally defined as an abnormal or excessive accumulation and storage of fat in the body to the extent that health may be impaired (WHO 2000). However, determining the level and location of adipose tissue when it is likely to affect health is a challenging task and requires accurate assessment of the body fat content. The fat content of the body can be measured in a number of ways. It can be measured by hydrostatic weighing (underwater weighing) or air-displacement plethysmography (Fields, Goran et al. 2002), which give a measure of the body density or volume that can be used to calculate body fat percentage. Or it can be assessed using simple field methods like skinfold thickness measurement or by bioelectric impedance assessment (BIA), which is based on the electrical conductivity of the body. The most accurate measures of the fat content and its distribution in the body are provided by contemporary imaging techniques such as dual-energy X-ray absorptiometry (DXA), computerized tomography (CT) and magnetic resonance imaging (MRI) (Fosbol and Zerahn 2015). In terms of percent body fat, obesity has been defined as 25% or greater in men and 35% or greater in women (Grundy 2004). However, assessing percent body fat using imaging techniques is inconvenient and costly. Furthermore, neither the world health organization (WHO) nor any other major society involved in obesity research has defined a normal value for percent body fat (Romero-Corral, Somers et al. 2010). For these reasons, percent body fat is rarely used to define obesity in clinical practice and large scale population studies.

In epidemiological surveys and clinical/public screening in adult populations, anthropometric measures such as body weight and height are the most frequently used method for assessing adiposity. Obviously, excessive adiposity cannot be defined solely from body weight, since a short, firmly-built person

may have the same body weight as a tall slim person. However, indices of body weight adjusted for stature are frequently used to estimate body fat content. Different combinations of weight and height are used to defined these indices (Colliver, Frank et al. 1983), but Body Mass Index (BMI), calculated as the weight in kilograms divided by the height in meters squared (Keys, Fidanza et al. 2014), is the most widely used anthropometric measure of adiposity (Heo, Kabat et al. 2013). The popularity of this method is due to its simplicity, reproducibility, and the relatively good correlation it has with body fatness (Gallagher, Visser et al. 1996; Blew, Sardinha et al. 2002; Flegal, Shepherd et al. 2009). Overweight is commonly defined as a BMI between 25 and 29.9, and BMI >30 indicate obesity (TABLE 1) (WHO 2000). These cut-off values are selected based on their approximate risk related to disease, and they apply for both men and women. However, a significant limitation of BMI is that it does not distinguish between fat and fat-free mass, nor does it provide any information about the location of fat in the body (Garn, Leonard et al. 1986). Thus, in essence BMI is a measure of excess weight, not necessarily of excess fat.

TABLE 1 Weight status based on Body Mass Index in adults and children (WHO 2000).

Weight status category	**BMI range in adults**	**BMI percentile range in children**
Underweight	less than 18.5	less than 5th percentile
Normal weight	18.5 to 24.9	5th percentile to 85th percentile
Overweight	25 to 29.9	85th to 95th percentile
Obese	30 or greater	above 95th percentile

In children and adolescents anthropometric measures are also the most commonly used methods to assess adiposity. However, determining the level of adiposity using these measures is slightly more problematic in children than adults because of rapid growth and development. During growth height increases and body composition undergoes considerable changes (Cheng, Volgyi et al. 2009), thus classification of overweight or obesity using a single measure is difficult. To overcome this problem many countries have developed national growth reference charts that indicate the normal changes in weight and height in children (Lahti-Koski 2004). These charts are constructed by observing the growth of large numbers of normal children over time. The changes in weight and height of a child can be compared to the expected values of children of the same sex and age, and to evaluate whether the child is growing appropriately (Saari, Sankilampi et al. 2011). Childhood and adolescent obesity has typically been defined based on these charts using specific cut-offs. A commonly used cut-off to classify obesity is ≥ 120% of the median weight for age, height and sex (Lahti-Koski 2004). Another way to classify obesity in children is to use certain percentiles in the reference growth curves. In this approach, the 85th percentile has often been used as the cut-off for overweight and the 97th percentile for obe-

sity (Lahti-Koski 2004). A more precise measure of weight status can be obtained by calculating the Z-score by subtracting the reference value from the measured weight and dividing by the standard deviation of the reference population. Using this method, obesity is usually defined as Z-score + 2 or more standard deviations. This approach is often used in research because it provides a comparable measure of weight status that is not influenced by gender and age (Lahti-Koski 2004).

BMI is also widely used to assess adiposity in children and adolescents (Chinn 2006). Whereas, in adults, the BMI cutoffs are based on fixed values related to health risk, in children finding risk related cutoffs is difficult because, in general, children are free from cardiovascular disease (Flegal and Ogden 2011). Therefore, statistical definition of BMI using the 85th and 95th percentiles for age and gender are commonly used cut-offs to classify overweight and obesity in childhood (TABLE 1) (Cole, Bellizzi et al. 2000; Cole and Lobstein 2012). In addition, WHO has defined overweight as a BMI Z-score greater than 2 and obesity as greater than 3 (WHO 2008). As with adults, the relationship between BMI and adiposity varies according to body composition in children (Pietrobelli, Faith et al. 1998), and therefore the diagnostic performance of BMI is not optimal to identify excessive adiposity in children either.

2.1.2 Prevalence

The prevalence of obesity in adults has increased substantially across the globe during the last three decades (FIGURE 1). Using the BMI criteria, over 1.9 billion adults, 18 years or older are currently considered overweight and over 600 million are obese (WHO 2015). The prevalence of obesity is highest in United States and lowest in Asian countries such as Japan. In the European region, the highest obesity rates are found in England, where one in four adults is obese (Doak, Wijnhoven et al. 2012). In Finland, BMI has continuously increased during the past three decades in adults (Vartiainen, Laatikainen et al. 2010), and currently, 20.7% of men and 24.1% of women are considered obese (Lahti-Koski, Seppanen-Nuijten et al. 2010). All indications are that the prevalence of overweight and obesity will continue to increase in the years to come (Finkelstein, Khavjou et al. 2012).

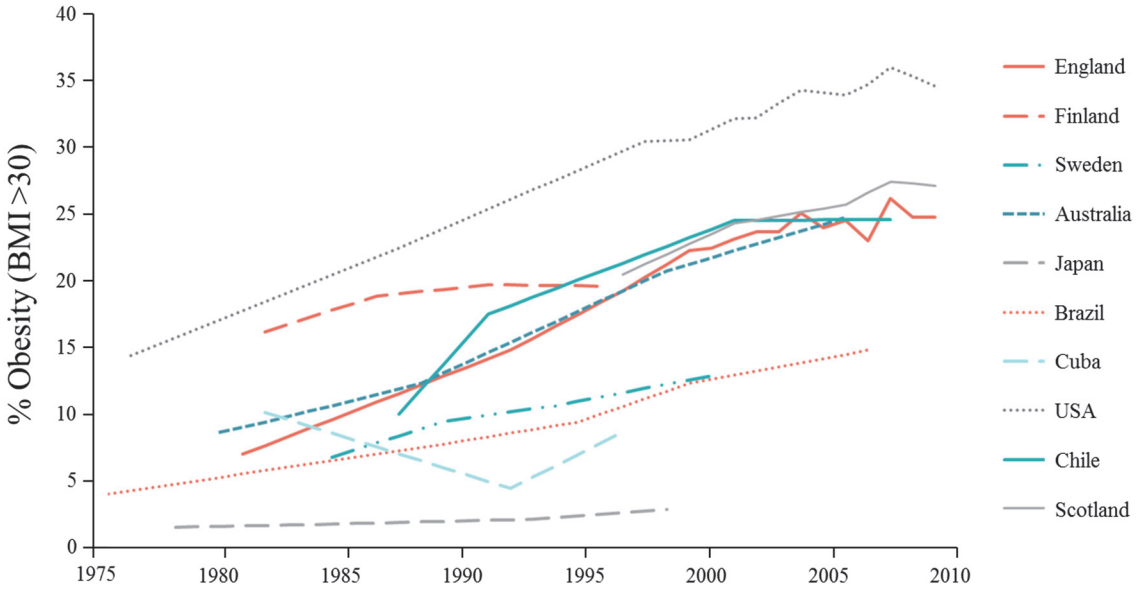


FIGURE 1 Percent changes in adult obesity prevalence over time in selected countries around the world (Trends in Global obesity 2016).

The prevalence of overweight in children has also increased considerably in recent decades (FIGURE 2) (Cunningham, Kramer et al. 2014; Lobstein, Jackson-Leach et al. 2015; Trends in Global obesity 2016). The prevalence has increased rapidly since the 1980s and 1990s in the United States and Europe, including Finland (Kautiainen, Rimpela et al. 2002; Vuorela, Saha et al. 2011), where the prevalence of overweight is currently high, particularly among children in lower social and economic groups (Kautiainen, Koivisto et al. 2009; Knai, Lobstein et al. 2012). It has been estimated that the worldwide prevalence of childhood overweight and obesity will increase in the following years (de Onis, Blossner et al. 2010), although recent data suggest that in some countries such as Germany childhood overweight and obesity may be leveling off (Olds, Maher et al. 2011).

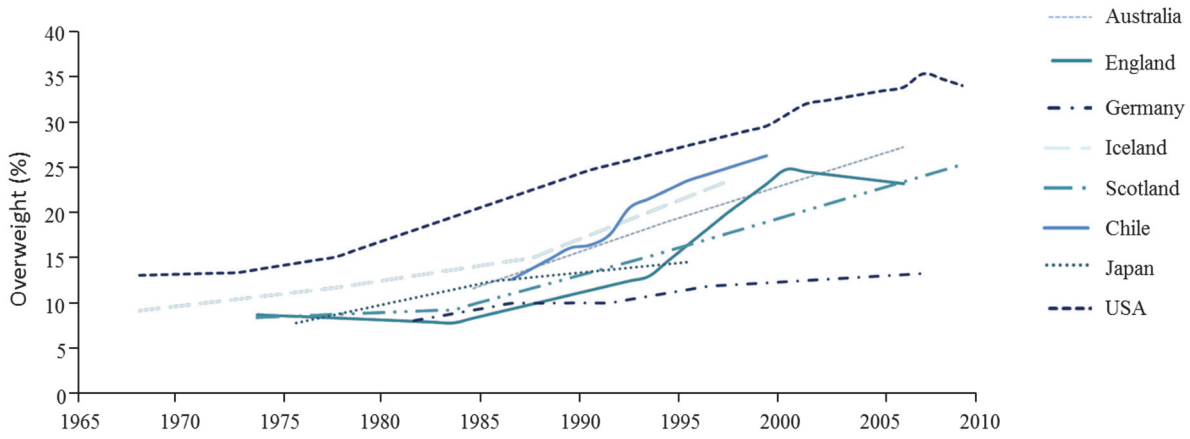


FIGURE 2 Percent changes in child overweight prevalence over time in selected countries around the world (Trends in Global obesity 2016).

2.2 Etiology of overweight and obesity

2.2.1 Physical inactivity and reduced energy expenditure

The growing obesity epidemic is occurring against the background of a continuous decline in the energy expenditure required for daily living (Brownson, Boehmer et al. 2005). A recent study estimated that in the United States, daily energy expenditure due to work related physical activity has decreased by more than 100 kcal during the last 50 years in both men and women, and this reduction was associated with the increase in mean body weight during this time frame (Church, Thomas et al. 2011). Similar trends have been observed also in other countries including Finland, where daily energy expenditure during work reportedly decreased by more than 50 kcal between 1982 and 1992, while the average body weights relentlessly crept upwards (Fogelholm, Männisto et al. 1996) Substantial reductions in daily energy expenditure have likely occurred also in developing countries such as China and Brazil, which have the highest absolute and relative rates of decline in total physical activity due to reductions in movement at work (Ng and Popkin 2012). For this reason, it is believed that the obesity epidemic has penetrated also the low-income countries, particularly in the urban areas, and will continue to spread for the foreseeable future (Prentice 2006).

There is also reason to believe that advances in transportation (motorized vehicle, elevators and escalators) and domestic mechanization (e.g. washing machines, dishwashers and vacuum cleaners) have reduced energy expenditure in physical activities over the years (Lanningham-Foster, Nysse et al. 2003). A recent study estimated that, in women, daily housework-related energy ex-

penditure has decreased by 360 kcal in the United States since the 1960s (Archer, Shook et al. 2013). The authors of the study concluded that such reductions in housework-related energy expenditure might be a substantial contributor to the rise in prevalence of obesity in women in the last five decades. Because such labor-saving devices are habitually used in affluent societies, their contribution to population energy balance in high-income countries has been considered substantive (Lanningham-Foster, Nysse et al. 2003). Domestic mechanization has also contributed to increased sedentariness, as time spent in house work has been replaced by sedentary activities such as watching television and use of other screen-based media (Archer, Lavie et al. 2013). According to recent studies, a third of the world´s adult population is currently physically inactive (Hallal, Andersen et al. 2012). More than 40% of middle-aged people spend over 4 hours per day sitting, and these numbers increase with age (Hallal, Andersen et al. 2012). Increased sedentariness is not limited to adults, as the majority of children and adolescents do not achieve the recommendations of daily physical activity (Kalman, Inchley et al. 2015). Consistent with this, studies indicate that participation in leisure-time physical activity (LTPA) has continuously declined in children and adolescents (Dollman, Norton et al. 2005; Huotari, Nupponen et al. 2010; Hills, Andersen et al. 2011). By contrast, in adults LTPA has progressively increased over the years (Bruce and Katzmarzyk 2002; Craig, Russell et al. 2004; Steffen, Arnett et al. 2006; Stamatakis, Ekelund et al. 2007; Knuth and Hallal 2009; Aadahl, Andreasen et al. 2013). However, it may be that on the secular basis the increase in LTPA has not been enough to offset increased sedentary behavior, as total physical activity has declined rapidly across the globe (Ng and Popkin 2012).

Based on the data presented above, it would seem intuitive that a decrease in daily energy expenditure is a major driver of the ongoing obesity epidemic. However, on closer scrutiny, this idea seems improbable, and there is fair amount of evidence to support that contention. Although, total physical activity has indisputably decreased over the years, doubly-labelled water studies (which provide the optimal method to measure energy expenditure in free-living individuals) show that daily energy expenditure has not declined between 1980 and 2005 in Europe or North America (Westerterp and Speakman 2008). Similarly, a recent meta-analysis of nearly 100 doubly-labelled water studies indicated that populations in industrialized countries do not have lower rates of daily energy expenditure compared with populations in developing countries (Dugas, Harders et al. 2011). This seeming paradox may be explained by the fact that obese individuals tend to have higher habitual energy expenditure compared with normal weight people (James, Davies et al. 1978; Ravussin, Burnand et al. 1982) (TABLE 2). Indeed, Leibel et al. demonstrated that a 10 percent weight gain (by overfeeding) increases daily energy expenditure (adjusted for metabolic mass) from 370 to 530 kcal, depending on the baseline weight (Leibel, Rosenbaum et al. 1995). In that study, weight gain resulted from a significant increase not only in fat but also in fat-free mass, suggesting that the higher energy expenditure in the obese is probably attributable to their greater

body size. Since a larger body requires more energy to move around, the obvious implication is that the rate of energy intake must also increase accordingly, otherwise weight loss will ensue. Thus, it appears unlikely that the obesity epidemic is attributable to decreases in daily energy expenditure alone.

TABLE 2 Twenty-four-hour energy expenditure (kcal/day) in lean, moderately obese and obese individuals measured in a respiration chamber, adapted from (Ravussin, Burnand et al. 1982).

Weight status	Resting metabolic rate	Total metabolic rate
Lean	1461	2016
Moderately obese	1588	2292
Obese	1813	2399

2.2.2 Physical activity, energy intake and weight management

Although decreases in daily energy expenditure may not be the primary cause of obesity that is not to say that physical activity or exercise has no role in energy balance. One theory holds that energy balance may be easier to achieve when energy flux is high. This concept was originally developed by (Mayer, Roy et al. 1956), and has subsequently been described (Blundell and King 1999) and (Hill, Wyatt et al. 2012). According to this theory, a threshold for physical activity exists above which people are in the so called "regulated zone" of energy balance (FIGURE 3). Those who are in the regulated zone are able to meet high energy expenditure needs with energy intake, thus maintaining body weight. However, those who are below the physical activity threshold have lower energy expenditure, and thus are in the "unregulated zone" without the matching decrements in energy intake. In other words, this theory suggests that appetite may not be appropriately regulated at low levels of physical activity. In support of this theory, Shook et al. examined the relationship between energy intake, physical activity, appetite, and weight gain during a 1 year follow-up, and found that individuals with low physical activity had higher levels of cravings for foods compared with those who had high levels of physical activity (Shook, Hand et al. 2015). Furthermore, the authors of that study noted that a threshold for achieving energy balance occurred at an activity level corresponding to 7116 steps per day. Thus, it may be that an increase in sedentariness allows for a much steeper trajectory in population weight gain than would have been otherwise possible.

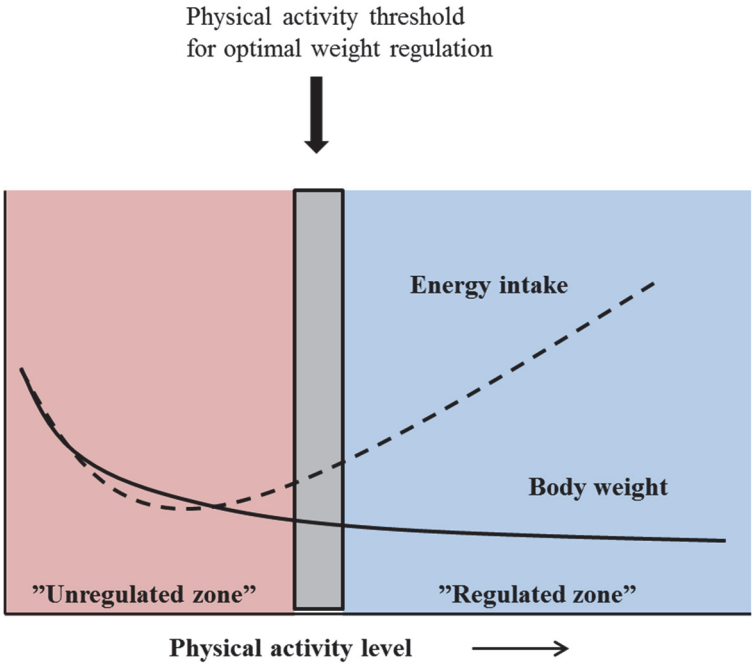


FIGURE 3 Schematic hypothesis that energy balance may be easier to achieve at higher levels physical activity and energy expenditure, adapted from (Hill, Wyatt et al. 2012).

Voluntary exercise is the most important discretionary component of total daily energy expenditure and therefore it has the potential to influence energy balance. This has been illustrated in many longitudinal studies. For example, a prospective study with 20 years of follow-up showed that maintaining high levels of physical activity mitigates weight gain significantly, particularly in women (Hankinson, Daviglus et al. 2010). Similar findings have been reported from the Finnish Twin Cohort (Leskinen and Kujala 2015). These studies show that twins who have been consistently discordant in LTPA for over 30 years differ significantly from each other in terms of body weight and fatness; physically active co-twins have lower body weight and smaller waist circumference (Waller, Kaprio et al. 2008), and they also have lower BMI (Piirtola, Kaprio et al. 2016) and much less (50%) visceral and hepatic fat compared with their inactive co-twin (Leskinen, Sipila et al. 2009). These findings indicate that a persistently higher physical activity level is associated with a lower rate of weight gain even after controlling for genetic susceptibility and childhood environment. There is also a wealth of evidence from controlled trials that exercise (or physical activity) carried out over longer periods of time can generate an energy deficit and thereby induce weight loss (Jakicic, Marcus et al. 2008; Rosenkilde, Auerbach et al. 2012; Donnelly, Honas et al. 2013). A series of reviews (Ballor and Keesey 1991; Catenacci and Wyatt 2007), including a Cochrane Review (Shaw, Gennat et al. 2006) (which is considered the gold standard in assessing evidence) indicate that exercise can induce weight loss, and the weight loss is greater when coupled with energy restriction. Thus, both diet and exercise are important components in programs intended for weight loss.

2.2.3 Food availability and energy intake

As physical labor has been technologically engineered out of the Western lifestyle, the food environment has also changed drastically. Powered agricultural machinery, improvements in cultivation techniques, fertilizers and agricultural subsidies have increased crop yields substantially (Cohen 2008). Consequently, food has become abundant and relatively inexpensive. Increased availability of food has most likely contributed to the obesity epidemic, because today people can eat many times a day and virtually as much as they want (Grundy 1998). Not only has food become abundant and readily available, the quality has also changed; the food and beverage industries produce highly-processed palatable foods and drinks that have high energy contents. Because these foods tend to be inexpensive, easily available and are often served in large portion sizes in fast food and other restaurants, they have most likely accelerated the increase in energy intake over the years.

Consistent with the increased availability of energy-dense food and beverages, Swinburn et al. showed that the estimated daily energy intake in adults increased on average by 500 kcal in the United States between 1970 and 2000 (Swinburn, Sacks et al. 2009). Subsequent studies using the national food availability data concur with these findings by showing that daily energy intake in the U.S increased slowly until the early 1980s, and then started to increased rapidly (Levitsky and Pacanowski 2012). Similar findings have been reported in several European countries (Silventoinen, Sans et al. 2004; Balanza, Garcia-Lorda et al. 2007). Moreover, a recent global analysis showed that increases in food-energy supplies is congruent with the dynamics of the population weight gain, particularly in high-income countries (Vandevijvere, Chow et al. 2015), suggesting that increased food intake is a major driver of the obesity epidemic.

2.2.4 Genetic susceptibility and heritability

Although food is now abundantly available in all affluent societies, not everyone develops obesity, thus obesity is likely a consequence of the complex interplay between the environment, behavior and genetic factors (Higginson, McNamara et al. 2016). In 1962 James Neel proposed that the prevalence of obesity is on the rise because human metabolism runs on ancient genes that are ill equipped for contemporary food environment (Neel 1999). The *thrifty genotype* hypothesis he put forward suggests that conditions of scarcity used to favor those with a parsimonious metabolism. In another words, populations that experienced periods of feast and famine, natural selection favored individuals carrying "thrifty alleles" that promote the storage of fat and energy. Barker and colleagues developed this hypothesis a little further, and suggested that instead of arising from genes, the thrifty metabolism developed as a direct result of the environment within the womb during development (Barker 1997). This conjecture was based on their observation of a relationship between low birth weight and increased adult cardio-metabolic risk. They attributed the low birth weight to fetal undernutrition, and proposed a *thrifty phenotype* hypothesis, which pos-

its that the metabolic adaptations adopted as a survival strategy by an undernourished fetus would lead to changes that are maladaptive in the affluent environment later in life. Thus, both hypotheses predict that in certain populations people are predisposed to eat excessively when food is abundant to prepare themselves for times of need, but in the contemporary environment this strategy entails a greater risk for obesity and its metabolic sequelae because food is continuously available and famine rarely experienced.

The Pima Indians, a group of Native Americans living in southern Arizona, are a good example of such a population (Schulz and Chaudhari 2015). For centuries, the Pima population practiced a traditional agrarian lifestyle; nearly the entire population was lean, and obesity was rare. But as they were forced to abandon their traditional way of living at the turn of the 19th century and adopt a modern way of life (characterized by a constant food supply and little physical labor), they quickly started to gain weight. By the mid-1970s the prevalence of obesity and type II diabetes among the Pima population was higher than in the Caucasian population (Knowler, Pettitt et al. 1991). Today, this tribe is notoriously famous for being among the fattest people in the world, with over 75% of the adults being obese (BMI >30) (Schulz and Chaudhari 2015). Given the low degree of admixture and high heritability of obesity in the Pima Indians, the population should represent a good source for genetic discovery. However, no convincing candidates for these thrifty genes have been discovered (Speakman 2006), thus the thrifty genes hypothesis remains little more than a nebulous concept, and obesity in the Pima appears to be determined mostly by environmental circumstances (Schulz, Bennett et al. 2006).

While obesity results mostly from changes in environment and the consequent change in lifestyle, it is also a highly heritable trait. This is evidenced by the fact that obese parents often have obese children, and the risk of a child becoming obese in adulthood is about two to three times higher if either the mother or the father is obese (Allison, Faith et al. 1996; Whitaker, Wright et al. 1997). Studies have shown heritability estimates varying between 25% and 60% for BMI and 25% and 40% for total body fat (Rankinen, Sarzynski et al. 2015). Twin studies have indicated that genetics and non-shared environment explain the variation in BMI to a significant degree (Schousboe, Willemsen et al. 2003), but common environmental factors seem to affect BMI only in mid-childhood, not later in life (Silventoinen, Rokholm et al. 2010). This is consistent with adoption studies, which have shown that the BMI of the adoptees is more closely related to their biological parents than to their adoptive parents (Stunkard, Sorensen et al. 1986; Sorensen, Price et al. 1989). Controlled feeding studies in monozygotic twins have shown that the tendency towards weight gain is comparable between twins, which supports the role of genetic factors in body weight regulation (Bouchard, Tremblay et al. 1990; Bouchard, Tremblay et al. 1996); and the fact that monozygotic twins discordant for obesity are relatively rare, underscores this notion (Pietiläinen, Naukkarinen et al. 2008).

Despite the high heritability of obesity, the genes that contribute to obesity are not well defined. Genome-wide studies (GWAS) have identified many gene

variants associated with BMI (Speliotes, Willer et al. 2010; Locke, Kahali et al. 2015). The first single nucleotide polymorphism (SNP) associated with increased BMI was mapped to a gene called FTO (Frayling, Timpson et al. 2007; Scuteri, Sanna et al. 2007). This gene acts by regulating appetite and energy expenditure. A number of other genetic variants have been identified to associate with risk of obesity and fat distribution (Loos and Yeo 2014), and although common allelic variants explain only a small proportion of the variance in BMI (<2%), these risk alleles contribute to obesity in a polygenic manner (Hofker and Wijmenga 2009). Only very rarely is obesity due to single gene mutations (e.g. leptin deficiency, leptin-receptor defects), and in these cases massive obesity in observed already in early childhood (Montague, Farooqi et al. 1997).

2.3 Metabolic disorders associated with obesity

2.3.1 Metabolic syndrome

Metabolic syndrome is defined by a constellation of interconnected physiological and metabolic abnormalities that increase the risk of cardiovascular disease and type II diabetes (Grundy 2015). From the historical point of view, the concept of metabolic syndrome originates from the 1920s when Eskil Kylin, a Swedish physician, noted the association of hypertension, hyperglycemia and gout (Nilsson 2001). Two decades later, Jean Vague, a physician from France, linked abdominal obesity with the metabolic abnormalities observed in type II diabetes and cardiovascular disease (Vague 1996). However, it was Gerald Phillips in 1978 who first suggested that aging, obesity and other metabolic disorders (that we now call metabolic syndrome) are associated with cardiovascular disease (Phillips 1978). In 1988, Gerald Reaven noted that dyslipidemia, hypertension and hyperglycemia commonly cluster together, and called it syndrome X (Reaven 1988). He further proposed that individuals who displayed this cluster of abnormalities tended to exhibit insulin resistance and compensatory hyperinsulinemia, hence the term insulin resistance syndrome is also used for this cluster of clinical manifestations. However, the term metabolic syndrome is commonly used in the literature because it avoids the implication of insulin resistance as the primary cause of the associated metabolic abnormalities. Different diagnostic criteria have been proposed for this condition (TABLE 3) (Alberti and Zimmet 1998; Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III) 2001; Alberti, Eckel et al. 2009), but the most recent version by the International Diabetes Federation (IDF) defines MetS as the presence of abdominal obesity and two or more of the following: elevated serum triglyceride or glucose, reduced high-density lipoprotein cholesterol and elevated blood pressure (Zimmet, Alberti et al. 2005).

MetS is common throughout the world, and its prevalence ranges between 10% and 40% depending on the criteria used (Grundy 2008). These rates are similar to those reported in middle aged Finnish men and women (Ilanne-Parikka, Eriksson et al. 2004; Hu, Lindstrom et al. 2008). In general, MetS seems to affect men and women at a similar rate, though in some countries a greater number of women than men meet the criteria of MetS (Cornier, Dabelea et al. 2008). This might be due to gender-specific cut-off points set for waist circumference and HDL-C (Regitz-Zagrosek, Lehmkuhl et al. 2006). Clustering of metabolic abnormalities is not limited to adults as the prevalence of MetS appears to be relatively high also in children and adolescents, particularly among those who are overweight or obese (Bokor, Frelut et al. 2008). A recent review indicated that 11.9% of overweight and 29.2% of obese children have MetS (Friend, Craig et al. 2013). However, the major problem with identifying children and adolescents with MetS is that there are no widely accepted criteria for the definition of pediatric metabolic syndrome (Kassi, Pervanidou et al. 2011). The rapid growth patterns in childhood and the effects of hormonal changes on glucose and lipid metabolism during puberty make such criteria difficult to establish (Zimmet, Alberti et al. 2007). Therefore, many studies in children have used metabolic risk scores, calculated from continuous variables of the constituent traits of MetS to represent clustering of metabolic risk factors (Andersen, Harro et al. 2006; Eisenmann 2008; Pandit, Chiplonkar et al. 2011). Using such continuous risk scores has been considered a valid tool for epidemiological research evaluating cardio-metabolic risk, not only in children but in all age groups, including older men and women (Viitasalo, Lakka et al. 2014).

Generally speaking, the prevalence of MetS increases with age (Park, Zhu et al. 2003; Ogbera 2010), and it is more common in obese than non-obese people (Park, Zhu et al. 2003). This suggests that excess adiposity and aging are among the primary risk factors for this condition. However, genetic susceptibility is also an important factor in the development of MetS (Bouchard 1995; Abou Ziki and Mani 2016). Studies have shown that children who have at least one parent with MetS are more likely to develop obesity and insulin resistance than children for whom neither parent has MetS (Pankow, Jacobs et al. 2004). Similarly, children of parents with premature coronary heart disease are more likely to be overweight in childhood and develop an adverse cardio-metabolic risk profile in early adulthood (Bao, Srinivasan et al. 1997). Other family and twin studies further support the contribution of genetics to the clustering of metabolic factors (Mitchell, Kammerer et al. 1996; Edwards, Newman et al. 1997; Hong, Pedersen et al. 1997). The heritability estimates of the different components of MetS range between 40% and 70%, with obesity and HDL cholesterol being most strongly heritable traits (Lusis, Attie et al. 2008). In addition to genetics, excess energy intake (Grundy 2015), low levels of physical activity and poor cardiorespiratory fitness (Laaksonen, Lakka et al. 2002; Lakka, Laaksonen et al. 2003; Laaksonen, Niskanen et al. 2004) are also important factors in the development of MetS.

TABLE 3 Different definitions of the metabolic syndrome.

World health Organization	**Adult Treatment Panel III**	**International Diabetes Federation**
Type II diabetes, impaired fasting glucose, impaired glucose tolerance, or insulin resistance plus any 2 of the following:	Any 3 of the following:	Waist circumference ≥94 cm in men, >80cm in women plus any 2 of the following:
BMI >30 and /or waist-to-hip ratio: >0.9 in men >0.85 in women	Waist circumference: >102 cm in men >88 cm in women	
Blood pressure ≥140/90 mmHg and/or antihypertensive medication	Blood pressure ≥130/85 mm Hg	Blood pressure ≥130/85 mmHg and/ or antihypertensive medication
Triglycerides ≥1.7 mmol/l	Triglycerides ≥1.7 mmol/l,	Triglycerides ≥1.7 mmol/l or lipid lowering medication
HDL cholesterol (mmol)l) <0.9 in men <1.0 in women	HDL cholesterol (mmol/l) <1.04 in men <1.3 in women	HDL-C (mmol/l) <1.03 in men <1.29 in women
Urinary albumin excretion rate ≥20 µg/min or albumin:creatinine ratio ≥3.4 mg/mmol	fasting glucose ≥6.1 mmol/l	fasting glucose ≥5.6 mmol/l or diagnosed type II diabetes
Adapted from (Alberti and Zimmet 1998)	Adapted from (Adult Treatment Panel III) 2001)	Adapted from (Zimmet, Alberti et al. 2005)

The pathogenesis of MetS remains incompletely understood (Simmons, Alberti et al. 2010). To date, there is no universally accepted single underlying mechanism, although insulin resistance (Reaven 1988), abdominal and ectopic fat accumulation (Rasouli, Molavi et al. 2007) have been suggested in this role. For this reason, the concept of MetS has been the subject of considerable criticism (Gale 2005; Kahn, Buse et al. 2005) and has sparked debate (Davidson 2006; Grundy 2006; Oda 2006). Nonetheless, studies show that individuals affected with MetS are at increased risk for cardiovascular disease and type II diabetes (Laaksonen, Lakka et al. 2002; Grundy, Cleeman et al. 2005; Grundy 2015), and the risk appears to be relatively higher in women compared with men (Galassi, Reynolds et al. 2006; Gami, Witt et al. 2007). Having MetS also doubles the risk for stroke (Ninomiya, L'Italien et al. 2004; Kurl, Laukkanen et al. 2006) and increases risk for myocardial infarction and premature mortality (Isomaa, Almgren et al. 2001). For these reasons, the definition of MetS is considered a useful tool in cardiovascular risk assessment in both public health screening

and clinical practice (Wijndaele, Beunen et al. 2006). Thus, the primary value of MetS should not be considered in pathophysiological terms, but as a pragmatic approach to identify individuals who are at increased risk for cardiovascular disease (Reaven 2005).

2.3.2 Abdominal obesity

Several prospective population studies have shown that abdominal obesity, defined as a large waist circumference (≥ 102 cm in men and ≥88 cm in women) is associated with increased risk for coronary heart disease (Rexrode, Carey et al. 1998; Canoy, Cairns et al. 2013; Hotchkiss, Davies et al. 2013; Klingberg, Mehlig et al. 2015). However, although waist circumference closely reflects both total and abdominal adiposity (Bouchard 2007), it cannot distinguish visceral from subcutaneous adiposity. Because of this, some researchers have claimed that measuring waist circumference provides little additional value over BMI in cardio-vascular risk prediction (Kiernan and Winkleby 2000; Farin, Abbasi et al. 2005; Bouchard 2007). Nonetheless, studies using computed tomography (Fox, Massaro et al. 2007; Rosito, Massaro et al. 2008; Liu, Fox et al. 2010; Preis, Massaro et al. 2010) and magnetic resonance imaging (Sironi, Petz et al. 2012; Chandra, Neeland et al. 2014; Gast, den Heijer et al. 2015) have consistently shown that excess visceral adiposity associates with various cardio-metabolic abnormalities independently of total fat mass, thus confirming that intra-abdominal fat carries more health risk than general fat accumulation.

The propensity to store fat inside the peritoneal cavity is highly variable among individuals, and several factors are known to contribute to this phenomenon (Tchernof and Despres 2013). However, gender and age are the most important determinants of fat distribution in the body (St-Onge and Gallagher 2010; Palmer and Clegg 2015). In men, fat is preferably stored in the upper body (trunk and abdomen), whereas in women fat tends to accumulate in the lower body (hips and thighs) (Blaak 2001). In women, accumulation of visceral fat accelerates after menopause (Toth, Tchernof et al. 2000), suggesting that the relative distribution of the fat in the body is influenced by sex hormones (Fried, Lee et al. 2015). This notion can be exemplified by transsexuals who have been treated with sex-hormones. Male-to-female transsexuals treated with estrogen show increased fat deposition in subcutaneous sites (Elbers, Asscheman et al. 1999), whereas females who receive testosterone treatment in order to become male show increases in the amount of visceral fat (Elbers, Asscheman et al. 1997).

Visceral fat increases also with age, and the increase is greater in men than in women (Lemieux, Prud'homme et al. 1993; Kotani, Tokunaga et al. 1994; Shen, Punyanitya et al. 2009). The reason for this is not well understood, but it may be attributable to loss of lean mass, the consequent decrease in resting energy expenditure (Hunter, Weinsier et al. 2001; Manini 2010), and to the inability of the body to develop and maintain adequate subcutaneous adipose tissue with age (Kuk, Saunders et al. 2009). The latter has been referred to as the adipose tissue overflow hypothesis (Sniderman, Bhopal et al. 2007) or the adipose

tissue expandability hypothesis (Virtue and Vidal-Puig 2010). These are two closely related features of one idea, which in essence, posit that all individuals have a maximum capacity for adipose tissue expansion. According to these hypotheses, once the adipose tissue expansion limit is reached, adipose tissue ceases to store energy efficiently, and (under conditions of positive energy balance) this leads to partitioning of fat away from subcutaneous adipose tissue, and accumulation of lipids in the visceral compartment and other ectopic sites. However, whether adipocytes lose their ability to expand in response to overfeeding with age remains to be confirmed. The capacity of subcutaneous adipocytes to sequester triglyceride may dwindle also because of decreased formation of new adipocytes. Spalding et al. investigated this phenomenon and demonstrated that there is a steady, maintained formation of new adipocytes in adulthood and that the lifespan of adipocytes is approximately 10 years on average (Spalding, Arner et al. 2008). Interestingly, however, and in support the of the adipose tissue expandability hypothesis, their data showed that the number of adipocytes in the subcutaneous adipose tissue depots decreased by 30% between the ages of 20 and 65 in obese individuals (Spalding, Arner et al. 2008). Such decrease in adipocyte number could well be expected to limit the triglyceride sequestering capacity of subcutaneous adipose tissue and thus contribute to increased fat deposition in the viscera.

In addition to sex steroids and age, regional fat distribution is also influenced by genetic factors. The role of genetics is supported by studies which have shown that the relative distribution of lower and upper body fat differs significantly between individuals of the same sex and age (Bouchard 1997). Segregation analyses have suggested that 51% of the variance in visceral adipose tissue accumulation is accounted by genetic factors (Bouchard, Rice et al. 1996). Consistent with this, overfeeding studies in twins have shown that the variance in abdominal visceral fat accumulation is about six times higher between twin pairs than within twin pairs (Bouchard, Tremblay et al. 1990; Bouchard, Tremblay et al. 1996). Although these findings provide compelling evidence for genetic regulation of body fat distribution under positive energy balance, the evidence for the contribution of specific genes to variation in visceral fat levels is not strong. This is probably due to the fact that visceral obesity is a polygenic trait caused by interactions of multiple genes and non-genetic factors (Schleinitz, Bottcher et al. 2014). Covering all the DNA sequence variants (most of which have a small effect size by themselves) requires very large sample sizes. In fact, recent large scale genome-wide meta-analysis identified 49 loci associated with waist-to-hip ratio, even after adjusting for BMI (Shungin, Winkler et al. 2015). This is consistent with earlier studies, which showed that multiple loci modulate body fat distribution independent of overall adiposity (Heid, Jackson et al. 2010).

The reason why fat stored in the viscera is much more detrimental to health than fat stored beneath the skin is not entirely clear (Wajchenberg 2000). Visceral adipocytes are smaller than abdominal subcutaneous adipocytes, and have greater transmembrane fluxes of fatty acids compared with subcutaneous

adipocytes (Marin, Andersson et al. 1992). Thus, from the metabolic point of view, subcutaneous adipose tissue is relatively inert, whereas visceral adipose tissue with its high fatty acid flux may contribute to dyslipidemia and impaired glycemia (Mathieu, Boulanger et al. 2014). Visceral adipose tissue may also contribute to systemic inflammation as it produces and secretes increased amounts of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF-α) and interleukin-6 (IL-6) (Gustafson 2010). A recent metabolomics study in twins discordant for obesity found that increased visceral fat was associated with atherogenic lipoproteins and more saturated fatty acid profile as well as higher levels of branched-chain and aromatic amino acids, and inflammatory-related glycoprotein (Bogl, Kaye et al. 2016). This suggests that the unfavorable metabolic profile associated with visceral fat is partly explained by shared genes, but also reflects mechanisms independent of genetic make-up. However, what these mechanisms are is not well defined.

2.3.3 Increased liver fat content

Increased accumulation of fat in the liver is a manifestation of the complex metabolic derangements associated with obesity (Fabbrini and Magkos 2015). The increase in liver fat (not due to alcohol or other known causes of steatosis e.g. viruses and drugs) is referred to as non-alcoholic fatty liver disease (NAFLD) (Yki-Jarvinen 2014). NAFLD represents a wide histological spectrum of disease from simple steatosis with no evidence of hepatocellular injury to nonalcoholic steatohepatitis (NASH) with hepatocyte injury with or without fibrosis (Kotronen and Yki-Jarvinen 2008). In the early phase NAFLD is usually asymptomatic, benign and reversible, whereas NASH can lead to a more severe form of liver disease, such as cirrhosis and hepatocellular carcinoma (Adams and Lindor 2007).

Definitive diagnosis of NAFLD requires a liver biopsy (which is considered the gold standard), but ultrasonography, computed tomography or magnetic resonance imaging (MRI) assessment are also frequently used to determine liver fat content. NAFLD is diagnosed if the proportion of hepatocytes containing fat droplets is greater than 5% (Chalasani, Younossi et al. 2012). The prevalence of NAFLD has increased considerably in recent years, currently affecting up to 30% of the adult population (Smith and Adams 2011; Younossi, Koenig et al. 2015). The prevalence of NAFLD increases with age (Bertolotti, Lonardo et al. 2014) and is more common in men than in women (Pan and Fallon 2014). In children and adolescents the prevalence of NAFLD varies between 3% and 10% (Della Corte, Mazzotta et al. 2016). However, estimation of the true prevalence of NAFLD in the general population is limited by the low accuracy of non-invasive tools feasible for large-scale population studies and the reliability of self-reported ethanol ingestion histories (Lidofsky 2008).

Family studies have shown that fatty liver is more common in children, whose siblings or parents have fatty liver, suggesting a genetic component to NAFLD (Schwimmer, Celedon et al. 2009). In a study of individuals with genetic dyslipidemia, the heritability of NAFLD as determined with plasma alanine

aminotransferase (ALT) was between 20% and 37% (Brouwers, Cantor et al. 2006), whereas in a study of twins the heritability of ALT was 55% (Makkonen, Pietilainen et al. 2009). Recent genome-wide association studies have shown that the rs738409 variant (single nucleotide polymorphism) of the adiponutrin/patatin-like phospholipase-3 (PNPLA3) gene is a major factor in the determination of liver fat content (Romeo, Kozlitina et al. 2008). Forty percent of Europeans carry this gene variant, which increases susceptibility to NAFLD, cirrhosis and hepatocellular carcinoma, independent of obesity and insulin resistance (Yki-Jarvinen and Luukkonen 2015; Luukkonen, Zhou et al. 2016). However, the exact mechanisms through which this gene variant contributes to the development NAFLD are not entirely clear.

The pathophysiology of NAFLD is complex and incompletely understood, but the traditional "two-hit" theory has been widely accepted (Day and James 1998). The "first hit" involves lipid accumulation in the hepatocytes, which predisposes the liver to the "second hit" of oxidative stress and subsequent lipid peroxidation, which, in turn, promotes progression of steatosis to steatohepatitis, and fibrosis (FIGURE 4). However, this pathophysiological theory has been criticized for being over simplistic (Tilg and Moschen 2010). New findings suggest that intrahepatic lipid accumulation results from lipid metabolism abnormalities such as increased whole body lipolysis, increased uptake of free fatty acids in the liver, synthesis of VLDL, and reduced fatty acid oxidation. In addition, abnormal adipokine production, such as leptin, adiponectin, resistin and visfatin, is associated with NAFLD (Katsiki, Mikhailidis et al. 2016). These metabolic alterations may be linked to dysfunctional adipose tissue (Cheng, Wiklund et al. 2015), increased muscle insulin resistance (Flannery, Dufour et al. 2012), impaired muscle mitochondrial activity (Szendroedi, Kaul et al. 2014), intestinal dysbiosis (Munukka, Pekkala et al. 2014; Jiang, Wu et al. 2015) and elevated plasma branched-chain amino acids (BCAA) (Iwasa, Ishihara et al. 2015; Rodriguez-Gallego, Guirro et al. 2015; Sunny, Kalavalapalli et al. 2015), which are also associated with NAFLD. However, causality has not been proven and mechanistic links require further study. Nonetheless, a new model has been proposed, which considers that multiple parallel hits are likely to cause NAFLD in genetically susceptible individuals (Buzzetti, Pinzani et al. 2016).

NAFLD is closely associated with MetS (Hamaguchi, Kojima et al. 2005; Kotronen, Westerbacka et al. 2007). This association is probably due to the fact that the liver is centrally involved in the production of two key components of MetS: fasting serum glucose and very-low density lipoprotein (VLDL), the latter containing most of the triglyceride present in serum. Under normal fasting conditions, insulin inhibits hepatic gluconeogenesis and lipogenesis. However, once the liver accumulates fat, the ability of insulin to suppress hepatic gluconeogenesis and triglyceride synthesis is impaired. This leads to overproduction of glucose and VLDL, leading to development of hyperglycemia, hyperinsulinemia and dyslipidemia (Yki-Jarvinen 2014). This may also be the primary reason why NAFLD predicts cardiovascular disease and type II diabetes (Anstee, Targher et al. 2013; Choi, Rhee et al. 2013; Park, Seo et al. 2013).

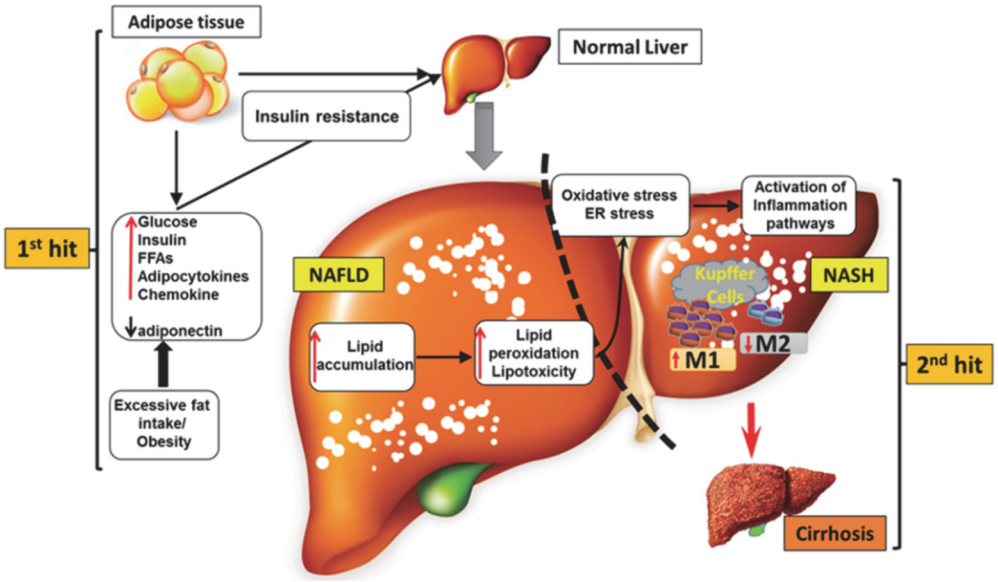


FIGURE 4 The "two-hit hypothesis" of NAFLD. Reproduced from (Xu, Kitade et al. 2015). Increased fat intake and obesity lead to increased flux of free fatty acids, which accumulate in the liver as triglycerides. The accumulation of fat predisposes the liver to oxidative stress, which activates inflammatory responses leading to development of NASH.

2.3.4 Dyslipidemia

Dyslipidemia is a well-established risk factor for cardiovascular disease (Rana, Visser et al. 2010). Dyslipidemia is generally characterized by high levels of serum triglyceride, and low-density lipoprotein (LDL) cholesterol, with low level of high-density lipoprotein (HDL) cholesterol (Grundy 2006; Musunuru 2010). A recent meta-analysis demonstrated that elevated serum triglyceride levels, in particular, are associated with increased risk of coronary heart disease (Sarwar, Danesh et al. 2007). In addition, lipoprotein particle subfractions have been proposed as potential risk factors of subclinical heart disease (Krauss 2010). In particular, alterations in LDL and HDL particle size and density have been associated with future cardiovascular events (Blake, Otvos et al. 2002; Cromwell, Otvos et al. 2007; Arsenault, Lemieux et al. 2009).

The reason why dyslipidemia is so closely associated with cardiovascular events is because dyslipidemia is a significant risk factor for atherosclerosis (Falk 2006; Nofer 2011). Atherosclerosis is a disease in which fat and cholesterol are deposited in the inner lining of arteries forming atherosclerotic plaques. These plaques may cause stenosis restricting blood flow, which can lead to ischemia or trigger thrombotic occlusion of coronary artery causing myocardial infarction, or a stroke, if the blood clot develops in the brain (Falk 2006). From the clinical point of view, serum triglyceride concentrations higher than 1.7 mmol/l are considered by many to be the point beyond which risk for coronary artery disease begins. Concomitantly with elevated triglycerides, LDL choles-

terol (in excess) tends to get trapped in the vessel wall, and this retention with subsequent oxidation is considered to be an important event in the early stages of an atherosclerotic lesion. The oxidized LDL promotes the recruitment of monocytes and lymphocytes (and conversion to macrophages) and increases the production of cytokines. HDL is also a key element in atherosclerosis because of its role in reverse cholesterol transport (Singh, Mengi et al. 2002). Atherosclerosis is a chronic disease that develops gradually over time. Both pathological and epidemiological studies suggest that atherogenesis begins early in life. Fatty streaks and fibrous plaque lesions, for example, have been found in young children and adolescents (McGill, McMahan et al. 2000).

Dyslipidemia can be due to hereditary factors (e.g. familial hyperlipidemia) (Brouwers, van Greevenbroek et al. 2012) or result from secondary causes such as diet, sedentary lifestyle, medical conditions (e.g., hypothyroidism, insulin resistance and type II diabetes) and use of certain medications, or a combination of the above (Vodnala, Rubenfire et al. 2012). Insulin resistance in the adipose tissue increases lipolysis and subsequent release of free fatty acids (Gastaldelli, Natali et al. 2010). Increased delivery of free fatty acids to the liver increases the secretion of triglyceride-abundant very low-density lipoprotein (VLDL) cholesterol (Lewis, Uffelman et al. 1995; Ginsberg, Zhang et al. 2005). Triglyceride accumulation in the liver may also increase hepatic insulin resistance and increase de-novo lipogenesis (Diraison, Moulin et al. 2003), thereby contributing to development of hypertriglyceridemia (Ginsberg, Zhang et al. 2006).

There has been increased effort to identify biomarkers that would allow for early prediction of hypertriglyceridemia. Recent genome-wide association studies have found numerous loci associated with triglyceride levels in adults (10). However, the predictive value of these gene variants is limited since they can only explain around 10% of the variation in lipid levels within the population. Recent metabolomic studies have revealed alterations in several circulating amino acid concentrations associated with obesity and dyslipidemia (Cheng, Rhee et al. 2012; Boulet, Chevrier et al. 2015). A longitudinal study in middle-aged and elderly men and women showed that increased levels of plasma branched-chain amino acids (BCAA: isoleucine, leucine and valine), were associated with an increased risk of hypertriglyceridemia after 7-year follow-up (Mook-Kanamori, Romisch-Margl et al. 2014). Another study in elderly men and women demonstrated that branched-chained amino acids and alanine predicted development of dyslipidemia after 4 years, even after controlling for BMI and HOMA-IR (Yamakado, Nagao et al. 2015). Furthermore, branched-chained amino acids and related metabolites have been associated with coronary artery disease (Bhattacharya, Granger et al. 2014; Yang, Wang et al. 2015) and found to predict cardiovascular events in individuals with cardiovascular disease (Shah, Bain et al. 2010). It is well established that serum lipid and lipoprotein levels continue to track from childhood into early adulthood (Webber, Srinivasan et al. 1991), and are associated with dyslipidemia, obesity and hypertension later in life (Nicklas, von Duvillard et al. 2002). A follow-up study in Finnish children revealed that dyslipidemia in childhood predicted increased carotid artery in-

tima-media thickness (IMT) in adulthood (Juonala, Viikari et al. 2008). Moreover, studies have shown that elevated triglyceride concentrations in childhood predict clinical cardiovascular events several decades later (Morrison, Glueck et al. 2009). However, it is not known if amino acid profiles in childhood predict the development of dyslipidemia in adulthood.

2.3.5 Insulin resistance and type II diabetes

Insulin is a hormone produced and secreted by the beta cells of pancreatic islets of Lagerhans in response to changes in plasma glucose concentration (Gastaldelli, Natali et al. 2010). Insulin affects metabolism in many ways, but its main physiologic effects are to facilitate glucose uptake in skeletal muscle and to suppress hepatic glucose and VLDL production and inhibit the release of free fatty acids from adipose tissue (lipolysis) (Gastaldelli, Natali et al. 2010; Samuel and Shulman 2012) (TABLE 4). Insulin resistance is a term that describes the condition when muscle, liver and fat cells do not respond properly to insulin (Laakso and Kuusisto 2014). Insulin resistance is not a disease as such, but a central metabolic defect that underlies development of many cardio-metabolic diseases. In a nondiabetic state, the impaired action of insulin is compensated by increased insulin secretion from the pancreas. But when the pancreas fails to secrete enough insulin to compensate for the impaired action of insulin, hyperglycemia ensues (Kahn and Porte 1988). Untreated insulin resistance and hyperglycemia can cause long-term complications, including type II diabetes, neuropathy, nephropathy and kidney failure, dyslipidemia, atherosclerosis and cardiovascular disease (Howard, O'Leary et al. 1996; Jellinger 2007; DeFronzo and Tripathy 2009; Reaven 2011). Thus, the primary value of the concept of insulin resistance is that it provides a conceptual framework with which to place a group of seemingly unrelated metabolic disorders into a pathophysiological construct (Reaven 2005).

TABLE 4 The main physiological effects of insulin in different target tissues, adapted from Samuel and Shulham (Samuel and Shulman 2012).

Target tissue	**Normal condition**	**Insulin resistance**
Muscle	increases glucose uptake stimulates protein synthesis	decreased glucose uptake/impaired glycogen synthesis/ stimulation of the process by which amino acids are incorporated into protein
Liver	increases lipogenesis, glycogen synthesis, inhibits gluconeogenesis	increases lipogenesis, impaired glycogen synthesis, impaired gluconeogenesis
Adipose cells	inhibits lipolysis	inhibition of lipolysis is attenuated
Blood vessels	dilation of blood vessels, anti-atherogenic effects in endothelial cells	impaired dilation of blood vessels, impaired anti-atherogenic effects in endothelial cells

It is believed that many of the metabolic abnormalities are more common in obese than lean individuals because obesity has the ability to engender insulin resistance (Kahn and Flier 2000). Therefore, studies on the etiology of insulin resistance have predominantly focused on lipid-induced mechanisms (Savage, Petersen et al. 2007). A commonly held view is that accumulation of lipids (free fatty acids) in skeletal muscle and liver impair insulin signaling and thereby contribute to whole body insulin resistance and deterioration of glucose tolerance (Galgani, Moro et al. 2008). Because only adipocytes can release free fatty acids into the circulation, elevated fatty acid concentrations are believed to be attributable to increased adipose tissue mass. It is also believed that the process of fatty acid mobilization from adipose tissue, which is normally suppressed by insulin, becomes insulin resistant, and thus, lipolysis is further increased, which can lead to a vicious cycle. However, the fatty acid theory of insulin resistance has recently been called into question. Karpe and colleagues reviewed over 40 studies where free fatty acid levels had been compared in obese/overweight versus lean subjects (Karpe, Dickmann et al. 2011). They found that the average increase in circulating free fatty acids in obesity was only around 0.07 mmol/l. Sophisticated tracer studies of free fatty acid turnover in lean and obese have also shown that the rate of fatty acid release per unit of fat mass is nearly halved in obese compared with lean individuals (Campbell, Carlson et al. 1994). Moreover, obese Pima men do not have elevated plasma free fatty acid levels like Caucasian obese men, despite having similar upper body obesity (Howard, Zech et al. 1980). They also have lower plasma triglyceride concentrations than obese Causasian men. In fact, obese Pima men have similar hepatic secretion rates for VLDL-TG and plasma triglyceride levels than non-obese Caucasians (Howard, Zech et al. 1980). Despite having lower plasma plasma free fatty acids and triglycerides, majority of obese Pima are insulin resistant and develop type II diabetes (Lillioja and Bogardus 1988). These findings cast doubt on the fatty acid theory of insulin resistance.

Insulin-mediated glucose uptake by muscle varies considerably in seemingly healthy, non-diabetic individuals (Yeni-Komshian, Carantoni et al. 2000). Approximately half of the variability in insulin action results from differences in physical fitness and degree of adiposity (Bogardus, Lillioja et al. 1985). The remaining half is likely to be of genetic origin. This supported by studies, which have shown that diabetes risk is three times higher for individuals whose either parent is diabetic and 6-fold higher if both parents are diabetic compared with offspring whose neither parent have diabetes (Meigs, Cupples et al. 2000). A meta-analysis of nearly 35,000 twin pairs data from the Discordant Twin (DISCOTWIN) consortium showed that monozygotic twins are more concordant for type II diabetes than dizygotic twins, with heritability estimates up to 70%, which provides compelling support for the role of genetics in the etiology of diabetes (Willemsen, Ward et al. 2015). Recent genome-wide association studies have identified many of common variants associated with type II diabetes; however, these explain only a fraction of the heritability of this disease, which

probably reflects the polygenic nature of this disease (Fuchsberger, Flannick et al. 2016).

Comprehensive metabolic profiling has also been increasingly applied to biomarker discovery in relation to insulin resistance and type II diabetes and the results have demonstrated amino acid-related metabolic signatures associated with these conditions (Wang, Larson et al. 2011; Wurtz, Makinen et al. 2012; Floegel, Stefan et al. 2013). These studies have consistently shown that branched-chain amino acids and aromatic amino acids are associated with insulin resistance (Wurtz, Makinen et al. 2012; Wurtz, Tiainen et al. 2012; Wurtz, Soininen et al. 2013) and predict development of type II diabetes (Wang, Larson et al. 2011). It is not clear whether these amino acids are associated with insulin resistance in a mechanistic manner. The seminal study of Newgard et al. showed that branched-chain amino acids contribute to development of obesity-associated insulin resistance in the context of a high fat diet through activation of the mechanistic target of rapamycin (mTOR) (Newgard, An et al. 2009). Earlier experimental studies in humans have suggested that elevated plasma amino acids induce insulin resistance in skeletal muscle by inhibiting glucose transport/phosphorylation (Krebs, Krssak et al. 2002; Tremblay, Brule et al. 2007). Despite these observations, the evidence that branched-chain amino acids cause or exacerbate insulin resistance is not yet conclusive (Adams 2011).

2.3.6 Elevated blood pressure

Elevated blood pressure is a significant risk factor for cardiovascular complications, such as stroke and ischemic heart disease (Kannel 2000; Black 2003). The association between obesity and elevated blood pressure has been recognized for decades (Kannel, Brand et al. 1967; Stamler, Stamler et al. 1978). Increased abdominal fat, in particular, is associated with elevated blood pressure (Siani, Cappuccio et al. 2002; Poirier, Lemieux et al. 2005). The mechanisms that underlie these associations are not entirely clear (Davy and Hall 2004). Studies have indicated that activation of the sympathetic nervous system plays an important role in the etiology of obesity-related hypertension (Kotsis, Stabouli et al. 2010). Physiological hyperinsulinemia stimulates the central nervous system, but does not increase arterial pressure (Rowe, Young et al. 1981; Anderson, Hoffman et al. 1991; Anderson, Balon et al. 1992). However, hyperinsulinemia increases sodium retention in the kidneys (DeFronzo 1981; Salonen, Lakka et al. 1998) and contributes to endothelial dysfunction (Deedwania 2004). Together with increased free fatty acids and leptin, insulin may synergistically stimulate sympathetic activity and promote vasoconstriction, thereby contributing to development of obesity-related hypertension (Montani, Antic et al. 2002).

2.4 Metabolic heterogeneity of obesity

2.4.1 Metabolically unhealthy obesity

Clearly, obesity is associated with adverse consequences for health. To suggest otherwise would seem counterintuitive since by definition obesity is a condition that is detrimental to health (WHO 2000). The detrimental effects of obesity on health are supported by a number of studies. The seminal Framingham Heart Study with 26 year follow-up, for example, showed that obesity is independently associated with development of heart disease (Hubert, Feinleib et al. 1983), and subsequent studies have provided evidence for the close connection between excess adiposity, cardiovascular disease (Kenchaiah, Evans et al. 2002) and mortality (Calle, Thun et al. 1999; Peeters, Barendregt et al. 2003). Furthermore, obesity is so closely associated with type II diabetes that the term "diabesity" has been coined (Golay and Ybarra 2005). The relationship between obesity and cardio-metabolic disease is most likely causal. This is supported by evidence that weight loss lowers disease risk; lifestyle interventions aimed at body weight control through physical activity and or diet modification significantly improve diabetes risk factors in healthy normoglycemic adults as shown in a recent meta-analysis (Appuhamy, Kebreab et al. 2014). The Finnish Diabetes Prevention Study (Tuomilehto, Lindstrom et al. 2001) and the U.S Diabetes Prevention Program (Knowler, Barrett-Connor et al. 2002) both showed significant reduction in diabetes incidence during a mean intervention period of about 3 years, and further analyses indicated that this was mostly attributable to weight loss (Hamman, Wing et al. 2006). The fact that bariatric surgery (and the weight loss that follows) reverses type II diabetes in most patients and improves other cardio-metabolic risk factors further supports the case for a causal relationship between obesity and cardio-metabolic disease (Buchwald, Estok et al. 2009; Brethauer, Aminian et al. 2013).

2.4.2 Metabolically healthy obesity

Despite the well-established link between obesity and cardio-metabolic disease, there appears to be considerable metabolic heterogeneity of obesity, suggesting that obesity is not synonymous with poor health (McLaughlin 2012). In fact, approximately 10 to 25% of obese individuals appear to be at least partially protected from the development of metabolic abnormalities that frequently accompany excessive adiposity (Bluher 2010). This phenomenon was first observed in 1965 when Albrink and Meigs noted studed the health of factory workers, and observed that many obese men had normal serum triglyceride levels (Albrink and Meigs 1965). Subsequently, Keyes in 1973 (Keyes 1973) and Andres in 1980 (Andres 1980) analyzed epidemiological data and concluded that for some people, obesity was not a risk factor for cardiovascular disease and mortality. Shortly after, Ethan Sims included the "healthy obese" phenotype as a subtype of obesity in his classification of obesity in 1982 (EAH 1982). Since then, much

research has been devoted to characterize this phenotype that in the current literature is collectively referred to as metabolically healthy obesity (MHO) (Karelis 2008).

In general terms, MHO describes an obese individual (BMI ≥ 30) with absence of any metabolic disorders, including insulin resistance, type II diabetes, dyslipidemia and hypertension (Bluher 2010). However, various, less strict criteria with precise definitions to classify healthy obesity have been suggested in the literature. These criteria are not considered here in detail, but the available definitions and specific cut-off values for each parameter have been reviewed elsewhere (Bluher and Schwarz 2014). Until very recently (van Vliet-Ostaptchouk, Nuotio et al. 2014) there has been a lack of consensus how to define metabolically healthy obese (Stefan, Haring et al. 2013; Plourde and Karelis 2014). This is probably the main reason why the reported prevalence of MHO has varied from 7% to 51% in different obese populations (Rey-Lopez, de Rezende et al. 2014; Wang, Zhuang et al. 2015). On the other hand, considerable variability in the prevalence of healthy obesity was reported between cohorts from different European regions despite the use of the same diagnostic criteria (van Vliet-Ostaptchouk, Nuotio et al. 2014). Thus, the range in prevalence may also be attributed in part to differences in ethnicity, genetics and lifestyle factors (Berezina, Belyaeva et al. 2015; Navarro, Funtikova et al. 2015). In Finland, metabolically healthy obesity was observed in 9.2% of obese men and in 16.4% of obese women (Pajunen, Kotronen et al. 2011). Importantly, it has been shown that the prevalence of MHO is higher when obesity is defined by percent body fat mass compared with BMI (Velho, Paccaud et al. 2010), suggesting that the true prevalence MHO may have been underestimated (Shea, Randell et al. 2011). Available data related to the MHO phenotype are mainly for adults as there are very limited studies that have assessed MHO in children. As in adults, there is considerable variety in MHO definitions for the pediatric population. Suggested definitions include BMI >95th percentile and quartiles of HOMA-IR (Vukovic, Mitrovic et al. 2013), or BMI z-score > 95th percentile and ≤ one cardio-metabolic risk factor based on age and gender-specific cut points of diastolic blood pressure, fasting plasma glucose, serum triglyceride and HDL cholesterol (Camhi, Waring et al. 2013; Senechal, Wicklow et al. 2013; Weghuber, Zelzer et al. 2013). The prevalence of MHO in these studies varied between 16% and 68%, depending on the criteria applied.

What accounts for the preserved metabolic function of MHO individuals is not well understood. Intra-abdominal fat is generally lower in MHO individuals compared to their unhealthy obese counterparts. Similarly, ectopic fat accumulation in the liver and skeletal muscle is lower in MHO compared with unhealthy obese (Karelis, St-Pierre et al. 2004). These findings support the notion that abdominal obesity and ectopic fat accumulation might be key determinants of metabolic health. Level of physical activity and cardio-respiratory fitness may also contribute to healthy obesity (Ortega, Cadenas-Sanchez et al. 2015). Higher cardiorespiratory fitness is associated with lower risk of type II diabetes in obese individuals (Lee, Sui et al. 2009), and attenuation of subclinical

carotid atherosclerosis is observed with increasing levels of cardiorespiratory fitness in MHO individuals (Jae, Franklin et al. 2015). It may be that the benefits of higher physical activity and fitness are mediated partly through lower visceral fat mass and liver fat (O'Donovan, Thomas et al. 2009).

As in the adult population, it is unclear why some obese children do not develop the metabolic abnormalities that typically accompany obesity, while others do. One explanation could be in the early-life growth characteristics and in particular in age at adiposity rebound. The adiposity rebound is the second rise in body mass index that occurs between 5 and 7 years of age (Rolland-Cachera, Deheeger et al. 2006). Recent studies have shown that early adiposity rebound (before the age of 5 years) predicts higher metabolic risk later in childhood and adulthood (Gonzalez, Corvalan et al. 2014; Peneau, Gonzalez-Carrascosa et al. 2016). The mechanism for this association is not clear, but it might be explained by increased adipose tissue dysfunction, which could arise from a rapid increase in fat mass that outpaces the normal adipose tissue development.

To date, few metabolomic studies, including one from our laboratory (Wiklund, Pekkala et al. 2014), have aimed to elucidate the molecular basis for metabolically healthy and unhealthy obese individuals. Batch et al. (Batch, Shah et al. 2013) identified several clusters of metabolites comprising branched-chain amino acids and acyl carnitines that distinguished metabolic health independent of BMI. Subsequently, Badoud et al. (Badoud, Lam et al. 2014) also showed lower levels of amino acids (including branched-chained amino acids) in MHO compared to metabolically unhealthy individuals. In that study adipose tissue gene expression profiling showed that genes related to branched-chain amino acid catabolism and the tricarboxylic acid cycle were less down-regulated in MHO individuals compared to metabolically unhealthy individuals, suggesting that increased circulating branched-chain amino acids may be attributable to reduced ability of adipose tissue to catabolize these amino acids. Recently, a metabolomics study in cultured human adipocytes collected from MHO and metabolically unhealthy subjects showed reduced intracellular levels of aspartate in metabolically unhealthy (insulin resistant) subjects, suggesting either a relative depletion of the TCA cycle or reduced aspartate uptake (Bohm, Halama et al. 2014). Taken together, these studies indicate that amino acid homeostasis is an important factor in cardio-metabolic health.

It is unclear whether obese people who are metabolically healthy will remain so over time. Several studies have suggested that the risk of type II diabetes (Meigs, Wilson et al. 2006; Appleton, Seaborn et al. 2013), cardiovascular disease and all-cause mortality (Kip, Marroquin et al. 2004; Song, Manson et al. 2007; Calori, Lattuada et al. 2011; Hosseinpanah, Barzin et al. 2011; Voulgari, Tentolouris et al. 2011; Hamer and Stamatakis 2012; Ogorodnikova, Kim et al. 2012) are not greater in MHO compared with metabolically healthy normal weight individuals in the long-term. Consistent with these reports, a recent Norwegian longitudinal population study with over 60,000 people found that the risk of myocardial infarction was not increased among MHO compared

with normal-weight, metabolically healthy subjects after 12 years follow-up, although the risk of heart failure was significantly increased (Morkedal, Vatten et al. 2014). However, a recent study with over 17 years follow-up showed that although individuals with MHO are at decreased risk for developing type II diabetes compared with unhealthy obese subjects, they are at increased risk for developing cardiovascular disease and type II diabetes compared with metabolically healthy normal weight individuals (Hinnouho, Czernichow et al. 2015). Other longitudinal studies have also reported that, regardless of their metabolic status, obese individuals have an increased risk for cardiovascular events and mortality (Meigs, Wilson et al. 2006; Song, Manson et al. 2007; Kuk and Ardern 2009; Arnlov, Ingelsson et al. 2010). Recent meta-analyses showed that MHO will eventually develop into unhealthy obesity if the follow-up period is long enough (Kramer, Zinman et al. 2013; Bell, Kivimaki et al. 2014; Eckel, Meidtner et al. 2015). This suggests that MHO may represent a snapshot in the time line of metabolic health; MHO might simply be a transient state.

2.4.3 Normal weight obesity

It is assumed that individuals with normal BMI have low body fat content, and thus are not at increased risk for cardio-metabolic diseases. However, as mentioned earlier, a major limitation of BMI is that it cannot differentiate lean mass from body fat. Therefore, people with low lean mass but high body fat content may have a low BMI. Indeed, recent meta-analysis suggested that half of the people with high body fat percentage may be misclassified as normal weight by BMI (Okorodudu, Jumean et al. 2010). These individuals are referred to as normal weight obese (NWO). Most studies define NWO as BMI<25 with body fat percent >30% for women and >25% for men. However, there is no consensus how to define excessive adiposity based on body fat percent, and thus the proposed cut-off points for obesity vary between 20 and 25% for men and 30 to 37% for women in different studies (Oliveros, Somers et al. 2014). In Finland, 34 % of men and 45 % of women have been reported to have NWO (Männistö, Harald et al. 2014). The lack of standardized definition may partly explain why the prevalence of NWO ranges from 3% to 34 in men and 2% to 45% in women (Marques-Vidal, Pecoud et al. 2008; Marques-Vidal, Pecoud et al. 2010; Männistö, Harald et al. 2014). Without such standardized definition, the true prevalence of NWO is difficult to quantify.

Despite the ambiguity of definitions, growing evidence indicates that individuals with NWO are at increased risk for cardio-metabolic dysregulation (Shea, King et al. 2012) and subclinical atherosclerosis (Kim, Kyung et al. 2015). They also have more cardio-metabolic risk factors (De Lorenzo, Martinoli et al. 2006; De Lorenzo, Del Gobbo et al. 2007; Marques-Vidal, Pecoud et al. 2010; Romero-Corral, Somers et al. 2010), coronary heart disease (Romero-Corral, Somers et al. 2007; Kosmala, Jedrzejuk et al. 2012), and they have a higher risk for cardiovascular and all-cause mortality (Coutinho, Goel et al. 2013; Sahakyan, Somers et al. 2015) compared to normal weight lean individuals. There are also individuals who are not obese based on any measure, but who are, like the un-

healthy obese, hyperinsulinemic and insulin resistant, and thus have the same risk for developing cardio-metabolic diseases (Ruderman, Schneider et al. 1981; Ruderman, Chisholm et al. 1998). Such individuals are common in the general population and it is likely that they represent a subset of all individuals with NWO. These observations conflict with the widely held belief that maintaining a normal body weight protects against cardio-metabolic diseases. Whereas NWO is known to associate with increased risk for cardio-metabolic disease in adults, there is considerable paucity of data in children and adolescents. A recent systematic review and meta-analysis indicated that more than 25% of children and adolescent with high percent body fat may be misclassified as normal weight when using only BMI to diagnose obesity (Javed, Jumean et al. 2015). However, to date, no study has evaluated whether NWO in early childhood tracks into adulthood, and whether it increases risk for cardiovascular disease later in life.

3 PURPOSE OF THE STUDY

Understanding the relationship between obesity and its metabolic sequelae is important because it may help to develop more tailored strategies to combat cardio-metabolic diseases. The aim of this study was to assess the relationship between adiposity and cardio-metabolic risk, and to identify biomarkers that associate with common obesity-related metabolic disorders in children and adults. More specifically, the objectives were the following:

1. To identify circulating biomarkers that discriminate middle-aged overweight and obese women with metabolic syndrome from those who are metabolically healthy using a cross-sectional study design (Study I).

2. To identify circulating biomarkers and gene expression profiles of adipose tissue and skeletal muscle that discriminate middle-aged women with and without NAFLD using a cross-sectional study design (Study II).

3. To identify circulating biomarkers and gene expression profiles of adipose tissue and skeletal muscle that are associated with insulin resistance in women with varying degrees of adiposity using cross-sectional study design (Study III).

4. To depict the development of relative body weight and body composition, and to assess the relationship between adiposity and cardio-metabolic risk among peripubertal girls with different body weight status using a longitudinal study design (Study IV).

5. To explore the patterns of longitudinal changes of serum amino acids and triglyceride, and to assess whether amino acid profiles in childhood predict triglyceride levels in early adulthood using a longitudinal study design (Study V).

4 MATERIAL AND METHODS

4.1 Subjects and study design

The original articles presented in this thesis are based on two separate studies: the EWI study and the Calex study. The recruitment of the study populations is presented schematically in FIGURE 5. In 2009, a short-term (6 weeks) aerobic exercise and weight control intervention (EWI) in overweight and obese women was performed in the University of Jyväskylä. The study participants were invited from Jyväskylä Central Finland Health District/Health Promotion Hospital initiative program. One hundred and sixty-one women (all hospital staff) responded to the invitation. One hundred met the screening criteria and were invited to participate in baseline measurements. After the baseline measurements, eligible participants were randomized into parallel exercise (EX) or diet intervention (DI) groups. Only the data obtained from baseline measurements was used in study I.

A description of the Calex study and participant recruitment is described in more detail elsewhere (Cheng, Lyytikainen et al. 2005). In brief, the Calex-study is a longitudinal study in girls with the aim of understanding how bone, fat, and muscle develop through childhood, puberty, and adolescence. The girls, (9-13 years old) were initially contacted in local schools via class teachers. Of 396 girls eligible, 258 (mean age at baseline 11.2 years) participated in measurements over a period of 8 years (mean duration of total follow-up was 7.5 years). Among the 258 girls, 200 participated in 1-year, 221 at 2-year, and 101 at 7-year follow-up. In addition, 135 girls from the original cohort were re-invited to participate in measurements in 2007 and 2008, thus resulting in 236 participants (mean age 18.3 years) at 7-year follow-up measurement. This data was used in studies IV and V.

In 2008-2009 an extension of the Calex study was performed to explore intrinsic linkages between adipose tissue, muscle, and bone in relation to systemic low-grade inflammation. For the purpose of this study, a subgroup of girls from the original cohort (n = 74) and their biological mothers (n=74) and fathers

(n=74) were invited to participate in the study. A total of 184 individuals responded to the invitation, of whom 163 (53 fathers, 53 mothers and 57 daughters) attended the laboratory tests. In study II, all the daughters were excluded from the analysis owing to the low number with NAFLD (n = 5), leaving only the mothers and fathers (n = 106). Valid measurements of liver fat were unavailable for two men. In addition, two men reported recent alcohol consumption of >21 drinks on average per week and one woman reported >14 drinks on average per week. These individuals were therefore excluded. Hence, the final numbers of participants were 49 men and 52 women. Thirty (n = 30) participants had NAFLD, as defined by the cut-off liver fat content value of >5.56% (Szczepaniak, Nurenberg et al. 2005). The remainder, with a liver fat content value of <5.56%, were assigned to the healthy control group (n = 71). However, this thesis focuses on body composition and obesity related cardio-metabolic disorders in women; therefore the results of the study II include only women. In study III, all the fathers were excluded from the analysis in order to reduce the variability in genetic architecture, leaving only the mothers and daughters (mothers = 53 and daughters = 57).

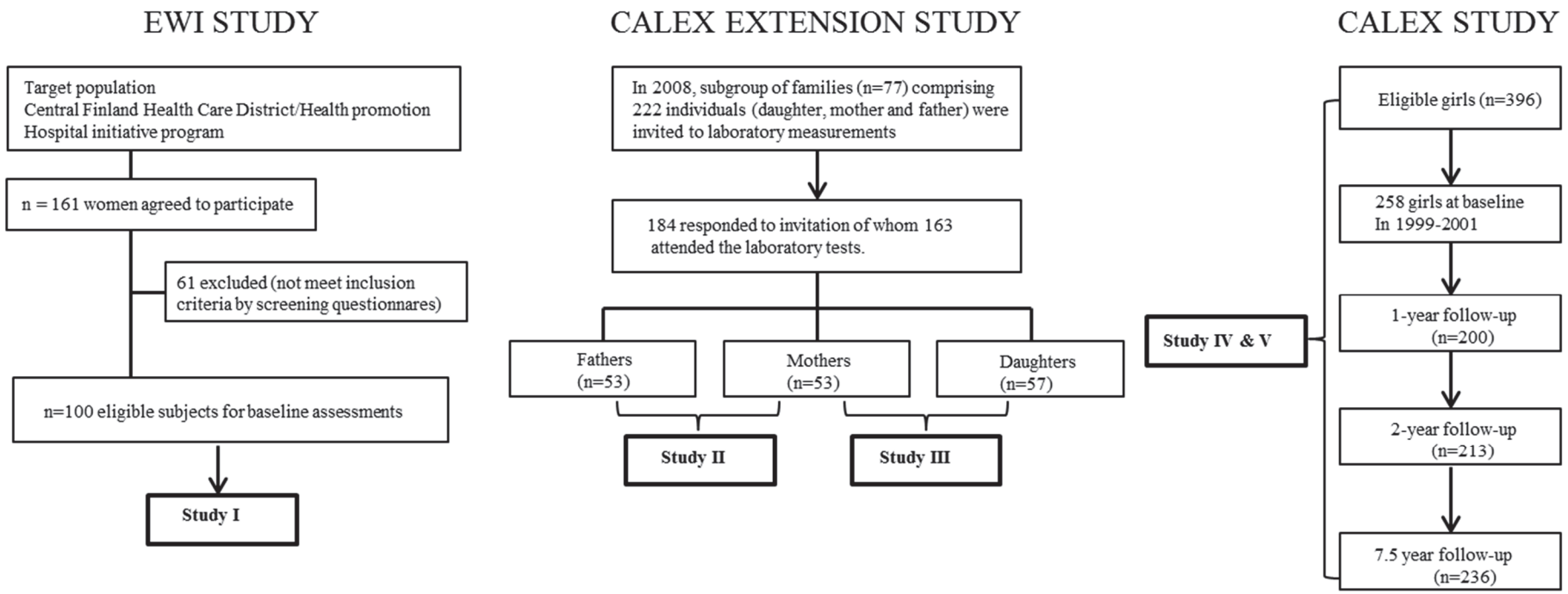


FIGURE 5 Flowchart of the study.

4.2 Measurements

4.2.1 Questionnaires

All variables assessed and methods used in the studies included in this thesis are presented in the TABLE 5. In studies I-V, health history and lifestyle characteristics were collected via self-administered questionnaires. Dietary intake of total energy and energy-yielding nutrients were assessed from three-day food (2 week days and 1 weekend day) records. Dietary intakes of energy, energy yielding nutrients were analyzed using Micro-Nutrica software (Social Insurance Institution, Turku, Finland). Leisure time physical activity (PA), including walking, jogging, running, gym fitness, ball games, swimming, etc., expressed as hours/week and times/week, was evaluated using a validated self-administered physical activity questionnaire, as previously described (Volgyi, Lyytikainen et al. 2010).

4.2.2 Anthropometry and body composition

In studies I-V, body height and weight were measured by using a stadiometer and an electronic scale, to the nearest 0.1 kg and 0.1 cm respectively, with subjects wearing light clothes and without shoes. BMI was calculated by dividing body weight in kilograms by the square of the body height in meters. In study I, waist circumference was measured on bare skin with a tape measure, midway between the top of the iliac crest and the bottom of the rib cage. Two independent measurements were performed and the mean value was used. Blood pressure (BP) in the right arm was recorded using an automated oscillometric device (OMRON M3 Intellisense, OMRON Healthcare, Co., Ltd, Kytoto, Japan) in a sitting position in the morning after a 10 minute rest. Two consecutive measurements were performed, and the mean of the measurements was used. In addition, growth charts of each participant in the Calex study were obtained from Finnish School Health Care System from birth to 17-20 years of age. The data obtained from growth charts was used in the study IV.

TABLE 5 Variables assessed in the studies and methods used.

Variable	Method	Study
Basic information		
Health history	Questionnare	I, II, III, IV, V
Growth chart	Official document	IV
Food records	Questionnare	I, II, III, IV, V
Physical activity	Questionnare	I, II, III, IV, V
Maximum oxygen uptake	Bicycle ergometer	I, III
Resting energy expenditure	Respiratory gas analysis	I
Anthropometry		
Body height	Stadiometer	I, II, III, IV, V
Body weight	Electronic scale	I, II, III, IV, V
Waist circumference	Tape measure	I
Blood pressure	Automated oscillometric device	I, IV
Body composition		
Body fat mass	DXA (bioimpedance in study I)	I, II, III, IV,V
Body lean mass	DXA (bioimpedance in study I)	I, II, III IV,V
Visceral fat	MRI spectroscopy	II, III
Liver fat	MRI spectroscopy	II, III
Myocellular lipids	MRI spectroscopy	II, III
Serum biochemistry		II, III
Glucose	Photometric assay	I, II, III, IV, V
Triglycerides	Photometric assay	I, II, III, IV, V
Total cholesterol	Photometric assay	I, V
HDL cholesterol	Photometric assay	I, IV
Free fatty acids	Photometric assay	II, III
S-ALAT	Photometric assay	I, II
S-ASAT	Photometric assay	I, II
GGT	Photometric assay	I, II
Insulin	immunofluorescence	I, II, III, V
hsCRP	ELISA	II, III
Leptin	ELISA	II, III
Adiponectin	ELISA	II, III
Biomarkers		
Metabolomics	NMR spectroscopy	I, II, III, IV, V
Adipose tissue gene expression	Microarray/qPCR	II, III
Muscle gene expression	Microarray/qPCR	II, III
Muscle protein expression	Western blot	II, III

In study I, body composition was assessed using bio-impedance (Inbody 720, Biospace Co. ltd Seoul, South Korea). Fat mass (FM) and fat-free mass (FFM) were used in this study. The coefficient of variation (CV) of two repeated measurements on the same day was on average 0.6 % for FM and 0.8% for FFM. In studies II, III, IV and V, body composition was assessed using dual-energy x-ray absorptiometry (DXA) (Prodigy; GE Linar Corp., Madison, WI, USA). Whole body FM and FFM were used in these studies. In study I, FM in android (abdominal region) and gynoid region (hips and thighs) was also determined (FIGURE 6). The CV of two repeated measurements on the same day was on average 2.2% for FM and 1.0% for FFM.

Intra-abdominal and liver fat content were assessed in studies II and III. The abdominal region and liver were scanned using a 1.5 Tesla MR-scanner (GE Sigma CV/i, General Electric Healthcare, Waukesha, WI, USA). Abdominal visceral adipose tissue (VAT) was quantified from a single slice image at the level of the L2-L3 intervertebral disc using the OsiriX software (OsiriX Foundation, Geneva, Switzerland). The results were converted into tissue fat mass in kilograms, taking into account slice thickness and an adipose tissue density of 0.9196 g/ml (Martin, Daniel et al. 1994; Ojanen, Borra et al. 2014). Liver fat content was assessed by ^{1}HMRS with a PRESS sequence and was analyzed using the Linear Combination of Model spectra software which is generally considered to be the gold standard for in-vivo spectroscopy analysis (Borra, Salo et al. 2009). In study II, NAFLD was defined by the cut-off liver fat content value of >5.56% (Szczepaniak, Nurenberg et al. 2005).

Muscle intra-myocellular lipid (IMCL) and extra-myocellular lipid (EMCL) from the tibialis anterior muscle were measured using a similar 1H MRS method with a surface coil placed over the middle part of the muscle (Furuyama, Nagarajan et al. 2014). In order to obtain maximal IMCL and EMCL separation the tibialis anterior muscle was aligned as optimally as possible with the direction of the magnetic field and the voxel was placed parallel to the muscle fibers (Furuyama, Nagarajan et al. 2014).

4.2.3 Resting energy expenditure

Resting energy expenditure (REE), expressed as kcal per day, was assessed by respiratory gas exchange analysis (GEA) using a ventilated-hood system (VIASYS Healthcare, Yorba Linda, CA, USA) in study I. Calibration of the GEA was carried out before each measurement according to the manufacturer's instructions. The subjects were instructed to avoid any strenuous physical activity and large, energy and protein rich meals for 24 h before the measurement. The subjects arrived at the laboratory in the morning after an overnight fast. After relaxing in a measurement bed for 30 min, a ventilated hood was placed over their heads. Oxygen consumption and carbon dioxide production were recorded for 20 min at 1 min intervals, in a supine position and in a thermoneutral (22–24 °C) environment. The first 5 min of the data were discarded as artefacts. The REE was calculated using the modified Weir equation (Weir 1990).

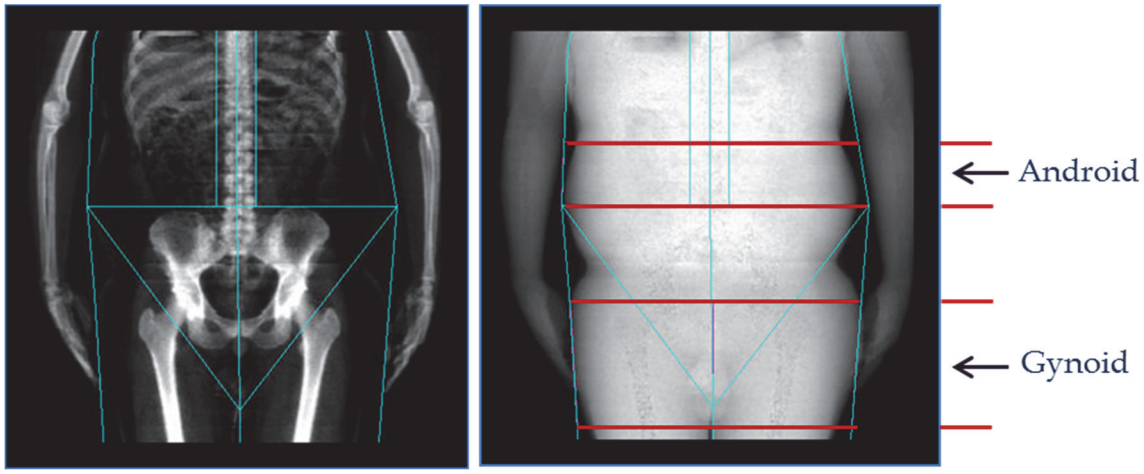


FIGURE 6 Android and gynoid regions obtained by DXA based on bony landmark. Android: Base located at top of pelvis. Height (H) = 20% of distance from top of pelvis to base of skull. Gynoid: Top located at 1.5 x height below base of android region, height = 2 x H.

4.2.4 Cardiorespiratory fitness

Maximal oxygen uptake (VO_{2max}, ml/kg/min) was measured by using a bicycle ergometer under physician's supervision in studies I and III. The test began with a 5-minute warm-up at an intensity of 50W. After that, the intensity was increased by 25W every second minute until exhaustion. Heart rate was measured using electrocardiograph. Blood pressure was monitored before, during and after the test to estimate cardiovascular risk. Oxygen uptake was measured using respiratory gas analyzer VIASYS (Healthcare Inc. USA). VO_{2max} was reached when the measured VO_2 reached a plateau or began to decrease, respiratory exchange ratio was over 1.0 or when the subject wanted to stop the tests because of exhaustion.

4.2.5 Biochemical analyses

In studies I-V, venous blood samples for biochemical analyses were drawn in standardized fasting conditions in the mornings between 7 am and 9 am. In the Calex study and the Calex extension study, the samples were collected 2 to 5 days after menstruation among girls and women with regular menses. Serum samples were stored frozen at -80°C until analyzed. Glucose was analyzed using the KONELAB 20XTi analyzer (Thermo Fischer Scientific inc. Waltham, MA, USA) and insulin was determined by immunofluorescence using the IMMULITE Analyzer (Diagnostic Products Corporation, Los Angeles). The inter- and intra-assay CVs were 2.0% and 3.7% for glucose, 11% and 3.4% for insulin, respectively. In study III, an oral glucose tolerance test with 75 g glucose solution was performed. Blood samples were drawn at fasting state and at 60 min and 120 minutes after glucose ingestion. Insulin sensitivity index (Matsuda index)

was calculated according to Matsuda and DeFronzo (Matsuda and DeFronzo 1999) using the following equation: 10000/sqrt(FPG x FPI x (mean PG x mean PI)), where 10,000 is a constant, FPG and FPI represent the fasting plasma glucose and serum insulin concentrations, PG represents the mean plasma glucose concentrations (60min +120min), and PI is the mean plasma insulin concentration (60min and 120 min), sqrt is the mathematical function to calculate the square root. HOMA-IR index (homeostatic model assessment of insulin resistance) was calculated as (fasting glucose x fasting insulin/22.5) in studies I-V.

Serum total cholesterol, high-density lipoprotein cholesterol, triacylglycerol, non-esterified fatty acids (NEFA), alanine amino transferase (S-ALAT), aspartate amino transferase (S-ASAT) and gamma glutamyltransferase (GGT) were analyzed using the KONELAB 20XTi analyzer (Thermo Fischer Scientific Inc. Waltham, MA, USA) in studies I and II. Serum leptin was assessed using human leptin (ELISA; Diagnostic Systems Laboratories, Inc., Webster, TX), and total adiponectin was measured by an enzyme immunoassay method using the Quantikine human total adiponectin/Acrp30 immunoassay (R&D Systems, Minneapolis, MN) in studies II and III. Inter- and intra-assay coefficients of variation (CVs) were 2.2% and 2.7% for leptin, 3.3% and 4.3% for adiponectin, respectively. In study II and III, serum high-sensitivity C-reactive protein (hsCRP) was assessed using an ELISA DuoSet (R&D Systems and Diagnostic Systems Laboratories, Inc.). The intra- and inter-assay CVs were 4.6% and 6.9%.

4.2.6 Definition of obesity subtypes and metabolic syndrome

In study IV, the participants were categorized based on their BMI and body fat percent at the age 18 as overweight or obese (OWOB, BMI >25 with fat% >30), normal weight obese (NWO, BMI; 18.5-24.9 with fat% >30), normal weight lean (NW, BMI; 18.5-24.9 with fat% <30) and Underweight (UW, BMI <18.5 with fat% <30). In study I, the metabolic syndrome was defined according to a "harmonized" definition (Alberti, Eckel et al. 2009) as the presence of at least three of the following five criteria: waist circumference ≥88 cm, fasting serum triacylglycerol ≥1.7 mmol/L, high density lipoprotein cholesterol (HDL-C) <1.30 mmol/L, glucose ≥5.6 mmol/L) and resting blood pressure ≥130/85 mmHg. In addition, in study I, subjects who were overweight or obese (BMI >25) but had no metabolic syndrome or any of the constituents of the syndrome (except waist circumference ≥88 cm) were categorized as metabolically healthy overweight and obese (MHO).

4.2.7 Cardio-metabolic risk score

In study IV, cardio-metabolic risk was assessed by constructing a standardized, continuously distributed variable for clustered metabolic risk similarly to previously published risk scores (Ekelund, Anderssen et al. 2007; Viitasalo, Lakka et al. 2014). The risk score was calculated by standardizing and then summing the following continuously distributed metabolic traits to create a z score: mean arterial pressure ([(2 x diastolic blood pressure)+systolic blood pressure]/3),

android fat mass, fasting plasma glucose, serum HDL cholesterol x -1, and fasting serum triglyceride z score. A higher score indicates a less favorable cardiometabolic risk profile. The purpose of using such continuously distributed risk score was to maximize statistical power (Ragland 1992) because average differences in metabolic traits tend to be relatively small in children and adolescents.

4.2.8 Serum metabolomics

In studies I-V, serum samples were analyzed using a high-throughput serum NMR metabonomics platform; the experimental protocols including sample preparation and NMR spectroscopy have been described in detail elsewhere (Soininen, Kangas et al. 2009). This methodology combines two molecular windows that contain the majority of the metabolic information available by ^{1}H NMR from native serum such as serum lipids, lipoprotein subclasses as well as various low-molecular-weight metabolites, including various amino acids, ketone bodies and glycolysis intermediates (Soininen, Kangas et al. 2009).

4.2.9 Tissue biopsies and RNA extraction and microarray analyses

Subcutaneous adipose tissue and skeletal muscle biopsies were obtained in studies II and III. Twenty-four participants agreed to donate subcutaneous adipose tissue biopsies, which were obtained under local anesthesia after overnight fasting. A region 5 cm lateral from the umbilicus either to the left or right side was sterilized. A small intracutaneous injection was made, and 2 ml of a local anesthetic agent (lidocaine) was injected. After 5 min, anesthesia was confirmed, skin was sterilized again and 16 G, 40 mm needle, was then adapted to a 50-ml syringe and 10ml of 0.9% sodium-chloride was aspirated. Approximately two-third of the length of the needle was inserted into the subcutaneous fat, and 5 ml of 0.9% sodium chloride was injected. The needle piston was then pulled back maximally and released until it was locked by a stopper, thereby creating a vacuum. Tissue resistance was created by gripping the abdominal skin with one hand while the other hand rotated the needle throughout the tissue in back and forth motion. Once the tissue was aspirated by the syringe, the needle was withdrawn, and the piston was removed. The adipose tissue samples were washed with saline solution, and were immediately frozen in liquid nitrogen and stored at - 80°C.

Twenty-four participants agreed to donate skeletal muscle biopsies, which were obtained under local anesthesia after overnight fasting. Biopsies were obtained from the vastus lateralis dx muscle with a 5-mm Bergström biopsy needle, midway between the patella and greater trochanter. The region and the optimum depth for muscle biopsy were confirmed by ultrasound imaging. The skin of the identified location was sterilized and 4 ml of local anesthetic agent (lidocaine) was injected into the procedure area. A cooling pack was then applied on the location. After 10 minutes, anesthesia was confirmed, skin was sterilized again and a small stab incision was made with a surgical scalpel. Subsequently, the biopsy needle attached to a syringe was introduced perpendicu-

larly into the incision. The piston was then pulled back maximally creating a vacuum and sample was obtained. The muscle sample was cleaned of any visible connective and adipose tissue, as well as blood, and was frozen immediately in liquid nitrogen and stored at −80°C.

Total RNA was extracted from biopsies using the FastPrep system (MP Biomedicals, France) and the RNeasy Lipid Tissue Mini Kit (QIAGEN, Gaithersburg, MD, USA) according to manufacturer's instructions. Total RNA was digested on column with the RNase-free DNase set (QIAGEN) during RNA isolation. The quality of the total RNA was studied using a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and Experion Automated Electrophoresis Station (BioRad, Hercules, CA, USA). The total RNA was amplified and processed using the Gene Chip 3´ IVT Express Kit (Affymetrix, Santa Clara, CA, USA) and hybridized on Affymetrix Human Genome U219 Array Plates. Total RNA was amplified and processed using the GeneChip 3´IVT Express Kit (Affymetrix, Santa Clara, CA, USA) and hybridized on Affymetrix Human Genome U219 Array Plates. Microarray data was pre-processed by the Robust Multiarray Averaging (RMA) algorithm in the R package affy (Gautier, Cope et al. 2004). Differentially expressed genes (DEG) were identified with the limma R package utilizing linear modeling and empirical Bayes methods. P-values were adjusted using the Benjamini and Hochberg multiple adjustment method (Benjamini and Heller 2008).

4.2.10 Gene enrichment analyses

The enriched Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for a given gene set were calculated using the R packages GOStats and KEGG.db. In the enrichment analysis, all human ENSEMBL genes were used as a background gene group and categories with a p-value lower than 0.05 were considered significantly enriched. Genes related to HOMA-IR were identified using the following two criteria: Genes were differentially expressed in our DEG – analysis between the low HOMA-IR and high HOMA-IR groups with adjusted p-value <0.05 or genes with a fold change >= 2 between the low and high HOMA-IR groups. For the gene pathways derived from KEGG enrichment analysis, the mean-centroid value representing the "activity" of the regulated part of the pathway was computed by normalizing the expression levels of all subset genes to a mean of zero and a variance of 1 across all individuals.

4.2.11 Protein extraction from muscle samples and Western blot analysis

Muscle biopsies were homogenized in ice-cold lysis buffer [20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150mM NaCl, 100 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM DTT, 1% Triton-X-100], supplemented with protease and phosphatase inhibitors inhibitors (Sigma Aldrich, St Louis, MO, USA). Thirty to sixty micrograms of muscle lysate samples were separated by SDS-Page using 4-20% gradient gels on Criterion electrophoresis cell (Bio-Rad Laboratories, Richmond,

CA). Proteins were transferred to nitrocellulose membranes at 300-mA constant current on ice at 4ºC. Membranes were blocked in TBS containing 5% nonfat dry milk for 1 hour at room temperature (RT), and then probed overnight at 4ºC with primary antibodies purchased from Cell Signaling Technology (Danvers, MA, USA) (p-Akt, p-IRβ and p-AS160), Sigma-Aldrich (anti-GAPDH) and Abcam (MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail, Abcam, Cambridge, MA, USA). All antibodies were diluted 1:1.000 (except anti-GAPDH (housekeeping, which was diluted 1:40.000) in TBS containing 5% non-fat dry milk. Membranes were then washed with TBS containing 0.1% Tween-20 (TBS-T) followed by 1 hour incubation with the secondary antibody. Odyssey anti-rabbit IRDye 800 and Odyssey anti-mouse IRDye 600 (LI-COR Biosciences, Lincoln, NE, USA) were used as a secondary antibody. Blots were visualized and quantified using Odyssey CLX Infrared Imager of Li-COR and manufacturer's software. When reprobing was needed, the membranes were incubated in 0.2 M NaOH for 10 min at RT, washed with TBS and reprobed with appropriate antibodies. All samples were run in the same gel to minimize the variability and the quantitative results for each protein were normalized to GAPDH.

4.3 Statistical analysis

All data were checked for normality using the Shapiro-Wilk's W-test (PASW Statistics 18). If data were not normally distributed, their natural logarithms were used. Nominal statistical significance was defined as $p<0.05$. For all analyses, the variables for adjustments were chosen based on their known relationships with either the dependent or independent variables.

In study I, clinical characteristics and serum metabolites were compared between MetS and MHO using an independent-samples t-test. To ensure that significant differences in metabolite levels between the groups were not confounded by age, waist circumference or BMI, analysis of covariance (ANCOVA) was used adjusting for the above-mentioned variables. Metabolites were denoted significant if the p-value was lower than 0.0005 to account for multiple testing of 100 independent tests. The metabolomics data was further clustered utilizing a hierarchical clustering algorithm. First, the metabolite and other values were standardized to have 0 as a mean and 1 as standard deviation. Second, the missing values within the data were imputed with k-nearest neighbor algorithm (k = 3). The resulted data values were clustered using correlation distance and average linkage methods.

Given the expected multicollinearity of metabolites, principal component analysis (PCA) was used to reduce the large number of correlated variables into fewer uncorrelated factors. PCA was performed on fasting levels of amino acids, fatty acids, phospholipids, glycoproteins, ketone bodies, and glycolysis and gluconeogenesis intermediates. Varimax rotated factors with an eigenvalue ≥ 1 were identified and metabolites with a factor load ≥ 0.4 were reported as com-

posing a given factor. Metabolomic factor scores were calculated for each individual based on the constructed scoring coefficients. Mean metabolite factor levels were compared between MHO and MetS groups with and without adjusting for age, BMI and waist circumference. Further, we assessed whether factor levels were predictors for MetS using logistic regression models in all subjects adjusted for age, waist circumference and BMI. Finally, the networks between the metabolite factors and clinical risk factors were computed with the Spearman correlation and illustrated using Himmeli software (Makinen, Forsblom et al. 2009).

In study II, clinical characteristics and serum metabolites were assessed in men and women with and without NAFLD. Since the data were from a family study, shared environmental (household) similarity was controlled for in the analysis. The linear mixed model was used to compare levels of the outcome variables between the NAFLD and healthy control groups. In addition, contrast tests were used in mixed models to assess the effect of gender while controlling for dependency among family members with random effects. Because this thesis focuses on body composition and obesity related cardio-metabolic disorders in women, the following results of study II included only women.

The metabolomics data was clustered utilizing a hierarchical clustering algorithm and p-values were adjusted to control for the false discovery rate (FDR) using the method of Benjamini and Hochberg when comparing metabolites between the groups (Benjamini and Heller 2008). Similarly to study I, PCA was used to reduce a large number of correlated variables to fewer uncorrelated factors and metabolite factor scores were calculated and compared between the groups. To exclude the possibility of misclassification, subjects were divided into quintiles based on their liver fat content and compared their mean metabolite factor levels adjusting for HOMA-IR, BMI and visceral fat mass. Pearson correlation analyses were performed to determine the relationship between the gene pathways and clinical characteristics.

In study III, clinical characteristic and serum metabolites were compared between insulin resistant and insulin sensitive women. The degree of insulin resistance was determined by the HOMA-IR index. According to their HOMA-IR values (median = 1.57), the subjects were divided into low (n = 55) and high (n = 55) groups. Since the data were from a family study, the familiarity (genetic and environmental (household) similarity) was controlled by using linear mixed model to compare levels of the outcome variables between the low and high HOMA-IR groups. Contrast tests were used in mixed models to assess the effect of generation while controlling for dependency among family members with random effects. P-values were adjusted to control for the false discovery rate (FDR) using the method of Benjamini and Hochberg when comparing metabolites between groups (Benjamini and Heller 2008). Pearson correlation analysis was used to determine the relationship between clinical characteristics, serum metabolites and adipose tissue gene expression.

In study IV, body composition and cardio-metabolic risk was compared between the OWOB, NWO, NW and UW groups at each time point using anal-

ysis of variance (ANOVA) with the Least Significant Difference post hoc test. In addition, for each single metabolite predictor and measure of fat mass (outcome) a path model was constructed linking the predictor measurements to the outcome measurements in three time-points (FIGURE 7). The model consists of outcome covariances (a-c), predictor effects (d-i) and predictor covariances (j-l). Fat mass and each predictor variable (metabolites), if necessary, were log-transformed to remove excess kurtosis and skewness. Due to varied ranges of measurement scales, all variables were standardized prior to modelling and modelled separately. For each model we grouped the predictors into 11 groups, each including measures from the same metabolic group, and included each group of variables in the same model. We report predictor path coefficients and their 95 % confidence intervals for each predictor in the model. Parameter estimation was conducted in Mplus, version 7.

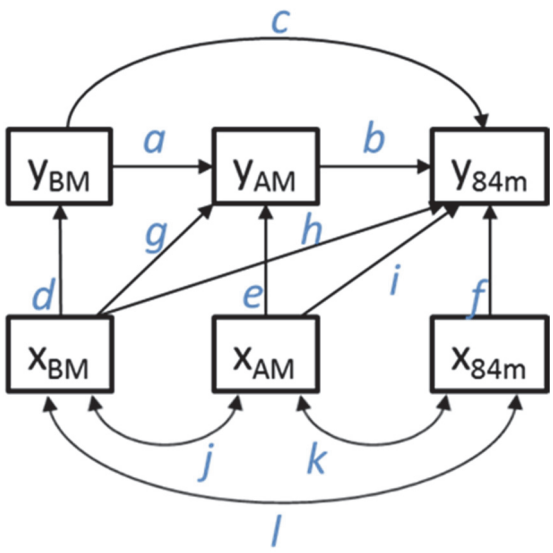


FIGURE 7 Path model for single metabolite predictor. The model consists of outcome covariances (a-c), predictor effects (d-i) and predictor covariances (j-l) for fat mass and each predictor variables. BM = before menarche (age 11), AM = after menarche (age 14), 84 = follow-up at 84 months (age 18).

In study V, longitudinal changes of serum amino acids and triglycerides across pubertal growth in girls were examined using hierarchical models. The data for different time points were compared with each other using the general linear model. A hierarchical (multilevel) non-linear model with random effects (MLwin2.20 software, Multiple Project, Institute of Education, University of London, London, United Kingdom) was used to explore the patterns of longitudinal changes of amino acids, triglyceride and HOMA-IR from pre-puberty to early adulthood. The hierarchical model allows inclusion of the data from every subject despite irregularity of temporally spaced follow-up or missing data (Goldstein 1986). Time relative to menarche was entered as the explanatory variable in the form of polynomial spline functions to explain the change of target

variables over time, as described in detail elsewhere (Cheng, Volgyi et al. 2009). The best model was determined by three criteria: the largest reduction in deviance test (2log likelihood by iterative generalized least squares), the lowest within-individual variance, and the necessary parsimony of the model.

To determine the associations of longitudinal changes in triglycerides with fat mass, HOMA-IR and amino acids before and after menarche, hierarchical models were used in which the outcome variable was triglyceride and the independent variables were amino acids. In this model, the time of menarche was selected as a shift knot for the model, which means that the coefficients of independent variables could be different before and after menarche. Thus, the associations between amino acids and triglyceride were assessed by regression coefficients before and after menarche, respectively. A t-test was used to assess whether the β coefficients were statistically different from 0. Furthermore, we divided subjects into quartiles based on their triglyceride levels at baseline and at 2-year and at 7 year follow-up and compared amino acid levels adjusting for fat mass, HOMA-IR and protein intake. Finally, we used receiver operating characteristics (ROC) curve analyses to determine the predictive effect of variables to identify hypertriglyceridemia in early adulthood. The area under the curve (AUC) is considered a measure of the usefulness of the predictor variable and represents the trade-off between the correct identification of individuals with hypertriglyceridemia (sensitivity) and the correct identification of normolipidemic individuals (specificity).

5 RESULTS

Characteristics of the subjects in separate studies are presented in TABLE 6.

TABLE 6 Characteristics of the study participants.

Study I	**MHO (n=42)**		**MetS (n=36)**	
	Mean	95%CI	Mean	95%CI
Age (years)	39.7	(37.3, 42.0)	44.1	(42.1, 46.1) †
Height (cm)	165.5	(163.7, 167.2)	164.7	(162.5, 166.8)
Weight (kg)	79.1	(76.0, 82.3)	83.1	(79.6, 86.6)
BMI (kg/m^2)	28.9	(27.9, 29.8)	30.6	(29.5, 31.7) *
FM (kg)	29.0	(26.5, 31.4)	32.2	(29.5, 34.9)
Study II	**Healthy controls (n=40)**		**NAFLD (n=12)**	
	Mean	95% CI	Mean	95% CI
Age (years)	50.1	(48.5, 51.6)	51.9	(48.6, 55.3)
Height (cm)	165.8	(164.2, 167.5	167.3	(163.6, 171.1)
Weight (kg)	67.1	(63.8, 70.5)	83.0	(75.9, 90.1) ‡
BMI (kg/m^2)	24.5	(23.4, 25.5	29.7	(27.4, 32.0) ‡
FM (kg)	22.1	(19.7, 24.6)	35.1	(30.0, 40.3) ‡
Study III	**Low HOMA-IR (n=55)**		**High HOMA-IR (n=55)**	
	Mean	95%CI	Mean	95%CI
Age (years)	35.1	(35.9, 36.3)	36.0	(35.0, 36.9)
Height (cm)	165.7	(163.9, 167.7)	166.1	(164.5, 167.6)
Weight (kg)	61.3	(58.4, 64.1)	67.3	(65.1, 69.5) †
BMI (kg/m^2)	22.3	(21.4, 23.2)	24.4	(23.7, 25.1) †
FM (kg)	17.8	(15.5, 20.1)	23.0	(21.9, 25.5) †
Study IV & V	**Baseline (n=230)**		**7-year follow-up (n=220)**	
	Mean	95% CI	Mean	95% CI
Age (years)	11.2	(11.1, 11.5)	18.10	(17.8, 18.4) ‡
Height (cm)	146.3	(145.0, 147.6)	165.10	(163.7, 166.5) ‡
Weight (kg)	39.7	(38.4, 41.1)	60.30	(59.0, 61.6) ‡
BMI (kg/m^2)	18.4	(18.0, 18.9)	21.90	(21.5, 22.3) ‡
FM (kg)	10.7	(10.0, 11.4)	19.5	(18.4, 20.7) ‡

*p<0.05, †p<0.01, ‡p<0.001

5.1 Serum biomarkers and cardio-metabolic risk (Study I)

To improve understanding of the association between excess adiposity and cardio-metabolic risk, we sought to describe and compare serum metabolic profiles in metabolically "healthy" and "unhealthy" overweight and obese women. Metabolically healthy (MHO) women were defined as (BMI>25) with no cardio-metabolic abnormalities (except increased waist circumference), and metabolically unhealthy (MetS) individuals were defined as having metabolic syndrome.

Metabolomics analysis showed higher levels of several fatty acid species, VLDL lipoprotein subclasses, and glycoprotein and branched-chain amino acids in subjects with MetS ($p<0.05$ for all). Principal component analysis of serum metabolites revealed eight metabolite factors composed of correlated metabolites. Metabolite factor 1 (branched-chain amino acids, phenylalanine, tyrosine and orosomucoid) and factor 2 (total fatty acids, omega-6 fatty acids, omega-7 and omega-9 fatty acids, linoleic acid, mono-unsaturated fatty acids, total phosphoglycerides, total phosphocholines) were significantly different between the metabolically healthy and unhealthy groups ($p<0.01$ for both). In a logistic regression analysis adjusted for age, waist circumference and BMI both factors increased the risk for metabolic syndrome with a similar magnitude (OR 2.90 vs. 2.67, $p<0.01$ for both).

Metabolite factor 2 correlated significantly with serum triglycerides ($r = 0.533$, $p<0.001$) and systolic, blood pressure ($r = 0.290$, $p = 0.36$), whereas metabolite factor 1 correlated significantly with serum triglycerides, fasting insulin, HOMA-IR, S-ALAT, systolic blood pressure and inversely with HDL cholesterol ($p<0.001$ for all), (FIGURE 8).

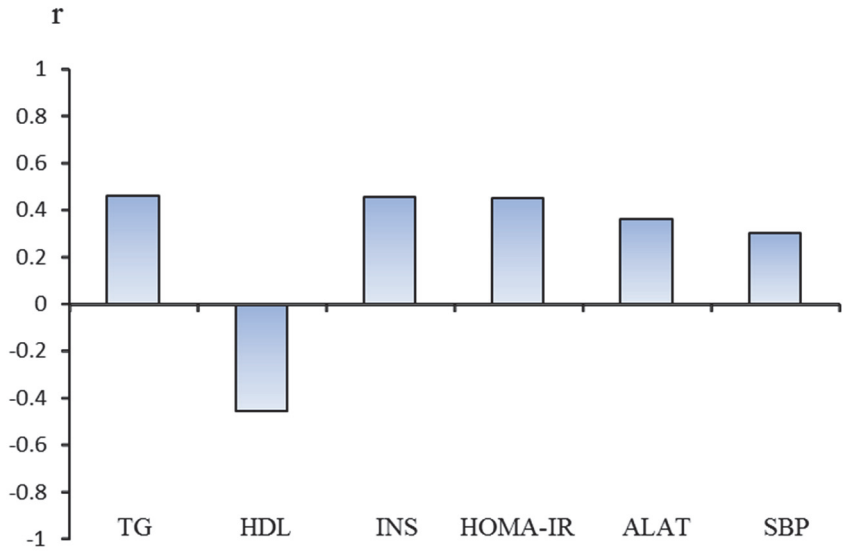


FIGURE 8 Associations between serum metabolite factor 1 (leucine, isoleucine, valine, tyrosine, phenylalanine, orosomucoid) and metabolic traits. TG (triglycerides), HDL (high-density lipoprotein cholesterol), INS (fasting insulin), ALAT (serum alanine amino transferase), SBP (systolic blood pressure).

TABLE 7 shows the correlations of individual metabolites of metabolite factor 1 with triglyceride, HDL-C, fasting insulin, HOMA-IR, S-ALAT and systolic blood pressure. Isoleucine, leucine and orosomucoid were strongly associated with serum triglyceride even after adjusting for age, BMI and waist circumference ($p<0.001$ for all). These metabolites displayed significant associations also with S-ALAT, insulin and HOMA-IR ($p<0.05$ for all). Phenylalanine and tyrosine correlated with S-ALAT and tyrosine with systolic blood pressure.

TABLE 7 Associations between individual metabolites in factor 1 and metabolic traits adjusted for age, BMI and waist circumference.

Metabolites	**TG**	**HDL-C**	**fs-insulin**	**HOMA-IR**	**S-ALAT**	**SBP**
Isoleucine	0.676‡	-0.300*	0.240*	0.285*	0.293†	ns
Leucine	0.572‡	ns	0.211*	0.303*	0.400‡	ns
Valine	ns	ns	ns	ns	ns	ns
Phenylalanine	ns	ns	ns	ns	0.357‡	ns
Tyrosine	ns	ns	ns	ns	0.251*	0.279*
Orosomucoid	0.558‡	ns	0.281*	0.323*	0.385‡	0.280*

TG (triglycerides), SBP (systolic blood pressure), S-ALT (serum alanine*$p<0.05$, †$p<0.01$, ‡$p<0.001$, ns = statistically not significant

5.2 Metabolic alterations associated with NAFLD (Study II)

The metabolites that distinguished metabolically unhealthy overweight and obese women from those who were metabolically healthy in study I displayed significant associations with triglycerides and liver enzymes. Since fatty liver is the most common cause of persistent elevation of liver enzyme levels, the results suggested that fatty liver might be a key determinant of metabolic abnormalities. Therefore, in study III we set out to identify systemic metabolic alterations associated with NAFLD.

The mean liver fat content in the NAFLD and healthy control groups was 15.6% vs. 1.7% respectively ($p<0.001$). Serum metabolomics analysis showed increased levels of VLDL subclasses, mono-unsaturated fatty acids, gluconeogenic substrates, orosomucoid and branched-chain amino acids, and decreased levels of HDL subclasses in participants with NAFLD ($p<0.05$ for all). Principal component analysis of serum metabolites revealed six metabolite factors composed of correlated metabolites. Each factor and their constituent metabolites are shown in the original article (table 2, page 9). Factor 1 (omega 7 and 9 and saturated fatty acids, total fatty acids and mono-unsaturated fatty acids), factor 2 (isoleucine, leucine, valine, phenylalanine, tyrosine and orosomucoid) and

factor 3 (acetate, alanine, lactate, pyruvate) were significantly higher in the NAFLD group compared with the healthy control group ($p = 0.004$ to $p<0.001$).

We further searched for signs of early changes in metabolic pathways in the adipose tissue and skeletal muscle. Gene expression analyses revealed 709 differential expressed genes in the subcutaneous adipose tissue of subjects with NAFLD, but no differentially expressed genes were found in the skeletal muscle. Gene enrichment analysis of the differentially expressed genes in the adipose tissue identified 6 down regulated pathways in NAFLD (TABLE 8).

TABLE 8 Pathways downregulated in subjects with NAFLD.

p-value	Count	Size	Pathway name
$4.6x10^{-9}$	18	44	Valine, leucine and isoleucine degradation
$3.0x10^{-7}$	13	30	Citrate cycle (TCA cycle)
$3.3x10^{-5}$	13	43	Fatty acid degradation
$7.1x10^{-3}$	20	132	Oxidative phosphorylation
$1.9x10^{-2}$	11	65	Glycolysis / Gluconeogenesis
$3.5x10^{-2}$	12	80	Glycerophospholipid metabolism

Count = number of differentially expressed genes that map in pathways, Size= total amount of genes involved in pathway.

Liver fat content in NAFLD was significantly associated with BCAA catabolism pathway, fatty acid degradation, TCA cycle, oxidative phosphorylation ($p<0.001$ for all) and serum metabolite factor 2 ($p<0.05$) (FIGURE 9). In a multiple linear regression analysis, only the serum metabolite factor 2 remained significantly associated with liver fat content ($\beta = 0.552$, $p = 0.046$).

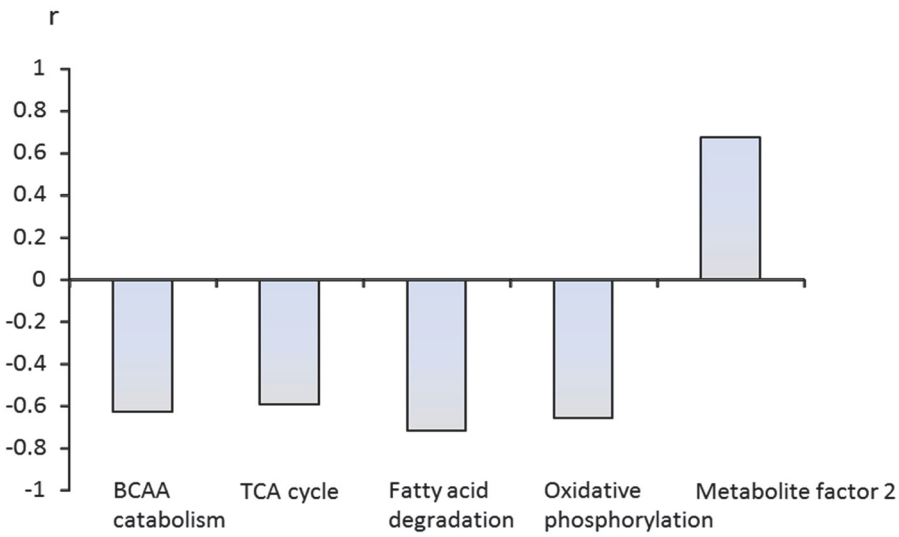


FIGURE 9 Associations for liver fat content with mean centroid of the down-regulated pathways in the adipose tissue and serum metabolite factor 2 (isoleucine, leucine, valine, phenylalanine, tyrosine and orosomucoid).

5.3 Metabolic alterations associated with insulin resistance (Study III)

Because metabolic syndrome and NAFLD are suggested to have common pathophysiological mechanisms, with a focus on insulin resistance and hyperinsulinemia as central factors, in study III we determined to investigate and identify systemic biomarkers associated with insulin resistance in women with varying degrees of adiposity.

Serum metabolite profile analysis revealed elevated levels of branched-chain amino acids (isoleucine, leucine and valine), aromatic amino acids (phenylalanine and tyrosine), glycerol and orosomucoid in the high HOMA-IR group compared with the low HOMA-IR group ($p<0.05$ for all). Differences in branched-chain amino acids and orosomucoid were also consistently present in normal weight individuals (Appendix 1). The associations for serum metabolites with fasting insulin, HOMA-IR and insulin sensitivity are shown in TABLE 9. Branched-chain amino acids, tyrosine and phenylalanine, orosomucoid and glycerol were associated with insulin and HOMA-IR ($p<0.05$ for all). Leucine, valine, orosomucoid and glycerol were associated with insulin sensitivity index ($p<0.05$ for all). Free fatty acids were not associated with insulin, HOMA-IR or insulin sensitivity index.

TABLE 9 Associations for serum metabolites with measures of insulin resistance and insulin sensitivity.

	fs-insulin	HOMA-IR	Insulin sensitivity index
Isoleucine	0.215*	0.210*	ns
Leucine	0.245*	0.276†	-0.396*
Valine	0.311†	0.321†	-0.395*
Total BCAA	0.388†	0.305†	-0.431†
Phenylalanine	0.211*	0.219*	ns
Tyrosine	0.237*	0.212*	ns
Orosomucoid	0.279†	0.329†	-0.444†
Glycerol	0.309†	0.357†	-0.429†
FFA	ns	ns	ns

*p<0.05, †p<0.01, ns=statistially not significant

To elucidate the metabolic pathways characterizing or contributing to insulin resistance, we studied global transcript profiles of adipose tissue and skeletal muscle. Gene expression analyses revealed 1 093 differentially expressed genes in the adipose tissue of subjects with high HOMA-IR, but no differentially expressed genes in the skeletal muscle was found. Gene enrichment analysis of the differentially expressed genes ($p<0.05$) identified 15 up-regulated pathways and 9 down-regulated and pathways (TABLE 10). Associations between gene

expression pathways and insulin sensitivity (Matsuda index) are shown in FIGURE 10. Lysine biosynthesis, propionate metabolism, TCA cycle and BCAA degradation pathways were positively associated with insulin sensitivity, whereas inflammatory-related pathways displayed negative associations (all $p<0.001$). The BCAA catabolism pathway correlated closely with mitochondrial respiration 0.947 and biogenesis, i.e., with the TCA cycle ($r = 0.947$, $p<0.001$) and VO2max ($r = 0.543$, $p<0.05$). The chemokine signaling pathway displayed significant associations with the TCA cycle and BCAA catabolism ($r = -0.812$ and $r = -0.788$, respectively, $p<0.001$ for both).

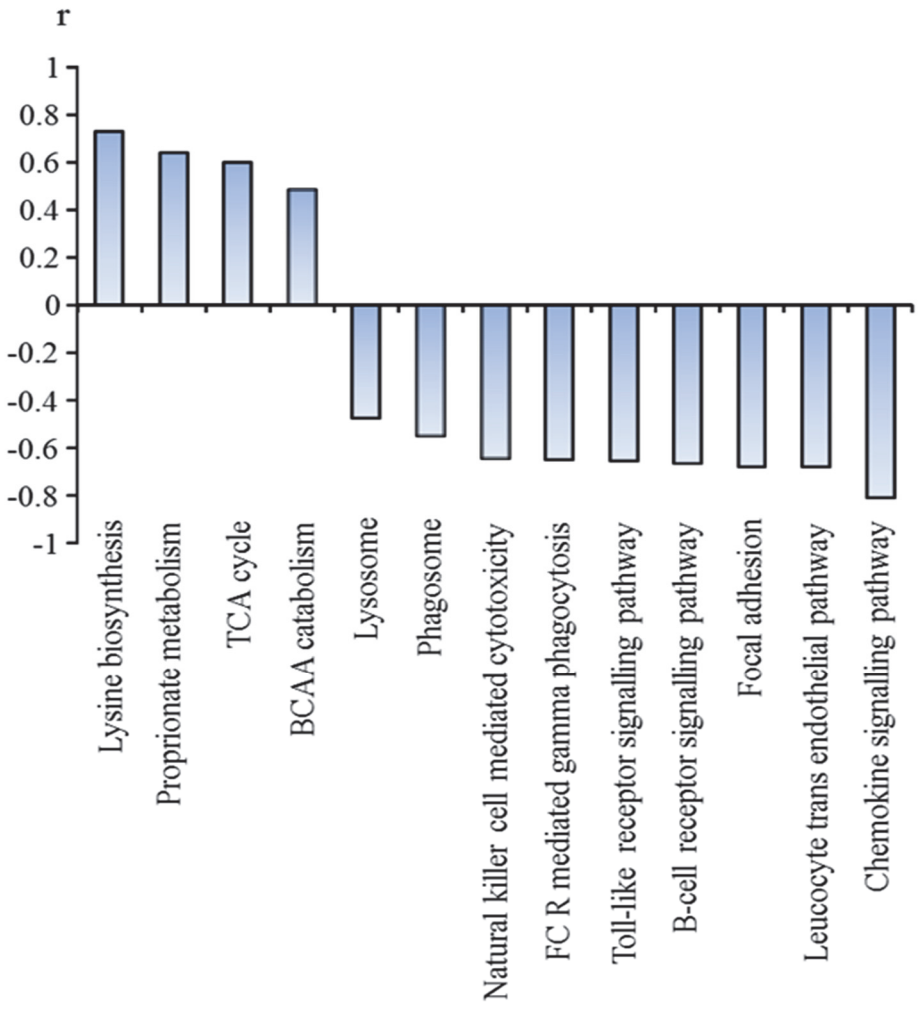


FIGURE 10 Associations for insulin sensitivity with mean centroids of the up and down-regulated pathways in adipose tissue.

TABLE 10 Up and down-regulated pathways in the adipose tissue of the high HOMA-IR group.

p-value	Count	Size	Pathway name
Up-regulated pathways			
$5.6x10^{-13}$	36	121	Lysosome
$2.2x10^{-5}$	28	156	Phagosome
$2.0x10^{-4}$	32	189	Chemokine signaling pathway
$7.4x10^{-4}$	21	117	Leukocyte transendothelial migration
$2.5x10^{-3}$	16	95	Fc gamma R-mediated phagocytosis
$1.9x10^{-3}$	17	102	Toll-like receptor signaling pathway
$2.8x10^{-3}$	10	65	Glycolysis / Gluconeogenesis
$7.8x10^{-3}$	5	17	Renin-angiotensin system
$3.3x10^{-3}$	6	17	Other glycan degradation
$1.3x10^{-2}$	12	75	B cell receptor signaling pathway
$1.3x10^{-2}$	5	19	Glycosaminoglycan degradation
$1.1x10^{-2}$	9	48	Amino sugar and nucleotide sugar metabolism
$1.7x10^{-2}$	11	69	Complement and coagulation cascades
$3.5x10^{-2}$	17	136	Natural killer cell mediated cytotoxicity
$1.2x10^{-2}$	25	200	Focal adhesion
Down-regulated pathways			
$1.1x10^{-7}$	17	44	Valine, leucine and isoleucine catabolism
$3.7x10^{-4}$	10	32	Proprionate metabolism
$2.9x10^{-3}$	6	17	Phenylalanine metabolism
$4.4x10^{-3}$	10	43	Fatty acid degradation
$1.2x10^{-2}$	9	42	Tryptophan metabolism
$1.6x10^{-2}$	7	30	Citrate cycle (TCA cycle)
$2.9x10^{-2}$	8	41	Tyrosine metabolism
$1.7x10^{-2}$	2	3	Lysine biosynthesis
$4.7x10^{-2}$	4	18	Glyoxylate and dicarboxylate metabolism
$1.9x10^{-2}$	12	68	Adipocytokine signaling pathway

Count = number of differentially expressed genes that map in pathways, Size= total amount of genes involved in pathway.

5.4 Body composition and cardio-metabolic risk (Study IV)

Studies I, II and III revealed underlying factors in both blood and adipose tissue that distinguished adults who differ in metabolic status. A particularly notable feature in all three studies was the key discriminatory role of serum branched amino acids for individuals with metabolic disorders. These studies raised new

questions that stimulated us to further explore whether increased cardio-metabolic risk in adulthood associated with adiposity originates from childhood, and whether the association between serum branched amino acids and cardio-metabolic risk found in adults exists already in children (Study IV and V).

In study IV, we investigated development of relative body weight and body composition and the relationship between adiposity and cardio-metabolic risk among individuals with different body weight status. The study subjects were first categorized as OWOB, NWO, NW and UW based on the combination of their BMI and body fat percent at the age of 18. Development of relative body weight between the groups was then compared retrospectively from birth to early adulthood using growth charts.

Longitudinal changes in relative body weight are shown in FIGURE 11. It can be seen that the relative body weight was higher in the OWOB group already at the age of four years compared with the NWO, NW and UW groups ($p<0.05$ for all). While the difference in relative body weight between the OWOB and the other weight groups continued to increase towards early adulthood, the NWO subjects were virtually indistinguishable from their NW peers throughout childhood and adolescence.

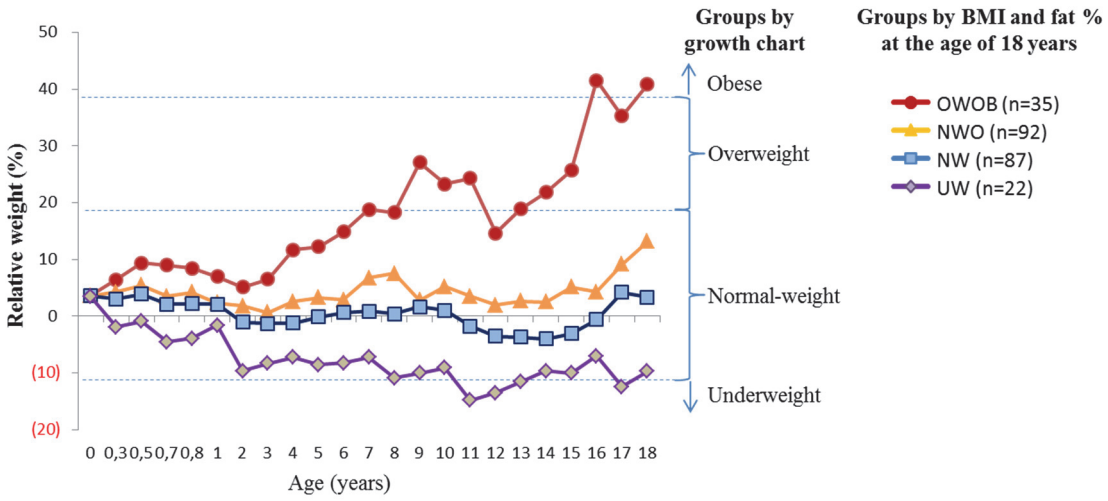


FIGURE 11 Longitudinal changes of relative body weight to height from birth to age of 18 years. Weight groups were defined by the combination of BMI and fat % at of 18 years and the comparison of relative body weight between the groups from birth to early adulthood were done retrospectively. OWOB = overweight and obese (BMI>25 and fat % >30), NWO = normal weight obese (BMI 18.5 - 24.9 and fat % >30), NW = normal weight lean (BMI 18.5 - 25 and fat% <30), UW = underweight (BMI<18.5).

Longitudinal changes in body composition are shown in FIGURE 12. At the age of 11, there was about a 10kg difference in total FM between the OWOB and NW group ($p<0.001$), and about 7kg between the NWO and NW group

(p<0.001) (FIGURE 12A). From age 11 to age 18, total and regional adiposity increased in all groups. The most rapid gain in FM was between the ages of 11 and 14 (FIGURE 12, A, C and D. In terms of FM distribution, the increase was greatest in the gynoid region in all groups (FIGURE 12 D). FM in the android region increased significantly only in the OWOB group (p<0.001) (FIGURE 12 C). Increase in LM was also greatest between the age of 11 and 14, the relative accrual being similar in all groups (FIGURE 12 E). The LM/FM ratio decreased in the NW and UW groups through childhood and adolescence, whereas in the OWOB and NWO groups the LM/FM ratio was relatively constant from childhood to early adulthood (FIGURE 12 F).

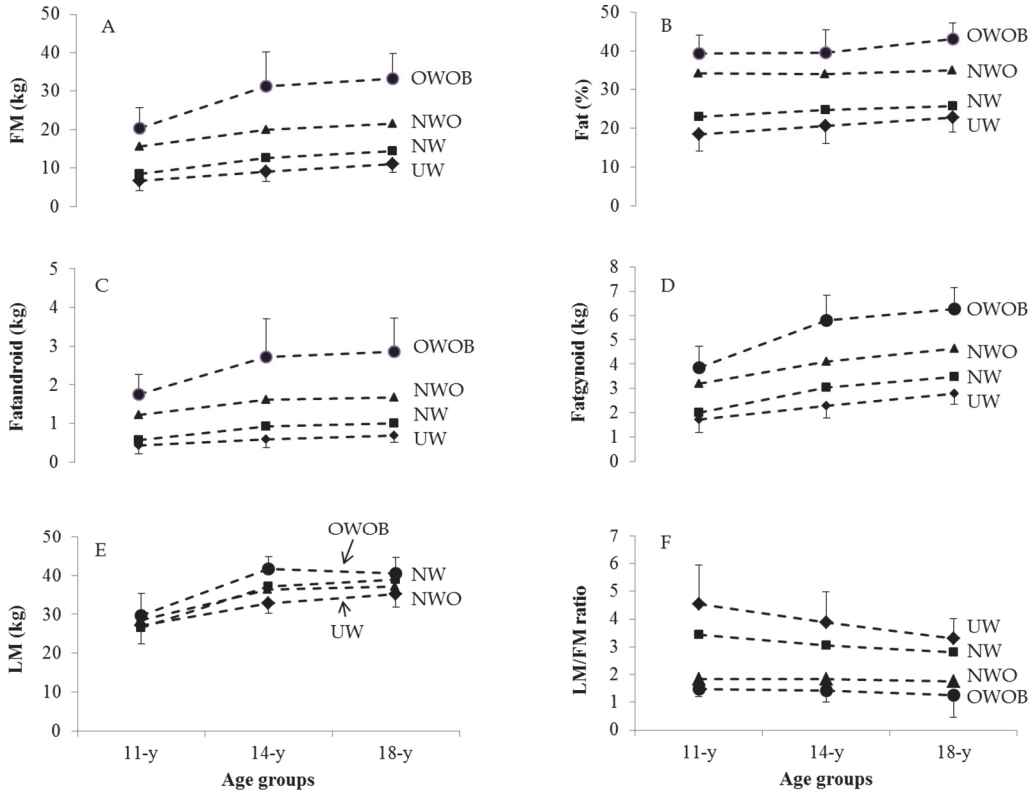


FIGURE 12 Longitudinal changes in total FM (A), fat percent body (B), android FM (C), gynoid FM (D), total LM (E), and LM to FM ratio (F) from age of 11 to age of 18. OWOB = overweight and obese (BMI>25 and fat % >30), NWO = normal weight obese (BMI 18.5 - 24.9 and fat % >30), NW = normal weight lean (BMI 18.5 - 25 and fat% <30), UW = underweight (BMI<18.5).

To estimate and compare cardio-metabolic risk between the body weight groups, constituent traits of the metabolic syndrome i.e. blood pressure, fasting plasma glucose and serum triglycerides and HDL cholesterol were assessed. Longitudinal changes in these metabolic traits are shown in FIGURE 13**.** In general, the OWOB group showed a tendency to have worse values in most traits, e.g., HDL cholesterol, systolic blood pressure and glucose (FIGURE 13, A, B and

C) compared with the other weight groups; however, statistical significance was not reached in all time points because of the relatively small differences. Similarly, the NWO group tended to have worse values in systolic blood pressure, glucose and triglycerides compared to the NW and UW groups (FIGURE 13, B, C and D), but mostly these differences were not statistically significant.

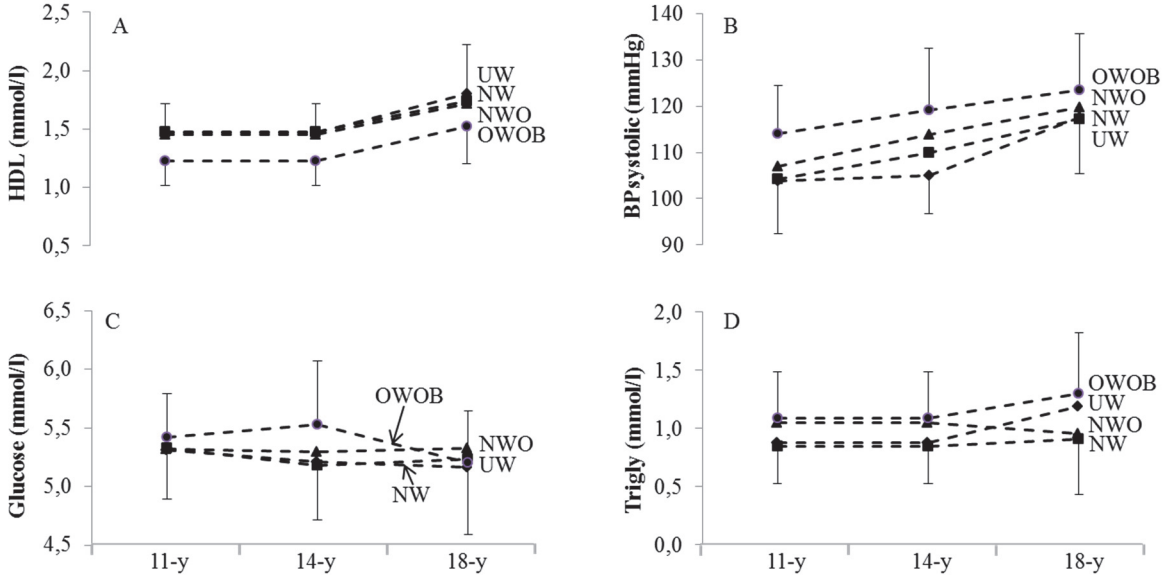


FIGURE 13 Longitudinal changes in serum HDL cholesterol (A), systolic blood pressure (B), fasting plasma glucose (C), and serum triglycerides (D) from age of 11 to age of 18. OWOB = overweight and obese (BMI>25 and fat % >30), NWO = normal weight obese (BMI 18.5 - 24.9 and fat % >30), NW = normal weight lean (BMI 18.5 - 25 and fat% <30), UW = underweight (BMI<18.5).

To be able to better compare cardio-metabolic risk between the groups, a continuously distributed variable for clustered metabolic risk was constructed. The MetS score was calculated using z scores for mean arterial pressure, android fat mass, fasting plasma glucose, serum triglycerides and HDL-cholesterol. Longitudinal changes in MetS scores are shown in FIGURE 14. The OWOB group had significantly higher MetS score compared with all other groups at all time points ($p<0.001$ for all). Similarly, the NWO group had higher MetS score compared with the NW and UW groups ($p<0.01$ for all), whereas, no difference in MetS score was found between the NW and UW groups.

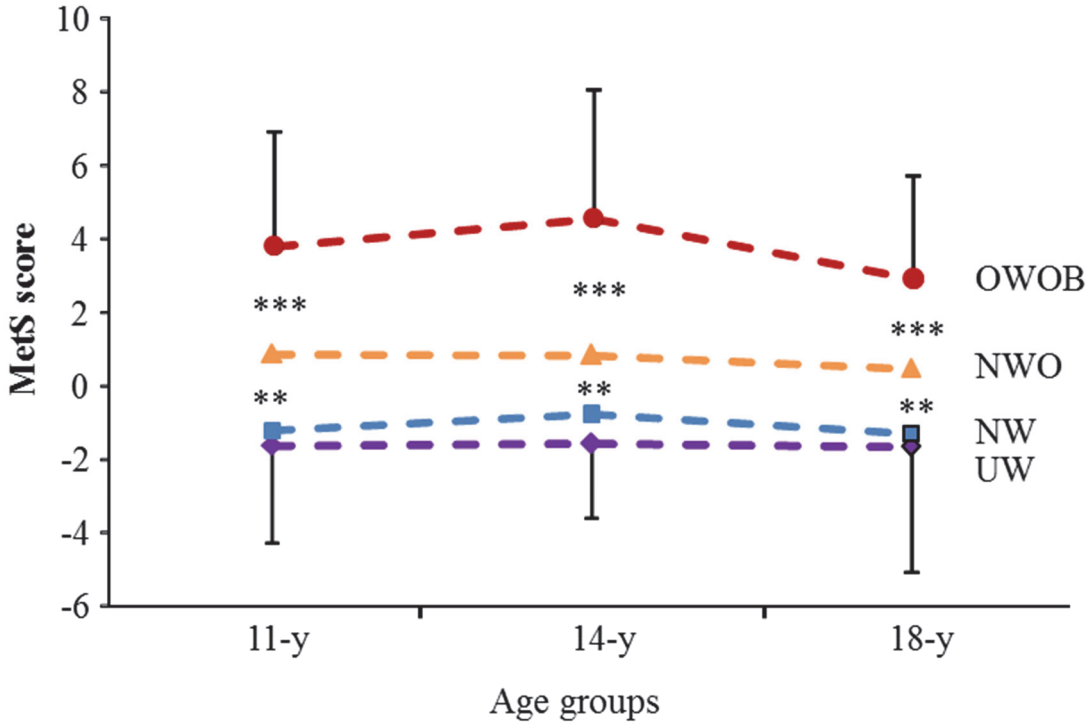


FIGURE 14 Longitudinal changes in MetS score from age of 11 to age of 18. Higher score indicates greater risk. OWOB = overweight and obese (BMI>25 and fat % >30), NWO = normal weight obese (BMI 18.5 - 24.9 and fat % >30), NW = normal weight lean (BMI 18.5 - 25 and fat% <30), UW = underweight (BMI<18.5). ***p<0.001 OWOB compared to NWO, NW and UW, and **p<0.01 NWO compared to NW and UW.

To identify biomarkers that predict fat mass and cardio-metabolic risk in adulthood, we constructed a path model that links the predictor measurements to the outcome measurements at three time-points. FIGURE 15 shows the premenarche (age 11) serum metabolites predicting cardio-metabolic risk score at age 18. We found that levels of medium size VLDL particles, isoleucine and pyruvate were positively and valine was inversely associated with MetS score at age of 18 years ($p<0.05$ for all).

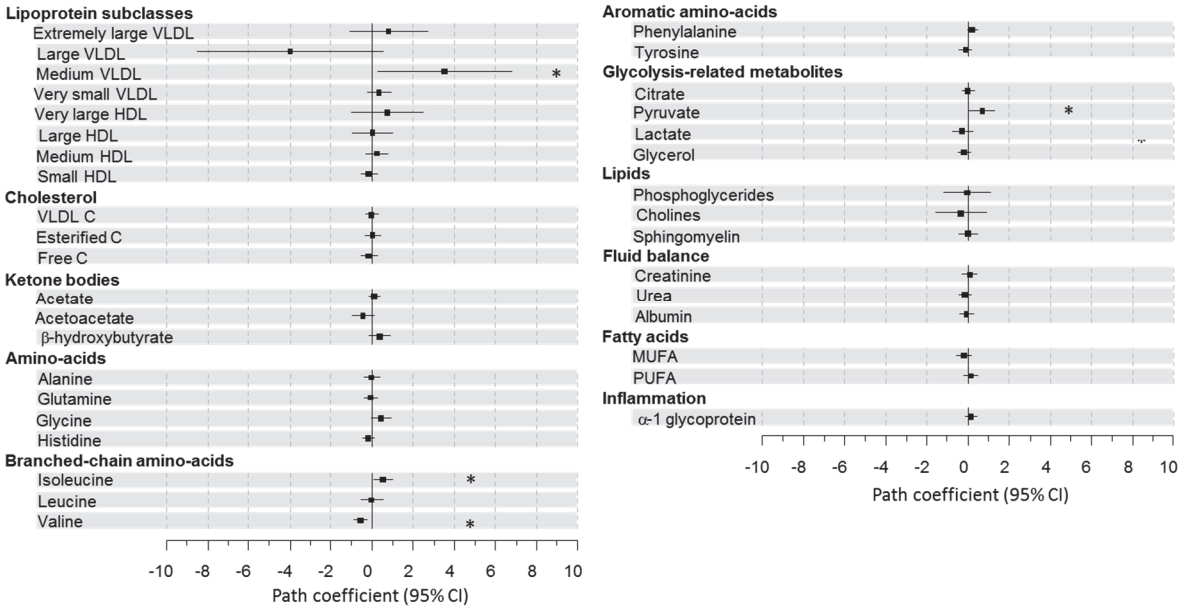


FIGURE 15 Prospective associations of serum metabolites at age 11 with MetS score at age 18. Associations magnitudes are in standardized units of 1 SD MetS per 1 SD difference in metabolite concentration. Error bars indicate 95% confident intervals, *p<0.05.

5.5 Relationship between amino acids and triglyceride (Study V)

The association of the amino acids with triglycerides and NAFLD in studies I and II led us to hypothesize that amino acid profiles in childhood could predict triglyceride levels in early adulthood. This hypothesis was tested in Study V.

We first assessed longitudinal change patterns of serum triglycerides and amino acid concentrations. Triglycerides increased steadily from pre-menarche into early adulthood, whereas amino acids tended to increase before menarche and peaked around menarche, and then decrease into early adulthood. The only exception was alanine, which plateaued around age of 15. Glutamine, isoleucine, and leucine levels were relatively constant before menarche and decreased gradually after menarche, whereas glycine, valine, phenylalanine, tyrosine, and histidine decreased steadily from pre-puberty until early adulthood ($p<0.05$ for all).

Triglyceride was positively associated with alanine ($r = 0.247$), isoleucine and ($r = 0.261$) leucine ($r = 0.235$) ($p<0.05$ for all), but no associations with other amino acids were found. Triglyceride levels at baseline (age of 11 years) predicted subsequent triglyceride levels at 2 year ($r = 0.386$) and at 7-year follow-up ($r = 0.703$, $p<0.01$ for both), but after adjusting for baseline leucine and isoleucine level, baseline triglyceride level was no longer associated with either 2 year or 7 year triglyceride levels (FIGURE 16). By contrast, baseline isoleucine

predicted 7-year triglycerides (r = 0.278, p = 0.026) and baseline leucine predicted 2-year triglycerides (r = 0.279, p = 0.01) even after adjusting for baseline triglyceride level.

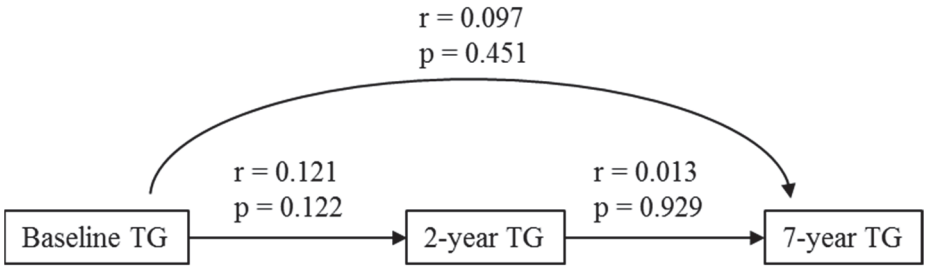


FIGURE 16 Correlations between earlier and subsequent triglyceride levels after adjusting for either baseline or 2-year leucine and isoleucine level.

In early adulthood (at age of 18 years), alanine and isoleucine were significant predictors of hypertriglyceridemia (AUC: 0.683 and 0.774, respectively, $p<0.01$ for both). Leucine was the most significant predictor of hypertriglyceridemia with an AUC of 0.822 ($p<0.001$) (FIGURE 17). By comparison, the AUCs for predicting hypertriglyceridemia were 0.528 for fat mass, 0.536 for fs-insulin, 0.542 for HOMA-IR (all $p>0.05$), respectively.

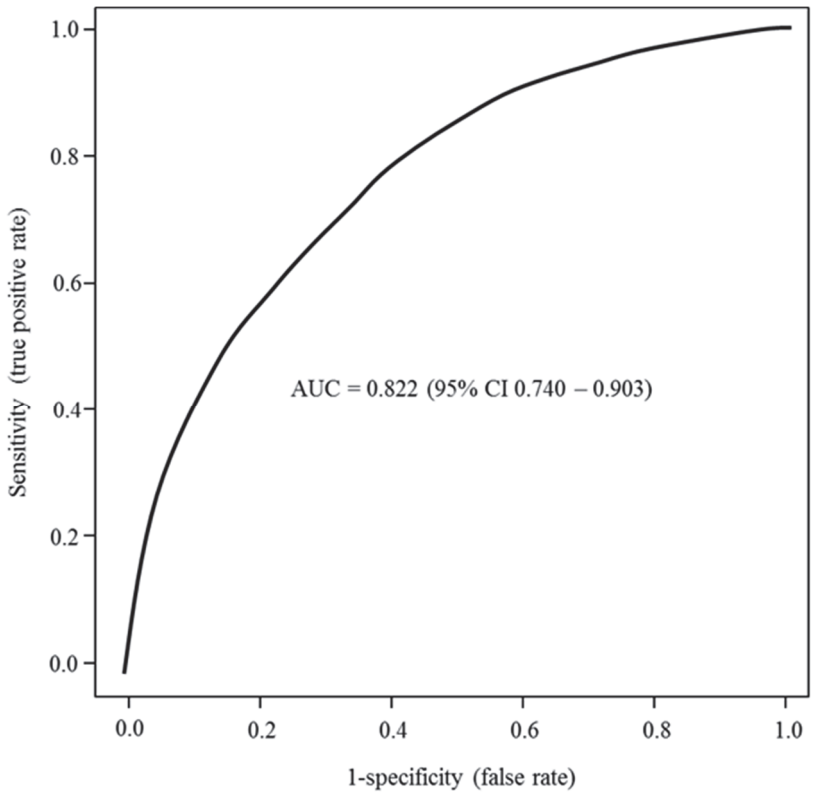


FIGURE 17 Receiver Operating Characteristic curve for leucine to predict hypertriglyceridemia.

6 DISCUSSION

The purpose of this thesis was to investigate and identify biomarkers that predict cardio-metabolic risk in children and adults. The study revealed a discriminatory role of circulating branched amino acids for adults with metabolic syndrome, NAFLD and insulin resistance. Not only were elevated serum branched-chain amino acid levels associated with poor metabolic health, but this was also reflected in subcutaneous adipose tissue gene expression profiles. These studies also showed for the first time that those children who have normal weight but high body fat percent are susceptible to increased cardio-metabolic risk in adulthood. Moreover, high levels of branched chain amino acids in childhood were predictive of increases in fat mass, triglycerides and cardio-metabolic risk later in life. Overall, this thesis provides evidence that support the view that branched-chain amino acids are viable biomarkers to assess cardio-metabolic health both in children and adults, and thus provides a rationale for continued investigation of the relationship between branched-chain amino acid metabolism, adipose tissue function, and metabolic health.

6.1 Metabolic profiles of healthy and unhealthy obese

Obese individuals often present a set of comorbidities such as hypertension, dyslipidemia, insulin resistance and hyperglycemia, which predisposes them to increased risk of type II diabetes and cardiovascular disease (Grundy 2015). Obesity is a condition brought about by chronic positive energy balance, but it is often defined in terms of excess body weight for height (Heo, Kabat et al. 2013). In reality, that excess weight usually reflects excess fat accumulation in adipose tissues, and therefore it is reasonable to assume that the increased risk of cardio-metabolic disease is a consequence of the excess fat. However, growing evidence indicates that not all obese individuals develop type II diabetes or cardiovascular disease; and conversely, not all lean people are metabolically healthy (Karelis, St-Pierre et al. 2004). This paradox allowed us to

hypothesize there might be specific factors that link obesity and its metabolic sequelae, which are not directly related to the amount of body fat *per se*.

Comparison between healthy and unhealthy obesity phenotypes represent a useful model to study the mechanisms linking obesity to its associated metabolic abnormalities (Bluher 2010). We used this approach in study I, and found that elevated serum branched-chain and aromatic amino acids, orosomucoid and several species of fatty acids distinguished overweight and obese women with MetS from those who were metabolically healthy. Although the number of subjects in this study was relatively small, the metabolic signature was highly significant, and the results are largely in agreement with other studies conducted in individuals classified as metabolically healthy and unhealthy obese (Batch, Shah et al. 2013; Badoud, Lam et al. 2014; Perreault, Zulyniak et al. 2014; Chen, Tseng et al. 2015).

It remains unclear why branched-chain amino acids and other metabolites were present in higher quantities in the circulation of metabolically unhealthy subjects. Studies have suggested that impaired metabolic pathways in adipose tissue could cause incomplete catabolism of amino acids, which would subsequently lead to increased circulating amino acid levels (She, Van Horn et al. 2007; Lackey, Lynch et al. 2013; Boulet, Chevrier et al. 2015). Skeletal muscle degradation may also increase due to sedentary behavior-induced insulin resistance (Dirks, Wall et al. 2016), which can result in a greater release of branched-chain amino acids into the circulation. Serum metabolite profile is also sensitive to food intake (Badoud, Lam et al. 2015) and physical activity (Kujala, Makinen et al. 2013). However, it is important to stress that differences in metabolites between the metabolically healthy and unhealthy obese women in the present study are unlikely to reflect dietary intake because metabolites were determined from the serum samples collected after 12 hour fasting to exclude the influences of immediate dietary intake. The study population was also selected to include only participants who were reportedly physically inactive. The fact that there was no difference in cardio-respiratory fitness between the healthy and unhealthy phenotypes suggests that the differences were not due to (inherent or acquired) aerobic fitness either. Thus, the metabolic signatures presented here likely represent prevailing metabolic status, and thus provides insight into differences in amino acid homeostasis between the metabolically healthy and unhealthy individuals.

When exploring the associations between metabolites and metabolic traits, we found that the metabolite factor 1, comprising branched-chain and aromatic amino acids and orosomucoid, was associated with all clinical risk factors for MetS, independent of body weight, fat mass and level of leisure-time physical activity. A closer analysis of the individual metabolites in factor 1 showed that isoleucine and leucine were positively associated with fasting insulin and HOMA-IR. This agrees with the seminal study of Felig et al. (Felig, Marliss et al. 1969) who discovered that elevated branched-chained amino acids in obesity correlated directly with serum insulin levels. Subsequently, several studies have reported associations between branched-chained amino acids and insulin re-

sistance in obese individuals (Huffman, Shah et al. 2009; Newgard, An et al. 2009; Cheng, Rhee et al. 2012; McCormack, Shaham et al. 2013; Wurtz, Soininen et al. 2013) and with the risk for future diabetes, suggesting that branched-chain amino acids might be important regulators of glucose metabolism. However, we noted that isoleucine and leucine were associated not only with insulin resistance, but also with serum triglyceride and liver enzymes (ALAT), suggesting that the relationship between branched amino acids and metabolic health is not confined to glucose metabolism alone.

6.2 Metabolic alterations associated with NAFLD

Since fatty liver is the most common cause of persistent elevation of liver enzymes (after excluding hepatitis C and other known causes of chronic liver disease) (Sonsuz, Basaranoglu et al. 2000), and because isoleucine and leucine are known to mediate activation of several important hepatic metabolic signaling pathways (Adeva, Calvino et al. 2012), we determined to identify systemic metabolic alterations associated with NAFLD. In study II, we found that the same cluster of serum metabolites as in study I (branched-chain and aromatic amino acid and orosomucoid) was significantly higher in subjects with NAFLD compared with healthy controls. Further analysis indicated that this metabolite cluster was significantly elevated at mean liver fat content levels of 2.4%, suggesting that systemic metabolic alterations associated with hepatic fat accretion can be observed early, well below the diagnostic clinical cut-off value for NAFLD. These results are largely in agreement with earlier studies, which have also found associations between elevated circulating branched-chained amino acids and NAFLD (Kalhan, Guo et al. 2011; Iwasa, Ishihara et al. 2015; Rodriguez-Gallego, Guirro et al. 2015).

Concomitantly with the increased serum branched-chain amino acids, we found that the genes involved in branched-chain amino acid degradation, TCA cycle and oxidative phosphorylation were significantly downregulated in the adipose tissue of subjects with NAFLD. The branched-chain amino acids degradation pathway was inversely associated with the serum branched-chain amino acids, insulin resistance and hepatic fat content. Similar findings have been reported in earlier studies which have shown that in obese individuals, the branched-chain amino acids degradation pathway is inversely associated with serum branched-chain amino acids, insulin resistance and hepatic fat content (Pietiläinen, Naukkarinen et al. 2008; Badoud, Lam et al. 2014). In the present study, TCA cycle, oxidative phosphorylation and fatty acid degradation pathways were also inversely associated hepatic fat content, suggesting that impaired adipose tissue function might play a role in the development of NAFLD.

The reason for down-regulation of energy metabolism-related genes in the adipose tissue is not clear, but it may be attributable to inflammation induced either by excessive enlargement of adipocytes or reduced adipocyte differentiation (Goossens 2008). Although a widespread induction of the inflammatory

cascade was not observed, the two most up-regulated genes in the adipose tissue were chitinase-3-like protein 1 (CHI3L1) and matrix metallopeptidase 9 (MMP9). These genes are related to cytoskeleton re-organization and degradation of the extracellular matrix, respectively and have been suggested to cause inflammatory cell infiltration, resulting in persistent inflammation in adipose tissue. However, we did not determine the stroma vascular fraction of the adipose tissue samples and therefore the evidence for the presence of low-grade inflammation in adipose tissue of subjects with NAFLD remains inferential.

We also found elevated intramuscular lipid content in subjects with NAFLD. Unexpectedly, however, no differentially-expressed genes in skeletal muscle were found. Similarly, there were no differences in the phosphorylation levels of several signaling proteins related to glucose metabolism. This may be explained by the fact that tissue samples were obtained after 12 hour fasting when subtle impairments in skeletal muscle metabolism are not observable. An earlier study with healthy, normal weight subjects demonstrated with high-carbohydrate feeding that skeletal muscle insulin resistance alters the distribution pattern of postprandial energy storage, promoting hepatic steatosis (Flannery, Dufour et al. 2012). Thus, our findings by no means suggest that skeletal muscle insulin resistance is not involved the development of systemic metabolic disorders. However, they do suggest that in the early stages of NAFLD, fasting skeletal muscle metabolism may not be altered irrespective of increased intra-myocellular lipid content.

6.3 Metabolic alteration associated with insulin resistance

Many of the adverse metabolic alterations, including NAFLD and metabolic syndrome are themselves mostly related to a reduction in insulin sensitivity (Reaven 2011). Insulin resistance is closely associated with obesity (Muoio and Newgard 2008), but the means by which excessive adiposity induces insulin resistance and glucose intolerance remain controversial. In study III, we aimed to identify systemic biomarkers associated with insulin resistance in women with varying degrees of adiposity.

We found that plasma free fatty acids were not associated with indices of insulin resistance in women with varying degree of adiposity. In addition, there was no difference in intramuscular lipids or liver fat content between the low and high HOMA-IR groups. These findings disagree with the widely accepted view that increases in plasma free fatty acids and accumulation of ectopic lipids are linked with the onset of peripheral and hepatic insulin resistance (Shulman 2014), but are in agreement with recent studies that have questioned the role of elevated free fatty acids in insulin resistance (Karpe, Dickmann et al. 2011; Arner and Ryden 2015).

Elevated serum branched-chain amino acids have long been implicated with obesity and insulin resistance (Felig, Marliss et al. 1969; Newgard, An et al. 2009). In our study, significant differences in these amino acids were found be-

tween the high and low HOMA-IR groups not only in overweight, but also in normal weight individuals. This suggests that perturbations in branched-chain amino acid homeostasis acids are related to insulin resistance rather than to obesity *per se*. Although the average difference in branched-chain amino acids between the low and high HOMA-IR groups was relatively small (~14% in whole study population and ~10% in normal weight individuals), a recent study suggested that such small but chronic increases in plasma branched-chain amino acids can disrupt signaling events in the mitochondria of the muscle and liver, thereby contributing to mitochondrial dysfunction and exacerbating insulin resistance (Sunny, Kalavalapalli et al. 2015).

The positive correlation of branched-chain amino acids with insulin concentration in our study suggests these amino acids may stimulate insulin secretion. This agrees with earlier studies, which have shown that branched-chain amino acids, leucine in particular, are potent stimulators of insulin secretion from the pancreas (Yang, Dolinger et al. 2012). On the other hand, elevated insulin may increase circulating branched-chain amino acids, possibly by decreasing degradation of these amino acids in adipose tissue, as suggested by earlier studies (Sunny, Kalavalapalli et al. 2015). Consistent with this notion, the branched-chain amino acid degradation pathway was downregulated in the adipose tissue of subjects with high insulin resistance in our study. The fact that there was no difference in average BMI or percent body fat between the low and high HOMA-IR groups suggests that down-regulation of the BCAA catabolism pathway was not attributable to increased adiposity. Thus, the decrease in the BCAA catabolism can probably be ascribed to reduced mitochondrial respiration and biogenesis (as indicated by the close correlation of the BCAA catabolism with the TCA cycle). It is possible that differences in aerobic fitness may have amplified the observed differences in gene expression since close correlation was found for VO_{2max} with branched-chain amino acid degradation pathways and the TCA cycle. Our study also complements the widely accepted idea that adipose tissue contributes to the development of insulin resistance (Masoodi, Kuda et al. 2015) by showing that up-regulated inflammation-related genes were closely associated with indices of insulin resistance and serum adiponectin. Furthermore, the chronic inflammation may also, in part, explain the observed impairments in adipose tissue energy metabolism (as indicated by the close inverse correlation of chemokine signaling genes with BCAA catabolism and the TCA cycle genes).

In contrast to earlier studies, we found no differently expressed genes in the skeletal muscle in individuals with high HOMA-IR. In addition, no significant differences in the phosphorylation levels of insulin receptor β and its downstream target Akt were found, nor was there any difference in the level of phosphorylated AS160, which promotes translocation of glucose transporters to the cell membrane. Also, whereas earlier studies have reported reduced muscle transcript levels related to oxidative metabolism in diabetic individuals compared to healthy controls (Patti, Butte et al. 2003), no difference in mitochondrial respiratory chain complex subunits between the low and high HOMA-IR

groups was found in our study. This may be explained by the fact that muscle biopsies were obtained in fasting condition when muscle metabolism is relatively inactive and alterations in glucose uptake are difficult to observe. Since both acute hyperinsulinemia and hyperglycemia have been shown to induce transcriptional and translational regulation of glucose and energy metabolism in the skeletal muscle (Rome, Clement et al. 2003; Meugnier, Faraj et al. 2007), it may be that significant differences could have existed during hyperinsulinemic-euglycemic clamp, glucose challenge or mixed meal feeding.

6.4 The relationship of adiposity and cardio-metabolic risk in children

Studies I, II and III investigated and identified biomarkers that discriminate adult individuals with metabolic perturbations. In study IV, we shifted our focus to children in an attempt to assess whether adiposity-related cardio-metabolic risk in adulthood originates from childhood, and whether serum metabolite levels in childhood predict fat mass and cardio-metabolic risk in adulthood.

We found that subjects who were overweight or obese in adulthood had higher relative body weight to height already at the age of four compared with those who were normal weight. Moreover, overweight and obese subjects had a worse cardio-metabolic risk profile than normal weight subjects in childhood, and this difference persisted through puberty into early adulthood. These findings suggest that the cumulative burden of excess adiposity and its adverse consequences may originate early in life. Supporting this view, Simmonds et al. reviewed large prospective cohort studies and showed that overweight and obese children defined by BMI were five times more likely to be obese in adulthood than those who were not obese (Simmonds, Llewellyn et al. 2016). Similarly, higher BMI during childhood have been shown to associate with an increased risk of cardiovascular disease in adulthood (Baker, Olsen et al. 2007; Franks, Hanson et al. 2010; Juonala, Magnussen et al. 2011; Tirosh, Shai et al. 2011).

Because the diagnostic performance of BMI is not optimal to identify excessive adiposity (Prentice and Jebb 2001), it has been suggested that a significant number of children might be at risk being misdiagnosed as lean if obesity is defined solely based on BMI (Javed, Jumean et al. 2015). Indeed, we showed that children who have normal body weight but high body fat percent are virtually indistinguishable from their normal weight lean peers in terms of relative body weight throughout childhood and adolescence. Moreover, we showed for the first time that these normal weight but obese children have a higher cardio-metabolic risk compared with their normal weight lean peers at all times points from childhood to early adulthood. These results conflict with the widely held

belief that maintaining a normal body weight in childhood protects against cardio-metabolic abnormalities later in life.

Direct comparison of our results with earlier studies is difficult, because normal weight obesity has not been studied before in children transiting from pre-puberty into early adulthood. Although there are few reports that describe children and adolescents with cardio-metabolic risk factors who are not obese according to BMI, most of the children with metabolic abnormalities in these studies had a family history of hypertension, atherogenic serum lipid profile or type II diabetes; however, whether they had low or high body fat percent is not clear because body composition was not assessed (Mahoney, Clarke et al. 1991; Burns, Moll et al. 1992; Gilliam, Liese et al. 2007; Rodriguez-Moran, Guerrero-Romero et al. 2013). In our study, normal weight obese children had no significant history of cardio-metabolic disease in their immediate family, suggesting that the increased cardio-metabolic risk is due to relatively high body fat content. Supporting this contention, studies in adults have shown that normal weight obesity is associated with abnormal serum lipid profile and metabolic syndrome (Conus, Allison et al. 2004), higher cardio-metabolic dysregulation (Shea, King et al. 2012), coronary heart disease (Romero-Corral, Somers et al. 2007) and cardiovascular mortality (Coutinho, Goel et al. 2013; Sahakyan, Somers et al. 2015). Taken together these findings indicate that elevated body fat content conveys increased cardio-metabolic risk, irrespective of body weight.

Four serum metabolites in childhood – pyruvate, isoleucine, valine and medium size VLDL particles - were prospectively associated with MetS score in early adulthood. These four biomarkers are implicated in metabolic homeostasis and can be interpreted as a reflection of mitochondrial dysfunction. Pyruvate is the end product of glycolysis, and it is either catabolized in the cytosol, or it enters into mitochondria to promote oxidative phosphorylation (Vanderperre, Bender et al. 2015). Mitochondrial metabolism of pyruvate plays an important role in energy production, and is essential for regulating glucose-stimulated insulin secretion (Patterson, Cousteils et al. 2014). In states of mitochondrial dysfunction or insufficient oxygen supply pyruvate is not imported into the mitochondria but is converted to lactate and alanine. Obesity is often associated with decreased fatty acid oxidation; therefore obese individuals tend to be more dependent on the glycolytic pathway for energy production, which results increased pyruvate production (Rogge 2009). Simultaneously, obesity is also associated with reduced mitochondrial biogenesis (Demine, Reddy et al. 2014) and diminished respiratory chain activity (Kelley, He et al. 2002).

Consistent with the above, it has been suggested that elevated levels of branched-chain amino acids in the circulation are due to their reduced mitochondrial oxidation (Lackey, Lynch et al. 2013). Elevated circulating levels of these amino acids are known to predict poor metabolic health in adults (Lynch and Adams 2014), and recent studies indicate that they are also elevated in obese children (Perng, Gillman et al. 2014; Butte, Liu et al. 2015) and future insulin resistance in adolescents (McCormack, Shaham et al. 2013). Interestingly, in the present study isoleucine was positively associated with future MetS score,

whereas valine displayed inverse association. Since valine is a strictly glucogenic amino acid, lower serum valine concentrations may reflect increased gluconeogenic activity, with valine being channeled to fuel gluconeogenesis through conversion to pyruvate. Finally, triglyceride-mediated lipoprotein metabolism is an important risk factor for cardiovascular disease (Do, Willer et al. 2013), and over production of VLDL is one of the characteristic features of dyslipidemia and MetS (Adiels, Olofsson et al. 2008). Experimentally-induced mitochondrial dysfunction was found to promote VLDL secretion in human hepatocytes (Mailloux, Lemire et al. 2007), suggesting a role of mitochondrial dysfunction as a further link between dyslipidemia and thus increased cardio-metabolic risk. Together, our results might suggest that mitochondrial dysfunction and metabolic inflexibility associated with cardio-metabolic risk start to develop already in childhood.

6.5 Relationship between amino acids and triglycerides

Following the clues provided by our earlier studies, we determined to explore the patterns of longitudinal changes of serum amino acids and triglyceride and examine whether serum amino acid profiles associate with triglyceride concentrations during pubertal growth and predict hypertriglyceridemia in early adulthood.

In general, the levels of amino acids were highest before menarche and then decreased after menarche until early adulthood (except alanine which increased from pre to post menarche). The decrease in amino acid concentrations after menarche is probably a reflection of somatic growth during puberty and adolescence, as well as changes in proteolysis and protein oxidation. Our results disagree with earlier studies, which have suggested that plasma amino acid levels tend to increase throughout childhood and adolescence (Gregory, Sovetts et al. 1986; Lepage, McDonald et al. 1997; Hammarqvist, Angsten et al. 2010). There may be several factors that could have contributed to these divergent observations, but we believe they may be due study design; other studies assessing amino acid levels in youth have been mainly cross-sectional, while our study is the first that has used longitudinal data from pre-puberty to early adulthood. Moreover, whereas in our study blood samples were drawn at standard phases of the menstrual cycle in girls with regular cycles, no previous study has taken into account the menstrual cycle in their analyses, although it has been demonstrated to affect plasma amino acid levels (Wallace, Hashim et al. 2010).

There was a positive association for serum triglyceride with isoleucine, leucine and alanine both before and after menarche. Further analysis showed that isoleucine and leucine (but not alanine) predicted future triglyceride level, even after adjusting for baseline triglyceride level. In contrast, earlier triglyceride level did not predict subsequent triglyceride levels after adjusting for baseline isoleucine and leucine level. Moreover, these amino acids were elevated in

subjects with high triglyceride throughout growth, and predicted hypertriglyceridemia in early adulthood, even after adjusting for fat mass and HOMA-IR. These results are in line with earlier longitudinal studies in middle-aged and elderly men and women showing a positive association between triglyceride and branched-chain amino acids (Mook-Kanamori, Romisch-Margl et al. 2014; Yamakado, Nagao et al. 2015). Thus, our findings substantiate the idea that amino acids are associated with development of hypertriglyceridemia and indicate that this relation may exist already in childhood and adolescence. Therefore these amino acid indices could be considered as biomarkers to identify individuals at high risk for developing hypertriglyceridemia and cardiovascular diseases later in life.

As with our earlier studies, the mechanisms underlying these associations cannot be clearly defined. Obesity or insulin resistance is unlikely to explain these associations as the association between amino acids and hypertriglyceridemia was not confounded by these variables. Physical activity level did not change considerably during the follow-up period so that is unlikely to explain the associations either. Interestingly, a recent animal study suggested that amino acids may be functionally involved in the development of obesity-related hypertriglyceridemia (Uno, Yamada et al. 2015). In that study, the authors demonstrated that elevated circulating amino acids activate the intracellular target of rapamycin complex-1 (mTORC1)/S6kinase (S6K) pathway in the liver, which modulates systemic lipid metabolism through neuronal inter-tissue communication (Uno, Yamada et al. 2015). However, whether branched-chain amino acids contribute to the development hypertriglyceridemia in a mechanistic manner also in humans remains to be established.

6.6 Implications and future directions

Obesity has reached epidemic -like proportions worldwide, and this has translated into mounted health care costs and increased mortality rates compared to normal weight people. The primary reason for the increases in these costs and mortality is cardiovascular disease, which in turn is ascribed to poor cardio-metabolic profile. This profile includes a constellation of metabolic abnormalities such as insulin resistance, dyslipidemia, fatty liver, and hypertension. However, the underlying mechanisms that trigger and exacerbate these metabolic disorders remain elusive.

In this thesis, I have described a comprehensive metabolic signature of obesity-related metabolic disorders. The overarching finding was the clear discriminatory role of branched-chain amino acids in adults with metabolic syndrome, NAFLD and insulin resistance. Moreover, the levels of branched-chain amino acids in young children were predictive of elevated triglycerides and increased cardio-metabolic risk adulthood. This metabolic signature is in accordance with the growing appreciation that obesity and many of the associated cardio-metabolic disorders are conditions of a broad perturbation not only in

glucose and lipid, but also in amino acid metabolism (Batch, Hyland et al. 2014; Lynch and Adams 2014). The results of the present study also support the idea that reduced expression of genes related to mitochondrial branched-chain amino acid catabolism and TCA cycle in the adipose tissue could underlie the patterns of circulating essential amino acids observed in obesity and insulin resistant states (Adams 2011), thus highlighting the possible contribution of adipose tissue in the development cardio-metabolic disorders (Badoud, Lam et al. 2014). Further research is needed to determine the clinical utility of branched-chain amino acid profiling, and the extent to which the observed associations reflect causal pathways in the adipose tissue.

Since branched-chain amino acids are essential amino acids and are not catabolized directly by the liver, their appearance in the bloodstream must be related to dietary intake. Therefore, one may consider whether diet rich in branched-chain amino acids is detrimental or beneficial to health. Population-based studies show that higher dietary branched-chain amino acid intake is associated with lower prevalence of overweight and obesity in Asian and Western populations (Qin, Xun et al. 2011). Supporting these findings, studies in animals and humans suggest that branched-chain amino acid supplementation has beneficial effect on health in terms in terms of improved glucose tolerance and lower adiposity (Balage and Dardevet 2010; Adams 2011). Animal studies have also shown that enzymes involved in branched-chain amino acid catabolism are reduced by low protein diets and increased by high-protein diets (Adams 2011). If this scenario is true also in humans, increased protein intake could in theory increase branched-chain amino acid oxidation, which would lead to lower blood branched-chain amino acid concentrations. However, Newgard et al. showed that branched-chain amino acids supplementation in the context of high fat diet leads to significant increase in isoleucine, valine and leucine concentrations and increased insulin resistance (Newgard, An et al. 2009). No negative effect on insulin sensitivity was observed when branched-chain amino acids were supplemented with low-fat diet, indicating interaction between branched-chain amino acids and high fat diet. These results highlight the need for further research to understand how dietary interventions alter the branched-chain amino acids oxidation and their circulating levels in humans, and whether they can improve mitochondrial function and metabolic health in obesity and states of insulin resistance and other metabolic disorders.

Finally, a commonly held belief is that excess adipose tissue is detrimental to health. However, an opposing view is that adipose tissue protects against metabolic consequences of over nutrition (Grundy 2015). If this is true, the protective capacity of adipose tissue probably lies in the fat storage capacity. The adipose tissue expandability hypothesis states that a failure in the capacity of adipose tissue to expand and accommodate excess energy rather than adipose tissue *per se* is the key factor linking obesity and positive energy balance to insulin resistance and other cardio-metabolic disorders (Virtue and Vidal-Puig 2010). This idea is intriguing and it could explain many of the findings presented in this thesis. For example, it could explain the differences in serum metabolite

profiles in metabolically healthy and unhealthy subjects with similar level of adiposity. Most certainly it could also explain the aberrant gene expression profiles in subcutaneous adipose tissue of subjects with NAFLD and insulin resistance. And perhaps the reason why normal weight obese subjects are at increased risk for cardio-metabolic disease is also attributable to insufficient fat storage capacity. These considerations suggest that there are aspects in adipose tissue function that could be targets for pharmacological manipulation. However, it I believe that for prevention or treatment of cardio-metabolic risk factors priority should be given to energy balance. This can be achieved either by restricting energy intake or enhancing its expenditure. Best outcome is probably achieved by combining both strategies.

6.7 Limitations

Studies presented in the current thesis are not without limitations. In general, due to their observational nature, studies presented in this thesis cannot determine causality, but rather serve to generate hypothesis for future studies. In all studies the number of study participants was relatively small and this limits the extent to which the results can be generalized beyond these groups. Furthermore, the fact that study participants were all women warrant cautions if seeking to generalize the results to men. However, the results were in line with findings reported in earlier reports. Therefore, I believe that the results are not biased.

There are also some limitations to biomarker discovery that requires consideration. The metabolites in circulation integrate the inputs of multiple organ systems, which make the source of a given signal difficult to ascertain. Although analysis of a serum metabolome can generate a snapshot of the metabolic status of an organism, it does not provide information on pathway flux. This means that it is practically impossible to determine whether a metabolite is increased because it is being produced in excess or because of decreased degradation. The number of subjects with adipose tissue and muscle tissue samples was also relatively small and replication cohorts are considered a requirement for all omics-studies nowadays. Despite this were able to get statistically significant results. Besides, both metabolic and transcriptomic signatures and their associations with cardio-metabolic risk factors were similar in all studies. Moreover, the results were in agreement with earlier reports and therefore I believe the results can be viewed with confidence.

Each study has also its own specific limitations. Considering the results of study I, it is important to note that there can be considerable heterogeneity of metabolic syndrome in terms of combination of constituent traits. For example, using the current criteria for metabolic syndrome, the syndrome can be diagnosed in ten different ways. However, the metabolic syndrome (no matter which way it is defined) is generally accepted as a sign of impaired metabolic health. Therefore, this concept can be used to study the molecular differences

between metabolically unhealthy and healthy individuals. In the study II, the interval between the different data collection points for each study participant varied to some extent, ranging from a few days to several months. However, there was no difference between the healthy control and NAFLD groups in the sampling time window. Further, no change in body weight or body composition was observed during the study period, nor was there any change in diet. In study III, homeostatic model of insulin resistance (HOMA-IR) is derived from fasting insulin and it has been shown that is not a strong estimate of what can be considered "true insulin resistance", especially among normoglycemic individuals. In study IV and V, the subjects were normoglycemic and hence the observations may not be applied to diabetic population. However, considering that the subjects in these studies are all Finnish girls, we believe that this cohort is appropriate for studying the relationships between amino acids and triglyceride metabolism and cardio-metabolic risk during growth from childhood to early adulthood. Strength of these studies was also the rigor exhibited in collecting blood samples in a strictly defined period of the menstrual cycle in girls with regular cycles.

7 MAIN FINDINGS AND CONCLUSIONS

On the basis of the results presented in this thesis, it can be concluded that:

1) Metabolically healthy and unhealthy obese individuals show distinct serum metabolic profiles. Metabolically unhealthy individuals have higher levels of branched-chain and aromatic amino acids and inflammatory-related glycoprotein. These findings imply that amino acid homeostasis is an important factor in cardio-metabolic health.

2) NAFLD is associated with elevated branched-chain amino acids, and this can be observed already well below the diagnostic cut-off value. NAFLD is also accompanied by down regulation of genes related to branched-chain amino acid degradation, TCA cycle and oxidative phosphorylation in the adipose tissue. These findings suggest that adipose tissue dysfunction and perturbed amino acid homeostasis may contribute to accumulation of fat in the liver.

3) Insulin resistance is associated with elevated branched-chain amino acids, downregulation of genes related to branched-chain amino acid catabolism and energy metabolism and up-regulation of wide range of inflammation related genes. These findings suggest that adipose tissue dysfunction and altered amino acid homeostasis are early events in the development of systemic insulin resistance.

4) Excess adiposity starts to develop early in life and this may be difficult to observe using traditional assessment methods such as relative body weight or BMI. High body fat percent in childhood, even when body weight is normal, is associated with poor cardio-metabolic profile in early adulthood. Serum metabolite profiles in childhood suggest that mitochondrial dysfunction and metabolic inflexibility associated with cardio-metabolic risk start to develop already in childhood.

5) Branched-chain amino acids isoleucine and leucine are elevated in subjects with high triglyceride and predict hypertriglyceridemia even after adjusting for fat mass and HOMA-IR. These findings indicate that amino acids are associated with development of hypertriglyceridemia, and therefore may serve as biomarkers to identify individuals at high risk for developing dyslipidemia and cardiovascular diseases later in life.

YHTEENVETO (FINNISH SUMMARY)

Ylipaino ja lihavuus ovat lisääntyneet merkittävästi viimeisen kolmen vuosikymmenen aikana. Lihavuudesta on tullut merkittävä kansanterveydellinen ongelma, koska se altistaa muun muassa metaboliselle oireyhtymälle, tyypin II diabetekselle sekä sydän- ja verisuonisairauksille. Vaikka lihavuuden tiedetään olevan epäedullista terveydelle, viimeaikaiset tutkimukset ovat osoittaneet, että kaikki lihavat eivät sairastu tyypillisiin lihavuuden liitännäissairauksiin. Toisaalta monet normaalipainoiset ihmiset sairastuvat tyypin II diabetekseen ja sydän- ja verisuonitauteihin. Tämä paradoksaaliselta vaikuttava ilmiö viittaa siihen, että lihavuus on heterogeeninen tila. Ylipainon ja lihavuuden liitännäissairauksien syntymekanismeja tunnetaankin vielä verrattain vähän.

Tämän tutkimuksen tarkoituksena oli selvittää lihavuuden ja sen tavallisimpien aineenvaihdunnanhäiriöiden taustalla olevia molekulaarisia muutoksia nuorilla ja keski-ikäisillä naisilla. Lisäksi selvitettiin kehon koostumuksen ja sydän- ja verisuonisairauksien riskitekijöiden kehittymistä lapsuudesta aikuisuuteen sekä arvioitiin kehon koostumuksen ja riskitekijöiden välistä yhteyttä. Ensimmäisessä artikkelissa tarkasteltiin seerumin molekulaarisia eroja keski-ikäisillä ylipainoisilla ja lihavilla naisilla, jotka olivat joko terveitä tai joilla oli metabolinen oireyhtymä. Toisessa artikkelissa selvitettiin ei-alkoholiperäiseen rasvamaksaan liittyviä seerumin molekulaarisia ja rasva- sekä lihaskudoksen geenien ilmentymisprofiilien muutoksia. Kolmannessa artikkelissa käsiteltiin muutoksia seerumin molekulaareissa sekä rasva- ja lihaskudoksen geenien ilmentymisprofiileissa, jotka ovat yhteydessä insuliiniresistenssiin. Neljännessä artikkelissa selvitettiin miten kehonkoostumuksen muutokset kasvuiässä liittyvät sydän- ja verisuonisairauksien riskitekijöiden esiintyvyyteen aikuisiällä. Viidennessä artikkelissa arvioitiin, miten seerumin aminohappojen pitoisuudet lapsuudessa ennustivat triglyseridien pitoisuutta aikuisuudessa.

Tutkimukseen osallistui 396 10–13-vuotiasta tyttöä, joista 258 osallistui 8 vuoden seurantatutkimukseen. Tutkimukseen rekrytoitiin lisäksi 74 tyttöä alkuperäisestä tutkimuskohortista sekä heidän biologiset vanhempansa, sekä 100 keski-ikäistä ylipanoista ja lihavaa naista. Tutkittavien kehonkoostumus määritettiin matalaenergiseen röntgensäteilyyn perustuvalla tekniikalla (DXA) ja lihaksen sekä vatsaontelon sisäisen rasvan määrä arvioitiin magneettiresonanssispektroskopian avulla. Seerumin metabolomiikan määrittämiseen käytettiin magneettiresonanssispektroskopiaa. Lisäksi osalta tutkittavista tutkittiin geenien ilmentymistä rasva- ja lihaskudoksesta.

Tutkimukset osoittivat, että nuorilla ja keski-ikäisillä naisilla kohonneet seerumin haaraketjuisten aminohappojen pitoisuudet olivat yhteydessä metaboliseen oireyhtymään, ei-alkoholiperäiseen rasvamaksaan sekä insuliiniresistenssiin. Ihonalaisessa rasvakudoksessa havaittiin samanaikaisesti muutoksia näiden aminohappojen aineenvaihduntaan liittyvien geenien ilmentymisessä. Tutkimustulokset viittasivat myös siihen, että nämä aineenvaihdunnan muutokset alkavat kehittyä jo lapsuudessa. Lisäksi havaittiin, että normaalipainoisilla lapsilla

voi olla liikaa rasvakudosta, joka lisää riskiä sairastua sydän- ja verisuonisairauksiin aikuisuudessa. Seerumin haaraketjuisten aminohappojen pitoisuudet lapsuudessa olivat myös yhteydessä korkeampiin seerumin triglyseridien pitoisuuksiin aikuisuudessa.

Yhteenvetona voidaan todeta, että nämä tutkimustulokset lisäävät ymmärrystä ja tukevat jo olemassa olevaa tietoa niiden aineenvaihduntahäiriöiden synnystä, joiden yleisesti ajatellaan liittyvän ylipainoon sekä lihavuuteen. Tätä tietoa voidaan käyttää lähtökohtana uusia tutkimuksia suunniteltaessa.

REFERENCES

Aadahl, M., A. H. Andreasen, et al. (2013). Recent temporal trends in sleep duration, domain-specific sedentary behaviour and physical activity. A survey among 25-79-year-old Danish adults. Scand J Public Health 41(7): 706-711.

Abou Ziki, M. D. and A. Mani (2016). Metabolic syndrome: genetic insights into disease pathogenesis. Curr Opin Lipidol.

Adams, L. A. and K. D. Lindor (2007). Nonalcoholic fatty liver disease. Ann Epidemiol 17(11): 863-869.

Adams, S. H. (2011). Emerging perspectives on essential amino acid metabolism in obesity and the insulin-resistant state. Adv Nutr 2(6): 445-456.

Adeva, M. M., J. Calvino, et al. (2012). Insulin resistance and the metabolism of branched-chain amino acids in humans. Amino Acids 43(1): 171-181.

Adiels, M., S. O. Olofsson, et al. (2008). Overproduction of very low-density lipoproteins is the hallmark of the dyslipidemia in the metabolic syndrome. Arterioscler Thromb Vasc Biol 28(7): 1225-1236.

Ala-Korpela, M., A. J. Kangas, et al. (2012). Quantitative high-throughput metabolomics: a new era in epidemiology and genetics. Genome Med 4(4): 36.

Alberti, K. G., R. H. Eckel, et al. (2009). Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation 120(16): 1640-1645.

Alberti, K. G. and P. Z. Zimmet (1998). Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 15(7): 539-553.

Albrink, M. J. and J. W. Meigs (1965). The relationship between serum triglycerides and skinfold thickness in obese subjects. Ann N Y Acad Sci 131(1): 673-683.

Allison, D. B., M. S. Faith, et al. (1996). Risch's lambda values for human obesity. Int J Obes Relat Metab Disord 20(11): 990-999.

Andersen, L. B., M. Harro, et al. (2006). Physical activity and clustered cardiovascular risk in children: a cross-sectional study (The European Youth Heart Study). Lancet 368(9532): 299-304.

Anderson, E. A., T. W. Balon, et al. (1992). Insulin increases sympathetic activity but not blood pressure in borderline hypertensive humans. Hypertension 19(6 Pt 2): 621-627.

Anderson, E. A., R. P. Hoffman, et al. (1991). Hyperinsulinemia produces both sympathetic neural activation and vasodilation in normal humans. J Clin Invest 87(6): 2246-2252.

Andres, R. (1980). Effect of obesity on total mortality. Int J Obes 4(4): 381-386.

Anstee, Q. M., G. Targher, et al. (2013). Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis. Nat Rev Gastroenterol Hepatol 10(6): 330-344.

Appleton, S. L., C. J. Seaborn, et al. (2013). Diabetes and cardiovascular disease outcomes in the metabolically healthy obese phenotype: a cohort study. Diabetes Care 36(8): 2388-2394.

Appuhamy, J. A., E. Kebreab, et al. (2014). Effects of diet and exercise interventions on diabetes risk factors in adults without diabetes: meta-analyses of controlled trials. Diabetol Metab Syndr 6: 127.

Archer, E., C. J. Lavie, et al. (2013). Maternal inactivity: 45-year trends in mothers' use of time. Mayo Clin Proc 88(12): 1368-1377.

Archer, E., R. P. Shook, et al. (2013). 45-Year trends in women's use of time and household management energy expenditure. PLoS One 8(2): e56620.

Arner, P. and M. Ryden (2015). Fatty Acids, Obesity and Insulin Resistance. Obes Facts 8(2): 147-155.

Arnlov, J., E. Ingelsson, et al. (2010). Impact of body mass index and the metabolic syndrome on the risk of cardiovascular disease and death in middle-aged men. Circulation 121(2): 230-236.

Arsenault, B. J., I. Lemieux, et al. (2009). HDL particle size and the risk of coronary heart disease in apparently healthy men and women: the EPIC-Norfolk prospective population study. Atherosclerosis 206(1): 276-281.

Badoud, F., K. P. Lam, et al. (2014). Serum and adipose tissue amino acid homeostasis in the metabolically healthy obese. J Proteome Res 13(7): 3455-3466.

Badoud, F., K. P. Lam, et al. (2015). Metabolomics Reveals Metabolically Healthy and Unhealthy Obese Individuals Differ in their Response to a Caloric Challenge. PLoS One 10(8): e0134613.

Badoud, F., M. Perreault, et al. (2015). Molecular insights into the role of white adipose tissue in metabolically unhealthy normal weight and metabolically healthy obese individuals. FASEB J 29(3): 748-758.

Baker, J. L., L. W. Olsen, et al. (2007). Childhood body-mass index and the risk of coronary heart disease in adulthood. N Engl J Med 357(23): 2329-2337.

Balage, M. and D. Dardevet (2010). Long-term effects of leucine supplementation on body composition. Curr Opin Clin Nutr Metab Care 13(3): 265-270.

Balanza, R., P. Garcia-Lorda, et al. (2007). Trends in food availability determined by the Food and Agriculture Organization's food balance sheets in Mediterranean Europe in comparison with other European areas. Public Health Nutr 10(2): 168-176.

Ballor, D. L. and R. E. Keesey (1991). A meta-analysis of the factors affecting exercise-induced changes in body mass, fat mass and fat-free mass in males and females. Int J Obes 15(11): 717-726.

Bao, W., S. R. Srinivasan, et al. (1997). Longitudinal changes in cardiovascular risk from childhood to young adulthood in offspring of parents with

coronary artery disease: the Bogalusa Heart Study. JAMA 278(21): 1749-1754.

Barker, D. J. (1997). Maternal nutrition, fetal nutrition, and disease in later life. Nutrition 13(9): 807-813.

Batch, B. C., K. Hyland, et al. (2014). Branch chain amino acids: biomarkers of health and disease. Curr Opin Clin Nutr Metab Care 17(1): 86-89.

Batch, B. C., S. H. Shah, et al. (2013). Branched chain amino acids are novel biomarkers for discrimination of metabolic wellness. Metabolism 62(7): 961-969.

Bell, J. A., M. Kivimaki, et al. (2014). Metabolically healthy obesity and risk of incident type 2 diabetes: a meta-analysis of prospective cohort studies. Obes Rev 15(6): 504-515.

Benjamini, Y. and R. Heller (2008). Screening for partial conjunction hypotheses. Biometrics 64(4): 1215-1222.

Berezina, A., O. Belyaeva, et al. (2015). Prevalence, Risk Factors, and Genetic Traits in Metabolically Healthy and Unhealthy Obese Individuals. Biomed Res Int 2015: 548734.

Bertolotti, M., A. Lonardo, et al. (2014). Nonalcoholic fatty liver disease and aging: epidemiology to management. World J Gastroenterol 20(39): 14185-14204.

Bhattacharya, S., C. B. Granger, et al. (2014). Validation of the association between a branched chain amino acid metabolite profile and extremes of coronary artery disease in patients referred for cardiac catheterization. Atherosclerosis 232(1): 191-196.

Blaak, E. (2001). Gender differences in fat metabolism. Curr Opin Clin Nutr Metab Care 4(6): 499-502.

Black, H. R. (2003). The burden of cardiovascular disease: following the link from hypertension to myocardial infarction and heart failure. Am J Hypertens 16(9 Pt 2): 4S-6S.

Blake, G. J., J. D. Otvos, et al. (2002). Low-density lipoprotein particle concentration and size as determined by nuclear magnetic resonance spectroscopy as predictors of cardiovascular disease in women. Circulation 106(15): 1930-1937.

Blew, R. M., L. B. Sardinha, et al. (2002). Assessing the validity of body mass index standards in early postmenopausal women. Obes Res 10(8): 799-808.

Bluher, M. (2010). The distinction of metabolically 'healthy' from 'unhealthy' obese individuals. Curr Opin Lipidol 21(1): 38-43.

Bluher, S. and P. Schwarz (2014). Metabolically healthy obesity from childhood to adulthood - Does weight status alone matter? Metabolism 63(9): 1084-1092.

Blundell, J. E. and N. A. King (1999). Physical activity and regulation of food intake: current evidence. Med Sci Sports Exerc 31(11 Suppl): S573-583.

Boden, G. and S. Salehi (2013). Why does obesity increase the risk for cardiovascular disease? Curr Pharm Des 19(32): 5678-5683.

Bogardus, C., S. Lillioja, et al. (1985). Relationship between degree of obesity and in vivo insulin action in man. Am J Physiol 248(3 Pt 1): E286-291.

Bogl, L. H., S. M. Kaye, et al. (2016). Abdominal obesity and circulating metabolites: A twin study approach. Metabolism 65(3): 111-121.

Bohm, A., A. Halama, et al. (2014). Metabolic signatures of cultured human adipocytes from metabolically healthy versus unhealthy obese individuals. PLoS One 9(4): e93148.

Bokor, S., M. L. Frelut, et al. (2008). Prevalence of metabolic syndrome in European obese children. Int J Pediatr Obes 3 Suppl 2: 3-8.

Borra, R. J., S. Salo, et al. (2009). Nonalcoholic fatty liver disease: rapid evaluation of liver fat content with in-phase and out-of-phase MR imaging. Radiology 250(1): 130-136.

Bouchard, C. (1995). Genetics and the metabolic syndrome. Int J Obes Relat Metab Disord 19 Suppl 1: S52-59.

Bouchard, C. (1997). Genetic determinants of regional fat distribution. Hum Reprod 12 Suppl 1: 1-5.

Bouchard, C. (2007). BMI, fat mass, abdominal adiposity and visceral fat: where is the 'beef'? Int J Obes (Lond) 31(10): 1552-1553.

Bouchard, C., T. Rice, et al. (1996). Major gene for abdominal visceral fat area in the Quebec Family Study. Int J Obes Relat Metab Disord 20(5): 420-427.

Bouchard, C., A. Tremblay, et al. (1996). Overfeeding in identical twins: 5-year postoverfeeding results. Metabolism 45(8): 1042-1050.

Bouchard, C., A. Tremblay, et al. (1990). The response to long-term overfeeding in identical twins. N Engl J Med 322(21): 1477-1482.

Boulet, M. M., G. Chevrier, et al. (2015). Alterations of plasma metabolite profiles related to adipose tissue distribution and cardiometabolic risk. Am J Physiol Endocrinol Metab 309(8): E736-746.

Brethauer, S. A., A. Aminian, et al. (2013). Can diabetes be surgically cured? Long-term metabolic effects of bariatric surgery in obese patients with type 2 diabetes mellitus. Ann Surg 258(4): 628-636; discussion 636-627.

Brouwers, M. C., R. M. Cantor, et al. (2006). Heritability and genetic loci of fatty liver in familial combined hyperlipidemia. J Lipid Res 47(12): 2799-2807.

Brouwers, M. C., M. M. van Greevenbroek, et al. (2012). The genetics of familial combined hyperlipidaemia. Nat Rev Endocrinol 8(6): 352-362.

Brownson, R. C., T. K. Boehmer, et al. (2005). Declining rates of physical activity in the United States: what are the contributors? Annu Rev Public Health 26: 421-443.

Bruce, M. J. and P. T. Katzmarzyk (2002). Canadian population trends in leisure-time physical activity levels, 1981-1998. Can J Appl Physiol 27(6): 681-690.

Buchwald, H., R. Estok, et al. (2009). Weight and type 2 diabetes after bariatric surgery: systematic review and meta-analysis. Am J Med 122(3): 248-256 e245.

Burns, T. L., P. P. Moll, et al. (1992). Increased familial cardiovascular mortality in obese schoolchildren: the Muscatine Ponderosity Family Study. Pediatrics 89(2): 262-268.

Butte, N. F., Y. Liu, et al. (2015). Global metabolomic profiling targeting childhood obesity in the Hispanic population. Am J Clin Nutr 102(2): 256-267.

Buzzetti, E., M. Pinzani, et al. (2016). The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). Metabolism.

Caballero, B. (2007). The global epidemic of obesity: an overview. Epidemiol Rev 29: 1-5.

Calle, E. E., M. J. Thun, et al. (1999). Body-mass index and mortality in a prospective cohort of U.S. adults. N Engl J Med 341(15): 1097-1105.

Calori, G., G. Lattuada, et al. (2011). Prevalence, metabolic features, and prognosis of metabolically healthy obese Italian individuals: the Cremona Study. Diabetes Care 34(1): 210-215.

Camhi, S. M., M. E. Waring, et al. (2013). Physical activity and screen time in metabolically healthy obese phenotypes in adolescents and adults. J Obes 2013: 984613.

Campbell, P. J., M. G. Carlson, et al. (1994). Fat metabolism in human obesity. Am J Physiol 266(4 Pt 1): E600-605.

Canoy, D., B. J. Cairns, et al. (2013). Coronary heart disease incidence in women by waist circumference within categories of body mass index. Eur J Prev Cardiol 20(5): 759-762.

Catenacci, V. A. and H. R. Wyatt (2007). The role of physical activity in producing and maintaining weight loss. Nat Clin Pract Endocrinol Metab 3(7): 518-529.

Chalasani, N., Z. Younossi, et al. (2012). The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. Gastroenterology 142(7): 1592-1609.

Chandra, A., I. J. Neeland, et al. (2014). The relationship of body mass and fat distribution with incident hypertension: observations from the Dallas Heart Study. J Am Coll Cardiol 64(10): 997-1002.

Chen, H. H., Y. J. Tseng, et al. (2015). The metabolome profiling and pathway analysis in metabolic healthy and abnormal obesity. Int J Obes (Lond) 39(8): 1241-1248.

Cheng, S., A. Lyytikainen, et al. (2005). Effects of calcium, dairy product, and vitamin D supplementation on bone mass accrual and body composition in 10-12-y-old girls: a 2-y randomized trial. Am J Clin Nutr 82(5): 1115-1126; quiz 1147-1118.

Cheng, S., E. P. Rhee, et al. (2012). Metabolite profiling identifies pathways associated with metabolic risk in humans. Circulation 125(18): 2222-2231.

Cheng, S., P. Wiklund, et al. (2015). Adipose Tissue Dysfunction and Altered Systemic Amino Acid Metabolism Are Associated with Non-Alcoholic Fatty Liver Disease. PLoS One 10(10): e0138889.

Cheng, S., E. Volgyi, et al. (2009). Trait-specific tracking and determinants of body composition: a 7-year follow-up study of pubertal growth in girls. BMC Med 7: 5.

Chinn, S. (2006). Definitions of childhood obesity: current practice. Eur J Clin Nutr 60(10): 1189-1194.

Choi, J. H., E. J. Rhee, et al. (2013). Increased risk of type 2 diabetes in subjects with both elevated liver enzymes and ultrasonographically diagnosed nonalcoholic fatty liver disease: a 4-year longitudinal study. Arch Med Res 44(2): 115-120.

Church, T. S., D. M. Thomas, et al. (2011). Trends over 5 decades in U.S. occupation-related physical activity and their associations with obesity. PLoS One 6(5): e19657.

Cohen, D. A. (2008). Obesity and the built environment: changes in environmental cues cause energy imbalances. Int J Obes (Lond) 32 Suppl 7: S137-142.

Cole, T. J., M. C. Bellizzi, et al. (2000). Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ 320(7244): 1240-1243.

Cole, T. J. and T. Lobstein (2012). Extended international (IOTF) body mass index cut-offs for thinness, overweight and obesity. Pediatr Obes 7(4): 284-294.

Colliver, J. A., S. Frank, et al. (1983). Similarity of obesity indices in clinical studies of obese adults: a factor analytic study. Am J Clin Nutr 38(4): 640-647.

Conus, F., D. B. Allison, et al. (2004). Metabolic and behavioral characteristics of metabolically obese but normal-weight women. J Clin Endocrinol Metab 89(10): 5013-5020.

Conus, F., R. Rabasa-Lhoret, et al. (2007). Characteristics of metabolically obese normal-weight (MONW) subjects. Appl Physiol Nutr Metab 32(1): 4-12.

Cornier, M. A., D. Dabelea, et al. (2008). The metabolic syndrome. Endocr Rev 29(7): 777-822.

Coutinho, T., K. Goel, et al. (2013). Combining body mass index with measures of central obesity in the assessment of mortality in subjects with coronary disease: role of "normal weight central obesity". J Am Coll Cardiol 61(5): 553-560.

Craig, C. L., S. J. Russell, et al. (2004). Twenty-year trends in physical activity among Canadian adults. Can J Public Health 95(1): 59-63.

Cromwell, W. C., J. D. Otvos, et al. (2007). LDL Particle Number and Risk of Future Cardiovascular Disease in the Framingham Offspring Study - Implications for LDL Management. J Clin Lipidol 1(6): 583-592.

Cunningham, S. A., M. R. Kramer, et al. (2014). Incidence of childhood obesity in the United States. N Engl J Med 370(5): 403-411.

Davidson, M. B. (2006). Does the metabolic syndrome exist: response to Grundy. Diabetes Care 29(11): 2565; author reply 2565-2566.

Davy, K. P. and J. E. Hall (2004). Obesity and hypertension: two epidemics or one? Am J Physiol Regul Integr Comp Physiol 286(5): R803-813.

Day, C. P. and O. F. James (1998). Steatohepatitis: a tale of two "hits"? Gastroenterology 114(4): 842-845.

De Lorenzo, A., V. Del Gobbo, et al. (2007). Normal-weight obese syndrome: early inflammation? Am J Clin Nutr 85(1): 40-45.

De Lorenzo, A., R. Martinoli, et al. (2006). Normal weight obese (NWO) women: an evaluation of a candidate new syndrome. Nutr Metab Cardiovasc Dis 16(8): 513-523.

de Onis, M., M. Blossner, et al. (2010). Global prevalence and trends of overweight and obesity among preschool children. Am J Clin Nutr 92(5): 1257-1264.

Deedwania, P. C. (2004). Diabetes is a vascular disease: the role of endothelial dysfunction in pathophysiology of cardiovascular disease in diabetes. Cardiol Clin 22(4): 505-509, v.

DeFronzo, R. A. (1981). Insulin and renal sodium handling: clinical implications. Int J Obes 5 suppl 1: 93-104.

DeFronzo, R. A. and D. Tripathy (2009). Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care 32 Suppl 2: S157-163.

Della Corte, C., A. R. Mazzotta, et al. (2016). Fatty liver disease and obesity in youth. Curr Opin Endocrinol Diabetes Obes 23(1): 66-71.

Demine, S., N. Reddy, et al. (2014). Unraveling biochemical pathways affected by mitochondrial dysfunctions using metabolomic approaches. Metabolites 4(3): 831-878.

Diraison, F., P. Moulin, et al. (2003). Contribution of hepatic de novo lipogenesis and reesterification of plasma non esterified fatty acids to plasma triglyceride synthesis during non-alcoholic fatty liver disease. Diabetes Metab 29(5): 478-485.

Dirks, M. L., B. T. Wall, et al. (2016). One week of bed rest leads to substantial muscle atrophy and induces whole-body insulin resistance in the absence of skeletal muscle lipid accumulation. Diabetes.

Do, R., C. J. Willer, et al. (2013). Common variants associated with plasma triglycerides and risk for coronary artery disease. Nat Genet 45(11): 1345-1352.

Doak, C. M., T. M. Wijnhoven, et al. (2012). Age standardization in mapping adult overweight and obesity trends in the WHO European Region. Obes Rev 13(2): 174-191.

Dollman, J., K. Norton, et al. (2005). Evidence for secular trends in children's physical activity behaviour. Br J Sports Med 39(12): 892-897; discussion 897.

Donnelly, J. E., J. J. Honas, et al. (2013). Aerobic exercise alone results in clinically significant weight loss for men and women: midwest exercise trial 2. Obesity (Silver Spring) 21(3): E219-228.

Dugas, L. R., R. Harders, et al. (2011). Energy expenditure in adults living in developing compared with industrialized countries: a meta-analysis of doubly labeled water studies. Am J Clin Nutr 93(2): 427-441.

EAH, S. (1982). Characterization of the syndromes of obesity. Baltimore, MD, Williams & Wilkins.

Eckel, N., K. Meidtner, et al. (2015). Metabolically healthy obesity and cardiovascular events: A systematic review and meta-analysis. Eur J Prev Cardiol.

Edwards, K. L., B. Newman, et al. (1997). Heritability of factors of the insulin resistance syndrome in women twins. Genet Epidemiol 14(3): 241-253.

Eisenmann, J. C. (2008). On the use of a continuous metabolic syndrome score in pediatric research. Cardiovasc Diabetol 7: 17.

Ekelund, U., S. A. Anderssen, et al. (2007). Independent associations of physical activity and cardiorespiratory fitness with metabolic risk factors in children: the European youth heart study. Diabetologia 50(9): 1832-1840.

Eknoyan, G. (2006). A history of obesity, or how what was good became ugly and then bad. Adv Chronic Kidney Dis 13(4): 421-427.

Elbers, J. M., H. Asscheman, et al. (1999). Effects of sex steroid hormones on regional fat depots as assessed by magnetic resonance imaging in transsexuals. Am J Physiol 276(2 Pt 1): E317-325.

Elbers, J. M., H. Asscheman, et al. (1997). Long-term testosterone administration increases visceral fat in female to male transsexuals. J Clin Endocrinol Metab 82(7): 2044-2047.

Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). (2001). JAMA 285(19): 2486-2497.

Fabbrini, E. and F. Magkos (2015). Hepatic Steatosis as a Marker of Metabolic Dysfunction. Nutrients 7(6): 4995-5019.

Fabbrini, E., S. Sullivan, et al. (2010). Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. Hepatology 51(2): 679-689.

Falk, E. (2006). Pathogenesis of atherosclerosis. J Am Coll Cardiol 47(8 Suppl): C7-12.

Farin, H. M., F. Abbasi, et al. (2005). Body mass index and waist circumference correlate to the same degree with insulin-mediated glucose uptake. Metabolism 54(10): 1323-1328.

Felig, P., E. Marliss, et al. (1969). Plasma amino acid levels and insulin secretion in obesity. N Engl J Med 281(15): 811-816.

Fields, D. A., M. I. Goran, et al. (2002). Body-composition assessment via air-displacement plethysmography in adults and children: a review. Am J Clin Nutr 75(3): 453-467.

Finkelstein, E. A., O. A. Khavjou, et al. (2012). Obesity and severe obesity forecasts through 2030. Am J Prev Med 42(6): 563-570.

Flannery, C., S. Dufour, et al. (2012). Skeletal muscle insulin resistance promotes increased hepatic de novo lipogenesis, hyperlipidemia, and hepatic steatosis in the elderly. Diabetes 61(11): 2711-2717.

Flegal, K. M. and C. L. Ogden (2011). Childhood obesity: are we all speaking the same language? Adv Nutr 2(2): 159S-166S.

Flegal, K. M., J. A. Shepherd, et al. (2009). Comparisons of percentage body fat, body mass index, waist circumference, and waist-stature ratio in adults. Am J Clin Nutr 89(2): 500-508.

Floegel, A., N. Stefan, et al. (2013). Identification of serum metabolites associated with risk of type 2 diabetes using a targeted metabolomic approach. Diabetes 62(2): 639-648.

Fogelholm, M., S. Männisto, et al. (1996). Determinants of energy balance and overweight in Finland 1982 and 1992. Int J Obes Relat Metab Disord 20(12): 1097-1104.

Fosbol, M. O. and B. Zerahn (2015). Contemporary methods of body composition measurement. Clin Physiol Funct Imaging 35(2): 81-97.

Fox, C. S., J. M. Massaro, et al. (2007). Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study. Circulation 116(1): 39-48.

Franks, P. W., R. L. Hanson, et al. (2010). Childhood obesity, other cardiovascular risk factors, and premature death. N Engl J Med 362(6): 485-493.

Frayling, T. M., N. J. Timpson, et al. (2007). A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science 316(5826): 889-894.

Fried, S. K., M. J. Lee, et al. (2015). Shaping fat distribution: New insights into the molecular determinants of depot- and sex-dependent adipose biology. Obesity (Silver Spring) 23(7): 1345-1352.

Friend, A., L. Craig, et al. (2013). The prevalence of metabolic syndrome in children: a systematic review of the literature. Metab Syndr Relat Disord 11(2): 71-80.

Fuchsberger, C., J. Flannick, et al. (2016). The genetic architecture of type 2 diabetes. Nature.

Furuyama, J. K., R. Nagarajan, et al. (2014). A pilot validation of multi-echo based echo-planar correlated spectroscopic imaging in human calf muscles. NMR Biomed 27(10): 1176-1183.

Galassi, A., K. Reynolds, et al. (2006). Metabolic syndrome and risk of cardiovascular disease: a meta-analysis. Am J Med 119(10): 812-819.

Gale, E. A. (2005). The myth of the metabolic syndrome. Diabetologia 48(9): 1679-1683.

Galgani, J. E., C. Moro, et al. (2008). Metabolic flexibility and insulin resistance. Am J Physiol Endocrinol Metab 295(5): E1009-1017.

Gallagher, D., M. Visser, et al. (1996). How useful is body mass index for comparison of body fatness across age, sex, and ethnic groups? Am J Epidemiol 143(3): 228-239.

Gami, A. S., B. J. Witt, et al. (2007). Metabolic syndrome and risk of incident cardiovascular events and death: a systematic review and meta-analysis of longitudinal studies. J Am Coll Cardiol 49(4): 403-414.

Garn, S. M., W. R. Leonard, et al. (1986). Three limitations of the body mass index. Am J Clin Nutr 44(6): 996-997.

Gast, K. B., M. den Heijer, et al. (2015). Individual contributions of visceral fat and total body fat to subclinical atherosclerosis: The NEO study. Atherosclerosis 241(2): 547-554.

Gastaldelli, A., A. Natali, et al. (2010). Insulin resistance, adipose depots and gut: interactions and pathological implications. Dig Liver Dis 42(5): 310-319.

Gautier, L., L. Cope, et al. (2004). affy--analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20(3): 307-315.

Gilliam, L. K., A. D. Liese, et al. (2007). Family history of diabetes, autoimmunity, and risk factors for cardiovascular disease among children with diabetes in the SEARCH for Diabetes in Youth Study. Pediatr Diabetes 8(6): 354-361.

Ginsberg, H. N., Y. L. Zhang, et al. (2005). Regulation of plasma triglycerides in insulin resistance and diabetes. Arch Med Res 36(3): 232-240.

Ginsberg, H. N., Y. L. Zhang, et al. (2006). Metabolic syndrome: focus on dyslipidemia. Obesity (Silver Spring) 14 Suppl 1: 41S-49S.

Golay, A. and J. Ybarra (2005). Link between obesity and type 2 diabetes. Best Pract Res Clin Endocrinol Metab 19(4): 649-663.

Goldstein, H. (1986). Efficient statistical modelling of longitudinal data. Ann Hum Biol 13(2): 129-141.

Gonzalez, L., C. Corvalan, et al. (2014). Early adiposity rebound is associated with metabolic risk in 7-year-old children. Int J Obes (Lond) 38(10): 1299-1304.

Goossens, G. H. (2008). The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. Physiol Behav 94(2): 206-218.

Gregory, D. M., D. Sovetts, et al. (1986). Plasma free amino acid values in normal children and adolescents. Metabolism 35(10): 967-969.

Grundy, S. M. (1998). Multifactorial causation of obesity: implications for prevention. Am J Clin Nutr 67(3 Suppl): 563S-572S.

Grundy, S. M. (2004). Obesity, metabolic syndrome, and cardiovascular disease. J Clin Endocrinol Metab 89(6): 2595-2600.

Grundy, S. M. (2006). Atherogenic dyslipidemia associated with metabolic syndrome and insulin resistance. Clin Cornerstone 8 Suppl 1: S21-27.

Grundy, S. M. (2006). Does the metabolic syndrome exist? Diabetes Care 29(7): 1689-1692; discussion 1693-1686.

Grundy, S. M. (2008). Metabolic syndrome pandemic. Arterioscler Thromb Vasc Biol 28(4): 629-636.

Grundy, S. M. (2015). Adipose tissue and metabolic syndrome: too much, too little or neither. Eur J Clin Invest 45(11): 1209-1217.

Grundy, S. M. (2015). Metabolic syndrome update. Trends Cardiovasc Med.

Grundy, S. M., J. I. Cleeman, et al. (2005). Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Circulation 112(17): 2735-2752.

Gustafson, B. (2010). Adipose tissue, inflammation and atherosclerosis. J Atheroscler Thromb 17(4): 332-341.

Hallal, P. C., L. B. Andersen, et al. (2012). Global physical activity levels: surveillance progress, pitfalls, and prospects. Lancet 380(9838): 247-257.

Hamaguchi, M., T. Kojima, et al. (2005). The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. Ann Intern Med 143(10): 722-728.

Hamer, M. and E. Stamatakis (2012). Metabolically healthy obesity and risk of all-cause and cardiovascular disease mortality. J Clin Endocrinol Metab 97(7): 2482-2488.

Hamman, R. F., R. R. Wing, et al. (2006). Effect of weight loss with lifestyle intervention on risk of diabetes. Diabetes Care 29(9): 2102-2107.

Hammarqvist, F., G. Angsten, et al. (2010). Age-related changes of muscle and plasma amino acids in healthy children. Amino Acids 39(2): 359-366.

Hankinson, A. L., M. L. Daviglus, et al. (2010). Maintaining a high physical activity level over 20 years and weight gain. JAMA 304(23): 2603-2610.

Heid, I. M., A. U. Jackson, et al. (2010). Meta-analysis identifies 13 new loci associated with waist-hip ratio and reveals sexual dimorphism in the genetic basis of fat distribution. Nat Genet 42(11): 949-960.

Heo, M., G. C. Kabat, et al. (2013). Optimal scaling of weight and waist circumference to height for maximal association with DXA-measured total body fat mass by sex, age and race/ethnicity. Int J Obes (Lond) 37(8): 1154-1160.

Higginson, A. D., J. M. McNamara, et al. (2016). Fatness and fitness: exposing the logic of evolutionary explanations for obesity. Proc Biol Sci 283(1822).

Hill, J. O., H. R. Wyatt, et al. (2012). Energy balance and obesity. Circulation 126(1): 126-132.

Hills, A. P., L. B. Andersen, et al. (2011). Physical activity and obesity in children. Br J Sports Med 45(11): 866-870.

Hinnouho, G. M., S. Czernichow, et al. (2015). Metabolically healthy obesity and the risk of cardiovascular disease and type 2 diabetes: the Whitehall II cohort study. Eur Heart J 36(9): 551-559.

Hofker, M. and C. Wijmenga (2009). A supersized list of obesity genes. Nat Genet 41(2): 139-140.

Hong, Y., N. L. Pedersen, et al. (1997). Genetic and environmental architecture of the features of the insulin-resistance syndrome. Am J Hum Genet 60(1): 143-152.

Hosseinpanah, F., M. Barzin, et al. (2011). Effect of different obesity phenotypes on cardiovascular events in Tehran Lipid and Glucose Study (TLGS). Am J Cardiol 107(3): 412-416.

Hotchkiss, J. W., C. A. Davies, et al. (2013). Adiposity has differing associations with incident coronary heart disease and mortality in the Scottish

population: cross-sectional surveys with follow-up. Int J Obes (Lond) 37(5): 732-739.

Howard, B. V., L. Zech, et al. (1980). Studied of very low density lipoprotein triglyceride metabolism in an obese population with low plasma lipids: lack of influence of body weight or plasma insulin. J Lipid Res 21(8): 1032-1041.

Howard, G., D. H. O'Leary, et al. (1996). Insulin sensitivity and atherosclerosis. The Insulin Resistance Atherosclerosis Study (IRAS) Investigators. Circulation 93(10): 1809-1817.

Hu, G., J. Lindstrom, et al. (2008). The increasing prevalence of metabolic syndrome among Finnish men and women over a decade. J Clin Endocrinol Metab 93(3): 832-836.

Hubert, H. B., M. Feinleib, et al. (1983). Obesity as an independent risk factor for cardiovascular disease: a 26-year follow-up of participants in the Framingham Heart Study. Circulation 67(5): 968-977.

Huffman, K. M., S. H. Shah, et al. (2009). Relationships between circulating metabolic intermediates and insulin action in overweight to obese, inactive men and women. Diabetes Care 32(9): 1678-1683.

Hunter, G. R., R. L. Weinsier, et al. (2001). Age-related decrease in resting energy expenditure in sedentary white women: effects of regional differences in lean and fat mass. Am J Clin Nutr 73(2): 333-337.

Huotari, P. R., H. Nupponen, et al. (2010). Secular trends in aerobic fitness performance in 13-18-year-old adolescents from 1976 to 2001. Br J Sports Med 44(13): 968-972.

Ilanne-Parikka, P., J. G. Eriksson, et al. (2004). Prevalence of the metabolic syndrome and its components: findings from a Finnish general population sample and the Diabetes Prevention Study cohort. Diabetes Care 27(9): 2135-2140.

Isomaa, B., P. Almgren, et al. (2001). Cardiovascular morbidity and mortality associated with the metabolic syndrome. Diabetes Care 24(4): 683-689.

Iwasa, M., T. Ishihara, et al. (2015). Elevation of branched-chain amino acid levels in diabetes and NAFL and changes with antidiabetic drug treatment. Obes Res Clin Pract 9(3): 293-297.

Jae, S. Y., B. Franklin, et al. (2015). Metabolically Healthy Obesity and Carotid Intima-Media Thickness: Effects of Cardiorespiratory Fitness. Mayo Clin Proc 90(9): 1217-1224.

Jakicic, J. M., B. H. Marcus, et al. (2008). Effect of exercise on 24-month weight loss maintenance in overweight women. Arch Intern Med 168(14): 1550-1559; discussion 1559-1560.

James, W. P., H. L. Davies, et al. (1978). Elevated metabolic rates in obesity. Lancet 1(8074): 1122-1125.

Javed, A., M. Jumean, et al. (2015). Diagnostic performance of body mass index to identify obesity as defined by body adiposity in children and adolescents: a systematic review and meta-analysis. Pediatr Obes 10(3): 234-244.

Jellinger, P. S. (2007). Metabolic consequences of hyperglycemia and insulin resistance. Clin Cornerstone 8 Suppl 7: S30-42.

Jiang, W., N. Wu, et al. (2015). Dysbiosis gut microbiota associated with inflammation and impaired mucosal immune function in intestine of humans with non-alcoholic fatty liver disease. Sci Rep 5: 8096.

Juonala, M., C. G. Magnussen, et al. (2011). Childhood adiposity, adult adiposity, and cardiovascular risk factors. N Engl J Med 365(20): 1876-1885.

Juonala, M., J. S. Viikari, et al. (2008). Associations of dyslipidemias from childhood to adulthood with carotid intima-media thickness, elasticity, and brachial flow-mediated dilatation in adulthood: the Cardiovascular Risk in Young Finns Study. Arterioscler Thromb Vasc Biol 28(5): 1012-1017.

Kahn, B. B. and J. S. Flier (2000). Obesity and insulin resistance. J Clin Invest 106(4): 473-481.

Kahn, R., J. Buse, et al. (2005). The metabolic syndrome: time for a critical appraisal: joint statement from the American Diabetes Association and the European Association for the Study of Diabetes. Diabetes Care 28(9): 2289-2304.

Kahn, S. E., R. L. Hull, et al. (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 444(7121): 840-846.

Kahn, S. E. and D. Porte, Jr. (1988). Islet dysfunction in non-insulin-dependent diabetes mellitus. Am J Med 85(5A): 4-8.

Kalhan, S. C., L. Guo, et al. (2011). Plasma metabolomic profile in nonalcoholic fatty liver disease. Metabolism 60(3): 404-413.

Kalman, M., J. Inchley, et al. (2015). Secular trends in moderate-to-vigorous physical activity in 32 countries from 2002 to 2010: a cross-national perspective. Eur J Public Health 25 Suppl 2: 37-40.

Kannel, W. B. (2000). Elevated systolic blood pressure as a cardiovascular risk factor. Am J Cardiol 85(2): 251-255.

Kannel, W. B., N. Brand, et al. (1967). The relation of adiposity to blood pressure and development of hypertension. The Framingham study. Ann Intern Med 67(1): 48-59.

Karelis, A. D. (2008). Metabolically healthy but obese individuals. Lancet 372(9646): 1281-1283.

Karelis, A. D., D. H. St-Pierre, et al. (2004). Metabolic and body composition factors in subgroups of obesity: what do we know? J Clin Endocrinol Metab 89(6): 2569-2575.

Karpe, F., J. R. Dickmann, et al. (2011). Fatty acids, obesity, and insulin resistance: time for a reevaluation. Diabetes 60(10): 2441-2449.

Kassi, E., P. Pervanidou, et al. (2011). Metabolic syndrome: definitions and controversies. BMC Med 9: 48.

Katsiki, N., D. P. Mikhailidis, et al. (2016). Non-alcoholic fatty liver disease and dyslipidemia: An update. Metabolism.

Kautiainen, S., A. M. Koivisto, et al. (2009). Sociodemographic factors and a secular trend of adolescent overweight in Finland. Int J Pediatr Obes 4(4): 360-370.

Kautiainen, S., A. Rimpela, et al. (2002). Secular trends in overweight and obesity among Finnish adolescents in 1977-1999. Int J Obes Relat Metab Disord 26(4): 544-552.

Kelley, D. E., J. He, et al. (2002). Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes 51(10): 2944-2950.

Kenchaiah, S., J. C. Evans, et al. (2002). Obesity and the risk of heart failure. N Engl J Med 347(5): 305-313.

Keyes, A. (1973). Overweight and the risk of sudden heart attack and sudden death, in obesity in perspective DHEW Publication No (NIH): 75–708.

Keys, A., F. Fidanza, et al. (2014). Indices of relative weight and obesity. Int J Epidemiol 43(3): 655-665.

Kiernan, M. and M. A. Winkleby (2000). Identifying patients for weight-loss treatment: an empirical evaluation of the NHLBI obesity education initiative expert panel treatment recommendations. Arch Intern Med 160(14): 2169-2176.

Kim, S., C. Kyung, et al. (2015). Normal-weight obesity is associated with increased risk of subclinical atherosclerosis. Cardiovasc Diabetol 14: 58.

Kip, K. E., O. C. Marroquin, et al. (2004). Clinical importance of obesity versus the metabolic syndrome in cardiovascular risk in women: a report from the Women's Ischemia Syndrome Evaluation (WISE) study. Circulation 109(6): 706-713.

Klingberg, S., K. Mehlig, et al. (2015). Increase in waist circumference over 6 years predicts subsequent cardiovascular disease and total mortality in nordic women. Obesity (Silver Spring) 23(10): 2123-2130.

Knai, C., T. Lobstein, et al. (2012). Socioeconomic patterning of childhood overweight status in Europe. Int J Environ Res Public Health 9(4): 1472-1489.

Knowler, W. C., E. Barrett-Connor, et al. (2002). Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N Engl J Med 346(6): 393-403.

Knowler, W. C., D. J. Pettitt, et al. (1991). Obesity in the Pima Indians: its magnitude and relationship with diabetes. Am J Clin Nutr 53(6 Suppl): 1543S-1551S.

Knuth, A. G. and P. C. Hallal (2009). Temporal trends in physical activity: a systematic review. J Phys Act Health 6(5): 548-559.

Kosmala, W., D. Jedrzejuk, et al. (2012). Left ventricular function impairment in patients with normal-weight obesity: contribution of abdominal fat deposition, profibrotic state, reduced insulin sensitivity, and proinflammatory activation. Circ Cardiovasc Imaging 5(3): 349-356.

Kotani, K., K. Tokunaga, et al. (1994). Sexual dimorphism of age-related changes in whole-body fat distribution in the obese. Int J Obes Relat Metab Disord 18(4): 207-202.

Kotronen, A., J. Westerbacka, et al. (2007). Liver fat in the metabolic syndrome. J Clin Endocrinol Metab 92(9): 3490-3497.

Kotronen, A. and H. Yki-Jarvinen (2008). Fatty liver: a novel component of the metabolic syndrome. Arterioscler Thromb Vasc Biol 28(1): 27-38.

Kotsis, V., P. Nilsson, et al. (2015). New developments in the pathogenesis of obesity-induced hypertension. J Hypertens 33(8): 1499-1508.

Kotsis, V., S. Stabouli, et al. (2010). Mechanisms of obesity-induced hypertension. Hypertens Res 33(5): 386-393.

Kramer, C. K., B. Zinman, et al. (2013). Are metabolically healthy overweight and obesity benign conditions?: A systematic review and meta-analysis. Ann Intern Med 159(11): 758-769.

Krauss, R. M. (2010). Lipoprotein subfractions and cardiovascular disease risk. Curr Opin Lipidol 21(4): 305-311.

Krebs, M., M. Krssak, et al. (2002). Mechanism of amino acid-induced skeletal muscle insulin resistance in humans. Diabetes 51(3): 599-605.

Kujala, U. M., V. P. Makinen, et al. (2013). Long-term leisure-time physical activity and serum metabolome. Circulation 127(3): 340-348.

Kuk, J. L. and C. I. Ardern (2009). Are metabolically normal but obese individuals at lower risk for all-cause mortality? Diabetes Care 32(12): 2297-2299.

Kuk, J. L., T. J. Saunders, et al. (2009). Age-related changes in total and regional fat distribution. Ageing Res Rev 8(4): 339-348.

Kurl, S., J. A. Laukkanen, et al. (2006). Metabolic syndrome and the risk of stroke in middle-aged men. Stroke 37(3): 806-811.

Laakso, M. and J. Kuusisto (2014). Insulin resistance and hyperglycaemia in cardiovascular disease development. Nat Rev Endocrinol 10(5): 293-302.

Laaksonen, D. E., H. M. Lakka, et al. (2002). Metabolic syndrome and development of diabetes mellitus: application and validation of recently suggested definitions of the metabolic syndrome in a prospective cohort study. Am J Epidemiol 156(11): 1070-1077.

Laaksonen, D. E., H. M. Lakka, et al. (2002). Low levels of leisure-time physical activity and cardiorespiratory fitness predict development of the metabolic syndrome. Diabetes Care 25(9): 1612-1618.

Laaksonen, D. E., L. Niskanen, et al. (2004). Epidemiology and treatment of the metabolic syndrome. Ann Med 36(5): 332-346.

Lackey, D. E., C. J. Lynch, et al. (2013). Regulation of adipose branched-chain amino acid catabolism enzyme expression and cross-adipose amino acid flux in human obesity. Am J Physiol Endocrinol Metab 304(11): E1175-1187.

Lahti-Koski, M. (2004). Defining Childhood Obesity. Pediatr Adolesc Med. 9: 1-19.

Lahti-Koski, M., E. Seppanen-Nuijten, et al. (2010). Twenty-year changes in the prevalence of obesity among Finnish adults. Obes Rev 11(3): 171-176.

Lakka, T. A., D. E. Laaksonen, et al. (2003). Sedentary lifestyle, poor cardiorespiratory fitness, and the metabolic syndrome. Med Sci Sports Exerc 35(8): 1279-1286.

Lanningham-Foster, L., L. J. Nysse, et al. (2003). Labor saved, calories lost: the energetic impact of domestic labor-saving devices. Obes Res 11(10): 1178-1181.

Lee, D. C., X. Sui, et al. (2009). Associations of cardiorespiratory fitness and obesity with risks of impaired fasting glucose and type 2 diabetes in men. Diabetes Care 32(2): 257-262.

Leibel, R. L., M. Rosenbaum, et al. (1995). Changes in energy expenditure resulting from altered body weight. N Engl J Med 332(10): 621-628.

Lemieux, S., D. Prud'homme, et al. (1993). Sex differences in the relation of visceral adipose tissue accumulation to total body fatness. Am J Clin Nutr 58(4): 463-467.

Lepage, N., N. McDonald, et al. (1997). Age-specific distribution of plasma amino acid concentrations in a healthy pediatric population. Clin Chem 43(12): 2397-2402.

Leskinen, T. and U. M. Kujala (2015). Health-Related Findings Among Twin Pairs Discordant for Leisure-Time Physical Activity for 32 Years: The TWINACTIVE Study Synopsis. Twin Res Hum Genet 18(3): 266-272.

Leskinen, T., S. Sipila, et al. (2009). Leisure-time physical activity and high-risk fat: a longitudinal population-based twin study. Int J Obes (Lond) 33(11): 1211-1218.

Lewis, G. F., K. D. Uffelman, et al. (1995). Interaction between free fatty acids and insulin in the acute control of very low density lipoprotein production in humans. J Clin Invest 95(1): 158-166.

Levitsky, D. A. and C. R. Pacanowski (2012). Free will and the obesity epidemic. Public Health Nutr 15(1): 126-141.

Lidofsky, S. D. (2008). Nonalcoholic fatty liver disease: diagnosis and relation to metabolic syndrome and approach to treatment. Curr Diab Rep 8(1): 25-30.

Lillioja, S. and C. Bogardus (1988). Obesity and insulin resistance: lessons learned from the Pima Indians. Diabetes Metab Rev 4(5): 517-540.

Liu, J., C. S. Fox, et al. (2010). Impact of abdominal visceral and subcutaneous adipose tissue on cardiometabolic risk factors: the Jackson Heart Study. J Clin Endocrinol Metab 95(12): 5419-5426.

Lobstein, T., R. Jackson-Leach, et al. (2015). Child and adolescent obesity: part of a bigger picture. Lancet 385(9986): 2510-2520.

Locke, A. E., B. Kahali, et al. (2015). Genetic studies of body mass index yield new insights for obesity biology. Nature 518(7538): 197-206.

Loos, R. J. and G. S. Yeo (2014). The bigger picture of FTO: the first GWAS-identified obesity gene. Nat Rev Endocrinol 10(1): 51-61.

Lusis, A. J., A. D. Attie, et al. (2008). Metabolic syndrome: from epidemiology to systems biology. Nat Rev Genet 9(11): 819-830.

Luukkonen, P. K., Y. Zhou, et al. (2016). Hepatic ceramides dissociate steatosis and insulin resistance in patients with non-alcoholic fatty liver disease. J Hepatol.

Lynch, C. J. and S. H. Adams (2014). Branched-chain amino acids in metabolic signalling and insulin resistance. Nat Rev Endocrinol 10(12): 723-736.

Mahoney, L. T., W. R. Clarke, et al. (1991). Childhood predictors of high blood pressure. Am J Hypertens 4(11): 608S-610S.

Mailloux, R., J. Lemire, et al. (2007). Aluminum-induced mitochondrial dysfunction leads to lipid accumulation in human hepatocytes: a link to obesity. Cell Physiol Biochem 20(5): 627-638.

Makinen, V. P., C. Forsblom, et al. (2009). Network of vascular diseases, death and biochemical characteristics in a set of 4,197 patients with type 1 diabetes (the FinnDiane Study). Cardiovasc Diabetol 8: 54.

Makkonen, J., K. H. Pietilainen, et al. (2009). Genetic factors contribute to variation in serum alanine aminotransferase activity independent of obesity and alcohol: a study in monozygotic and dizygotic twins. J Hepatol 50(5): 1035-1042.

Manini, T. M. (2010). Energy expenditure and aging. Ageing Res Rev 9(1): 1-11.

Marin, P., B. Andersson, et al. (1992). The morphology and metabolism of intraabdominal adipose tissue in men. Metabolism 41(11): 1242-1248.

Marques-Vidal, P., A. Pecoud, et al. (2008). Prevalence of normal weight obesity in Switzerland: effect of various definitions. Eur J Nutr 47(5): 251-257.

Marques-Vidal, P., A. Pecoud, et al. (2010). Normal weight obesity: relationship with lipids, glycaemic status, liver enzymes and inflammation. Nutr Metab Cardiovasc Dis 20(9): 669-675.

Martin, A. D., M. Z. Daniel, et al. (1994). Adipose tissue density, estimated adipose lipid fraction and whole body adiposity in male cadavers. Int J Obes Relat Metab Disord 18(2): 79-83.

Masoodi, M., O. Kuda, et al. (2015). Lipid signaling in adipose tissue: Connecting inflammation & metabolism. Biochim Biophys Acta 1851(4): 503-518.

Mathieu, P., M. C. Boulanger, et al. (2014). Ectopic visceral fat: a clinical and molecular perspective on the cardiometabolic risk. Rev Endocr Metab Disord 15(4): 289-298.

Matsuda, M. and R. A. DeFronzo (1999). Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetes Care 22(9): 1462-1470.

Mayer, J., P. Roy, et al. (1956). Relation between caloric intake, body weight, and physical work: studies in an industrial male population in West Bengal. Am J Clin Nutr 4(2): 169-175.

McCormack, S. E., O. Shaham, et al. (2013). Circulating branched-chain amino acid concentrations are associated with obesity and future insulin resistance in children and adolescents. Pediatr Obes 8(1): 52-61.

McGill, H. C., Jr., C. A. McMahan, et al. (2000). Origin of atherosclerosis in childhood and adolescence. Am J Clin Nutr 72(5 Suppl): 1307S-1315S.

McLaughlin, T. (2012). Metabolic heterogeneity of obesity: role of adipose tissue. Int J Obes Suppl 2(Suppl 1): S8-S10.

Meigs, J. B., L. A. Cupples, et al. (2000). Parental transmission of type 2 diabetes: the Framingham Offspring Study. Diabetes 49(12): 2201-2207.

Meigs, J. B., P. W. Wilson, et al. (2006). Body mass index, metabolic syndrome, and risk of type 2 diabetes or cardiovascular disease. J Clin Endocrinol Metab 91(8): 2906-2912.

Meugnier, E., M. Faraj, et al. (2007). Acute hyperglycemia induces a global downregulation of gene expression in adipose tissue and skeletal muscle of healthy subjects. Diabetes 56(4): 992-999.

Mitchell, B. D., C. M. Kammerer, et al. (1996). Genetic analysis of the IRS. Pleiotropic effects of genes influencing insulin levels on lipoprotein and obesity measures. Arterioscler Thromb Vasc Biol 16(2): 281-288.

Montague, C. T., I. S. Farooqi, et al. (1997). Congenital leptin deficiency is associated with severe early-onset obesity in humans. Nature 387(6636): 903-908.

Montani, J. P., V. Antic, et al. (2002). Pathways from obesity to hypertension: from the perspective of a vicious triangle. Int J Obes Relat Metab Disord 26 Suppl 2: S28-38.

Mook-Kanamori, D. O., W. Romisch-Margl, et al. (2014). Increased amino acids levels and the risk of developing of hypertriglyceridemia in a 7-year follow-up. J Endocrinol Invest 37(4): 369-374.

Morkedal, B., L. J. Vatten, et al. (2014). Risk of myocardial infarction and heart failure among metabolically healthy but obese individuals: HUNT (Nord-Trondelag Health Study), Norway. J Am Coll Cardiol 63(11): 1071-1078.

Morrison, J. A., C. J. Glueck, et al. (2009). Pediatric triglycerides predict cardiovascular disease events in the fourth to fifth decade of life. Metabolism 58(9): 1277-1284.

Munukka, E., S. Pekkala, et al. (2014). Gut-adipose tissue axis in hepatic fat accumulation in humans. J Hepatol 61(1): 132-138.

Muoio, D. M. and C. B. Newgard (2008). Mechanisms of disease:Molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. Nat Rev Mol Cell Biol 9(3): 193-205.

Musunuru, K. (2010). Atherogenic dyslipidemia: cardiovascular risk and dietary intervention. Lipids 45(10): 907-914.

Männistö, S., K. Harald, et al. (2014). Dietary and lifestyle characteristics associated with normal-weight obesity: the National FINRISK 2007 Study. Br J Nutr 111(5): 887-894.

Navarro, E., A. N. Funtikova, et al. (2015). Can metabolically healthy obesity be explained by diet, genetics, and inflammation? Mol Nutr Food Res 59(1): 75-93.

Neel, J. V. (1999). Diabetes mellitus: a "thrifty" genotype rendered detrimental by "progress"? 1962. Bull World Health Organ 77(8): 694-703; discussion 692-693.

Newgard, C. B., J. An, et al. (2009). A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab 9(4): 311-326.

Ng, M., T. Fleming, et al. (2014). Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a

systematic analysis for the Global Burden of Disease Study 2013. Lancet 384(9945): 766-781.

Ng, S. W. and B. M. Popkin (2012). Time use and physical activity: a shift away from movement across the globe. Obes Rev 13(8): 659-680.

Nicklas, T. A., S. P. von Duvillard, et al. (2002). Tracking of serum lipids and lipoproteins from childhood to dyslipidemia in adults: the Bogalusa Heart Study. Int J Sports Med 23 Suppl 1: S39-43.

Nilsson, S. (2001). [Research contributions of Eskil Kylin]. Sven Med Tidskr 5(1): 15-28.

Ninomiya, J. K., G. L'Italien, et al. (2004). Association of the metabolic syndrome with history of myocardial infarction and stroke in the Third National Health and Nutrition Examination Survey. Circulation 109(1): 42-46.

Nofer, J. R. (2011). Hyperlipidemia and cardiovascular disease: triglycerides - a revival of cardiovascular risk factor? Curr Opin Lipidol 22(4): 319-321.

O'Donovan, G., E. L. Thomas, et al. (2009). Fat distribution in men of different waist girth, fitness level and exercise habit. Int J Obes (Lond) 33(12): 1356-1362.

Oda, E. (2006). The metabolic syndrome (emperor) wears no clothes: response to Kahn. Diabetes Care 29(11): 2566.

Ogbera, A. O. (2010). Prevalence and gender distribution of the metabolic syndrome. Diabetol Metab Syndr 2: 1.

Ogorodnikova, A. D., M. Kim, et al. (2012). Incident cardiovascular disease events in metabolically benign obese individuals. Obesity (Silver Spring) 20(3): 651-659.

Ojanen, X., R. J. Borra, et al. (2014). Comparison of vertebral bone marrow fat assessed by 1H MRS and inphase and out-of-phase MRI among family members. Osteoporos Int 25(2): 653-662.

Okorodudu, D. O., M. F. Jumean, et al. (2010). Diagnostic performance of body mass index to identify obesity as defined by body adiposity: a systematic review and meta-analysis. Int J Obes (Lond) 34(5): 791-799.

Olds, T., C. Maher, et al. (2011). Evidence that the prevalence of childhood overweight is plateauing: data from nine countries. Int J Pediatr Obes 6(5-6): 342-360.

Oliveros, E., V. K. Somers, et al. (2014). The concept of normal weight obesity. Prog Cardiovasc Dis 56(4): 426-433.

Ortega, F. B., C. Cadenas-Sanchez, et al. (2015). Role of Fitness in the Metabolically Healthy but Obese Phenotype: A Review and Update. Prog Cardiovasc Dis 58(1): 76-86.

Pajunen, P., A. Kotronen, et al. (2011). Metabolically healthy and unhealthy obesity phenotypes in the general population: the FIN-D2D Survey. BMC Public Health 11: 754.

Palmer, B. F. and D. J. Clegg (2015). The sexual dimorphism of obesity. Mol Cell Endocrinol 402: 113-119.

Pan, J. J. and M. B. Fallon (2014). Gender and racial differences in nonalcoholic fatty liver disease. World J Hepatol 6(5): 274-283.

Pandit, D., S. Chiplonkar, et al. (2011). Efficacy of a continuous metabolic syndrome score in Indian children for detecting subclinical atherosclerotic risk. Int J Obes (Lond) 35(10): 1318-1324.

Pankow, J. S., D. R. Jacobs, Jr., et al. (2004). Insulin resistance and cardiovascular disease risk factors in children of parents with the insulin resistance (metabolic) syndrome. Diabetes Care 27(3): 775-780.

Park, S. K., M. H. Seo, et al. (2013). Clinical availability of nonalcoholic fatty liver disease as an early predictor of type 2 diabetes mellitus in Korean men: 5-year prospective cohort study. Hepatology 57(4): 1378-1383.

Park, Y. W., S. Zhu, et al. (2003). The metabolic syndrome: prevalence and associated risk factor findings in the US population from the Third National Health and Nutrition Examination Survey, 1988-1994. Arch Intern Med 163(4): 427-436.

Patterson, J. N., K. Cousteils, et al. (2014). Mitochondrial metabolism of pyruvate is essential for regulating glucose-stimulated insulin secretion. J Biol Chem 289(19): 13335-13346.

Patti, M. E., A. J. Butte, et al. (2003). Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. Proc Natl Acad Sci U S A 100(14): 8466-8471.

Peeters, A., J. J. Barendregt, et al. (2003). Obesity in adulthood and its consequences for life expectancy: a life-table analysis. Ann Intern Med 138(1): 24-32.

Peneau, S., R. Gonzalez-Carrascosa, et al. (2016). Age at adiposity rebound: determinants and association with nutritional status and the metabolic syndrome at adulthood. Int J Obes (Lond).

Perng, W., M. W. Gillman, et al. (2014). Metabolomic profiles and childhood obesity. Obesity (Silver Spring) 22(12): 2570-2578.

Perreault, M., M. A. Zulyniak, et al. (2014). A distinct fatty acid profile underlies the reduced inflammatory state of metabolically healthy obese individuals. PLoS One 9(2): e88539.

Phillips, G. B. (1978). Sex hormones, risk factors and cardiovascular disease. Am J Med 65(1): 7-11.

Pi-Sunyer, F. X. (2002). The obesity epidemic: pathophysiology and consequences of obesity. Obes Res 10 Suppl 2: 97S-104S.

Pietiläinen, K. H., J. Naukkarinen, et al. (2008). Global transcript profiles of fat in monozygotic twins discordant for BMI: pathways behind acquired obesity. PLoS Med 5(3): e51.

Pietrobelli, A., M. S. Faith, et al. (1998). Body mass index as a measure of adiposity among children and adolescents: a validation study. J Pediatr 132(2): 204-210.

Piirtola, M., J. Kaprio, et al. (2016). Leisure-time physical inactivity and association with body mass index: a Finnish Twin Study with a 35-year follow-up. Int J Epidemiol.

Plourde, G. and A. D. Karelis (2014). Current issues in the identification and treatment of metabolically healthy but obese individuals. Nutr Metab Cardiovasc Dis 24(5): 455-459.

Poirier, P., I. Lemieux, et al. (2005). Impact of waist circumference on the relationship between blood pressure and insulin: the Quebec Health Survey. Hypertension 45(3): 363-367.

Preis, S. R., J. M. Massaro, et al. (2010). Abdominal subcutaneous and visceral adipose tissue and insulin resistance in the Framingham heart study. Obesity (Silver Spring) 18(11): 2191-2198.

Prentice, A. M. (2006). The emerging epidemic of obesity in developing countries. Int J Epidemiol 35(1): 93-99.

Prentice, A. M. and S. A. Jebb (2001). Beyond body mass index. Obes Rev 2(3): 141-147.

Qin, L. Q., P. Xun, et al. (2011). Higher branched-chain amino acid intake is associated with a lower prevalence of being overweight or obese in middle-aged East Asian and Western adults. J Nutr 141(2): 249-254.

Ragland, D. R. (1992). Dichotomizing continuous outcome variables: dependence of the magnitude of association and statistical power on the cutpoint. Epidemiology 3(5): 434-440.

Rana, J. S., M. E. Visser, et al. (2010). Metabolic dyslipidemia and risk of future coronary heart disease in apparently healthy men and women: the EPIC-Norfolk prospective population study. Int J Cardiol 143(3): 399-404.

Rankinen, T., M. A. Sarzynski, et al. (2015). Are there genetic paths common to obesity, cardiovascular disease outcomes, and cardiovascular risk factors? Circ Res 116(5): 909-922.

Rasouli, N., B. Molavi, et al. (2007). Ectopic fat accumulation and metabolic syndrome. Diabetes Obes Metab 9(1): 1-10.

Ravussin, E., B. Burnand, et al. (1982). Twenty-four-hour energy expenditure and resting metabolic rate in obese, moderately obese, and control subjects. Am J Clin Nutr 35(3): 566-573.

Reaven, G. M. (1988). Banting lecture 1988. Role of insulin resistance in human disease. Diabetes 37(12): 1595-1607.

Reaven, G. M. (2005). The metabolic syndrome: requiescat in pace. Clin Chem 51(6): 931-938.

Reaven, G. M. (2011). Insulin resistance: the link between obesity and cardiovascular disease. Med Clin North Am 95(5): 875-892.

Regitz-Zagrosek, V., E. Lehmkuhl, et al. (2006). Gender differences in the metabolic syndrome and their role for cardiovascular disease. Clin Res Cardiol 95(3): 136-147.

Rexrode, K. M., V. J. Carey, et al. (1998). Abdominal adiposity and coronary heart disease in women. JAMA 280(21): 1843-1848.

Rey-Lopez, J. P., L. F. de Rezende, et al. (2014). The prevalence of metabolically healthy obesity: a systematic review and critical evaluation of the definitions used. Obes Rev 15(10): 781-790.

Roberts, L. D. and R. E. Gerszten (2013). Toward new biomarkers of cardiometabolic diseases. Cell Metab 18(1): 43-50.

Rodriguez-Gallego, E., M. Guirro, et al. (2015). Mapping of the circulating metabolome reveals alpha-ketoglutarate as a predictor of morbid obesity-associated non-alcoholic fatty liver disease. Int J Obes (Lond) 39(2): 279-287.

Rodriguez-Moran, M., F. Guerrero-Romero, et al. (2013). Atherogenic indices and prehypertension in obese and non-obese children. Diab Vasc Dis Res 10(1): 17-24.

Rogge, M. M. (2009). The role of impaired mitochondrial lipid oxidation in obesity. Biol Res Nurs 10(4): 356-373.

Rolland-Cachera, M. F., M. Deheeger, et al. (2006). Early adiposity rebound: causes and consequences for obesity in children and adults. Int J Obes (Lond) 30 Suppl 4: S11-17.

Rome, S., K. Clement, et al. (2003). Microarray profiling of human skeletal muscle reveals that insulin regulates approximately 800 genes during a hyperinsulinemic clamp. J Biol Chem 278(20): 18063-18068.

Romeo, S., J. Kozlitina, et al. (2008). Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 40(12): 1461-1465.

Romero-Corral, A., V. K. Somers, et al. (2007). Diagnostic performance of body mass index to detect obesity in patients with coronary artery disease. Eur Heart J 28(17): 2087-2093.

Romero-Corral, A., V. K. Somers, et al. (2010). Normal weight obesity: a risk factor for cardiometabolic dysregulation and cardiovascular mortality. Eur Heart J 31(6): 737-746.

Rosenkilde, M., P. Auerbach, et al. (2012). Body fat loss and compensatory mechanisms in response to different doses of aerobic exercise--a randomized controlled trial in overweight sedentary males. Am J Physiol Regul Integr Comp Physiol 303(6): R571-579.

Rosito, G. A., J. M. Massaro, et al. (2008). Pericardial fat, visceral abdominal fat, cardiovascular disease risk factors, and vascular calcification in a community-based sample: the Framingham Heart Study. Circulation 117(5): 605-613.

Rowe, J. W., J. B. Young, et al. (1981). Effect of insulin and glucose infusions on sympathetic nervous system activity in normal man. Diabetes 30(3): 219-225.

Ruderman, N., D. Chisholm, et al. (1998). The metabolically obese, normal-weight individual revisited. Diabetes 47(5): 699-713.

Ruderman, N. B., S. H. Schneider, et al. (1981). The "metabolically-obese," normal-weight individual. Am J Clin Nutr 34(8): 1617-1621.

Saari, A., U. Sankilampi, et al. (2011). New Finnish growth references for children and adolescents aged 0 to 20 years: Length/height-for-age, weight-for-length/height, and body mass index-for-age. Ann Med 43(3): 235-248.

Sahakyan, K. R., V. K. Somers, et al. (2015). Normal-Weight Central Obesity: Implications for Total and Cardiovascular Mortality. Ann Intern Med 163(11): 827-835.

Salonen, J. T., T. A. Lakka, et al. (1998). Hyperinsulinemia is associated with the incidence of hypertension and dyslipidemia in middle-aged men. Diabetes 47(2): 270-275.

Samuel, V. T. and G. I. Shulman (2012). Mechanisms for insulin resistance: common threads and missing links. Cell 148(5): 852-871.

Sarwar, N., J. Danesh, et al. (2007). Triglycerides and the risk of coronary heart disease: 10,158 incident cases among 262,525 participants in 29 Western prospective studies. Circulation 115(4): 450-458.

Savage, D. B., K. F. Petersen, et al. (2007). Disordered lipid metabolism and the pathogenesis of insulin resistance. Physiol Rev 87(2): 507-520.

Schleinitz, D., Y. Bottcher, et al. (2014). The genetics of fat distribution. Diabetologia 57(7): 1276-1286.

Schousboe, K., G. Willemsen, et al. (2003). Sex differences in heritability of BMI: a comparative study of results from twin studies in eight countries. Twin Res 6(5): 409-421.

Schulz, L. O., P. H. Bennett, et al. (2006). Effects of traditional and western environments on prevalence of type 2 diabetes in Pima Indians in Mexico and the U.S. Diabetes Care 29(8): 1866-1871.

Schulz, L. O. and L. S. Chaudhari (2015). High-Risk Populations: The Pimas of Arizona and Mexico. Curr Obes Rep 4(1): 92-98.

Schwimmer, J. B., M. A. Celedon, et al. (2009). Heritability of nonalcoholic fatty liver disease. Gastroenterology 136(5): 1585-1592.

Scuteri, A., S. Sanna, et al. (2007). Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. PLoS Genet 3(7): e115.

Senechal, M., B. Wicklow, et al. (2013). Cardiorespiratory fitness and adiposity in metabolically healthy overweight and obese youth. Pediatrics 132(1): e85-92.

Shah, S. H., J. R. Bain, et al. (2010). Association of a peripheral blood metabolic profile with coronary artery disease and risk of subsequent cardiovascular events. Circ Cardiovasc Genet 3(2): 207-214.

Shaw, K., H. Gennat, et al. (2006). Exercise for overweight or obesity. Cochrane Database Syst Rev(4): CD003817.

She, P., C. Van Horn, et al. (2007). Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. Am J Physiol Endocrinol Metab 293(6): E1552-1563.

Shea, J. L., M. T. King, et al. (2012). Body fat percentage is associated with cardiometabolic dysregulation in BMI-defined normal weight subjects. Nutr Metab Cardiovasc Dis 22(9): 741-747.

Shea, J. L., E. W. Randell, et al. (2011). The prevalence of metabolically healthy obese subjects defined by BMI and dual-energy X-ray absorptiometry. Obesity (Silver Spring) 19(3): 624-630.

Shen, W., M. Punyanitya, et al. (2009). Sexual dimorphism of adipose tissue distribution across the lifespan: a cross-sectional whole-body magnetic resonance imaging study. Nutr Metab (Lond) 6: 17.

Shook, R. P., G. A. Hand, et al. (2015). Low levels of physical activity are associated with dysregulation of energy intake and fat mass gain over 1 year. Am J Clin Nutr 102(6): 1332-1338.

Shulman, G. I. (2014). Ectopic fat in insulin resistance, dyslipidemia, and cardiometabolic disease. N Engl J Med 371(12): 1131-1141.

Shungin, D., T. W. Winkler, et al. (2015). New genetic loci link adipose and insulin biology to body fat distribution. Nature 518(7538): 187-196.

Siani, A., F. P. Cappuccio, et al. (2002). The relationship of waist circumference to blood pressure: the Olivetti Heart Study. Am J Hypertens 15(9): 780-786.

Silventoinen, K., B. Rokholm, et al. (2010). The genetic and environmental influences on childhood obesity: a systematic review of twin and adoption studies. Int J Obes (Lond) 34(1): 29-40.

Silventoinen, K., S. Sans, et al. (2004). Trends in obesity and energy supply in the WHO MONICA Project. Int J Obes Relat Metab Disord 28(5): 710-718.

Simmonds, M., A. Llewellyn, et al. (2016). Predicting adult obesity from childhood obesity: a systematic review and meta-analysis. Obes Rev 17(2): 95-107.

Simmons, R. K., K. G. Alberti, et al. (2010). The metabolic syndrome: useful concept or clinical tool? Report of a WHO Expert Consultation. Diabetologia 53(4): 600-605.

Singh, R. B., S. A. Mengi, et al. (2002). Pathogenesis of atherosclerosis: A multifactorial process. Exp Clin Cardiol 7(1): 40-53.

Sironi, A. M., R. Petz, et al. (2012). Impact of increased visceral and cardiac fat on cardiometabolic risk and disease. Diabet Med 29(5): 622-627.

Smith, B. W. and L. A. Adams (2011). Non-alcoholic fatty liver disease. Crit Rev Clin Lab Sci 48(3): 97-113.

Sniderman, A. D., R. Bhopal, et al. (2007). Why might South Asians be so susceptible to central obesity and its atherogenic consequences? The adipose tissue overflow hypothesis. Int J Epidemiol 36(1): 220-225.

Soininen, P., A. J. Kangas, et al. (2015). Quantitative serum nuclear magnetic resonance metabolomics in cardiovascular epidemiology and genetics. Circ Cardiovasc Genet 8(1): 192-206.

Soininen, P., A. J. Kangas, et al. (2009). High-throughput serum NMR metabonomics for cost-effective holistic studies on systemic metabolism. Analyst 134(9): 1781-1785.

Song, Y., J. E. Manson, et al. (2007). Comparison of usefulness of body mass index versus metabolic risk factors in predicting 10-year risk of cardiovascular events in women. Am J Cardiol 100(11): 1654-1658.

Sonsuz, A., M. Basaranoglu, et al. (2000). Relationship between aminotransferase levels and histopathological findings in patients with nonalcoholic steatohepatitis. Am J Gastroenterol 95(5): 1370-1371.

Sorensen, T. I., R. A. Price, et al. (1989). Genetics of obesity in adult adoptees and their biological siblings. BMJ 298(6666): 87-90.

Spalding, K. L., E. Arner, et al. (2008). Dynamics of fat cell turnover in humans. Nature 453(7196): 783-787.

Speakman, J. R. (2006). Thrifty genes for obesity and the metabolic syndrome--time to call off the search? Diab Vasc Dis Res 3(1): 7-11.

Speliotes, E. K., C. J. Willer, et al. (2010). Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. Nat Genet 42(11): 937-948.

St-Onge, M. P. and D. Gallagher (2010). Body composition changes with aging: the cause or the result of alterations in metabolic rate and macronutrient oxidation? Nutrition 26(2): 152-155.

Stamatakis, E., U. Ekelund, et al. (2007). Temporal trends in physical activity in England: the Health Survey for England 1991 to 2004. Prev Med 45(6): 416-423.

Stamler, R., J. Stamler, et al. (1978). Weight and blood pressure. Findings in hypertension screening of 1 million Americans. JAMA 240(15): 1607-1610.

Stefan, N., H. U. Haring, et al. (2013). Metabolically healthy obesity: epidemiology, mechanisms, and clinical implications. Lancet Diabetes Endocrinol 1(2): 152-162.

Steffen, L. M., D. K. Arnett, et al. (2006). Population trends in leisure-time physical activity: Minnesota Heart Survey, 1980-2000. Med Sci Sports Exerc 38(10): 1716-1723.

Stunkard, A. J., T. I. Sorensen, et al. (1986). An adoption study of human obesity. N Engl J Med 314(4): 193-198.

Subramanian, S. and A. Chait (2012). Hypertriglyceridemia secondary to obesity and diabetes. Biochim Biophys Acta 1821(5): 819-825.

Sunny, N. E., S. Kalavalapalli, et al. (2015). Cross-talk between branched-chain amino acids and hepatic mitochondria is compromised in nonalcoholic fatty liver disease. Am J Physiol Endocrinol Metab 309(4): E311-319.

Sunny, N. E., S. Kalavalapalli, et al. (2015). Crosstalk between branched chain amino acids and hepatic mitochondria is compromised in nonalcoholic fatty liver disease. Am J Physiol Endocrinol Metab: ajpendo 00161 02015.

Swinburn, B., G. Egger, et al. (1999). Dissecting obesogenic environments: the development and application of a framework for identifying and prioritizing environmental interventions for obesity. Prev Med 29(6 Pt 1): 563-570.

Swinburn, B., G. Sacks, et al. (2009). Increased food energy supply is more than sufficient to explain the US epidemic of obesity. Am J Clin Nutr 90(6): 1453-1456.

Szczepaniak, L. S., P. Nurenberg, et al. (2005). Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. Am J Physiol Endocrinol Metab 288(2): E462-468.

Szendroedi, J., K. Kaul, et al. (2014). Lower fasting muscle mitochondrial activity relates to hepatic steatosis in humans. Diabetes Care 37(2): 468-474.

Tchernof, A. and J. P. Despres (2013). Pathophysiology of human visceral obesity: an update. Physiol Rev 93(1): 359-404.

Tilg, H. and A. R. Moschen (2010). Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. Hepatology 52(5): 1836-1846.

Tirosh, A., I. Shai, et al. (2011). Adolescent BMI trajectory and risk of diabetes versus coronary disease. N Engl J Med 364(14): 1315-1325.

Toth, M. J., A. Tchernof, et al. (2000). Menopause-related changes in body fat distribution. Ann N Y Acad Sci 904: 502-506.

Tremblay, F., S. Brule, et al. (2007). Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance. Proc Natl Acad Sci U S A 104(35): 14056-14061.

Trends in Global obesity. (2016). Retrieved 20.3, 2016, from http://www.worldobesity.org/resources/obesity-data-repository/resources/trends/12/.

Tuomilehto, J., J. Lindstrom, et al. (2001). Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. N Engl J Med 344(18): 1343-1350.

Uno, K., T. Yamada, et al. (2015). A hepatic amino acid/mTOR/S6K-dependent signalling pathway modulates systemic lipid metabolism via neuronal signals. Nat Commun 6: 7940.

Vague, J. (1996). Sexual differentiation. A determinant factor of the forms of obesity. 1947. Obes Res 4(2): 201-203.

Wajchenberg, B. L. (2000). Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. Endocr Rev 21(6): 697-738.

Wallace, M., Y. Z. Hashim, et al. (2010). Effects of menstrual cycle phase on metabolomic profiles in premenopausal women. Hum Reprod 25(4): 949-956.

Waller, K., J. Kaprio, et al. (2008). Associations between long-term physical activity, waist circumference and weight gain: a 30-year longitudinal twin study. Int J Obes (Lond) 32(2): 353-361.

van Vliet-Ostaptchouk, J. V., M. L. Nuotio, et al. (2014). The prevalence of metabolic syndrome and metabolically healthy obesity in Europe: a collaborative analysis of ten large cohort studies. BMC Endocr Disord 14: 9.

Vanderperre, B., T. Bender, et al. (2015). Mitochondrial pyruvate import and its effects on homeostasis. Curr Opin Cell Biol 33: 35-41.

Vandevijvere, S., C. C. Chow, et al. (2015). Increased food energy supply as a major driver of the obesity epidemic: a global analysis. Bull World Health Organ 93(7): 446-456.

Wang, B., R. Zhuang, et al. (2015). Prevalence of Metabolically Healthy Obese and Metabolically Obese but Normal Weight in Adults Worldwide: A Meta-Analysis. Horm Metab Res 47(11): 839-845.

Wang, T. J., M. G. Larson, et al. (2011). Metabolite profiles and the risk of developing diabetes. Nat Med 17(4): 448-453.

Vartiainen, E., T. Laatikainen, et al. (2010). Thirty-five-year trends in cardiovascular risk factors in Finland. Int J Epidemiol 39(2): 504-518.

Webber, L. S., S. R. Srinivasan, et al. (1991). Tracking of serum lipids and lipoproteins from childhood to adulthood. The Bogalusa Heart Study. Am J Epidemiol 133(9): 884-899.

Weghuber, D., S. Zelzer, et al. (2013). High risk vs. "metabolically healthy" phenotype in juvenile obesity - neck subcutaneous adipose tissue and serum uric acid are clinically relevant. Exp Clin Endocrinol Diabetes 121(7): 384-390.

Weir, J. B. (1990). New methods for calculating metabolic rate with special reference to protein metabolism. 1949. Nutrition 6(3): 213-221.

Velho, S., F. Paccaud, et al. (2010). Metabolically healthy obesity: different prevalences using different criteria. Eur J Clin Nutr 64(10): 1043-1051.

Westerterp, K. R. and J. R. Speakman (2008). Physical activity energy expenditure has not declined since the 1980s and matches energy expenditures of wild mammals. Int J Obes (Lond) 32(8): 1256-1263.

Whitaker, R. C., J. A. Wright, et al. (1997). Predicting obesity in young adulthood from childhood and parental obesity. N Engl J Med 337(13): 869-873.

WHO (2000). Obesity: preventing and managing the global epidemic. Report of a WHO consultation. World Health Organ Tech Rep Ser 894: i-xii, 1-253.

WHO. (2008). Training course on child growth assessment. from http://www.who.int/childgrowth/training/module_c_interpreting_indicators.pdf.

WHO. (2015, 15.1). Obesity and overweight, Fact sheet N°311. Retrieved 20.3, 2016, from http://www.who.int/mediacentre/factsheets/fs311/en/.

Viitasalo, A., T. A. Lakka, et al. (2014). Validation of metabolic syndrome score by confirmatory factor analysis in children and adults and prediction of cardiometabolic outcomes in adults. Diabetologia 57(5): 940-949.

Wijndaele, K., G. Beunen, et al. (2006). A continuous metabolic syndrome risk score: utility for epidemiological analyses. Diabetes Care 29(10): 2329.

Wiklund, P. K., S. Pekkala, et al. (2014). Serum metabolic profiles in overweight and obese women with and without metabolic syndrome. Diabetol Metab Syndr 6(1): 40.

Willemsen, G., K. J. Ward, et al. (2015). The Concordance and Heritability of Type 2 Diabetes in 34,166 Twin Pairs From International Twin Registers: The Discordant Twin (DISCOTWIN) Consortium. Twin Res Hum Genet 18(6): 762-771.

Virtue, S. and A. Vidal-Puig (2010). Adipose tissue expandability, lipotoxicity and the Metabolic Syndrome--an allostatic perspective. Biochim Biophys Acta 1801(3): 338-349.

Vodnala, D., M. Rubenfire, et al. (2012). Secondary causes of dyslipidemia. Am J Cardiol 110(6): 823-825.

Volgyi, E., A. Lyytikainen, et al. (2010). Long-term leisure-time physical activity has a positive effect on bone mass gain in girls. J Bone Miner Res 25(5): 1034-1041.

Voulgari, C., N. Tentolouris, et al. (2011). Increased heart failure risk in normal-weight people with metabolic syndrome compared with metabolically healthy obese individuals. J Am Coll Cardiol 58(13): 1343-1350.

Vukovic, R., K. Mitrovic, et al. (2013). Insulin-sensitive obese children display a favorable metabolic profile. Eur J Pediatr 172(2): 201-206.

Vuorela, N., M. T. Saha, et al. (2011). Change in prevalence of overweight and obesity in Finnish children - comparison between 1974 and 2001. Acta Paediatr 100(1): 109-115.

Wurtz, P., V. P. Makinen, et al. (2012). Metabolic signatures of insulin resistance in 7,098 young adults. Diabetes 61(6): 1372-1380.

Wurtz, P., P. Soininen, et al. (2013). Branched-chain and aromatic amino acids are predictors of insulin resistance in young adults. Diabetes Care 36(3): 648-655.

Wurtz, P., M. Tiainen, et al. (2012). Circulating metabolite predictors of glycemia in middle-aged men and women. Diabetes Care 35(8): 1749-1756.

Xu, L., H. Kitade, et al. (2015). Roles of Chemokines and Chemokine Receptors in Obesity-Associated Insulin Resistance and Nonalcoholic Fatty Liver Disease. Biomolecules 5(3): 1563-1579.

Yamakado, M., K. Nagao, et al. (2015). Plasma Free Amino Acid Profiles Predict Four-Year Risk of Developing Diabetes, Metabolic Syndrome, Dyslipidemia, and Hypertension in Japanese Population. Sci Rep 5: 11918.

Yang, J., M. Dolinger, et al. (2012). Leucine stimulates insulin secretion via down-regulation of surface expression of adrenergic alpha2A receptor through the mTOR (mammalian target of rapamycin) pathway: implication in new-onset diabetes in renal transplantation. J Biol Chem 287(29): 24795-24806.

Yang, R. Y., S. M. Wang, et al. (2015). Association of branched-chain amino acids with coronary artery disease: A matched-pair case-control study. Nutr Metab Cardiovasc Dis 25(10): 937-942.

Yeni-Komshian, H., M. Carantoni, et al. (2000). Relationship between several surrogate estimates of insulin resistance and quantification of insulin-mediated glucose disposal in 490 healthy nondiabetic volunteers. Diabetes Care 23(2): 171-175.

Yki-Jarvinen, H. (2014). Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. Lancet Diabetes Endocrinol 2(11): 901-910.

Yki-Jarvinen, H. and P. K. Luukkonen (2015). Heterogeneity of non-alcoholic fatty liver disease. Liver Int 35(12): 2498-2500.

Younossi, Z. M., A. B. Koenig, et al. (2015). Global Epidemiology of Non-Alcoholic Fatty Liver Disease-Meta-Analytic Assessment of Prevalence, Incidence and Outcomes. Hepatology.

Zimmet, P., G. Alberti, et al. (2007). The metabolic syndrome in children and adolescents. Lancet 369(9579): 2059-2061.

Zimmet, P. Z., K. G. Alberti, et al. (2005). Mainstreaming the metabolic syndrome: a definitive definition. Med J Aust 183(4): 175-176.

APPENDICES

Appendix 1

General characteristics and serum metabolites in normal weight individuals stratified by low and high HOMA-IR groups (MIXED model estimated marginal means with 95% confidence intervals are given taking into account genetic similarity and shared environment (daughter and mother) and contrast estimates' p-values were used to localize the significant differences between the two groups and group by generation interaction).

	Low HOMA-IR n=20		High HOMA-IR n=20			
	Mean	95% CI	Mean	95% CI	p-value	Group by generation
Age	35.6	(34.2, 37.1)	36.9	(35.3, 38.5)	0.245	0.192
Height (cm)	168.1	(165.4, 170.8)	166.0	(163.1, 168.8)	0.267	0.163
Weight (kg)	59.2	(56.3, 62.2)	58.8	(55.7, 61.9)	0.826	0.301
BMI (kg/height^2)	21.0	(20.2, 21.8)	21.3	(20.4, 22.1)	0.580	0.907
Percent body fat	23.5	(21.4, 25.2)	25.7	(23.7, 27.7)	0.471	0.927
FM (kg)	16.9	(14.8, 19.0)	21.2	(19.1, 23.3)	0.006	0.227
FFM (kg)	40.9	(39.5, 42.3)	40.5	(39.1, 42.0)	0.968	0.599
VAT (kg)	0.55	(0.47, 0.62)	0.60	(0.53, 0.67)	0.328	0.056
Liver fat (%)	2.5	(1.0, 4,0)	1.9	(0.4, 3.4)	0.440	0.766
HOMA-IR	0.9	(0.7, 1.2)	2.4	(2.1, 2.7)	<0.001	0.234
Metabolites (mmol/l)						
Betahydroxybutyrate	0.049	(0.028, 0.069)	0.071	(0.053, 0.089)	0.115	0.057
Acetate	0.040	(0.036, 0.043	0.041	(0.038, 0.045)	0.186	0.788
Acetoacetate	0.038	(0.031, 0.045)	0.035	(0.029, 0.042)	0.876	0.926
Alanine	0.380	(0.355, 0.405)	0.400	(0.377, 0.423)	0.218	0.494
Citrate	0.100	(0.093, 0.107)	0.104	(0.097, 0.111)	0.300	0.191
Creatinine	0.050	(0.045, 0.054)	0.051	(0.047, 0.055)	0.222	0.853
Glutamine	0.512	(0.492, 0.532)	0.540	(0.522, 0.557)	0.091	0.881
Glycerol	0.058	(0.048, 0.068)	0.069	(0.060, 0.078)	0.089	0.065
Glycine	0.256	(0.237, 0.276)	0.279	(0.261, 0.296)	0.508	0.486
Orosomucoid	1.221	(1.155, 1.288)	1.299	(1.238, 1.360)	0.026	0.845
Histidine	0.050	(0.040, 0.054)	0.056	(0.053, 0.059)	0.089	0.933
Isoleucine	0.035	(0.032, 0.045)	0.043	(0.038, 0.044)	0.010	0.598
Leucine	0.061	(0.056, 0.065)	0.067	(0.063, 0.071)	0.016	0.887
Valine	0.154	(0.145, 0.168)	0.170	(0.160, 0.181)	0.046	0.472
BCAA_sum	0.255	(0.236, 0.275	0.282	(0.262, 0.298)	0.029	0.714
Tyrosine	0.042	(0.038, 0.046)	0.046	(0.042, 0.049)	0.121	0.109
Phenylalanine	0.062	(0.058, 0.065)	0.064	(0.061, 0.067)	0.275	0.192
Pyruvate	0.067	(0.060, 0.075)	0.074	(0.067, 0.081)	0.147	0.703
Lactate	0.888	(0.796, 0.980)	0.936	(0.850, 1.022)	0.333	0.259
Urea	0.052	(0.045, 0.060)	0.050	(0.043, 0.057)	0.408	0.065

FM = fat mass; FFF = fat-free mass; VAT = visceral adipose tissue; HOMA-IR = homeostatic model assessment of insulin resistance.

Appendix 2

General characteristics of the participants with adipose tissue biopsies

	Low HOMA-IR n= 12		High HOMA-IR n=12			
	Mean	**95% CI**	**Mean**	**95% CI**	**p-value**	**Group by generation**
Anthropometry						
Age (years)	34.5	(31.8, 37.3)	36.5	(34.2, 38.9)	0.257	0.077
Height (cm)	162.4	(158.2, 166.6)	164.3	(160.7, 168.0)	0.484	0.033
Weight (kg)	59.4	(51.3, 67.6)	66.2	(59.1, 73.3)	0.201	0.966
BMI (kg/height(m)^2)	22.5	(19.8, 25.2)	24.5	(22.2, 28.7)	0.263	0.308
Percent body fat	34.8	(29.2, 40.4)	37.1	(30.8, 43,4)	0.567	0.189
Body composition						
FM (kg)	19.6	(13.0, 26.2)	24.7	(18.9, 30.5)	0.233	0.423
FFM (kg)	37.4	(34.8, 40.0)	38.6	(36.3, 40.8)	0.473	0.070
VAT (kg)	0.51	(0.34, 0.67)	0.67	(0.52, 0.81)	0.141	0.809
Liver fat (%)	4.3	(-5.6, 14.2)	4.9	(-2.1, 11.9)	0.912	0.714
Metabolic biomarkers						
Glucose (mmol/l)	5.3	(4.8, 5.8)	6.2	(5.7, 6.6)	0.011	0.112
Insulin (IU/l)	3.3	(-1.9, 6.9)	11.0	(7.9, 14.0)	0.003	0.372
HOMA-IR	0.8	(-0.4, 1.9)	3.1	(2.2, 4.1)	0.004	0.253
Matsuda index	155	(63, 248)	134	(63, 204)	0.694	0.162
Lipids						
FFA (mmol/l)	5.2	(4.1, 6.3)	3.8	(2.6, 4.8)	0.051	0.053
Triglyceride (mmol/l)	1.3	(0.8, 1.8)	1.1	(0.7, 1.6)	0.607	0.398
Adipokines						
Leptin (ng/ml)	18.2	(3.4, 33.0)	31.5	(18.8, 44.2)	0.168	0.443
Adiponectin (µg/ml)	12.9	(7.1, 18.7)	15.0	(9.9, 20.1)	0.571	0.834
Diet						
Energy (kcal)	1490	(1121, 1860)	1717	(1342, 2091)	0.374	0.235
Protein (E%)	17.7	(14.9, 20.7)	18.8	(15.8, 21.8)	0.600	0.796
Fat (E%)	36.5	(30.6, 42.5)	34.4	(28.4, 40.4)	0.604	0.304
Carbohydrates (E%)	45.8	(44.5, 49.6)	46.8	(45.6, 50.5)	0.574	0.227
Aerobic fitness						
LTPA (hours/week)	4.8	(3.4, 6.3)	4.0	(2.7, 5.2)	0.350	0.624
VO2max (ml/kg/min)	38.9	(32.8, 44.9)	37.7	(31.5, 43.8)	0.771	0.497

Data are given as mean ± SD. FM = fat mass; VAT = visceral adipose tissue; HOMA-IR = homeostatic model assessment of insulin resistance; Matsuda index = insulin sensitivity index; FFA = free fatty acids; E% = percentage of total energy intake.

ORIGINAL PAPERS

I

SERUM METABOLIC PROFILES IN OVERWEIGHT AND OBESE WOMEN WITH AND WITHOUT METABOLIC SYNDROME

by

Wiklund P, Pekkala S, Autio R, Munukka E, Xu L, Saltevo J,
Cheng S, Kujala UM, Alen M, Cheng S.

Diabetol Metab Syndr. 2014 Mar 20;6(1):40

Wiklund et al. Diabetology & Metabolic Syndrome 2014, 6:40
http://www.dmsjournal.com/content/6/1/40

DIABETOLOGY & METABOLIC SYNDROME

RESEARCH Open Access

Serum metabolic profiles in overweight and obese women with and without metabolic syndrome

Petri K Wiklund[1,2], Satu Pekkala[1], Reija Autio[3], Eveliina Munukka[1], Leiting Xu[4], Juha Saltevo[5], ShuMei Cheng[1], Urho M Kujala[1], Markku Alen[2,6] and Sulin Cheng[1*]

Abstract

Objective: To identify serum biomarkers through metabolomics approach that distinguishes physically inactive overweight/obese women with metabolic syndrome from those who are metabolically healthy, independent of body weight and fat mass.

Methods: We applied nuclear magnetic resonance spectroscopy-based profiling of fasting serum samples to examine the metabolic differences between 78 previously physically inactive, body weight and fat mass matched overweight/obese premenopausal women with and without MetS. MetS was defined as the presence of at least three of the following five criteria: waist circumference ≥88 cm, serum triacylglycerol ≥1.7 mmol/L, and high density lipoprotein cholesterol (HDL-C) <1.30 mmol/L, blood pressure ≥ 130/85 mmHg and fasting glucose ≥5.6 mmol/L). Principal component analysis was used to reduce the large number of correlated variables to fewer uncorrelated factors.

Results: Two metabolic factors were associated with MetS independent of BMI, fat mass, waist circumference and physical activity/fitness. Factor comprising branched-chain amino acids (BCAA) and aromatic amino acids (AAA) and orosomucoid was associated with all clinical risk factors ($p < 0.01$ for all).

Conclusion: Two metabolic factors distinguish overweight/obese women with metabolic syndrome from those who are metabolically healthy independent of body weight, fat mass and physical activity/fitness. In particular, factor comprising BCAA, AAA and orosomucoid seems auspicious biomarker determining metabolic health as it was associated with all clinical risk factors. Further research is needed to determine the public health and clinical significance of these results in terms of screening to identify those at greatest cardio-metabolic risk for whom appropriate intervention strategies should be developed.

Keywords: Obesity, Metabolic syndrome, Metabolomics, Women

Background

Excess fat mass is often seen in conjunction with a constellation of other cardiovascular risk factors such as hypertension, dyslipidemia and hyperglycemia, so-called metabolic syndrome (MetS) [1]. In recent years the prevalence of MetS has increased directly with the epidemic of obesity [2]. Comparisons of obese and lean subjects have evoked several hypotheses to explain the pathophysiological pathways of obesity associated metabolic disorders including insulin resistance, systemic low-grade inflammation [3], abdominal and ectopic fat accumulation [4], and intestinal microbiota composition [5]. Experimental evidence show that dysfunctional adipose tissue have an unfavorable effect on metabolism and thereby seem to underlie some of the obesity associated metabolic morbidities such as insulin resistance and type 2 diabetes [6]. Furthermore, nutritional factors [7], poor aerobic fitness [8] and physical inactivity [9] may also contribute to the development of MetS.

* Correspondence: shulin.cheng@jyu.fi
[1]Department of Health Sciences, University of Jyväskylä, Jyväskylä FIN-40014, Finland
Full list of author information is available at the end of the article

However, not all obese people develop metabolic disorders. In fact, preliminary evidence suggests that 16% of the Finnish obese women [10] and ~ 20% of the general obese population [11,12] are free from metabolic disorders. Discovery of specific biomarkers in the blood associated with MetS may reveal etiological pathways and help to identify obese individuals at risk for disease. In this study, we applied nuclear magnetic resonance (NMR) spectroscopy to analyze circulating metabolites to identify biomarkers that distinguish individuals who are metabolically healthy from individuals with MetS, independent of fat mass and physical activity/fitness.

Materials and methods

Study subjects

One hundred and three participants were recruited from the city of Jyväskylä and its surroundings to participate in EWI-study (Exercise and weight control intervention to study aerobic exercise intervention for improving physical fitness and weight control in overweight and obese women, ISRCTN87529813). A study physician examined the physical condition of the subjects and ensured that they met the inclusion criteria: 25–50 year old premenopausal woman with a body mass index between 25 and 40 kg/m^2, with a history of physically inactive lifestyle (participating in regular exercise ≤ 2 times/wk and ≤ 45 min/time), and without diagnosed musculoskeletal, hypertensive or cardiovascular conditions or type I/II diabetes and without any medication affecting glucose or lipid metabolism. The study protocol was approved by the ethics committee of Central Finland Health Care District. An informed consent was obtained from all subjects prior to the assessments.

From those subjects who fulfilled the basic inclusion criteria we identified individuals who had MetS defined as the presence of at least three of the following five criteria [13]: waist circumference ≥88 cm, fasting serum triacylglycerol ≥1.7 mmol/L, high density lipoprotein cholesterol (HDL-C) <1.30 mmol/L, glucose ≥5.6 mmol/L) and resting blood pressure ≥ 130/85 mmHg. Women who had none of the above (except waist circumference ≥88 cm) were categorized as metabolically healthy overweight/obese (MHO). Thirty-six out of 103 overweigth/obese women were characterized as MetS and forty-two as MHO. Twenty-five had one of the above (in addition to waist circumference ≥88 cm) and were discarded from the analysis.

Background information

Background information including medical history and current health status was collected via self-administered questionnaires. Food consumption and intakes of total energy and energy-yielding nutrients were assessed from three day food records and analyzed using Micro-Nutrica software developed by the Social Insurance Institution of Finland and updated with data for new foodstuffs by the study nutritionist [14]. Leisure time physical activity (LTPA) of hours/week (participating in exercise such as walking, jogging, running, gym fitness, ball games, swimming, etc.) and physical inactivity hours per day (PIA, including lying down and sitting time) were evaluated using a validated self-administrated physical activity questionnaire described previously [15].

Fitness test

Maximum oxygen uptake (VO2max, ml/kg/min) was assessed by bicycle ergometer. During tests, heart rates were assessed using ECG and respiratory gases and ventilation was measured using respiratory gas analyzer VIASYS (Healthcare Inc. USA). A specialist physician was responsible for monitoring ECG and blood pressure responses during the test and recording subject's signs and symptoms throughout the test.

Respiratory gas exchange analysis

The REE (kcal/day) was assessed by respiratory gas exchange analysis (GEA) using a ventilated-hood system (VIASYS Healthcare, Yorba Linda, CA, USA). Calibration of the GEA was carried out before each measurement according to the manufacturer's instructions. The subjects were instructed to avoid any strenuous physical activity and large, energy and protein rich meals for 24 h before the laboratory visit. The subjects arrived at the laboratory in the morning after an overnight fast. After relaxing in a measurement bed for 30 min, a ventilated hood was placed over their heads. Their oxygen consumption and carbon dioxide production were measured for 20 min at 1 min intervals, in a supine position and in a thermoneutral (22–24°C) environment. The first 5 min of the data were discarded as artefacts. The REE was calculated using the modified Weir equation [16].

Anthropometrical and body composition assessments

Body height (cm) was measured by using standardized protocols (a wall-fixed measuring device). Body weight (kg) and fat mass (FM, kg) were assessed using bioimpedance (Inbody 720, Biospace Co. ltd Seoul, South Korea). Precision of the repeated measurements expressed as coefficient of variation was, on average, 0.6% for FM. Body mass index (BMI) was calculated as weight/height^2 ($kg \cdot m^{-2}$). Blood pressure (BP) was measured twice by manual oscillometric methods in the morning after sitting for 10 minutes after the subjects arrived at the laboratory. Standing waist circumference was measured twice with a tape measure and the mean value was used.

Blood samples

Venous blood samples for biochemical analyses were taken in standardized fasting conditions in the mornings

between 7 am and 9 am. Serum samples were stored frozen at -80°C until analyzed. Serum glucose, total cholesterol, HDL, triacylglycerol, alanine amino transferase (S-ALAT); aspartate amino transferase (S-ASAT) and gamma glutamyltransferase (GGT) were analyzed using the KONELAB 20XTi analyzer (Thermo Fischer Scientific inc. Waltham, MA, USA). Insulin was determined by immunofluorescence using the IMMULITE Analyser (Diagnostic Products Corporation, Los Angeles). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as (fasting insulin concentration × fasting glucose concentration)/22.5. The inter- and intra-assay CVs were 2.0% and 3.7% for glucose and 11% and 3.4% for insulin, respectively.

Serum metabolomics

All serum samples were analysed using a high-throughput serum NMR metabolomics platform; the experimental protocols including sample preparation and NMR spectroscopy have been described in detail elsewhere [17,18]. This methodology has recently been applied in various large-scale epidemiological and genetic studies [19,20]. The NMR metabolomics methodology provides comprehensive quantitative information on various amino acids, glycolysis intermediates, fatty acid composition and degree of saturation and lipoprotein subclass distributions.

Data analyses

All data were checked for normality using the Shapiro-Wilk's W-test (PASW Statistics 18). If data were not normally distributed, the natural logarithms were used. Clinical characteristics and serum metabolites were compared using an independent-samples t-test. To ensure that the significant differences in metabolite levels between the groups was not confounded by age, waist circumference or BMI, analysis of covariance (ANCOVA) was used adjusting for the above-mentioned variables. Metabolites were denoted significant if the p-value was below 0.0005 to account for multiple testing of 100 independent tests. All assayed metabolites are shown in Additional file 1: Table S1 and Additional file 2: Table S2.

The metabolomics data was clustered utilizing hierarchical clustering algorithm. First, the metabolite and other values were metabolite-wise standardized to have 0 as a mean and 1 as standard deviation. Second, the missing values within the data were imputed with k-nearest neighbour algorithm (k = 3). The resulted data values were clustered using correlation distance and average linkage methods (Figure 1).

Given the expected multicollinearity of metabolites, we used principal component analysis (PCA) to reduce the large number of correlated variables into fewer uncorrelated factors. PCA was performed on fasting levels of amino acids, fatty acids, phospholipids, glycoproteins, ketone bodies, and glycolysis and gluconeogenesis intermediates. Varimax rotated factors with an eigenvalue ≥ 1 were identified and metabolites with a factor load ≥ 0.4 were reported as composing a given factor. Metabolomic factor scores were calculated for each individual based on the constructed scoring coefficients. Mean metabolite factor levels were compared between MHO and MetS groups with and without adjusting for age, BMI and

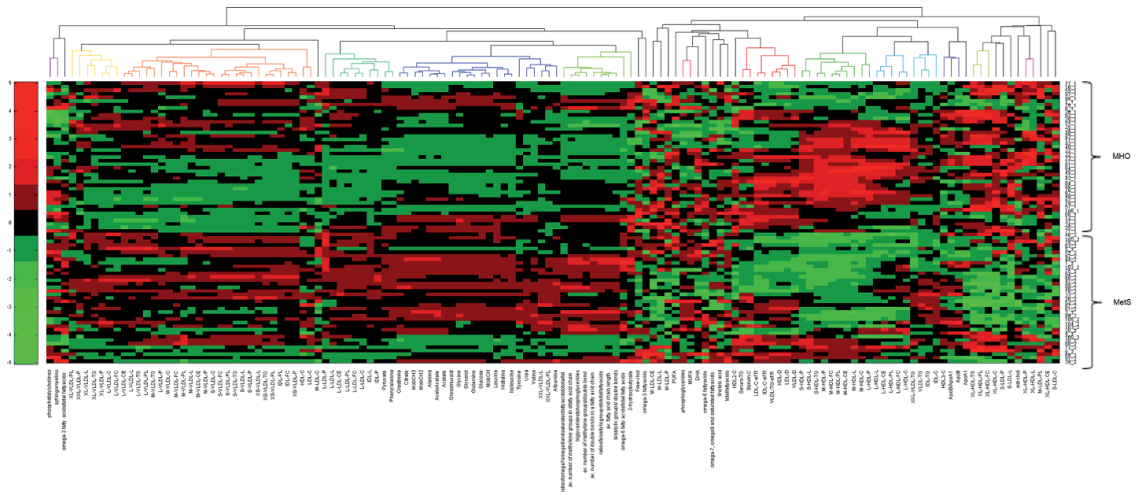


Figure 1 Hierarchical clustering of metabolomics data values in MHO and MetS groups. The heat map shows changes of x-fold standard deviation from the overall mean concentration of the metabolite in each individual belonging to either MHO or MetS group. Green squares represent a decrease, and red squares an increase. Metabolite names are shown on x-axis and individual subjects with adherent groups on (right) y-axis.

waist circumference. Further, we assessed whether factor levels were predictors for MetS using logistic regression models in all subjects adjusted for age, waist circumference and BMI. Finally, the networks between the metabolite factors and clinical risk factors were computed with the Spearman correlation and illustrated using Himmeli software [21]. Nominal statistical significance was defined as $p < 0.05$.

Results

Clinical characteristics

The general characteristics of the study subjects are given in Table 1. MetS group were older ($p < 0.005$), and had higher BMI ($p = 0.018$) but no significant differences in other anthropometric measures, REE, VO2max, LTPA or dietary intakes of total energy and energy-yielding nutrients between the groups were found. Systolic and diastolic blood pressure, glucose, insulin, HOMA-IR, triacylglycerol, HDL, total cholesterol, S-ASAT and S-ALAT were all higher in MetS compared to MHO ($p < 0.05$ for all). After controlling for age, BMI and waist circumference, the statistical significance remained for all.

Serum metabolites

A cluster analysis of serum metabolites implicated accumulation of several fatty acid species, VLDL lipoprotein subclasses, and glycoprotein and branched-chain amino acids in subjects with MetS (Figure 1). All metabolite and lipoprotein subclass quantities and statistics are shown in Additional file 1: Table S1 and Additional file 2: Table S2.

To further identify relevant biomarkers associated with Mets, we used principal component analysis. Eight metabolic factors were identified composed of correlated metabolites (Additional file 3: Table S3). Mean metabolite component levels are shown in Table 2. There were significant differences between MHO and MetS for factor 1 (branched-chain amino acids, phenylalanine, tyrosine and orosomucoid) ($p = 0.001$) and factor 2 (total fatty acids, omega-6 fatty acids, omega-7 and omega-9 fatty acids, linoleic acid, mono-unsaturated fatty acids, total phosphoglycerides, total phosphocholines) ($p = 0.003$). After adjusting for age, waist circumference and BMI, the level of statistical significance remained for both factors.

To verify that the association between metabolite factors with MetS was not confounded by differences in age and body fat, we performed a logistic regression analysis adjusted for age, waist circumference and BMI with MetS as the dependent (outcome) variable. The results showed that both factors were significantly associated with MetS ($p < 0.01$ for both).

Finally, we performed a network analysis to explore relationships between metabolite factors and clinical risk factors (Figure 2). When examining all subjects together, factor 1 was associated with HOMA-IR, insulin, triacylglycerol, SBP, VLDL, BMI, waist circumference, S-ALAT and inversely with HDL ($p < 0.01$ for all). Factor 2 and factor 3 were associated with IDL, LDL and VLDL, while factor 2 was also associated with SBP and triacylglycerol ($p < 0.01$ for all). Factor 7 was inversely associated with BMI and waist circumference, while factors 5 and 8 were inversely associated with insulin and triacylglycerol, respectively ($p < 0.01$ for all). All correlation coefficients and p-values are given in Additional file 3: Table S3.

Discussion

In this study, we aimed to identify metabolite profiles that distinguish physically inactive individuals who are metabolically healthy from those who have MetS independent of fat mass. We found that two metabolite factors composed of 1) branched-chain amino acids (BCAAs), aromatic amino acids (AAAs), orosomucoid and 2) several species of fatty acids and phospholipids were associated with MetS. Factor 1 was associated with all clinical risk factors suggesting that serum amino acids and orosomucoid may be relevant biomarkers of obesity associated cardiometabolic disorders.

The risk for developing metabolic disorders is proportional to the degree of obesity [22]. However, a subset of obese individuals seems to be protected from metabolic disorders, despite having excess fat mass [23]. Consequently, factors or mechanisms that explain the development of MetS remain poorly understood, and are under intense investigation since their understanding may help design novel therapeutic strategies. The large variation in susceptibility and age of onset in individuals with a similar risk profile, suggests both genetic and environmental factors contribute to development of metabolic disorders [24]. Emerging evidence suggests several potential mechanisms contributing to MetS including dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis due to chronic stress [25], dysregulation of the adipose tissue and increased cytokine production [26], the consequent systemic low-grade inflammatory state [27] and increased cellular oxidative stress [28]. Recent studies suggest that all of these mechanisms may be acting at different time during gestation, permanently reprogramming the structure and physiology of the offspring toward the development of metabolic disorders gradually progressing into a constellation of metabolic disorders in adulthood [29].

Recent studies have found that and elevated serum BCAAs [30,31] are associated with metabolic disorders independent of body weight. Our results are in agreement with the above-cited studies by showing that two factors (factor 1: BCAA, AAA and orosomucoid and factor 2: several species of fatty acids and phospholipids) were significantly different between MHO and MetS, independent of

Table 1 General characteristics of the study population

	MHO n = 42		MetS n = 36		p-value
Anthropometry					
Age (years)	39.7	(7.6)	44.1	(6.1)	0.005
Height (cm)	165.5	(5.8)	164.7	(6.4)	0.565
Weight (kg)	79.1	(10.3)	83.1	(10.5)	0.095
BMI (weight (kg)/height (m)2)	28.9	(3.2)	30.6	(3.4)	0.018
Fat mass (kg)	29.0	(7.9)	32.2	(8.1)	0.071
Fat free mass (kg)	50.2	(5.5)	50.8	(5.5)	0.596
Waist circumference (cm)	95.7	(9.2)	99.2	(6.5)	0.061
Metabolic					
SBP (mmHg)	122.0	(7.4)	136.4	(11.3)	<0.0001
DBP (mmHg)	77.7	(6.1)	84.4	(6.7)	<0.0001
GLUC (mmol/l)	5.1	(0.3)	5.5	(0.7)	0.0001
Insulin (μIU/l)	6.4	(2.9)	9.5	(3.6)	0.0001
HOMA-IR	1.6	(1.0)	2.3	(0.9)	0.002
HDL-C (mmol/l)	1.6	(0.3)	1.4	(0.3)	0.001
TRIGLY (mmol/l)	1.0	(0.3)	2.0	(0.9)	<0.0001
CHOLtot (mmol/l)	4.7	(0.6)	5.6	(0.9)	<0.0001
ALAT (IU/l)	13.3	(5.4)	18.8	(8.9)	0.003
ASAT (IU/l)	16.5	(5.6)	19.3	(6.8)	0.038
GGT (IU/l)	22.3	(13.8)	31.5	(13.6)	0.098
Energy expenditure and physical fitness					
RMR (kcal/day)	1547	(200)	1505	(113)	0.337
VO2max (ml/kg/min)	31.7	(4.8)	31.3	(5.6)	0.758
LTPA (≤ ½ h/wk, %)	16		11		0.250
LTPA (1 h/wk, %)	62		46		0.671
LTPA (2 h/wk, %)	22		43		0.399
PIA (h/day)	16.1	(2.9)	16.1	(3.5)	0.965
Diet					
Energy (kcal)	1791	(526)	1811	(555)	0.896
Protein (E%)	18.7	(3.9)	19.1	(3.9)	0.704
Carbohydrate (E%)	44.1	(11.2)	44.5	(8.7)	0.906
Fat (E%)	33.0	(6.4)	34.5	(11.4)	0.551
Saturated fat (E%)	12.4	(3.3)	13.0	(3.1)	0.537
Monounsaturated fat (E%)	11.1	(2.5)	11.2	(4.8)	0.914
Polyunsaturated fat (E%)	6.0	(2.2)	7.1	(4.7)	0.317

Data are given as mean (SD). P-values are for 2-tailed t-tests. MHO = healthy overweight/obese; MetS = metabolic syndrome; SBP = systolic blood pressure; DBP = diastolic blood pressure; HOMA-IR = homeostatic model assessment of insulin resistance; S-ALAT = alanine amino transferase; S-ASAT = aspartate amino transferase; S-GGT = gamma glutamyltransferase; RMR = resting metabolic rate, VO2max (maximum oxygen uptake); LTPA = leisure time physical activity, PIA = physical inactivity, E% = percentage of total energy intake.

age, waist circumference and BMI. The two factors increased the risk for MetS with similar magnitude (OR 2.90 vs. 2.67, Table 3) in the present study. However, the network analysis (Figure 2) showed that factor 2 correlated only with systolic blood pressure and serum lipids and lipoproteins, whereas factor 1 was associated with all risk determinants, with most pronounced associations with triacylglycerol, insulin, HOMA-IR, S-ALAT and HDL. These findings indicate that elevated serum BCAAs and AAAs are not only associated with insulin resistance as shown in prior studies [32-34] but that they are also closely related with lipid metabolism. This notion is in agreement with a

Table 2 Mean metabolite factor levels in MHO and MetS groups

Factor	MHO n = 42		MetS n = 36		p-value	adj p-value
1) Amino acids and glycoproteins	−0.34	(0.98)	0.43	(0.86)	0.001	0.001
2) Fatty acids and phospholipids	−0.31	(0.82)	0.39	(1.08)	0.003	0.002
3) PUFA	−0.16	(1.06)	0.21	(0.89)	0.120	0.631
4) Ketone bodies	0.07	(1.05)	−0.09	(0.94)	0.497	0.377
5) Gluconeogenic intermediates	0.06	(0.97)	−0.08	(1.04)	0.555	0.944
6) Miscellaneous	0.04	(0.97)	−0.05	(1.05)	0.726	0.464
7) Miscellaneous	0.08	(0.99)	−0.10	(1.01)	0.431	0.700
8) Miscellaneous	0.15	(0.75)	−0.18	(1.23)	0.167	0.228

MHO = metabolically healthy overweight/obese; MetS = metabolic syndrome. Values are given as mean (SD); Factor 1(leucine, isoleucine, valine, tyrosine, phenylalanine, orosomucoid); Factor 2 (total fatty acids, omega-6 fatty acids, omega7 and 9 fatty acids, linoleic acid, mono-unsaturated fatty acids, total phosphoglycerides, total phosphocholines); Factor 3 (docosahexaenoic acid, polyunsaturated fatty acids, omega-3 fatty acids); Factor 4 (acetoacetate, 3-hydroxybutyrate); Factor 5 (glutamine, glycine, pyruvate); Factor 6 (acetate, histidine); Factor 7 (creatinine, citrate); Factor 8 (urea). P-values are for the difference in mean metabolite factor levels between MHO and MetS groups with and without adjustment for age, fat mass and waist circumference.

recent animal study, which showed that oral administration of BCAAs increased lipogenic gene expression and synthesis of triacylglycerol in the liver [35]. Furthermore, the association of Factor 1 with S-ALAT also suggest potential relationship for BCAAs and AAAs with fatty liver [36], which is an important predictor of several components of metabolic syndrome [37]. However, whether the amino acids are causally implicated with increased serum lipids and fatty liver in humans remains uncertain and warrants further investigation.

It is unclear why factor 1 and its various components are present in higher concentrations in individuals with MetS. The acute phase protein (orosomucoid) is induced by infection and inflammation, and elevated plasma levels have been found in patients with type 2 diabetes [38]. Although the role of orosomucoid in the circulation is not well understood, it has been suggested to modulate immune responses to protect adipose tissue from inflammation and metabolic dysfunction [39]. As glucose [40] and lipid metabolites [41] are potential stimulants for inflammatory pathways in both adipocytes and macrophages, it is possible that the higher serum orosomucoid concentration in MetS group is attributed to higher level of these metabolic risk factors.

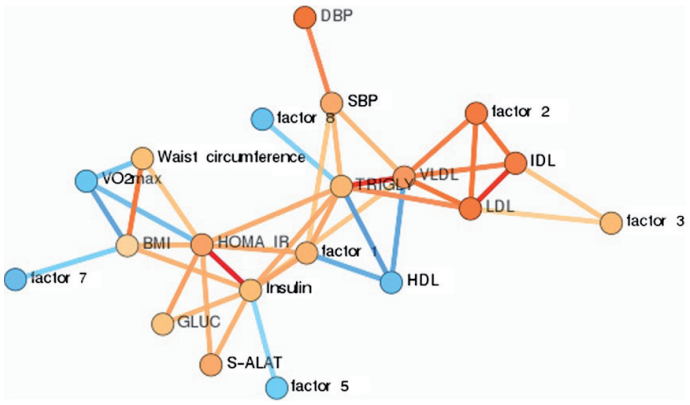


Figure 2 A pruned visualization of the correlation network from un-adjusted Spearman correlation analysis. Each variable was converted to a surrogate linear predictor before computations. The color of the edges indicate the association magnitude as shown in the legend. The vertices are colored as red and blue if all edges of the vertex are positive or negative correlations, respectively. In the cases where the vertex has both negative and positive correlations with its neighbor, the vertex is colored orange. Abbreviations: SBP = systolic blood pressure; DBP = diastolic blood pressure; VLDL = triacylglycerol and cholesterol in very-low density lipoprotein particles; LDL = triacylglycerol and cholesterol in low density lipoprotein particles; IDL = triacylglycerol and cholesterol in intermediate-density lipoprotein particles; Factor 1 (leucine, isoleucine, valine, tyrosine, phenylalanine, orosomucoid); Factor 2 (total fatty acids, omega-6 fatty acids, omega7 and 9 fatty acids, linoleic acid, mono-unsaturated fatty acids, total phosphoglycerides, total phosphocholines); Factor 3 (docosahexaenoic acid, polyunsaturated fatty acids, omega-3 fatty acids); Factor 5 (glutamine, glycine, pyruvate); Factor 6 (acetate, histidine); Factor 7 (creatinine, citrate); Factor 8 (urea).

Table 3 Logistic regression model for individual factors

Factor	OR	95% CI	p-value
1) Amino acids and glycoproteins	2.90	1.40 - 6.03	0.004
2) Fatty acids and phospholipids	2.67	1.34 - 5.32	0.005
3) PUFA	1.14	0.62 - 2.09	0.667
4) Ketone bodies	0.77	0.43 - 1.39	0.385
5) Gluconeogenic intermediates	1.00	0.57 - 1.75	0.991
6) Miscellaneous	0.80	0.46 - 1.40	0.437
7) Miscellaneous	0.93	0.53 - 1.63	0.804
8) Miscellaneous	0.70	0.39 - 1.25	0.227

Component levels as predictors of MetS adjusted for age, BMI and waist circumference.
Factor 1(leucine, isoleucine, valine, tyrosine, phenylalanine, orosomucoid); Factor 2 (total fatty acids, omega-6 fatty acids, omega7 and 9 fatty acids, linoleic acid, mono-unsaturated fatty acids, total phosphoglycerides, total phosphocholines); Factor 3 (docosahexaenoic acid, polyunsaturated fatty acids, omega-3 fatty acids); Factor 4 (acetoacetate, 3-hydroxybutyrate); Factor 5 (glutamine, glycine, pyruvate); Factor 6 (acetate, histidine); Factor 7 (creatinine, citrate); Factor 8 (urea).
OR = odds ratio.

Recent omics-studies have shown that increased long-term leisure-time physical activity is associated with low BCAA concentration [42], and high muscle BCAA degradation [43]. Furthermore, it has been shown that high intrinsic aerobic endurance capacity is associated with higher resting metabolic rate, improved signature of muscle BCAA degradation and lower risk for MetS [44]. In the current study, all participants were physically inactive, had similar aerobic fitness and resting metabolic rate. Furthermore, although VO2max was inversely associated with triacylglycerol, HOMA-IR, BMI and waist circumference, no associations with metabolite factors were found. Dietary patterns are also significant determinants of the circulating levels of metabolites during fast. In the current study, no differences were found in dietary energy or energy yielding nutrient intakes. Thus, it is unlikely that the higher level of factor 1 and it various components can be explained by low level of physical activity or poor cardiorespiratory fitness or dietary intake. However, it could be that some biological/genetic difference in BCAA catabolism reflects the circulating BCAA concentrations in the present study. Since tissue biopsies were not obtained in the present study, we were unable to measure genetic variations of the genes encoding BCAA catabolic enzymes in skeletal muscle or adipose tissues, and thus we cannot verify whether the differences in amino acid concentrations between the groups were attributable to subtle alterations in expression of the genes in BCAA catabolic pathway.

Although, the biological basis and clinical feasibility of MetS are still debatable [45], in the present study serum metabolite profiles were significantly different between the MHO and MetS. Consequently, our results tend to suggest that serum BCAA, AAA, orosomucoid and fatty acids may be relevant determinants of metabolic health independent of fat mass and physical activity. However, our results must be interpreted in the light of the study limitations. First, the cross-sectional study design does not show temporal relationship between the studied clinical risk factors and serum metabolites and therefore causal relationship cannot be deduced but rather serves to generate hypotheses. It is also important to note that in general population, all individuals with the MetS do not necessarily have all the features described in the present study. Moreover, this study is also limited by the relatively small number of participants and the fact that the study participants only consist of Finnish women. Finally, although HOMA-IR is a widely accepted measure of insulin resistance, other methods such as hyperinsulinemic-euglycemic clamp technique is considered more robust method to measure insulin resistance [46].

In summary, our results showed that two metabolite factors were associated with MetS independent of BMI, fat mass, waist circumference and physical activity/fitness. Especially factor comprising BCAA, AAA and orosomucoid seems auspicious biomarker determining metabolic health as it was associated with all clinical risk factors. Further research is needed to determine the public health and clinical significance of these results in terms of screening to identify those at greatest cardio-metabolic risk for whom appropriate intervention strategies should be developed.

Additional files

Additional file 1: Table S1. Serum lipid constituents and low molecular-weight metabolites of study population stratified by MHO and MetS categories.

Additional file 2: Table S2. Serum lipoprotein subclasses in study population stratified by MHO and MetS categories.

Additional file 3: Table S3. Correlations between metabolite factors and clinical risk factors.

Competing interests
The authors have nothing to declare.

Authors' contributions
The authors contributed to this manuscript in a following manner: PW participated in data collection, analyzed data and wrote the manuscript. SP participated in writing and editing the manuscript. RA carried out the clustering and network analyses and participated in writing the manuscript. EM coordinated the study, participated in measurements and carried out biochemical analyses. SMC carried out biochemical analyses and participated in measurements. LX, JS and UMK participated in writing and editing manuscript. MA participated in study design and writing and editing the manuscript, SC designed the study and participated in data analysis and writing and editing the manuscript. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Acknowledgements
This study was supported financially by the Academy of Finland, Ministry of Education of Finland and University of Jyväskylä. We would like to thank Ms. Arja Lyytikäinen, and Mr. Erkki Helkala for their valuable work and technical assistance on this project.

Author details
[1]Department of Health Sciences, University of Jyväskylä, Jyväskylä FIN-40014, Finland. [2]Department of Medical Rehabilitation, Oulu University Hospital, Oulu, Finland. [3]Department of Signal Processing, Tampere University of Technology, Tampere, Finland. [4]Ningbo University School of Medicine, Ningbo, China. [5]Central Hospital Central Finland, Jyväskylä, Finland. [6]Institute of Health Sciences, University of Oulu, Oulu, Finland.

Received: 5 March 2014 Accepted: 11 March 2014
Published: 20 March 2014

References

1. Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC Jr, Spertus JA, Costa F: **Diagnosis and management of the metabolic syndrome: an American heart association/national heart, lung, and blood institute scientific statement.** *Curr Opin Cardiol* 2006, **21**(1):1–6.
2. Park YW, Zhu S, Palaniappan L, Heshka S, Carnethon MR, Heymsfield SB: **The metabolic syndrome: prevalence and associated risk factor findings in the US population from the third national health and nutrition examination survey, 1988–1994.** *Arch Intern Med* 2003, **163**(4):427–436.
3. Muoio DM, Newgard CB: **Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes.** *Nat Rev Mol Cell Biol* 2008, **9**(3):193–205.
4. Rasouli N, Molavi B, Elbein SC, Kern PA: **Ectopic fat accumulation and metabolic syndrome.** *Diabetes Obes Metab* 2007, **9**(1):1–10.
5. Munukka E, Wiklund P, Pekkala S, Volgyi E, Xu L, Cheng S, Lyytikainen A, Marjomaki V, Alen M, Vaahtovuo J, Keinänen-Kiukaanniemi S, Cheng S: **Women with and without metabolic disorder differ in their gut microbiota composition.** *Obesity (Silver Spring)* 2012, **20**(5):1082–1087.
6. Guilherme A, Virbasius JV, Puri V, Czech MP: **Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes.** *Nat Rev Mol Cell Biol* 2008, **9**(5):367–377.
7. Abete I, Astrup A, Martinez JA, Thorsdottir I, Zulet MA: **Obesity and the metabolic syndrome: role of different dietary macronutrient distribution patterns and specific nutritional components on weight loss and maintenance.** *Nutr Rev* 2010, **68**(4):214–231.
8. Gill JM, Malkova D: **Physical activity, fitness and cardiovascular disease risk in adults: interactions with insulin resistance and obesity.** *Clin Sci (Lond)* 2006, **110**(4):409–425.
9. Kujala UM, Jokelainen J, Oksa H, Saaristo T, Rautio N, Moilanen L, Korpi-Hyovalti E, Saltevo J, Vanhala M, Niskanen L, Peltonen M, Tuomilehto J, Uusitupa M, Keinänen-Kiukaannemi S: **Increase in physical activity and cardiometabolic risk profile change during lifestyle intervention in primary healthcare: 1-year follow-up study among individuals at high risk for type 2 diabetes.** *BMJ Open* 2011, **1**(2):e000292.
10. Pajunen P, Kotronen A, Korpi-Hyovalti E, Keinanen-Kiukaanniemi S, Oksa H, Niskanen L, Saaristo T, Saltevo JT, Sundvall J, Vanhala M, Uusitupa M, Peltonen M: **Metabolically healthy and unhealthy obesity phenotypes in the general population: the FIN-D2D survey.** *BMC Public Health* 2011, **11**:754.
11. Karelis AD, St-Pierre DH, Conus F, Rabasa-Lhoret R, Poehlman ET: **Metabolic and body composition factors in subgroups of obesity: what do we know?** *J Clin Endocrinol Metab* 2004, **89**(6):2569–2575.
12. Bluher M: **The distinction of metabolically 'healthy' from 'unhealthy' obese individuals.** *Curr Opin Lipidol* 2010, **21**(1):38–43.
13. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WP, Loria CM, Smith SC Jr: **Harmonizing the metabolic syndrome: a joint interim statement of the international diabetes federation task force on epidemiology and prevention; national heart, lung, and blood institute; american heart association; world heart federation; international atherosclerosis society; and international association for the study of obesity.** *Circulation* 2009, **120**(16):1640–1645.
14. Lyytikainen A, Lamberg-Allardt C, Kannas L, Cheng S: **Food consumption and nutrient intakes with a special focus on milk product consumption in early pubertal girls in central Finland.** *Public Health Nutr* 2005, **8**(3):284–289.
15. Volgyi E, Lyytikainen A, Tylavsky FA, Nicholson PH, Suominen H, Alen M, Cheng S: **Long-term leisure-time physical activity has a positive effect on bone mass gain in girls.** *J Bone Miner Res* 2010, **25**(5):1034–1041.
16. Weir JB: **New methods for calculating metabolic rate with special reference to protein metabolism: 1949.** *Nutrition* 1990, **6**(3):213–221.
17. Inouye M, Kettunen J, Soininen P, Silander K, Ripatti S, Kumpula LS, Hamalainen E, Jousilahti P, Kangas AJ, Männisto S, Savolainen MJ, Jula M, Leiviskä J, Palotie A, Salomaa V, Perola M, Ala-Korpela M, Peltonen L: **Metabonomic, transcriptomic, and genomic variation of a population cohort.** *Mol Syst Biol* 2010, **6**:441.
18. Soininen P, Kangas AJ, Wurtz P, Tukiainen T, Tynkkynen T, Laatikainen R, Jarvelin MR, Kahonen M, Lehtimaki T, Viikari J, Raitakari OT, Savolainen MJ, Alakorpela M: **High-throughput serum NMR metabonomics for cost-effective holistic studies on systemic metabolism.** *Analyst* 2009, **134**(9):1781–1785.
19. Kettunen J, Tukiainen T, Sarin AP, Ortega-Alonso A, Tikkanen E, Lyytikainen LP, Kangas AJ, Soininen P, Wurtz P, Silander K, Dick DM, Rose RJ, Savolainen MJ, Viikari J, Kähönen M, Lehtimäki T, Pietiläinen KH, Inouye M, McCarthy MI, Jula M, Eriksson J, Raitakari OT, Salomaa V, Kaprio J, Järvelin MR, Peltonen L, Perola M, Freimer NB, Ala-korpela M, Palotie A, *et al.*: **Genome-wide association study identifies multiple loci influencing human serum metabolite levels.** *Nat Genet* 2012, **44**(3):269–276.
20. Stancakova A, Paananen J, Soininen P, Kangas AJ, Bonnycastle LL, Morken MA, Collins FS, Jackson AU, Boehnke ML, Kuusisto J, Ala-korpela M, Kuusisto M: **Effects of 34 risk loci for type 2 diabetes or hyperglycemia on lipoprotein subclasses and their composition in 6,580 nondiabetic Finnish men.** *Diabetes* 2011, **60**(5):1608–1616.
21. Makinen VP, Forsblom C, Thorn LM, Waden J, Kaski K, Ala-Korpela M, Groop PH: **Network of vascular diseases, death and biochemical characteristics in a set of 4,197 patients with type 1 diabetes (the FinnDiane study).** *Cardiovasc Diabetol* 2009, **8**:54.
22. Despres JP, Lemieux I, Bergeron J, Pibarot P, Mathieu P, Larose E, Rodes-Cabau J, Bertrand OF, Poirier P: **Abdominal obesity and the metabolic syndrome: contribution to global cardiometabolic risk.** *Arterioscler Thromb Vasc Biol* 2008, **28**(6):1039–1049.
23. Primeau V, Coderre L, Karelis AD, Brochu M, Lavoie ME, Messier V, Sladek R, Rabasa-Lhoret R: **Characterizing the profile of obese patients who are metabolically healthy.** *Int J Obes (Lond)* 2011, **35**(7):971–981.
24. Ordovas JM: **Genetic links between diabetes mellitus and coronary atherosclerosis.** *Curr Atheroscler Rep* 2007, **9**(3):204–210.
25. Chrousos GP: **The role of stress and the hypothalamic-pituitary-adrenal axis in the pathogenesis of the metabolic syndrome: neuro-endocrine and target tissue-related causes.** *Int J Obes Relat Metab Disord* 2000, **24**(Suppl 2):S50–S55.
26. Matsuzawa Y: **The metabolic syndrome and adipocytokines.** *FEBS Lett* 2006, **580**(12):2917–2921.
27. Lasselin J, Capuron L: **Chronic low-grade inflammation in metabolic disorders: relevance for behavioral symptoms.** *Neuroimmunomodulation* 2014, **21**(2–3):95–101.
28. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I: **Increased oxidative stress in obesity and its impact on metabolic syndrome.** *J Clin Invest* 2004, **114**(12):1752–1761.
29. Lakshmy R: **Metabolic syndrome: role of maternal undernutrition and fetal programming.** *Rev Endocr Metab Disord* 2013, **14**(3):229–240.
30. Batch BC, Shah SH, Newgard CB, Turer CB, Haynes C, Bain JR, Muehlbauer M, Patel MJ, Stevens RD, Appel LJ, Newby LK, Svetkey LP: **Branched chain amino acids are novel biomarkers for discrimination of metabolic wellness.** *Metabolism* 2013, **62**(7):961–969.
31. Lackey DE, Lynch CJ, Olson KC, Mostaedi R, Ali M, Smith WH, Karpe F, Humphreys S, Bedinger DH, Dunn TN, Thomas AP, Oort PJ, Kieffer DA, Amin R, Bettaieb A, Haj FG, Permana P, Anthony TG, Adams SH: **Regulation of adipose branched-chain amino acid catabolism enzyme expression and cross-adipose amino acid flux in human obesity.** *Am J Physiol Endocrinol Metab* 2013, **304**(11):E1175–E1187.
32. Felig P, Marliss E, Cahill GF Jr: **Plasma amino acid levels and insulin secretion in obesity.** *N Engl J Med* 1969, **281**(15):811–816.
33. Huffman KM, Shah SH, Stevens RD, Bain JR, Muehlbauer M, Slentz CA, Tanner CJ, Kuchibhatla M, Houmard JA, Newgard CB, Kraus WE: **Relationships between circulating metabolic intermediates and insulin action in overweight to obese, inactive men and women.** *Diabetes Care* 2009, **32**(9):1678–1683.
34. Tai ES, Tan ML, Stevens RD, Low YL, Muehlbauer MJ, Goh DL, Ilkayeva OR, Wenner BR, Bain JR, Lee JJ, Lim SC, Khoo CM, Shah SH, Newgard CB: **Insulin resistance is associated with a metabolic profile of altered protein metabolism in Chinese and Asian-Indian men.** *Diabetologia* 2010, **53**(4):757–767.

35. Higuchi N, Kato M, Miyazaki M, Tanaka M, Kohjima M, Ito T, Nakamuta M, Enjoji M, Kotoh K, Takayanagi R: **Potential role of branched-chain amino acids in glucose metabolism through the accelerated induction of the glucose-sensing apparatus in the liver.** *J Cell Biochem* 2011, **112**(1):30–38.
36. Razavizade M, Jamali R, Arj A, Talari H: **Serum parameters predict the severity of ultrasonographic findings in non-alcoholic fatty liver disease.** *Hepatobiliary Pancreat Dis Int* 2012, **11**(5):513–520.
37. Kotronen A, Yki-Jarvinen H, Sevastianova K, Bergholm R, Hakkarainen A, Pietilainen KH, Juurinen L, Lundbom N, Sorensen TI: **Comparison of the relative contributions of intra-abdominal and liver fat to components of the metabolic syndrome.** *Obesity (Silver Spring)* 2011, **19**(1):23–28.
38. Fournier T, Medjoubi NN, Porquet D: **Alpha-1-acid glycoprotein.** *Biochim Biophys Acta* 2000, **1482**(1–2):157–171.
39. Lee YS, Choi JW, Hwang I, Lee JW, Lee JH, Kim AY, Huh JY, Koh YJ, Koh GY, Son HJ, Masuzaki H, Hotta K, Alfadda AA, Kim JB: **Adipocytokine orosomucoid integrates inflammatory and metabolic signals to preserve energy homeostasis by resolving immoderate inflammation.** *J Biol Chem* 2010, **285**(29):22174–22185.
40. Lin Y, Berg AH, Iyengar P, Lam TK, Giacca A, Combs TP, Rajala MW, Du X, Rollman B, Li W, Hawkins M, Barzilai N, Rhodes CJ, Fantus IG, Brownlee M, Scherer PE: **The hyperglycemia-induced inflammatory response in adipocytes: the role of reactive oxygen species.** *J Biol Chem* 2005, **280**(6):4617–4626.
41. Nguyen MT, Satoh H, Favelyukis S, Babendure JL, Imamura T, Sbodio JI, Zalevsky J, Dahiyat BI, Chi NW, Olefsky JM: **JNK and tumor necrosis factor-alpha mediate free fatty acid-induced insulin resistance in 3 T3-L1 adipocytes.** *J Biol Chem* 2005, **280**(42):35361–35371.
42. Kujala UM, Makinen VP, Heinonen I, Soininen P, Kangas AJ, Leskinen TH, Rahkila P, Wurtz P, Kovanen V, Cheng S, Sipilä S, Hirvensalo M, Telama R, Tammelin T, Savolainen MJ, Pouta A, O'Reilly PF, Mäntyselkä P, Viikari J, Kähönen M, Lehtimäki T, Elliott P, Vanhala MJ, Raitakari OT, Järvelin MR, Kaprio J, Kainulainen H, Ala-Korpela M: **Long-term leisure-time physical activity and serum metabolome.** *Circulation* 2013, **127**(3):340–348.
43. Leskinen T, Rinnankoski-Tuikka R, Rintala M, Seppanen-Laakso T, Pollanen E, Alen M, Sipila S, Kaprio J, Kovanen V, Rahkila P, Oresic M, Kainulainen H, Kujala UM: **Differences in muscle and adipose tissue gene expression and cardio-metabolic risk factors in the members of physical activity discordant twin pairs.** *PLoS One* 2010, **5**(9):e12609.
44. Kivela R, Silvennoinen M, Lehti M, Rinnankoski-Tuikka R, Purhonen T, Ketola T, Pullinen K, Vuento M, Mutanen N, Sartor MA, Reunanen H, Koch LG, Britton SL, Kainulainen H: **Gene expression centroids that link with low intrinsic aerobic exercise capacity and complex disease risk.** *FASEB J* 2010, **24**(11):4565–4574.
45. Kahn R, Buse J, Ferrannini E, Stern M: **The metabolic syndrome: time for a critical appraisal. Joint statement from the American Diabetes Association and the European Association for the Study of Diabetes.** *Diabetologia* 2005, **48**(9):1684–1699.
46. Hosker JP, Matthews DR, Rudenski AS, Burnett MA, Darling P, Bown EG, Turner RC: **Continuous infusion of glucose with model assessment: measurement of insulin resistance and beta-cell function in man.** *Diabetologia* 1985, **28**(7):401–411.

doi:10.1186/1758-5996-6-40
Cite this article as: Wiklund *et al.*: Serum metabolic profiles in overweight and obese women with and without metabolic syndrome. *Diabetology & Metabolic Syndrome* 2014 **6**:40.

II

ADIPOSE TISSUE DYSFUNCTION AND ALTERED SYSTEMIC AMINO ACID METABOLISM ARE ASSOCIATED WITH NON-ALCOHOLIC FATTY LIVER DISEASE

by

Cheng S, Wiklund P, Autio R, Borra R, Ojanen X, Xu L, Törmäkangas T, Alen M.

PLoS One. 2015 Oct 6;10(10):e0138889

PLOS | ONE

RESEARCH ARTICLE

Adipose Tissue Dysfunction and Altered Systemic Amino Acid Metabolism Are Associated with Non-Alcoholic Fatty Liver Disease

Sulin Cheng[1,2☯]*, Petri Wiklund[2☯], Reija Autio[3,8], Ronald Borra[4], Xiaowei Ojanen[5], Leiting Xu[1,6], Timo Törmäkangas[2], Markku Alen[2,7]

1 Exercise Health and Technology Centre, Shanghai Jiao Tong University, Shanghai, China, **2** Department of Health Sciences, University of Jyvaskyla, Jyvaskyla, Finland, **3** Department of Signal Processing, Tampere University of Technology, Tampere, Finland, **4** Department of Diagnostic Radiology, University of Turku and Turku University Hospital, Turku, Finland, **5** Department of Applied Physics, University of Eastern Finland, Kuopio, Finland, **6** Medical School, Ningbo University, Ningbo, China, **7** Department of Medical Rehabilitation, Oulu University Hospital, Oulu, Finland, **8** School of Health Sciences, University of Tampere, Tampere, Finland

☯ These authors contributed equally to this work.
* sulin.cheng@sjtu.edu.cn; sulin.cheng@jyu.fi

OPEN ACCESS

Citation: Cheng S, Wiklund P, Autio R, Borra R, Ojanen X, Xu L, et al. (2015) Adipose Tissue Dysfunction and Altered Systemic Amino Acid Metabolism Are Associated with Non-Alcoholic Fatty Liver Disease. PLoS ONE 10(10): e0138889. doi:10.1371/journal.pone.0138889

Editor: Patricia Aspichueta, University of Basque Country, SPAIN

Received: June 16, 2015

Accepted: September 4, 2015

Published: October 6, 2015

Data Availability Statement: ArrayExpress data are available via the EMBL-EBI (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-3616).

Funding: The study funding is supported by various sources: The academy of Finland SKID-KIDS program (No. 135038); Shanghai Jiao Tong University Zhiyuan Foundation (CP2014013); China State Sport General Adiministration (No: 2013B040); and EVO research grants 2012/2013 from Oulu University Hospital.

Abstract

Background

Fatty liver is a major cause of obesity-related morbidity and mortality. The aim of this study was to identify early metabolic alterations associated with liver fat accumulation in 50- to 55-year-old men (n = 49) and women (n = 52) with and without NAFLD.

Methods

Hepatic fat content was measured using proton magnetic resonance spectroscopy (^{1}H MRS). Serum samples were analyzed using a nuclear magnetic resonance (NMR) metabolomics platform. Global gene expression profiles of adipose tissues and skeletal muscle were analyzed using Affymetrix microarrays and quantitative PCR. Muscle protein expression was analyzed by Western blot.

Results

Increased branched-chain amino acid (BCAA), aromatic amino acid (AAA) and orosomucoid were associated with liver fat accumulation already in its early stage, independent of sex, obesity or insulin resistance ($p<0.05$ for all). Significant down-regulation of BCAA catabolism and fatty acid and energy metabolism was observed in the adipose tissue of the NAFLD group ($p<0.001$for all), whereas no aberrant gene expression in the skeletal muscle was found. Reduced BCAA catabolic activity was inversely associated with serum BCAA and liver fat content ($p<0.05$ for all).

Competing Interests: The authors have declared that no competing interests exist.

Abbreviations: NAFLD, non-alcoholic fatty liver disease; ^{1}H MRS, = proton magnetic resonance; NMR, nuclear magnetic resonance; BCAA, branched-chain amino acids; AAA, aromatic amino acids; NASH, non-alcoholic steatohepatitis; LFC, liver fat content; BMI, body mass index; PA, participation in leisure time physical activity; FM, fat mass; SAT, abdominal subcutaneous adipose tissue; VAT, visceral adipose tissue; RAT, retroperitoneal adipose tissue; IMCL, Muscle intra-myocellular lipid; EMCL, extra-myocellular lipid; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transferase; NEFA, non-esterified fatty acids; HOMA-IR index, homeostatic model assessment of insulin resistance; TG, triglycerides; VLDL, very low density lipoprotein; E, energy; SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; Ch, carbohydrate; FM, fat mass; hsCRP, high-sensitivity C-reactive protein; Factor 1, Omega 7and 9 and saturated fatty acids, total fatty acids, mono-unsaturated fatty acids; Factor 2, isoleucine, leucine, valine, phenylalanine, tyrosine and orosomucoid; Factor 3, acetate, alanine, lactate, pyruvate; Factor 4, esterified cholesterol, free cholesterol, omega 6 fatty acids, phosphoglycerides, phosphocholines and sphingomyelines; Factor 5, beta-hydroxybutyrate, citrate, histidine; Factor 6, acetoacetate, glutamine; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Conclusions

Liver fat accumulation, already in its early stage, is associated with increased serum branched-chain and aromatic amino acids. The observed associations of decreased BCAA catabolism activity, mitochondrial energy metabolism and serum BCAA concentration with liver fat content suggest that adipose tissue dysfunction may have a key role in the systemic nature of NAFLD pathogenesis.

Introduction

The prevalence of non-alcoholic fatty liver (NAFLD) is on the rise, currently affecting up to 30% of the adult population and an increasing number of children in the developed countries [1]. In the early phase, NAFLD is asymptomatic, benign and often reversible, but if not controlled, it may progress to non-alcoholic steatohepatitis (NASH), cirrhosis and ultimately liver failure [2]. Therefore, metabolic perturbations contributing to the development of the disease should be identified before clinical manifestations occur. The metabolic abnormalities related to fatty liver are reflected in the level of circulating metabolites. Thus, comprehensive metabolic profiling holds potential for identifying specific disease-related patterns and non-invasive biomarkers [3]

Metabolic profiling studies have commonly noted increased free fatty acids and other lipid species in the plasma or serum of subjects with NAFLD [4]. In particular, acylcarnitines, lysophosphatidylcholines and triacylglycerol with low carbon number and double-bond content, have emerged as auspicious liver fat biomarkers [5,6]. Recent studies have also demonstrated increased circulating branched-chain amino acids (BCAAs) and their metabolic intermediates in subjects with NAFLD [7–9] and NASH[9,10] compared to healthy controls, but the underlying mechanisms of these associations remain to be established. These findings indicate that metabolic profiling can provide important information about etiology of the development and progression of NAFLD. However, the influence of extra-hepatic tissues on hepatic steatosis is incompletely understood.

Since NAFLD is closely associated with obesity, insulin resistance and type 2 diabetes[11–13], it is suspected that adipose tissue and skeletal muscle may play an important role in the development of NAFLD. Indeed, growing evidence indicates that adipose tissue dysfunction [14] and increased secretion of adipokines [15] are implicated in the systemic nature of NAFLD pathogenesis. Experimental studies have demonstrated that skeletal muscle insulin resistance promotes liver fat accumulation by altering the distribution pattern of postprandial energy storage [16]. Skeletal muscle has also been shown to modulate adipose tissue metabolism [17]. The newly discovered myokine, irisin, which has been proposed to convey the inter-organ signaling between skeletal muscle and adipose tissue, has recently been associated with NAFLD [18,19]. Furthermore, both skeletal muscle and adipose tissue are significant regulators of systemic amino acid metabolism, as most of the catabolic activity of BCAAs resides in these tissues [20]. Therefore, tissue-specific alterations in BCAA metabolism may contribute to the elevated levels of circulating BCAAs associated with fatty liver [21,22].

The aim of our study was to identify early systemic metabolic alterations associated with liver fat accumulation in healthy middle-aged men and women with and without NAFLD. In addition, we studied global gene expression profiles of adipose tissues and skeletal muscle, the purpose being to describe the early changes in the metabolic pathways that accompany liver fat accumulation and relate these to the serum metabolite profiles and associated clinically relevant factors.

Materials and Methods

Study participants

This article is a part of the Calex family study (n = 282 families), which has been described elsewhere [23,24]. For the purpose of this report, a subgroup of families (n = 74), comprising 222 individuals (daughter, mother and father) with no history of liver, pancreas or heart disease, or of heavy drinking, were contacted by letters for an additional study aimed at identifying the early metabolic alterations associated with liver fat accumulation. A total of 184 individuals responded to our invitation, of whom 163 (53 fathers, 53 mothers and 57 daughters) attended the laboratory tests. For this report, all the daughters were excluded owing to the low number with NAFLD (n = 5), leaving only the mothers and fathers (n = 106). Valid measurements of liver fat were unavailable for two men. In addition, two men reported recent alcohol consumption of >21 drinks on average per week and one woman reported >14 drinks on average per week. These individuals were therefore excluded. Hence, the final numbers of participants were 49 men and 52 women. Thirty (n = 30) participants had NAFLD, as defined by the cut-off LFC value of >5.56% [25]. The remainder, with a LFC value of <5.56%, were assigned to the healthy control group (n = 71). Of these 101 subjects, 32 (11 with NAFLD and 21 healthy controls) agreed to donate subcutaneous and skeletal muscle (vastus lateralis) biopsies.

Health history and current status was checked by the study physician. No major liver (cancer, hepatitis), pancreas (type I/II diabetes) or cardiac diseases were found. However, five men (3 healthy controls and 2 NAFLD) and two women (1 healthy control and 1 NAFLD) were using statins, and thirteen men (6 healthy controls and 7 NAFLD) and four women (3 controls and 1 NAFLD) were using hypertension medication. In addition, one man and three women were using thyroxine for hypothyreosis. All the other subjects were clinically euthyreotic. Twenty-two women were in early post menopause (15 healthy controls and 7 NAFLD), but there was no difference between the two groups in menopausal age, and none of the women were on hormonal replacement therapy. Including or excluding these women did not influence the results, and hence they were included in the final analysis. The study protocol was approved by the ethics committee of the Central Finland Health Care District. A written informed consent was obtained from all participants.

Background information, liver fat content, abdominal fat mass and myocellular lipid assessment

Body height, weight and body mass index (BMI) were assessed and the results reported elsewhere [23,26]. Dietary intake of total energy and energy-yielding nutrients were assessed from three-day food records and analyzed using Micro-Nutrica software developed by the Social Insurance Institution of Finland and updated with data for new foodstuffs by the study nutritionist [27]. Leisure time physical activity (PA), including walking, jogging, running, gym fitness, ball games, swimming, etc., expressed as hours/week and times/week, was evaluated using a validated self-administered physical activity questionnaire, as described previously [28].

Whole body fat mass (FM) was assessed by Dual-energy X-ray absorptiometry (DXA Prodigy, GE Lunar Corp., Madison, WI USA). In this study, two repeated measurements of FM showed a coefficient of variation (CV) of 2.2% [23].

Liver and abdominal regions were scanned using a 1.5 Tesla MR scanner (GE Signa CV/i, General Electric Healthcare, Waukesha, WI, USA). LFC was assessed by ^{1}HMRS with a PRESS sequence and was analysed using the Linear Combination of Model spectra software which is generally considered to be the gold standard for in-vivo spectroscopy analysis [29,30].

Abdominal adipose tissue compartments (subcutaneous = SAT, visceral = VAT, retroperitoneal = RAT) were quantified from a single slice image at the level of the L2-L3 intervertebral

disc using OsiriX software (OsiriX Foundation, Geneva, Switzerland). The results were converted into tissue fat mass in kg taking slice thickness into account and assuming an adipose tissue density of 0.9196 g/ml [31].

Muscle intra-myocellular lipid (IMCL) and extra-myocellular lipid (EMCL) from the tibialis anterior muscle were measured using a similar ^{1}H MRS method with a surface coil placed over the middle part of the muscle[32]. In order to obtain maximal IMCL and EMCL separation, the tibialis anterior muscle was aligned as closely as possible with the direction of the magnetic field and the voxel was placed parallel to the muscle fibers[32].

Biochemical assessments

Blood samples were collected in the morning between 7:00 and 9:00 am after overnight fasting. Among the women with regular menses, the samples were collected between 2 and 5 days after menstruation onset. Plasma glucose, serum alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT) and non-esterified fatty acids (NEFA) were assessed by a KONELAB 20XTi analyzer (Thermo Fischer Scientific inc. Waltham, MA, USA). Plasma insulin was assessed by chemiluminescent immunoassay (Diagnostic Products Corporation, Los Angeles). The intra- and inter-assay CVs were 3.4% and 2.0% for glucose, 11% and 3.4% for insulin, and 7.4% and 8.4% for NEFA. The HOMA-IR index (homeostatic model assessment of insulin resistance) was calculated as (fasting glucose x fasting insulin/22.5). Serum leptin was assessed using human leptin (ELISA; Diagnostic Systems Laboratories, Inc., Webster, TX). Total adiponectin was measured by an enzyme immunoassay method using the Quantikine human total adiponectin/Acrp30 immunoassay (R&D Systems, Minneapolis, MN). The inter- and intra-assay coefficients of variation (CVs) were 2.2% and 2.7% for leptin, and 3.3% and 4.3% for adiponectin. Serum high-sensitivity C-reactive protein (hsCRP) was assessed using an ELISA DuoSet (R&D Systems and Diagnostic Systems Laboratories, Inc). The intra- and inter-assay CVs were 4.6% and 6.9%.

Serum NMR spectroscopy

All serum samples were analyzed using a high-throughput serum NMR metabolomics platform; the experimental protocols, including sample preparation and NMR spectroscopy, have been described in detail elsewhere [33,34]. Altogether 130 metabolites were assessed.

Subcutaneous adipose tissue biopsies

Subcutaneous adipose tissue biopsies were obtained from 16 men and 16 women under local anesthesia after an overnight fast. A region 5 cm lateral from the umbilicus either to the left side or right side was sterilized. A small intracutaneous injection was made, and 2 ml of a local anesthetic agent (lidocaine) was injected. After 5 min, anesthesia was confirmed. The skin was then sterilized again and 10ml of 0.9% sodium-chloride was aspirated using a 16 G x 40 mm needle fitted to a 50-mL syringe. Approximately two-thirds of the length of the needle was inserted into the subcutaneous fat, and 5 ml of 0.9% sodium chloride was injected. The needle piston was then pulled back maximally and released until it was locked by a stopper, thereby creating a vacuum. Tissue resistance was created by gripping the abdominal skin with one hand while the other hand rotated the needle (back and forth) throughout the tissue (by a back and forth motion). Once the tissue had been aspirated by the syringe, the needle was withdrawn, and the piston removed. The adipose tissue samples were washed with saline solution, and were immediately frozen in liquid nitrogen and stored at—80°C.

Skeletal muscle biopsies

Vastus lateralis biopsies were taken from 16 men and 16 women under local anesthesia after an overnight fast. Biopsies were taken from the vastus lateralis dx muscle with a 5-mm Bergström biopsy needle, midway between the patella and greater trochanter. The location and optimum depth for the muscle biopsy were confirmed by ultrasound imaging. The skin covering the identified location was sterilized and 4 ml of local anesthetic agent (lidocaine) was injected into the subdermal tissue. A cooling pack was then applied to the location. After 10 minutes, anesthesia was confirmed, the skin was sterilized again and a small stab incision made with a surgical scalpel. Next, the biopsy needle, attached to a syringe, was introduced perpendicularly into the incision. The piston was then pulled back maximally, creating a vacuum, and the sample was obtained. After this, pressure was applied to the incision site to induce hemostasis. The muscle sample was cleaned of any visible connective and adipose tissue, as well as blood, and was frozen immediately in liquid nitrogen (−180°C) and stored at −80°C.

RNA extraction

Total RNA was extracted from the biopsies using the FastPrep system (MP Biomedicals, France) and a RNeasy Lipid Tissue Mini Kit (QIAGEN, Gaithersburg, MD, USA) according to manufacturer's instructions. Total RNA was digested on column with a RNase-free DNase set (QIAGEN) during RNA isolation. The quality of the total RNA was studied using a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and Experion Automated Electrophoresis Station (BioRad, Hercules, CA, USA). The total RNA was amplified and processed using a Gene Chip 3′ IVT Express Kit (Affymetrix, Santa Clara, CA, USA) and hybridized on Affymetrix Human Genome U219 Array Plates. The samples of this study have been submitted to ArrayExpress.

Protein extraction and Western blot from skeletal muscle biopsies

The muscle biopsies were homogenized in ice-cold lysis buffer [20 mM HEPES (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM Mg2Cl, 100 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM DTT, 1% Triton-X-100], supplemented with protease and phosphatase inhibitors inhibitors (Sigma Aldrich, St Louis, MO, USA).

Fifty to sixty micrograms of muscle lysate samples were separated by SDS-Page using 4–20% gradient gels on a Criterion electrophoresis cell (Bio-Rad Laboratories, Richmond, CA). Proteins were transferred to nitrocellulose membranes at 300-mA constant current on ice at 4°C. Membranes were blocked in TBS containing 5% nonfat dry milk for 1 hour at room temperature (RT), and then probed overnight at 4°C with commercially available primary antibodies. All antibodies were diluted 1:1000 (except anti-GAPDH (housekeeping, which was diluted 1:40,000) in TBS containing 5% nonfat dry milk. Membranes were then washed with TBS containing 0.1% Tween-20 (TBS-T) followed by 1 hour incubation with the secondary antibody. Blots were visualized and quantified using an Odyssey CLX Infrared Imager of (Li-COR Biosciences) and the manufacturer's software. When re-probing was needed, the membranes were incubated in 0.2 M NaOH for 10 min at RT, washed with TBS and re-probed with appropriate antibodies. All samples were run in the same gel to minimize variability and the quantitative results for each protein were normalized to GAPDH.

Transcriptomics analysis

Analysis of the transcriptomics data have been reported earlier [1]. Briefly, the gene values of the expression measurements were analyzed by using the Robust Multiarray Averaging (RMA) algorithm, as implemented in the R package affy. We ran the differentially expressed genes

(DEG) analysis with the LimmaR package utilizing linear modeling and empirical Bayes methods. Raw p-values were adjusted using the Benjamini and Hochberg multiple adjustment method.

Quantitative PCR

The results of the adipose tissue microarray analyses were confirmed by qPCR as described earlier [1]. Briefly, qPCR was performed on MMP9 and VAV1 from the same RNA samples. A High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA) was used to reverse transcribe 230 ng of RNA. Real-time PCR analysis was performed using iQ SYBR Supermix and CFX96™ Real-time PCR Detection System (Bio-Rad Laboratories, Richmond, CA, USA).

The primer sequences were as follows:

MMP9 sense: 50-GAGTGGCAGGGGGAAGATGC-30, and antisense 50-CCTCAGGG CACTGCAGGATG-30

VAV1 sense: 50-AGCAGTGGGAAGCACAAAGTATT-30, and antisense 50-GTCAC GGGCGCAGAAGTC-30

GAPDH sense: 50-CCACCCATGGCAAATTCC-30 and antisense: 50-TGGGATTTCCAT TGATGACAA- 30

Relative expression levels for MMP9 and VAV1 were calculated with the DDCt method and normalized to the expression of GAPDH. The fold changes in each gene between the groups were similar to those detected in the microarray analysis (data not shown).

Gene enrichment analysis

The enriched Gene Ontology (GO) terms or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for a given gene set were calculated by utilizing the R packages GOStats [2]. In the enrichment analysis, all the human ENSEMBL genes were used as a background gene group. Categories with a p-value lower than 0.05 are considered significantly enriched. We detected the genes whose expression was related to liver fat content by utilizing the following two criteria: 1) the DEG analysis with an adjusted p-value of <0.05 identified genes that were differentially expressed in the healthy control and NAFLD groups, and 2) the genes had a fold change of > 1 in the NAFLD group compared to the healthy controls. The mean-centroid value representing the "activity" of the regulated part of the pathway was computed by normalizing the expression levels of all the genes in the subset to a mean of zero across all individuals. Mean centroids have previously been shown to correlate with various metabolic and physiologic parameters [22,35], and may therefore be used to assess gene expression patterns that are associated with metabolic diseases. Correlation analyses with liver fat and serum metabolites were performed as described the next section.

Statistical methods

Continuous data were checked for normality by Shapiro-Wilk's test before each analysis using PASW statistics version 21 (IBM Corporation, USA). If data were not normally distributed, their natural logarithms were used. Since the data were from a family study, shared environmental (household) similarity was controlled for in the analysis. The linear mixed model was used to compare levels of the outcome variables between the NAFLD and healthy control groups. Contrast tests were used in mixed models to assess the effect of gender while controlling for dependency among family members with random effects.

The metabolomics data were clustered utilizing a hierarchical clustering algorithm which has been reported elsewhere [34]. The results are expressed as means with standard deviation

(SD). P-values were adjusted to control for the false discovery rate (FDR) using the method of Benjamini and Hochberg when comparing metabolites between the healthy control and NAFLD groups [36]. To identify relevant metabolites associated with LFC, we used principal component analysis to reduce a large number of correlated variables to fewer uncorrelated factors. Metabolite factor scores were calculated for each individual based on the constructed scoring coefficients. The principal component score is a transformation on the values of the metabolites, which can be considered as a weighted measure of the variability shared by the variables. Fasting levels of amino acids, fatty acids, phospholipids, glycoproteins, ketone bodies, and glycolysis and gluconeogenesis intermediates were included in the principal component analysis. Mean metabolite factor levels were compared between the NAFLD and healthy control groups. To exclude the possibility of misclassification, we divided the participants into quintiles based on liver fat content and compared their mean metabolite factor levels adjusting for HOMA-IR, BMI and visceral fat mass. Pearson correlation analyses were performed to determine the relationship between the gene pathways and clinical characteristics. Statistical significance was set at $p < 0.05$.

Results

Anthropometry, fat depots, lifestyle factors and conventional serum biomarkers

The mean liver fat content in the NAFLD and healthy control groups were 13.6% vs. 1.9% ($p<0.001$). No group by gender interaction was observed in liver fat content. The NAFLD participants were heavier and had higher BMI, total, visceral and retroperitoneal FM and IMCL compared to the healthy controls ($p \leq 0.005$ for all, Table 1). The NAFLD participants had higher fasting glucose, insulin, HOMA-IR, triglycerides, hsCRP, NEFA, leptin and liver enzymes but lower adiponectin and HDL-C levels than the healthy controls. ($p<0.05$ for all, Table 1). No differences were found in physical activity, dietary energy or energy yield nutrient intakes between the NAFLD and healthy control groups.

Serum metabolites

A cluster analysis of serum metabolites is illustrated in S1 Fig**.** The analysis revealed increased levels of very-low density lipoprotein (VLDL) subclasses, mono-unsaturated fatty acids, gluconeogenic substrates, orosomucoid and branched-chain amino acids, and decreased levels of high-density lipoprotein subclasses in participants with NAFLD. All essayed metabolites and lipoprotein subclass quantities and statistics are shown in S1 Table.

To further identify relevant metabolites associated with NAFLD, we used principal component analysis. Mean metabolite component levels are shown in Table 2. Factor 1 (omega 7and 9 and saturated fatty acids, total fatty acids and mono-unsaturated fatty acids), factor 2 (isoleucine, leucine, valine, phenylalanine, tyrosine and orosomucoid) and factor 3 (acetate, alanine, lactate, pyruvate) were significantly higher in the NAFLD group compared to the healthy control group ($p = 0.008$ to $p<0.001$). No group by gender interaction was found in any of the factors.

The mean metabolite component levels were further compared between the quintiles of liver fat content adjusting for gender, BMI, visceral fat mass, leptin and adiponectin (S2 Table). Factor 1 was significantly higher in the 5^{th} quintile compared to the 1^{st} and 2^{nd} quintiles. Factor 2 was significantly higher in the 3^{rd}, 4^{th} and 5^{th} quintile compared to the 1^{st} quintile, and factor 4 was significantly higher in the 5^{th} quintile compared to 1^{st} quintile. No significant difference in the other factors was observed between the highest and lowest quintile groups.

Table 1. Physical characteristics, fat mass distribution, glucose metabolism hormones and liver enzymes in the healthy controls and NAFLD group (MIXED model estimated marginal means with 95% confidence intervals are given taking into account shared environment within family (husband and wife) and contrast estimates' p-values were used to localize the significant differences between the two groups and group by gender interaction).

	Healthy controls (n = 71)		NAFLD (n = 30)			
	Mean	95% CI	Mean	95% CI	p	Group by gender
Men/women (n)	31/40		18/12		0.372	
Age (years)	51.7	(50.5, 52.9)	52.9	(50.9, 54.8)	0.244	0.479
Height (cm)	171.4	(170.1, 172.8)	171.6	(169.5, 173.8)	0.480	0.382
Weight (kg)	73.2	(70.7, 75.7)	87.3	(83.3, 91.3)	<0.001	0.493
BMI	24.9	(24.0, 25.7)	29.6	(28.3, 30.9)	<0.001	0.516
FM (kg)	19.8	(18.0, 21.6)	30.8	(27.9, 33.8)	<0.001	0.209
SAT (kg)	2.82	(2.49, 3.15)	4.61	(4.08, 5.15)	<0.001	0.002
VAT (kg)	0.749	(0.679, 0.818)	1.1	(0.99, 1.21)	0.005	0.293
RAT (kg)	1.13	(0.978, 1.28)	2.2	(2.00, 2.44)	<0.001	0.470
IMCL (%)	0.16	(0.142, 0.179)	0.24	(0.212, 0.268)	0.001	0.780
EMCL (%)	0.345	(0.281, 0.409)	0.408	(0.300, 0.515)	0.139	0.216
Energy (kcal/day)	1979	(1963, 2095)	1958	(1759, 2157)	0.674	0.947
Protein (E%)	18.1	(17.4, 18.8)	18.8	(17.5, 20.0)	0.557	0.980
Fat_{tot} (E%)	33.5	(31.8, 35.2)	31.7	(28.7, 34.5)	0.256	0.509
SAFA (E%)	12.9	(12.1, 13.7)	12.5	(11.1, 13.8)	0.588	0.766
MUFA (E%)	11.4	(10.6, 12.3)	10.4	(8.96, 11.9)	0.300	0.618
PUFA (E%)	6.00	(5.53, 6.45)	5.43	(4.63, 6.23)	0.100	0.170
Ch (E%)	45.8	(43.9, 47.7)	47.2	(43.9, 50.5)	0.359	0.484
Sucrose (E%)	5.02	(4.09, 5.95)	6.72	(5.12, 8.31)	0.515	0.419
PA (time/week)	2.94	(2.53, 3.35)	2.2	(1.55, 2.86)	0.143	0.806
PA (hour/week)	3.81	(3.36, 4.26)	3.25	(2.54, 3.97)	0.183	0.525
Glucose (mmol/l)	5.46	(5.33, 5.59)	5.71	(5.49, 5.92)	0.030	0.239
Insulin (μIU/ml)	6.22	(5.12, 7.32)	10.3	(8.58, 12.1)	0.001	0.306
HOMA-IR	1.53	(1.24, 1.82)	2.68	(2.21, 3.15)	<0.001	0.215
hsCRP (ng/ml)	722	(376, 1068)	1562	(1020, 2103)	0.006	0.709
NEFA (μmol/l)	395	(353, 438)	453	(383, 524)	0.032	0.069
Serum-TG	1.03	(0.93, 1.12)	1.45	(1.30, 1.59)	<0.001	0.067
Leptin (ng/ml)	11.9	(7.8, 16.0)	30.9	(24.8, 37.0)	<0.001	0.001
Adiponectin (μg/ml)	9.4	(7.8, 11.0)	5.1	(2.6, 7.6)	0.011	0.103
ALP (IU/l)	60.5	(56.9, 64.1)	64.9	(58.9, 70.9)	0.299	0.771
ALT (IU/l)	18.9	(16.0, 21.8)	30.9	(26.3, 35.5)	<0.001	0.129
AST (IU/l)	20.1	(18.6, 21.7)	23.0	(20.6, 24.4)	0.003	0.020
GGT (IU/l)	29.6	(25.0, 34.2)	39.4	(32.0, 46.8)	0.009	0.103

NAFLD = non-alcohol fatty liver disease; BMI = body mass index; FM = fat mass of the whole body; SAT = abdominal subcutaneous adipose tissue; VAT = visceral adipose tissue; RAT = retroperitoneal adipose tissue; IMCL = intra-myocellular lipids; EMCL = extra-myocellular lipids; E = energy; SAFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; Ch = carbohydrate; PA = physical activity; hsCRP = high-sensitivity C-reactive protein; NEFA = non-esterified fatty acids; TG = triglycerides; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; GGT = γ-glutamyltransferase.

doi:10.1371/journal.pone.0138889.t001

Adipose tissue gene expression

To elucidate the metabolic pathways associated with NAFLD, we studied global transcript profiles of adipose tissue and skeletal muscle. Microarray analysis revealed 709 differential

Table 2. Mean metabolite component levels stratified by the healthy control and NAFLD groups (MIXED model estimated marginal means with 95% confidence intervals are given taking into account shared environment within family, and contrast estimates' p-values were used to localize the significant differences between the two groups and group by gender interaction).

	Healthy Controls (n = 71)		NAFLD (n = 30)			
	Mean	95% CI	Mean	95% CI	p	Group by Gender
Factor 1	-0.233	(-0.471, 0.006)	0.640	(0.268, 1.011)	<0.001	0.171
Factor 2	-0.235	(-0.450, -0.019)	0.610	(0.277, 0.943)	0.001	0.450
Factor 3	-0.216	(-0.463, 0.031)	0.492	(0.111, 0.874)	0.008	0.368
Factor 4	-0.049	(-0.303, 0.204)	-0.051	(-0.446, 0.343)	0.996	0.988
Factor 5	0.015	(-0.260, 0.290)	-0.054	(-0.479, 0.371)	0.938	0.879
Factor 6	0.093	(-0.179, 0.365)	-0.173	(-0.593, 0.248)	0.753	0.558

NAFLD = non-alcohol fatty liver disease; values are given as mean and 95% confident interval (CI). Factor 1 (Omega 7 and 9 and saturated fatty acids, total fatty acids, mono-unsaturated fatty acids); Factor 2 (isoleucine, leucine, valine, phenylalanine, tyrosine and orosomucoid); Factor 3 (acetate, alanine, lactate, pyruvate); Factor 4 (esterified cholesterol, free cholesterol, omega 6 fatty acids, phosphoglycerides, phosphocholines and sphingomyelines); Factor 5 (beta-hydroxybutyrate, citrate, histidine); Factor 6 (acetoacetate, glutamine)

doi:10.1371/journal.pone.0138889.t002

expressed genes (adjusted $p<0.05$) in the adipose tissue of the NAFLD group. Of these 709 genes, 255 were up-regulated and 454 were down-regulated (S3 Table). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the differentially expressed genes ($p < 0.05$) identified 6 down regulated pathways (Table 3). The most down-regulated pathway was valine, leucine and isoleucine degradation ($p = 4.6x10^{-9}$). Down-regulated genes in this pathway included the mitochondrial components (BCKDHB and DLD) that are common to the degradation of all BCAAs, i.e., isoleucine, leucine and valine. Genes specific for the degradation of leucine (AUH), isoleucine (PCCA and PCCB) and valine (HIBADH) were also down-regulated in the subjects with NAFLD.

The mean centroid of the BCAA degradation pathway correlated negatively with liver fat content and serum metabolite factor 2 (Fig 1), serum total BCAA ($r = -0.471$, $p = 0.023$) and fasting insulin concentrations ($r = -0.550$, $p = 0.008$) and HOMA-IR ($r = -0.542$, $p = 0.009$). In a multiple linear regression analysis, including total FM, visceral FM, retroperitoneal FM, HOMA-IR and hsCRP, only the BCAA degradation pathway and serum metabolite factor 2

Table 3. KEGG pathway enrichment analysis of differentially expressed genes in adipose tissue.

P-value	Count	Size	Pathway name	Genes
4.6x10-9	18	44	Valine, leucine and isoleucine degradation	ACADM, ACADSB, ALDH7A1, ALDH9A1, AUH, BCKDHB, DLD, HADH, HADHA, HADHB, HIBADH, HIBCH, MCCC1, MCEE, MUT, OXCT1, PCCA, PCCB
3.0x10-7	13	30	Citrate cycle (TCA cycle)	CS, DLD, DLST, FH, IDH1, IDH3A, IDH3B, PCK1, PDHB, SDHB, SUCLA2, SUCLG1, SUCLG2
3.3x10-5	13	43	Fatty acid degradation	ACADM, ACADSB, ACADVL, ACSL1, ADH1B, ADH5, ALDH7A1, ALDH9A1, DCI, HADH, HADHA, HADHB, PECI
7.1x10-3	20	132	Oxidative phosphorylation	ATP5A1, ATP5B, ATP5G3, ATP5L, ATP6AP1, ATP6V1C1, COX5A, COX5B, CYC1, NDUFA10, NDUFA12, NDUFA6, NDUFB4, NDUFB5, NDUFB6, NDUFS1, NDUFS2, NDUFS4, SDHB, UQCRC2
1.9x10-2	11	65	Glycolysis / Gluconeogenesis	ADH1B, ADH5, ALDH7A1, ALDH9A1, DLD, ENO1, PCK1, PDHB, PFKP, PGK1, PGM1
3.5x10-2	12	80	Glycerophospholipid metabolism	AGPAT6, AGPAT9, CEPT1, CHKA, CHPT1, CRLS1, ETNK2, GNPAT, GPD1L, GPD2, LPCAT1, PGS1

Count = Amount of differentially expressed genes that mapped on pathway. Size = Total amount of genes involved in pathway.

doi:10.1371/journal.pone.0138889.t003

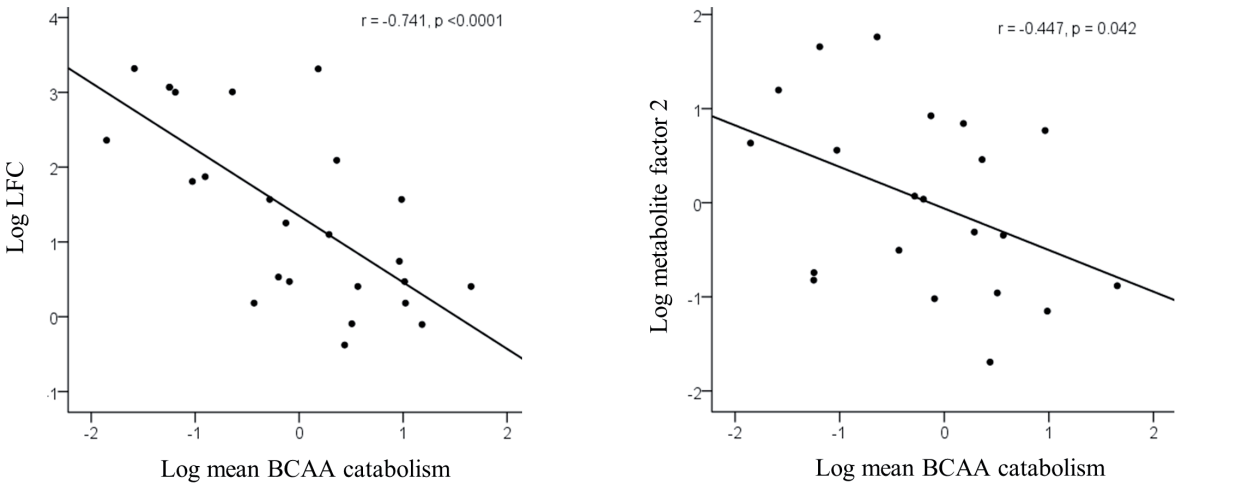


Fig 1. Correlations between liver fat content (LFC) assessed by ^{1}H MRS and different adipose tissue gene expression clusters in certain pathways. The LFC was transformed into a normal distribution by natural logarithms. Each dot represents an individual and the line is a linear regression fit line.

doi:10.1371/journal.pone.0138889.g001

remained significantly associated with liver fat (β = -0.791, and β = 0.992, respectively, $p<0.05$ for both). Non-alcoholic fatty liver was also associated with significant down-regulation of the energy metabolism in the adipose tissue. Fatty acid degradation, citric acid cycle and oxidative phosphorylation were associated with liver fat (r values ranged from -0.684 to -0.767, $p<0.001$ for all, Fig 2). No significant associations were found between the gene pathways and other serum metabolite factors (data not shown).

Skeletal muscle gene expression and signaling protein phosphorylation

Unexpectedly, no differentially expressed genes were found in the skeletal muscle. However, since skeletal muscle is the primary site of insulin-stimulated glucose disposal, we further studied whether there were differences in the phosphorylation levels of several signaling proteins related to glucose metabolism. No differences in the phosphorylation levels of insulin receptor

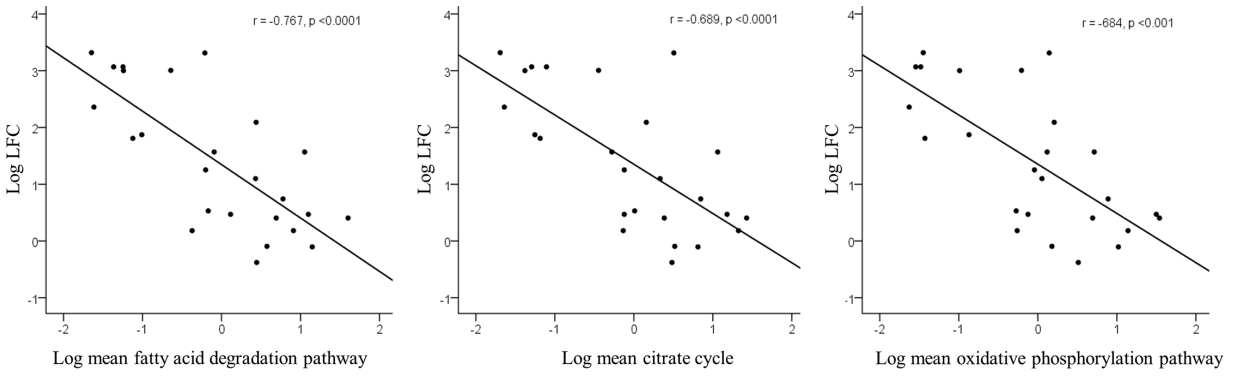


Fig 2. Correlations between serum metabolites factor 2 (isoleucine, leucine, valine, phenylalanine, tyrosine and orosomucoid) and different adipose tissue gene expression clusters in certain pathways. Each dot represents an individual and the line is a linear regression fit line.

doi:10.1371/journal.pone.0138889.g002

β and its downstream targets of Akt, ERK1/2, or mTOR and 4EBP1 were found. The level of phosphorylated AS160, which promotes translocation of glucose transporters to the cell membrane, was also similar between the groups.

Discussion

In this study, we showed that increased serum branched-chain and aromatic amino acids are already present well-below the clinical cutoff value for NAFLD. In addition, reduced BCAA catabolism and mitochondrial energy metabolism were observed in the adipose tissue of the NAFLD group, whereas no significant between-group differences in skeletal muscle gene expression were found.

The pathophysiology of NAFLD is complex and has not been fully elucidated. However, there is growing evidence that obesity is an important factor in its causation [37]. Recent studies suggest that the major basis for this link is the ability of obesity to engender insulin resistance [12]. The current theory suggests that as the adipose tissue mass expands, chronic inflammation in the adipose tissue ensues [38]. The inflamed adipose tissue becomes insulin resistant, and the impaired ability of insulin to suppress lipolysis leads to increased flux of non-esterified fatty acids, which accumulate in the liver as triglycerides [39]. The excessive accumulation of intrahepatic triglycerides gradually attenuates the ability of insulin to suppress hepatic gluconeogenesis and triglyceride synthesis, resulting in the development of hyperglycemia, hyperinsulinemia and dyslipidemia [40]. However, studies have suggested that NAFLD can also develop in the absence of marked insulin resistance and increased adipose tissue lipolysis [41], and that hepatokines may be involved in the cross-talk between liver and extra-hepatic tissues [42]. The results of the present study showed that all the measures of adiposity, serum glucose, insulin and HOMA-IR as well as free fatty acids, triglycerides and CRP were higher in the NAFLD group compared to healthy controls. Elevated concentrations of gluconeogenetic substrates, VLDL triglycerides and various species of fatty acids further supported the notion of increased gluconeogenetic activity and imbalanced lipid metabolism in the subjects with NAFLD.

Significant increases in serum BCAA and aromatic amino acid concentrations were found in the subjects with NAFLD compared to those with low liver fat content. These findings demonstrate the perturbations in systemic amino acid homeostasis that accompany liver fat accretion. Recent studies have also shown increased serum BCAA in subjects with NAFLD [7,9,43]. However, these studies were conducted in morbidly obese patients undergoing bariatric surgery [9], or in subjects with type 2 diabetes using non-specific ultrasonography [7], which is substantially limited by its low sensitivity to mild steatosis and inability to provide reliable quantitative information on liver fat infiltration [43]. Another study found increased BCAA in the plasma of subjects with NASH but not NAFLD when compared to healthy controls [44]. However, no significant difference in BCAA was found between NAFLD and NASH, which is not surprising given that steatosis and steatohepatitis are defects on a continuum. Furthermore, although biopsy is considered the "golden standard" in comparative studies of fatty liver disease, sampling errors have been shown to limit its diagnostic accuracy [45]. Thus, one should bear in mind the possibility of methodological errors when interpreting dichotomized data, especially when the variable under investigation is a continuous variable such as liver fat [46]. Here, we used ^{1}H magnetic resonance spectroscopy, which provides a highly specific estimation of hepatic fat *in vivo* [30]. To overcome the possibility of misclassification, we also divided our participants into quintile groups based on liver fat content. The results showed that the level of the metabolite factor consisting of BCAA and aromatic amino acids was significantly elevated at mean liver fat content of 2.4% (**S2 Table**), which is well below the clinical diagnostic

cut-off value. This finding suggests that clinically meaningful hepatic steatosis could be present even at less than 5% liver fat content. Importantly, we have previously demonstrated with another study cohort that the same metabolites as in metabolite factor 2 (BCAA and tyrosine and phenylalanine) were auspicious biomarkers determining metabolic health independent of obesity and physical activity [34]. Other studies have also reported associations between systemic BCAA and metabolic health [47–49]. Although we cannot fully explain the discrepancy between our study and the other studies in te the state and progression of NAFLD, our results suggest that perturbations in systemic BCAA homeostasis could be an early event in the development of NAFLD. This notion is supported by a recent study which demonstrated that chronic elevation of circulating BCAA induces hepatic mitochondrial dysfunction in NAFLD [50].

We further searched for signs of early changes in metabolic pathways in the adipose tissue and skeletal muscle. We found a significant reduction in the adipose tissue BCAA catabolism pathway in subjects with NAFLD compared to those with low liver fat content. The decrease in BCAA catabolism was inversely associated with the serum total BCAA, serum metabolite factor 2, fasting insulin, HOMA-IR and liver fat content. These findings are in line with an earlier study in monozygotic twins discordant for obesity, which showed that down-regulation of BCAA catabolism in subcutaneous adipose tissue was associated with increased insulin resistance and liver fat content [22]. In our study, liver fat accumulation was also associated with significant down-regulation of the energy metabolism in the adipose tissue. Thus it is possible that decreased BCAA catabolism and impaired mitochondrial function in subcutaneous adipose tissue could link excess adiposity to the development of insulin resistance and liver fat accumulation [51].

The down-regulation of BCAA catabolism in the present study could be ascribed to reduced mitochondrial respiration, as indicated by the concurrent significant down-regulation of the TCA cycle, oxidative phosphorylation and decreased fatty acid degradation in the adipose tissue. These metabolic impairments could also be attributable to local inflammation induced by excessive enlargement of adipocytes or diminished adipocyte differentiation [52]. A study with monozygotic twins showed marked inflammation in the subcutaneous adipose tissue concurrently with decreased BCAA catabolism in obese subjects [22]. Inflammation-induced regulation of BCAA metabolism in visceral, but not subcutaneous adipose tissue was also recently reported [53]. In the present study, inflammatory pathways were not significantly up-regulated in the adipose tissue of the participants with NAFLD. However, the two most over-expressed genes in the adipose tissue were chitinase-3-like protein 1 (CHI3L1) and matrix metallopeptidase 9 (MMP9) (**S3 Table**). These genes are related to cytoskeleton re-organization and degradation of the extracellular matrix and have been suggested to cause inflammatory cell infiltration, resulting in persistent inflammation in the adipose tissue [54]. These results are consistent with the higher serum hsCRP and orosomucoid observed in subjects with NAFLD, indicating the presence of subclinical low grade inflammation.

In addition to adipose tissue dysfunction, recent studies have implicated increased intramyocellular lipid content [55], increased muscle insulin resistance [16] and impaired skeletal muscle energy metabolism [56] in hepatic steatosis. In the present study, significantly higher intramuscular lipid content was observed in the NAFLD group compared to the low liver fat content group. Unexpectedly, no difference between the groups in gene expression profiles in the skeletal muscle was found. To confirm these findings with respect to glucose metabolism, we further studied whether there were differences in the phosphorylation levels of several signaling proteins. No significant differences in the phosphorylation levels of insulin receptor β and its downstream targets of Akt, ERK1/2, or mTOR and 4EBP1 were found. Nor was there any difference in the level of phosphorylated AS160, which promotes translocation of glucose

transporters to the cell membrane. These findings by no means negate the role of skeletal muscle insulin resistance in the development of systemic metabolic disorders. However, these findings do suggest that, at least in the fasting state, skeletal muscle glucose metabolism is not altered in the early stages of NAFLD, irrespective of increased intra-myocellular lipid content.

Our study is not without limitations. We acknowledge that our approach cannot determine causality and that the results can indicate only a general pattern. The study was conducted during 2009 to 2010. The interval between the different data collection points for each study participant varied to some extent, ranging from a few days to several months. However, there was no difference between the healthy control and NAFLD groups in the sampling time window. Further, no change in body weight or body composition was observed during the study period, nor was there any change in diet. The study participants were married couples drawn from a comprehensive and carefully performed family study. The couples shared the same family environment and living conditions. They were carefully selected in order to minimize confounding factors. For these reasons, we are confident that our results are not biased by background characteristics and health history. However, the narrow age range of the subjects in our study (50–55 years) may partly explain the lack of agreement between some of our findings and data from previous studies, and thus suggest that our results should be interpreted in the context of age. It should also be noted that this study is limited by the fact that adipose tissue and skeletal muscle biopsies were only available for a relatively small group of subjects (11 with and 21 without NAFLD). However, we used the state-of-the-art method ^{1}H MRS to quantify the ectopic fat content and serum metabolites. Furthermore, all the NAFLD subjects were in early or in moderate stage of fatty liver, which gave us the possibility to identify biomarkers associated with NAFLD in its early stage.

In summary, we demonstrate that already in its early stage liver fat accumulation is associated with increased serum branched-chain and aromatic amino acids. Significant down-regulation of BCAA catabolism and mitochondrial energy metabolism in the adipose tissue was found in participants with NAFLD, whereas no aberrant gene expression in skeletal muscle was observed. The observed associations between decreased BCAA catabolic activity and serum metabolite factor 2, total BCAA concentration and liver fat content suggest that adipose tissue dysfunction may play a key role in the systemic nature of NAFLD pathogenesis. However, whether BCAAs are involved in the development of NAFLD in a functional manner is unclear and warrants further study.

Supporting Information

S1 Fig. The heat map shows changes in the x-fold standard deviation from the overall mean concentration of the metabolite in each individual from either the non-alcohol fatty liver (NAFLD) or healthy control group. Green squares represent a decrease and red squares an increase in values. Metabolite names are shown on the x-axis and individual subjects with adherent groups on the (right) y-axis. The metabolite names are shown in S4 Table.
(PPTX)

S1 Table. Metabolites between the healthy control and NAFLD groups in men and women.
(DOCX)

S2 Table. Mean metabolite component levels stratified by the quintiles of liver fat content (General linear model estimated marginal means with 95% confidence intervals adjusted for BMI, visceral fat mass, leptin and adiponectin).
(DOCX)

S3 Table. Differentially expressed genes in the adipose tissue of the NAFL group.
(DOCX)

S4 Table. Abbreviations and full names of metabolites.
(DOCX)

Acknowledgments

We thank Ms. Shu Mei Cheng, Arja Lyytikainen, Eveliina Munukka, Satu Pekkala and other researchers in the Calex-family study for their technical support and assistance with the data collection, and Riitta Lahesmaa and Omid Rasool for performing the microarray assessment.

The study has been funded with support from the following: the Academy of Finland SKIDKIDS program (No. 135038), the Shanghai Jiao Tong University Zhiyuan Foundation (CP2014013), the China State Sport General Administration (2013B040), and EVO research grants 2012/2013 from Oulu University Hospital.

Author Contributions

Conceived and designed the experiments: SC PW MA. Performed the experiments: SC PW RA RB XO LX TT. Analyzed the data: PW SC RA TT. Contributed reagents/materials/analysis tools: XO RB LX. Wrote the paper: SC PW MA. Principal investigator: SC.

References

1. Smith BW, Adams LA (2011) Non-alcoholic fatty liver disease. Crit Rev Clin Lab Sci 48: 97–113. doi: 10.3109/10408363.2011.596521 PMID: 21875310
2. Tilg H, Moschen AR (2010) Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. Hepatology 52: 1836–1846. doi: 10.1002/hep.24001 PMID: 21038418
3. Cano A, Alonso C (2014) Deciphering non-alcoholic fatty liver disease through metabolomics. Biochem Soc Trans 42: 1447–1452. doi: 10.1042/BST20140138 PMID: 25233430
4. Beyoglu D, Idle JR (2013) The metabolomic window into hepatobiliary disease. J Hepatol 59: 842–858. doi: 10.1016/j.jhep.2013.05.030 PMID: 23714158
5. Hyysalo J, Gopalacharyulu P, Bian H, Hyotylainen T, Leivonen M, Jaser N, et al. (2013) Circulating triacylglycerol signatures in nonalcoholic fatty liver disease associated with the I148M variant in PNPLA3 and with obesity. Diabetes 63: 312–322. doi: 10.2337/db13-0774 PMID: 24009255
6. Lehmann R, Franken H, Dammeier S, Rosenbaum L, Kantartzis K, Peter A, et al. (2013) Circulating lysophosphatidylcholines are markers of a metabolically benign nonalcoholic fatty liver. Diabetes Care 36: 2331–2338. doi: 10.2337/dc12-1760 PMID: 23514731
7. Iwasa M, Ishihara T, Mifuji-Moroka R, Fujita N, Kobayashi Y, Hasegawa H, et al. (2015) Elevation of branched-chain amino acid levels in diabetes and NAFL and changes with antidiabetic drug treatment. Obes Res Clin Pract.
8. Lehmann R, Franken H, Dammeier S, Rosenbaum L, Kantartzis K, Peter A, et al. Circulating lysophosphatidylcholines are markers of a metabolically benign nonalcoholic fatty liver. Diabetes Care 36: 2331–2338. doi: 10.2337/dc12-1760 PMID: 23514731
9. Rodriguez-Gallego E, Guirro M, Riera-Borrull M, Hernandez-Aguilera A, Marine-Casado R, Fernandez-Arroyo S, et al. (2015) Mapping of the circulating metabolome reveals alpha-ketoglutarate as a predictor of morbid obesity-associated non-alcoholic fatty liver disease. Int J Obes (Lond) 39: 279–287.
10. Lake AD, Novak P, Shipkova P, Aranibar N, Robertson DG, Reily MD, et al. (2014) Branched chain amino acid metabolism profiles in progressive human nonalcoholic fatty liver disease. Amino Acids 47: 603–615. doi: 10.1007/s00726-014-1894-9 PMID: 25534430
11. Stefan N, Kantartzis K, Haring HU (2008) Causes and metabolic consequences of Fatty liver. Endocr Rev 29: 939–960. doi: 10.1210/er.2008-0009 PMID: 18723451
12. Yki-Jarvinen H (2014) Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. Lancet Diabetes Endocrinol. doi: 10.1016/S2213-8587(14)70032-4 PMID: 24731669

13. Gastaldelli A, Natali A, Vettor R, Corradini SG (2010) Insulin resistance, adipose depots and gut: interactions and pathological implications. Dig Liver Dis 42: 310–319. doi: 10.1016/j.dld.2010.01.013 PMID: 20194050

14. Tsochatzis EA, Papatheodoridis GV, Archimandritis AJ (2009) Adipokines in nonalcoholic steatohepatitis: from pathogenesis to implications in diagnosis and therapy. Mediators Inflamm 2009: 831670. doi: 10.1155/2009/831670 PMID: 19753129

15. Duval C, Thissen U, Keshtkar S, Accart B, Stienstra R, Boekschoten MV, et al. (2010) Adipose tissue dysfunction signals progression of hepatic steatosis towards nonalcoholic steatohepatitis in C57BL/6 mice. Diabetes 59: 3181–3191. doi: 10.2337/db10-0224 PMID: 20858684

16. Flannery C, Dufour S, Rabol R, Shulman GI, Petersen KF (2012) Skeletal muscle insulin resistance promotes increased hepatic de novo lipogenesis, hyperlipidemia, and hepatic steatosis in the elderly. Diabetes 61: 2711–2717. doi: 10.2337/db12-0206 PMID: 22829450

17. Argiles JM, Lopez-Soriano J, Almendro V, Busquets S, Lopez-Soriano FJ (2005) Cross-talk between skeletal muscle and adipose tissue: a link with obesity? Med Res Rev 25: 49–65. PMID: 15389734

18. Liu WY, Lu da J, Du XM, Sun JQ, Ge J, Wang RW, et al. (2014) Effect of aerobic exercise and low carbohydrate diet on pre-diabetic non-alcoholic fatty liver disease in postmenopausal women and middle aged men—the role of gut microbiota composition: study protocol for the AELC randomized controlled trial. BMC Public Health 14: 48. doi: 10.1186/1471-2458-14-48 PMID: 24438438

19. Choi ES, Kim MK, Song MK, Kim JM, Kim ES, Chung WJ, et al. (2014) Association between serum irisin levels and non-alcoholic fatty liver disease in health screen examinees. PLoS One 9: e110680. doi: 10.1371/journal.pone.0110680 PMID: 25343462

20. Brosnan JT, Brosnan ME (2006) Branched-chain amino acids: enzyme and substrate regulation. J Nutr 136: 207S–211S. PMID: 16365084

21. Herman MA, She P, Peroni OD, Lynch CJ, Kahn BB (2010) Adipose tissue branched chain amino acid (BCAA) metabolism modulates circulating BCAA levels. J Biol Chem 285: 11348–11356. doi: 10.1074/jbc.M109.075184 PMID: 20093359

22. Pietilainen KH, Naukkarinen J, Rissanen A, Saharinen J, Ellonen P, Keranen H, et al. (2008) Global transcript profiles of fat in monozygotic twins discordant for BMI: pathways behind acquired obesity. PLoS Med 5: e51. doi: 10.1371/journal.pmed.0050051 PMID: 18336063

23. Cheng S, Volgyi E, Tylavsky FA, Lyytikainen A, Tormakangas T, Xu L, et al. (2009) Trait-specific tracking and determinants of body composition: a 7-year follow-up study of pubertal growth in girls. BMC Med 7: 5. doi: 10.1186/1741-7015-7-5 PMID: 19171028

24. Wang Q, Xu L, Chen D, Tian H, Lu C, Cheng S, et al. (2011) Is bone loss the reversal of bone accrual? Evidence from a cross-sectional study in daughter-mother-grandmother trios. J Bone Miner Res 26: 934–940. doi: 10.1002/jbmr.291 PMID: 21541995

25. Szczepaniak LS, Nurenberg P, Leonard D, Browning JD, Reingold JS, Grundy S, et al. (2005) Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. Am J Physiol Endocrinol Metab 288: E462–468. PMID: 15339742

26. Lu C, Ivaska KK, Alen M, Wang Q, Tormakangas T, Xu L, et al. (2012) Serum osteocalcin is not associated with glucose but is inversely associated with leptin across generations of nondiabetic women. J Clin Endocrinol Metab 97: 4106–4114. doi: 10.1210/jc.2012-2045 PMID: 22948754

27. Cheng S, Lyytikainen A, Kroger H, Lamberg-Allardt C, Alen M, Koistinen A, et al. (2005) Effects of calcium, dairy product, and vitamin D supplementation on bone mass accrual and body composition in 10-12-y-old girls: a 2-y randomized trial. Am J Clin Nutr 82: 1115–1126; quiz 1147–1118. PMID: 16280447

28. Volgyi E, Lyytikainen A, Tylavsky FA, Nicholson PH, Suominen H, Alen M, et al. (2010) Long-term leisure-time physical activity has a positive effect on bone mass gain in girls. J Bone Miner Res 25: 1034–1041. doi: 10.1359/jbmr.091115 PMID: 19929440

29. Provencher SW (2001) Automatic quantitation of localized in vivo 1H spectra with LCModel. NMR Biomed 14: 260–264. PMID: 11410943

30. Borra RJ, Salo S, Dean K, Lautamaki R, Nuutila P, Komu M, et al. (2009) Nonalcoholic fatty liver disease: rapid evaluation of liver fat content with in-phase and out-of-phase MR imaging. Radiology 250: 130–136. doi: 10.1148/radiol.2501071934 PMID: 19017926

31. Munukka E, Pekkala S, Wiklund P, Rasool O, Borra R, Kong L, et al. (2014) Gut-adipose tissue axis in hepatic fat accumulation in humans. J Hepatol. doi: 10.1016/j.jhep.2014.02.020 PMID: 24613361

32. Furuyama JK, Nagarajan R, Roberts CK, Lee CC, Hahn TJ, Thomas MA (2014) A pilot validation of multi-echo based echo-planar correlated spectroscopic imaging in human calf muscles. NMR Biomed 27: 1176–1183. doi: 10.1002/nbm.3171 PMID: 25132520

33. Soininen P, Kangas AJ, Wurtz P, Tukiainen T, Tynkkynen T, Laatikainen R, et al. (2009) High-throughput serum NMR metabonomics for cost-effective holistic studies on systemic metabolism. Analyst 134: 1781–1785. doi: 10.1039/b910205a PMID: 19684899

34. Wiklund PK, Pekkala S, Autio R, Munukka E, Xu L, Saltevo J, et al. (2014) Serum metabolic profiles in overweight and obese women with and without metabolic syndrome. Diabetol Metab Syndr 6: 40. doi: 10.1186/1758-5996-6-40 PMID: 24650495

35. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, et al. (2003) PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet 34: 267–273. PMID: 12808457

36. Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B 57: 289–300.

37. Emilsson V, Thorleifsson G, Zhang B, Leonardson AS, Zink F, Zhu J, et al. (2008) Genetics of gene expression and its effect on disease. Nature 452: 423–428. doi: 10.1038/nature06758 PMID: 18344981

38. Trayhurn P (2013) Hypoxia and adipose tissue function and dysfunction in obesity. Physiol Rev 93: 1–21. doi: 10.1152/physrev.00017.2012 PMID: 23303904

39. Masoodi M, Kuda O, Rossmeisl M, Flachs P, Kopecky J (2014) Lipid signaling in adipose tissue: Connecting inflammation & metabolism. Biochim Biophys Acta 1851: 503–518. doi: 10.1016/j.bbalip.2014.09.023 PMID: 25311170

40. Chen G, Xu R, Zhang S, Wang Y, Wang P, Edin ML, et al. (2015) CYP2J2 overexpression attenuates nonalcoholic fatty liver disease induced by high-fat diet in mice. Am J Physiol Endocrinol Metab 308: E97–E110. doi: 10.1152/ajpendo.00366.2014 PMID: 25389366

41. Stefan N, Haring HU (2011) The metabolically benign and malignant fatty liver. Diabetes 60: 2011–2017. doi: 10.2337/db11-0231 PMID: 21788578

42. Charakida M, Masi S, Deanfield JE (2013) The Year in Cardiology 2012: focus on cardiovascular disease prevention. Eur Heart J 34: 314–317. doi: 10.1093/eurheartj/ehs429 PMID: 23284098

43. Schwenzer NF, Springer F, Schraml C, Stefan N, Machann J, Schick F (2009) Non-invasive assessment and quantification of liver steatosis by ultrasound, computed tomography and magnetic resonance. J Hepatol 51: 433–445. doi: 10.1016/j.jhep.2009.05.023 PMID: 19604596

44. Kalhan SC, Guo L, Edmison J, Dasarathy S, McCullough AJ, Hanson RW, et al. (2011) Plasma metabolomic profile in nonalcoholic fatty liver disease. Metabolism 60: 404–413. doi: 10.1016/j.metabol.2010.03.006 PMID: 20423748

45. Ratziu V, Charlotte F, Heurtier A, Gombert S, Giral P, Bruckert E, et al. (2005) Sampling variability of liver biopsy in nonalcoholic fatty liver disease. Gastroenterology 128: 1898–1906. PMID: 15940625

46. MacCallum RC, Zhang S, Preacher KJ, Rucker DD (2002) On the practice of dichotomization of quantitative variables. Psychol Methods 7: 19–40. PMID: 11928888

47. Batch BC, Shah SH, Newgard CB, Turer CB, Haynes C, Bain JR, et al. (2013) Branched chain amino acids are novel biomarkers for discrimination of metabolic wellness. Metabolism 62: 961–969. doi: 10.1016/j.metabol.2013.01.007 PMID: 23375209

48. Huffman KM, Shah SH, Stevens RD, Bain JR, Muehlbauer M, Slentz CA, et al. (2009) Relationships between circulating metabolic intermediates and insulin action in overweight to obese, inactive men and women. Diabetes Care 32: 1678–1683. doi: 10.2337/dc08-2075 PMID: 19502541

49. Wurtz P, Makinen VP, Soininen P, Kangas AJ, Tukiainen T, Kettunen J, et al. (2012) Metabolic signatures of insulin resistance in 7,098 young adults. Diabetes 61: 1372–1380. doi: 10.2337/db11-1355 PMID: 22511205

50. Sunny NE, Kalavalapalli S, Bril F, Garrett TJ, Nautiyal M, Mathew JT, et al. (2015) Crosstalk between branched chain amino acids and hepatic mitochondria is compromised in nonalcoholic fatty liver disease. Am J Physiol Endocrinol Metab: ajpendo 00161 02015.

51. Heilbronn L, Smith SR, Ravussin E (2004) Failure of fat cell proliferation, mitochondrial function and fat oxidation results in ectopic fat storage, insulin resistance and type II diabetes mellitus. Int J Obes Relat Metab Disord 28 Suppl 4: S12–21. PMID: 15592481

52. Goossens GH (2008) The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. Physiol Behav 94: 206–218. PMID: 18037457

53. Burrill JS, Long EK, Reilly B, Deng Y, Armitage IM, Scherer PE, et al. (2015) Inflammation and ER Stress Regulate Branched Chain Amino Acid Uptake and Metabolism in Adipocytes. Mol Endocrinol: me20141275. doi: 10.1210/me.2014-1275 PMID: 25635940

54. Libreros S, Garcia-Areas R, Shibata Y, Carrio R, Torroella-Kouri M, Iragavarapu-Charyulu V (2011) Induction of proinflammatory mediators by CHI3L1 is reduced by chitin treatment: decreased tumor

metastasis in a breast cancer model. Int J Cancer 131: 377–386. doi: 10.1002/ijc.26379 PMID: 21866546

55. Petersen KF, Dufour S, Savage DB, Bilz S, Solomon G, Yonemitsu S, et al. (2007) The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. Proc Natl Acad Sci U S A 104: 12587–12594. PMID: 17640906

56. Szendroedi J, Kaul K, Kloock L, Strassburger K, Schmid AI, Chmelik M, et al. (2013) Lower fasting muscle mitochondrial activity relates to hepatic steatosis in humans. Diabetes Care 37: 468–474. doi: 10.2337/dc13-1359 PMID: 24026561

III

INSULIN RESISTANCE IS ASSOCIATED WITH ALTERED AMINO ACID METABOLISM AND ADIPOSE TISSUE DYSFUNCTION INNORMOGLYCEMIC WOMEN

by

Wiklund P, Zhang X, Pekkala S, Autio R, Kong L, Yanga Y,
Keinänen-Kiukaanniemi S, Alen M, Cheng S,

Scientific Reports 2016 Apr 15; 6:24540

SCIENTIFIC REPORTS

OPEN

Insulin resistance is associated with altered amino acid metabolism and adipose tissue dysfunction in normoglycemic women

Received: 26 November 2015
Accepted: 31 March 2016
Published: 15 April 2016

Petri Wiklund[1,2], Xiaobo Zhang[1], Satu Pekkala[2], Reija Autio[3], Lingjia Kong[4], Yifan Yang[1], Sirkka Keinänen-Kiukaanniemi[5], Markku Alen[5,6] & Sulin Cheng[1,2]

Insulin resistance is associated adiposity, but the mechanisms are not fully understood. In this study, we aimed to identify early metabolic alterations associated with insulin resistance in normoglycemic women with varying degree of adiposity. One-hundred and ten young and middle-aged women were divided into low and high IR groups based on their median HOMA-IR (0.9 ± 0.4 vs. 2.8 ± 1.2). Body composition was assessed using DXA, skeletal muscle and liver fat by proton magnetic resonance spectroscopy, serum metabolites by nuclear magnetic resonance spectroscopy and adipose tissue and skeletal muscle gene expression by microarrays. High HOMA-IR subjects had higher serum branched-chain amino acid concentrations (BCAA) ($p < 0.05$ for both). Gene expression analysis of subcutaneous adipose tissue revealed significant down-regulation of genes related to BCAA catabolism and mitochondrial energy metabolism and up-regulation of several inflammation-related pathways in high HOMA-IR subjects ($p < 0.05$ for all), but no differentially expressed genes in skeletal muscle were found. In conclusion, in normoglycemic women insulin resistance was associated with increased serum BCAA concentrations, down-regulation of mitochondrial energy metabolism and increased expression of inflammation-related genes in the adipose tissue.

One of the earliest detectable defects in the metabolic continuum leading to type 2 diabetes is insulin resistance[1]. Impaired glucose homeostasis is associated with obesity[2], but the means by which excessive adiposity induces insulin resistance and glucose intolerance remain controversial. Indeed, only about a quarter of the variance of insulin resistance is explained by BMI in the general population[3]. Studies have shown that increased risk of heart disease is independent of central obesity in individuals with metabolic syndrome[4], and lean individuals may be as insulin resistant as those with type 2 diabetes[5]. On the other hand, not all overweight and obese individuals develop insulin resistance or other metabolic disorders[6], suggesting that the quantitative impact of obesity *per se* on insulin sensitivity may not be as large as previously thought[7].

Obesity-associated increase in plasma free fatty acids and accumulation of ectopic lipids are linked with the onset of peripheral and hepatic insulin resistance[8]. Dysregulated amino acid metabolism is also associated with obesity-related impaired insulin sensitivity[9] and increased risk for future diabetes[10]. We have recently used high-through put metabolite quantification to identify metabolic differences between sedentary obese individuals with and without metabolic syndrome. We found that branched-chain and aromatic amino acids were strongly associated with insulin resistance and other metabolic risk factors, independent of fat mass and waist circumference[11]. This finding suggests that excess fat mass alone does not explain the associations of these amino acids with insulin resistance. Thus, the source of increased branched-chain and aromatic amino acids, and the mechanisms by which they might contribute to impaired insulin sensitivity remain incompletely understood.

Recent evidence suggests that obesity is associated with altered adipose tissue metabolism, which in turn affects systemic glucose homeostasis and induces insulin resistance in skeletal muscle[12,13]. There is little

[1]Shanghai Jiao Tong University, Shanghai, China. [2]Department of Health Sciences, University of Jyväskylä, Jyväskylä, Finland. [3]School of Health Sciences, University of Tampere, Tampere, Finland. [4]Department of Signal Processing, Tampere University of Technology, Tampere, Finland. [5]Center for Life Course Health Research, University of Oulu, Oulu, Finland. [6]Department of Medical Rehabilitation, Oulu University Hospital, Oulu, Finland. Correspondence and requests for materials should be addressed to S.C. (email: sulin.cheng@jyu.fi)

	low HOMA-IR (n = 55)		high HOMA-IR (n = 55)		p-value	Group by generation
	Mean	95% CI	Mean	95% CI		
Anthropometry						
Age (years)	35.1	(35.9, 36.3)	36.0	(35.0, 36.9)	0.269	0.096
Height (cm)	165.7	(163.9, 167.7)	166.1	(164.5, 167.6)	0.837	0.562
Weight (kg)	61.3	(58.4, 64.1)	67.3	(65.1, 69.5)	0.001	0.604
BMI (weight(kg)/height(m)2)	22.3	(21.4, 23.2)	24.4	(23.7, 25.1)	0.002	0.497
Body composition						
Percent body fat	29.5	(27.3, 31.6)	35.0	(32.9, 37.1)	0.001	0.209
FFM (kg)	40.6	(39.3, 42.0)	40.7	(39.6, 41.8)	0.944	0.417
FM (kg)	17.8	(15.5, 20.1)	23.7	(21.9, 25.5)	0.001	0.266
VAT (kg)	0.5	(0.47, 0.62)	0.7	(0.59, 0.70)	0.044	0.164
Liver fat (%)	2.5	(0.9, 4.1)	2.7	(1.5, 4.0)	0.787	0.577
IMCL (%)	0.2	(0.16, 0.25)	0.2	(0.17, 0.23)	0.796	0.261
EMCL (%)	0.2	(0.15, 0.31)	0.3	(0.27, 0.39)	0.068	0.925
Metabolic biomarkers						
fs-glucose (mmol/l)	5.2	(5.0, 5.4)	5.5	(5.3, 5.6)	0.042	0.982
fs-insulin (μU/ml)	4.1	(3.0, 5.1)	9.9	(9.0, 10.8)	<0.001	0.110
HOMA-IR	0.9	(0.6, 1.2)	2.4	(2.2, 2.7)	<0.001	0.112
Lipids						
FFA (mmol/l)	3.6	(3.0, 4.2)	4.0	(3.5, 4.5)	0.308	0.968
Triglycerides (mmol/l)	0.9	(0.7, 1.0)	1.1	(1.0, 1.2)	0.013	0.250
Adipokines						
Leptin (ng/ml)	14.6	(9.0, 20.2)	27.4	(23.1, 31.7)	0.001	0.490
Adiponectin (μg/ml)	10.4	(18.2, 20.6)	10.1	(8.4, 11.8)	0.830	0.375
Diet						
Energy (kcal)	1840.0	(1690, 1990)	1780.0	(1650, 1900)	0.523	0.583
Protein (E%)	17.7	(16.3, 18.8)	18.3	(17.3, 19.3)	0.324	0.545
Fat (E%)	34.5	(31.4, 36.0)	32.3	(30.1, 34.0)	0.283	0.564
Carbohydrates (E%)	47.8	(44.8, 49.5)	49.4	(46.4, 50.3)	0.414	0.868
Physical activity and fitness						
LTPA (hours/week)	4.5	(3.8, 5.2)	3.6	(3.0, 4.1)	0.049	0.466
VO_{2max} (ml/kg/min)	41.1	(36.8, 45.5)	35.1	(31.3, 38.9)	0.042	0.540

Table 1. General characteristics in the low and high HOMA-IR groups (MIXED model estimated marginal means with 95% confidence intervals are given taking into account genetic similarity and shared environment (daughter and mother) and contrast estimates' p-values were used to localize the significant differences between the two groups and group by generation interaction). FFM = fat-free mass; FM = fat mass; SAT = subcutaneous adipose tissue; VAT = visceral adipose tissue; IMCL = intra-myocellular lipids; EMCL = extra-myocellular lipids; HOMA-IR = homeostatic model assessment of insulin resistance; OGTT = oral glucose tolerance test; Matsuda index = insulin sensitivity index; FFA = free fatty acids; E% = percentage of total energy intake; LTPA = leisure-time physical activity; VO_{2max} = maximum oxygen uptake.

knowledge on the alterations in systemic, adipose tissue and skeletal muscle metabolism in relation to insulin resistance in normoglycemic individuals with varying degree of adiposity. Therefore, we aimed to investigate the systemic metabolite and gene expression profiles of subcutaneous adipose tissue and skeletal muscle in (fasting) normoglycemic women but differing in insulin resistance.

Results

Clinical characteristics of the low and high HOMA-IR groups. The high HOMA-IR group had higher body weight and BMI than the low HOMA-IR group ($p < 0.01$ for both, Table 1). High HOMA-IR subjects also had higher percent body fat, total and visceral fat mass ($p < 0.01$ for both). No significant difference in hepatic or intra-muscular fat was found. Fasting glucose concentrations were within the normoglycemic range in the majority of the subjects (92%). HOMA-IR indices were almost three times higher in individuals in the high HOMA-IR group than in those in the low HOMA-IR group. This difference was largely due to the significantly higher fasting insulin levels in the high HOMA-IR group ($p < 0.001$). Serum triglyceride and leptin concentrations were higher and physical activity and aerobic fitness was lower in the high HOMA-IR than low HOMA-IR group ($p < 0.05$ for all), but no difference in adiponectin was found. Dietary intake did not differ between the groups, nor was there a significant difference in plasma free fatty acids. No group by generation interaction was found on any of the variables.

	low HOMA-IR (n = 55)		high HOMA-IR (n = 55)		pBH	Group by generation
	Mean	95% CI	Mean	95% CI		
Low-molecular weight metabolites						
betahydroxybutyrate	0.049	(0.033, 0.066	0.061	(0.046, 0.077)	0.357	0.253
acetate	0.039	(0.036, 0.042)	0.043	(0.040, 0.046)	0.081	0.785
acetoacetate	0.038	(0.032, 0.044)	0.034	(0.029, 0.040)	0.961	0.793
alanine	0.374	(0.353, 0.394)	0.399	(0.380, 0.418)	0.261	0.305
citrate	0.098	(0.092, 0.104)	0.102	(0.096, 0.108)	0.406	0.923
creatinine	0.049	(0.046, 0.053)	0.051	(0.048, 0.055)	0.197	0.311
glutamine	0.511	(0.494, 0.529)	0.533	(0.517, 0.550)	0.685	0.174
glycerol	0.056	(0.047, 0.064)	0.074	(0.066, 0.082)	0.003	0.134
glycine	0.257	(0.239, 0.276)	0.276	(0.259, 0.292)	0.685	0.211
orosomucoid	1.225	(1.168, 1.281)	1.344	(1.291, 1.397)	0.045	0.443
histidine	0.050	(0.047, 0.053)	0.056	(0.053, 0.058)	0.362	0.329
isoleucine	0.038	(0.035, 0.041)	0.043	(0.040, 0.046)	0.040	0.959
leucine	0.061	(0.057, 0.064)	0.069	(0.066, 0.073)	0.005	0.813
valine	0.154	(0.144, 0.163)	0.174	(0.165, 0.183)	0.014	0.351
BCAAsum	0.253	(0.237, 0.268)	0.288	(0.273, 0.302)	0.009	0.666
phenylalanine	0.061	(0.058, 0.064)	0.066	(0.063, 0.069)	0.035	0.045
tyrosine	0.041	(0.037, 0.044)	0.047	(0.044, 0.050)	0.030	0.089
pyruvate	0.068	(0.061, 0.075)	0.077	(0.070, 0.083)	0.064	0.915
lactate	0.891	(0.799, 0.984)	0.967	(0.877, 1.057)	0.253	0.101
urea	0.052	(0.045, 0.058)	0.049	(0.043, 0.055)	0.731	0.131
Lipid extract constituents						
esterified cholesterol	2.691	(2.528, 2.855)	2.831	(2.675, 2.987)	0.147	0.422
free cholesterol	1.020	(0.952, 0.1087)	1.097	(1.032, 1.161)	0.127	0.386
omega3 fatty acids	0.361	(0.316, 0.406)	0.369	(0.326, 0.411)	0.699	0.551
omega6 fatty acids	2.789	(2.626, 2.952)	2.920	(2.824, 3.136)	0.328	0.683
omega7and 9 fatty acids	4.918	(4.510, 5.325)	5.422	(5.032, 5.812)	0.253	0.446
total fatty acids	8.068	(7.505, 8.630)	8.770	(8.233, 9.308)	0.259	0.699
linoleic acid	2.303	(2.157, 2.449)	2.501	(2.362, 2.641)	0.328	0.738
polyunsaturated fatty acids	1.647	(1.493, 1.801)	1.690	(1.543, 1.837)	0.792	0.747
docosahexanoic acid	0.143	(0.122, 0.164)	0.145	(0.125, 0.165)	0.716	0.872
monounsaturated fatty acids	2.183	(1.976, 2.389)	2.429	(2.232, 2.626)	0.253	0.370
total phosphoglycerides	0.620	(0.567, 0.673)	0.671	(0.620, 0.721)	0.685	0.763
phosphocholines	1.526	(1.413, 1.639)	1.622	(1.514, 1730)	0.685	0.700
sphingomyelines	0.202	(0.187, 0.216)	0.207	(0.193, 0.220)	0.685	0.520
omega3/total fatty acid ratio	4.453	(4.066, 4.840)	4.165	(3.795, 4.536)	0.458	0.173
omega6/total fatty acid ratio	34.87	(33.80, 35.95)	34.40	(33.36, 35.42)	0.676	0.206
omega7and9/total fatty acid ratio	60.67	(59.61, 61.73)	61.45	(60.43, 62.46)	0.458	0.076
fatty acid length	17.92	(17.84, 18.00)	17.94	(17.87, 18.01)	0.857	0.264

Table 2. Serum low-molecular weight metabolites and lipid extract constituents in the low and high HOMA-IR groups(MIXED model estimated marginal means with 95% confidence intervals are given taking into account genetic similarity and shared environment (daughter and mother) and contrast estimates' p-values were used to localize the significant differences between the two groups and group by generation interaction). P-values are adjusted for multiple comparisons using Benjamin-Hochberg correction. All metabolites are in mmol/l.

Serum metabolites. Serum metabolite profile analysis revealed many similarities but also significant differences between the high and low HOMA-IR groups (Table 2). There were no significant differences in the fatty acid and phospholipid (Table 2) and lipoprotein subclass concentrations (Supplementary Table S1) between the groups. However, concentrations of branched-chain amino acids (BCAA = isoleucine, leucine and valine), aromatic amino acids (AAA = phenylalanine and tyrosine), glycerol and orosomucoid were significantly higher in the high HOMA-IR group than the low HOMA-IR group ($p < 0.05$ for all). Only phenylalanine showed group by generation interaction. The differences between the groups remained significant after adjusting for age, total or visceral fat mass, physical activity and aerobic fitness.

To confirm that the circulating metabolite levels were not confounded by difference in adiposity, we investigated whether the differences in metabolites between the HOMA-IR groups were also consistently present

in normal weight individuals. We found that serum BCAAs and orosomucoid were significantly higher in the high HOMA-IR group than the low HOMA-IR group ($p < 0.05$ for all) (Supplementary Table S2). No difference between the groups in the other metabolites was found. Total serum BCAA correlated with fs insulin ($r = 0.388$), even after adjusting for age, percent fat mass and visceral adipose tissue. The concentration of plasma free fatty acids was not associated with fs insulin ($r = 0.108$), HOMA-IR ($r = 0.168$), fat mass ($r = 0.093$) or visceral adipose tissue ($r = 0.153$) ($p > 0.05$ for all).

Adipose tissue gene expression. To elucidate the metabolic pathways characterizing or contributing to insulin resistance, we studied global transcript profiles of adipose tissue and skeletal muscle. Microarray analysis revealed 1093 differentially expressed genes (688 up-regulated and 405 down-regulated) in the adipose tissue of the high HOMA-IR group (Supplementary Table S4). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the differentially expressed genes ($p < 0.05$) identified 9 down-regulated and 15 up-regulated pathways.

The up-regulated pathways in the adipose tissue revealed increased activation of immune response involving both innate and adaptive immune systems (Table 3). Lysosome was the most up-regulated pathway ($p = 5.6 \times 10^{-13}$), with contributions by genes involving all aspects of lysosome biogenesis and function, including structural genes (*LAMP1*, *LAMP2* and *LAPTM5*), several lysosomal acid hydrolases and transport proteins of lipids and cholesterol (*MCOLN1* and *NPC2*) as well as proteins required for lysosome acidification (*ATP6V0D2* and *ATP6AP1*). Several chemokines (e.g., *CCL2*, *CCL3*, *CCL4* and *CCL5*), which are produced by innate immune cells, and also by pre-adipocytes and mature adipocytes[14] were also present in the up-regulated pathways. Consistently, leukocyte trans-endothelial migration, Fc gamma R-mediated phagocytosis, toll-like receptor signaling, and the complement system, (complement *C1* [*C1S*, *C1QA*, *C1QB*, *C1QC*]) were up-regulated in the high HOMA-IR subjects. Up-regulation of the B cell receptor signaling pathway was also observed, providing further evidence for the involvement of the adaptive immune system and infiltration of inflammatory cells to the adipose tissue in insulin resistance.

The most down-regulated pathway in the adipose tissue was valine, leucine and isoleucine degradation ($p = 1.1 \times 10^{-7}$, Table 4). The genes that mapped to this pathway encode the cytosolic and mitochondrial components of the pathway and included both genes common to the degradation of all BCAAs, namely isoleucine, leucine and valine (*BCAT1*, *BCKDHB*) and those specific for the degradation of leucine (*MCC2*, *AUH*), isoleucine (*PCCA*, *PCCB*) and valine (*HIBADH*, *ALDH6A1*). Further, we found that systemic insulin resistance was also associated with significant down-regulation of the genes encoding proteins that play important roles in cellular energy homeostasis. These included a master regulator of mitochondrial biogenesis and function, peroxisome proliferator-activated receptor-gamma coactivator-1α (*PPARGC1A*) and mitochondrial acetyl-coenzyme A carboxylase beta (*ACACB*), which is an important regulator of fatty acid oxidation and synthesis. Further, peroxisome proliferator-activated receptor alpha (*PPARA*), which promotes the uptake, utilization, and catabolism of fatty acids, was also down-regulated in the high HOMA-IR group. Accordingly, down-regulation of the fatty acid degradation pathway and tricarboxylic acid cycle (TCA cycle) was observed. Other down-regulated pathways involved aromatic amino acid (phenylalanine and tryptophan) and short-chain fatty acid (propanoate) metabolism, and lysine biosynthesis.

To ensure our observations were not biased, we validated our results with two other independent experiments in obese insulin-resistant subjects by using gene expression omnibus (GEO) and the GSE26637[15] and GSE20950[16] and data sets, respectively. The up-regulated inflammation-related genes in the adipose tissue were similar in our study than in the study of Soronen *et al.*[15] and Hardy *et al.*[16] (Fig. 1). In addition, the inflammation and energy metabolism-related pathways in the adipose tissue were similarly up- and down-regulated than in obese insulin resistant women in the study of Soronen *et al.*[15].

We then assessed the associations between adipose tissue gene expressions and clinical traits in non-obese individuals. Characteristics of the study participants are presented in Supplementary Table S3. The mean centroid of the BCAA catabolism pathway in the adipose tissue was associated with insulin sensitivity (Matsuda index) and fs-insulin ($p < 0.05$ for all) (Fig. 2). These associations remained significant after adjusting for age and percent fat mass or visceral fat mass. The BCAA catabolism pathway correlated closely with mitochondrial respiration and biogenesis, i.e., with the TCA cycle and PPARGC1A ($p < 0.001$) (Fig. 3). Maximum oxygen uptake (VO_{2max}) correlated with the BCAA catabolism ($r = 0.543$) and the TCA cycle ($r = 0.522$) ($p < 0.05$ for all). Inflammation pathways were coordinately up-regulated with insulin resistance and adipokines, e.g., the chemokine signaling pathway correlated with insulin sensitivity (Matsuda index) ($r = -0.807$), fs-insulin ($r = 0.858$), fs-adiponectin ($r = -0.598$) and leptin ($r = 0.428$) ($p < 0.001$ for all). The chemokine signaling pathway displayed significant associations with the TCA cycle and BCAA catabolism ($r = -0.812$ and $r = -0.788$, respectively, $p < 0.001$ for both).

Skeletal muscle gene expression and signaling protein phosphorylation. Unexpectedly, when the transcriptomic data of skeletal muscle were studied, no differentially expressed genes were found between the low and high HOMA-IR samples. However, since skeletal muscle is the primary site of insulin-stimulated glucose disposal, we studied whether there were differences in the phosphorylation levels of several proteins related to glucose uptake, insulin signaling and mitochondrial energy metabolism. No differences in the phosphorylation levels of insulin receptor β or its downstream target Akt were found (Supplementary Figure S1). The level of phosphorylated AS160, which promotes translocation of glucose transporters to the cell membrane, was also similar between the groups. In addition, no differences in the expression of mitochondrial respiratory chain complex subunits, namely ATP5A, UQCRC2, MTCO1, SDHB and NDUFF88 between the groups were found (Supplementary Figure S1).

P-value	Count	Size	Pathway name	Gene Names
5.6×10^{-13}	36	121	Lysosome	*ACP5, AP1B1, ARSB, ATP6AP1, ATP6V0B, ATP6V0D2, CD68, CTSA, CTSB, CTSG, CTSH, CTSS, CTSZ, DNASE2B, FUCA1, GAA, GBA, GLA, GLB1, GM2A, GUSB, HEXB, LAMP1, LAPTM5, LGMN, MAN2B1, MCOLN1, NAGA, NPC2, PLA2G15, PPT1, PSAP, LAMP2, SLC11A2, SMPD1, TCIRG1*
2.2×10^{-5}	28	156	Phagosome	*ACTB, ACTG1, ATP6AP1, ATP6V0B, ATP6V0D2, ATP6V1B2, C1R, CD14, CLEC7A, CORO1A, CTSS, CYBA, FCGR2A, FCGR2B, FCGR3A, ITGB2, ITGB5, LAMP1, MARCO, MSR1, NCF2, NCF4, SEC61A1, TCIRG1, TUBA1C, TUBB2A, TUBB2B, VAMP3*
2.0×10^{-4}	32	189	Chemokine signaling pathway	*ADCY6, ADCY7, ADRBK2, ARRB2, CCL13, CCL18, CCL19, CCL2, CCL22, CCL3, CCL4, CCL5, CCR1, CXCL10, CXCL16, CXCR4, DOCK2, FGR, GNAI1, GNB4, GNG2, GRB2, HCK, PIK3R5, PREX1, PRKCB, PRKX, RAC2, STAT2, STAT3, TIAM1, VAV1*
7.4×10^{-4}	21	117	Leukocyte transendothelial migration	*ACTB, ACTG1, ACTN1, CXCR4, CYBA, EZR, F11R, GNAI1, ICAM1, ITGAL, ITGB2, MMP9, MSN, MYL9, NCF2, NCF4, PIK3R5, PRKCB, RAC2, THY1, VAV1*
2.5×10^{-3}	16	95	Fc gamma R-mediated phagocytosis	*ARF6, CFL2, DOCK2, FCGR2A, FCGR2B, FCGR3A, HCK, LAT, PIK3R5, PPAP2B, PRKCB, PTPRC, RAC2, SCIN, SYK, VAV1*
1.9×10^{-3}	17	102	Toll-like receptor signaling pathway	*CCL3, CCL4, CCL5, CD14, CD86, CXCL10, IRAK1, LBP, LY96, MAP2K3, MAPK10, PIK3R5, SPP1, TLR1, TLR5, TLR7, TLR8*
2.8×10^{-3}	10	65	Glycolysis/Gluconeogenesis	*ADH1B, ALDH2, ALDH3B1, ALDH7A1, ENO1, GALM, HK3, PDHB, PFKP, PGAM2*
$7.8.0 \times 10^{-3}$	5	17	Renin-angiotensin system	*AGTR1, ANPEP, CTSA, CTSG, NLN*
3.3×10^{-3}	6	17	Other glycan degradation	*FUCA1, FUCA2, GBA, GLB1, HEXB, MAN2B1*
1.3×10^{-2}	12	75	B cell receptor signaling pathway	*BLNK, DAPP1, FCGR2B, GRB2, NFKBIE, PIK3AP1, PIK3R5, PRKCB, PTPN6, RAC2, SYK, VAV1*
1.3×10^{-2}	5	19	Glycosaminoglycan degradation	*ARSB, GLB1, GUSB, HEXB, HPSE*
1.1×10^{-2}	9	48	Amino sugar and nucleotide sugar metabolism	*CHI3L1, CHIT1, GNE, GNPDA1, HEXB, HK3, NAGK, NPL, PMM1*
1.7×10^{-2}	11	69	Complement and coagulation cascades	*C1QA, C1QB, C1QC, C1R, C1S, C3AR1, C5AR1, F13A1, PLAU, PLAUR, SERPINE1*
3.5×10^{-2}	17	136	Natural killer cell mediated cytotoxicity	*BID, CD48, FCER1G, FCGR3A, GRB2, HCST, ICAM1, ITGAL, ITGB2, LAT, PIK3R5, PRKCB, PTPN6, RAC2, SYK, TYROBP, VAV1*
1.2×10^{-2}	25	200	Focal adhesion	*ACTB, ACTG1, ACTN1, BIRC3, CCND2, COL6A1, COL6A2, COL6A6, FLNA, GRB2, ITGB5, LAMB3, MAPK10, MYL9, PDGFA, PIK3R5, PPP1CA, PRKCB, PTEN, RAC2, SPP1, TNC, VAV1, VEGFA, ZYX*

Table 3. Up-regulated pathways in the adipose tissue of high HOMA-IR group. Count = Amount of differentially expressed genes that map in pathway. Size = Total amount of genes involved in pathway.

Discussion

In this study with young and middle-aged normoglycemic women, we found that insulin resistance was associated with increased serum BCAA levels, independent of obesity. Consistent with this, we found a significant down-regulation of genes related to BCAA catabolism and mitochondrial energy metabolism, concurrently with increased expression of inflammation-related genes in the adipose tissue.

Plasma free fatty acids are commonly elevated in obese individuals due to increased adipose tissue lipolysis[17]. Elevated circulating free fatty acids may accumulate in other insulin-responsive tissues, such as skeletal muscle and liver (where they interfere with insulin signaling and cause insulin resistance)[18]. However, we found that in normoglycemic women with varying degree of adiposity, insulin resistance was not associated with increased plasma free fatty acids, but instead with increased serum amino acid concentrations. Our observation partly agrees with a recent study in normal weight subjects discordant for insulin sensitivity[19], and a meta-analysis by Karpe *et al.*[20]. It is tempting, therefore, to suggest that the contribution of circulating free fatty acids to insulin resistance may be relatively small.

Increased serum BCAA concentrations have been associated with obesity-related insulin resistance in earlier studies[21–23]. Our results suggest that perturbations in systemic BCAA homeostasis are related to insulin resistance rather than to obesity *per se,* since significant difference in these amino acids was observed between the high and low HOMA-IR groups also in normal weight individuals (Table S2). The average difference in total BCAA between low and high HOMA-IR groups was ~14% in whole study population and ~10% in normal weight individuals. Whether such difference is physiologically meaningful is not clear. However, Sunny *et al.*[24] recently demonstrated that insulin-stimulated increases (10–20%) in plasma BCAA correlated significantly with insulin resistance indices in humans. They concluded that such small but chronic increase in circulating BCAA with insulin resistance may be sufficient to disrupt signaling events in the mitochondria of the muscle and liver thereby contributing to mitochondrial dysfunction. However, our study cannot show temporal relationships, although our results are compatible with previous studies[25,26], which have suggested that BCAAs associate with insulin resistance.

It is unclear why serum BCAA is elevated in obesity and insulin resistance. Differences in diet[23], protein turnover (muscle loss)[27] or liver fat (fatty liver disease) can affect circulating levels of amino acids. However, in our study no difference in diet (protein intake), fat-free mass or liver fat content was observed between the low and high HOMA-IR groups. The positive correlation of BCAA with insulin concentration in our study is line

P-value	Count	Size	Pathway name	Genes
1.1×10^{-7}	17	44	Valine, leucine and isoleucine catabolism	*ACADM, ACADSB, ALDH2, ALDH6A1, ALDH7A1, AUH, BCAT1, BCKDHB, DLD, HADH, HIBADH, IL4I1, MCCC2, MUT, OXCT1, PCCA, PCCB*
3.7×10^{-4}	10	32	Proprionate metabolism	*ACACB, ACADM, ACSS3, ALDH2, ALDH6A1, ALDH7A1, MUT, PCCA, PCCB, SUCLG2*
2.9×10^{-3}	6	17	Phenylalanine metabolism	*ALDH3B1, AOC2, IL4I1, MAOB, MIF, PRDX6*
4.4×10^{-3}	10	43	Fatty acid degradation	*ACADL, ACADM, ACADSB, ADH1B, ADH1C, ALDH2, ALDH7A1, CPT1A, HADH, PECI*
1.2×10^{-2}	9	42	Tryptophan metabolism	*ALDH2, ALDH7A1, CAT, CYP1B1, HADH, IL4I1, KMO, KYNU, MAOB*
1.6×10^{-2}	7	30	Citrate cycle (TCA cycle)	*DLD, DLST, IDH3B, PC, PDHB, SDHB, SUCLG2*
2.9×10^{-2}	8	41	Tyrosine metabolism	*ADH1B, ADH1C, ALDH3B1, AOC2, COMT, IL4I1, MAOB, MIF*
1.7×10^{-2}	2	3	Lysine biosynthesis	*AASS, ALDH7A1*
4.7×10^{-2}	4	18	Glyoxylate and dicarboxylate metabolism	*HYI, MUT, PCCA, PCCB*
1.9×10^{-2}	12	68	Adipocytokine signaling pathway	*ACACB, ADIPOQ, ADIPOR1, CPT1A, MAPK10, NFKBIE, PPARA, PPARGC1A, PRKAG1, SLC2A4, STAT3, TNFRSF1B*

Table 4. Down-regulated pathways in the adipose tissue of high HOMA-IR group. Count = Amount of differentially expressed genes that map in pathway. Size = Total amount of genes involved in pathway.

with earlier reports, which have suggested that these amino acids may stimulate insulin secretion from the pancreas[21,28,29]. On the other hand, elevated insulin may increase circulating BCAA, possibly by attenuating BCAA catabolism in different tissues as suggested by Sunny *et al.*[24]. Indeed, obesity-related increases in circulating BCAAs have been associated with decreased BCAA catabolism in adipose tissue[30]. Here, we showed significant down-regulation of the BCAA degradation pathway genes in the adipose tissue of normoglycemic subjects with high insulin resistance. The fact that there was no difference in average BMI or percent body fat between the low and high HOMA-IR groups suggests that down-regulation of the BCAA catabolism pathway was not attributable to adiposity alone. Thus, the decrease in the BCAA catabolism can probably be ascribed to reduced mitochondrial respiration and biogenesis (as indicated by the close correlation of the BCAA catabolism with the TCA cycle and PPARGC1A genes). Since physical activity and aerobic fitness are known to improve insulin sensitivity and energy metabolism[31], it is possible that differences in physical activity and aerobic fitness may have amplified the observed differences in gene expression between the low and high HOMA-IR groups. The close correlation between VO_{2max} and BCAA catabolism and the TCA cycle further support this notion.

Growing evidence indicates that obesity-associated low-grade inflammation of adipose tissue contributes to the development of insulin resistance[32]. Our results complement this notion by showing that up-regulated inflammation-related genes were closely associated with insulin resistance, serum adiponectin and leptin also in normoglycemic individuals, even after adjusting for measures of adiposity. Earlier studies have demonstrated that plasma adiponectin and leptin levels are associated with insulin resistance independent of fat mass[33–35], and they may play a key role in the regulation of inflammation and immunity[36]. In line with earlier studies[37,38], we found that the innate inflammatory component related to macrophages was coordinately up-regulated with insulin resistance. The physiological role of macrophages is probably to clear adipose debris through the process of phagocytosis and activate the adaptive immune system[39]. Accordingly, we found that in terms of over-expressed genes the most up-regulated pathways in the adipose tissue were lysosome and phagosome pathways. These findings, (together with activation and infiltration of lymphocytes, a toll-like receptor signaling pathway, and the complement system) suggest a state of chronic low-grade inflammation in the adipose tissue. Furthermore, the chronic inflammation may also in part explain the observed impairments in adipose tissue energy metabolism (as indicated by the close inverse correlation of chemokine signaling genes with BCAA catabolism and the TCA cycle genes).

Impaired insulin-mediated skeletal muscle glucose uptake[40] and intramyocellular lipid concentrations[41] are major contributors to insulin resistance and type 2 diabetes. In our study, intramuscular triglycerides were not increased in subjects with high HOMA-IR. Consistent with this, no aberrant gene expression in individuals with high HOMA-IR was found. To confirm these findings, we further studied whether there were differences in the phosphorylation levels of several signaling proteins related to glucose metabolism. No significant differences in the phosphorylation levels of insulin receptor β and its downstream target Akt, were found, nor was there any significant difference in the level of phosphorylated AS160, which promotes translocation of glucose transporters to the cell membrane. Previous studies have reported reduced muscle transcript levels related to oxidative metabolism in diabetic individuals compared to healthy controls[42]. We found no difference in mitochondrial respiratory chain complex subunits between the low and high HOMA-IR groups. These findings suggest that in the fasting state glucose and mitochondrial energy metabolism is not significantly altered in the skeletal muscle in early stages of insulin resistance. However, since both acute hyperinsulinemia[43] and hyperglycemia[44] have been shown to induce transcriptional and translational regulation of glucose and energy metabolism in the skeletal muscle, it may be that significant differences could exist during hyperinsulinemic-euglycemic clamp, glucose challenge or mixed meal feeding.

This study has some limitations. First, the number of participants in our study was relatively small and consisted solely of women. Despite this we were able to identify statistically significant differences in serum

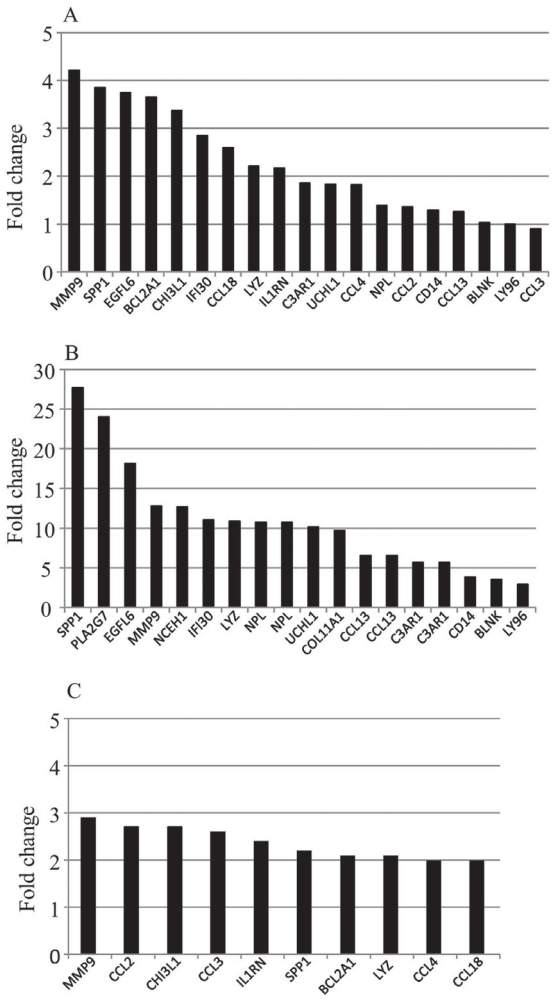


Figure 1. Up-regulated inflammation-related genes in the adipose tissue in insulin resistant compared to insulin sensitive group. The y-axis shows the fold change of gene expression of up-regulated genes in high HOMA-IR subjects of the present study (**A**) and obese insulin resistant subjects in the study of Soronen *et al.* (**B**)[15] and Hardy *et al.*[16] (**C**).

metabolites and gene expressions in adipose tissue between the groups. In addition, the participants were carefully selected in order to minimize confounding factors and genetic variability. Furthermore, gene expression in the adipose tissue of participants with high HOMA-IR was validated by two independent experiments with non-obese type 2 diabetic and obese insulin-resistant subjects. Thus, we believe that our results are not biased and that the gene expression data can be viewed with confidence. Finally, the majority of the participants were within the normal fasting glycemic range and exclusion of those with impaired fasting glucose did not change the results. This gave us the possibility to identify the biomarkers associated with systemic insulin resistance in its early stage.

Our data demonstrate that serum fatty acids, intra-myocellular lipids and liver fat content were not elevated in normoglycemic women with high HOMA-IR. Instead, we show that impaired insulin sensitivity was associated with a significant increase in serum BCAA concentration, up-regulation of inflammation-related genes and down-regulation of genes related to BCAA catabolism and mitochondrial energy metabolism in adipose tissue. These findings suggest that adipose tissue inflammation and mitochondrial dysfunction may be early events in the development of systemic insulin resistance. Further studies are needed to determine the initial factor(s) that trigger the transcriptional changes that lead to these metabolic alterations.

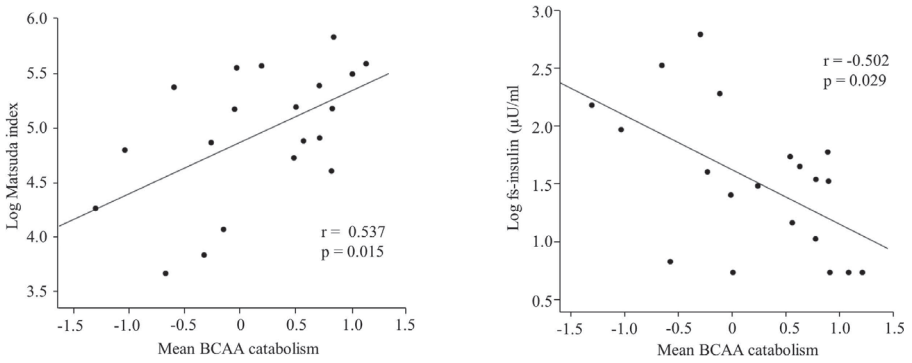


Figure 2. Correlations between the mean centroids of the BCAA catabolism pathway and fs-insulin and insulin sensitivity (Matsuda index). The values of fs-insulin and the Matsuda index were transformed to normal distribution by natural logarithms. Each dot represents an individual and the line is a linear regression fit line.

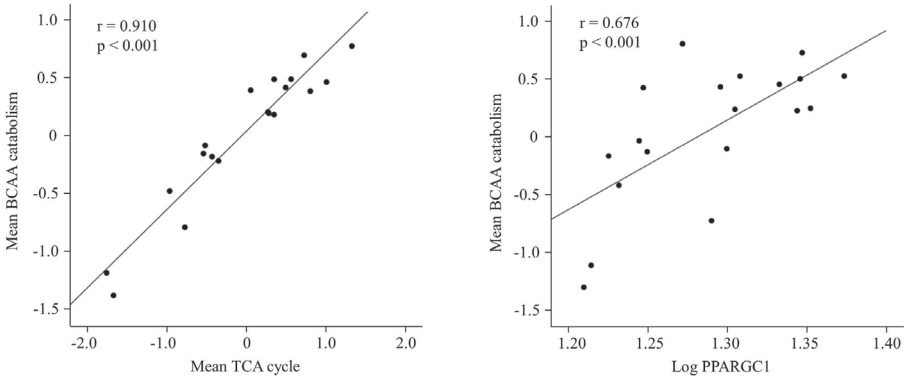


Figure 3. Correlation between the mean centroids of the BCAA catabolism pathway and the TCA cycle and PPARGC1A. Each dot represents an individual and the line is a linear regression fit line.

Materials and Methods

Study subjects. This article is part of a large family study with 282 participating families and has been described elsewhere[45]. A subgroup of families (n = 74), comprising 222 individuals (daughter, mother and father) with no type I/II diabetes or family history (first degree relative) of diabetes, cardiac diseases, autoimmune diseases or major liver (cancer, hepatitis) diseases were contacted by letter for an additional study aimed at identifying biomarkers associated with insulin resistance and liver fat accumulation. A total of 184 individuals responded to our invitation, of whom 163 (53 fathers, 53 mothers and 57 daughters) attended the laboratory tests. For this report, all the fathers were excluded in order to reduce the variability in genetic architecture, leaving only the mothers and daughters (mothers = 53 and daughters = 57). Mothers and daughters did not differ in measures of adiposity, insulin resistance, serum triglycerides, fatty acids or amino acids. Therefore, all data including the metabolome and microarray data were pooled before the phenotypic analysis and all results were adjusted for age and familiarity. Further, to minimize the metabolic alterations occurring at different stages of the menstrual cycle, blood samples were collected from the women with regular menses between 2 and 5 days after (menstruation). Twenty-two participants were in early post menopause but none were on hormonal replacement therapy. Including or excluding these participants did not influence the results. All subjects were clinically euthyreotic. The study protocol was approved by the ethics committee of the Central Finland Health Care District. A written informed consent was obtained from all participants, and all experiments were performed in accordance with relevant guidelines and regulations.

Methods. A detailed description of the background information and methods are provided in supplementary text S1. In short, background information including medical history, current health status and physical activity was collected via self-administered questionnaires. Food consumption and intakes of total energy and energy-yielding nutrients were assessed from three-day food records. All measurements were performed in the morning after overnight fasting. Venous blood samples were obtained for the analyses of glucose, insulin,

non-esterified fatty acids, leptin, and adiponectin. The HOMA-IR index (homeostatic model assessment of insulin resistance) was calculated as (fasting glucose x fasting insulin/22.5). According their HOMA-IR values (median = 1.57), the subjects were divided into low (n = 55) and high (n = 55) groups. Body composition was assessed by DXA, subcutaneous and intra-abdominal adipose tissue by MRI[46], and ectopic fat of liver, muscle intra-myocellular lipid (IMCL) and extra-myocellular lipid (EMCL) by ^{1}HMRS[47]. Serum metabolites were assessed by NMR spectroscopy[48]. Maximum oxygen uptake (VO_{2max}, ml/kg/min) was assessed by a bicycle ergometer test. In addition, superficial abdominal subcutaneous adipose tissue and skeletal muscle (vastus lateralis) biopsies were obtained from 24 individuals to assess the differences in global gene expression profiles and muscle protein expression between the low and high HOMA-IR groups. Furthermore, a 75-g oral glucose tolerance test (OGTT) was performed for subjects with tissue biopsies to assess whole body insulin sensitivity[49]. Microarray measurements were analyzed by using the Robust Multiarray Averaging (RMA) algorithm in the Bioconductor R package affy[50–52]. The Limma R package was used for differentially expressed genes (DEGs). Raw p values were adjusted to control for the false discovery rate (FDR) using the method of Benjamini and Hochberg[53] (for more detailed information on microarray and gene enrichment analysis see supplementary text S1).

Statistical methods. Before each analysis, continuous data were checked for normality by Shapiro-Wilk's test using PASW statistics version 21 (IBM Corporation, USA). If data were not normally distributed, their natural logarithms were used. Clinical characteristics and serum metabolites were compared using an independent-samples t-test. Since the data were from a family study, the familiarity (genetic and environmental (household) similarity) was controlled by using linear mixed model to compare levels of the outcome variables between the low and high HOMA-IR groups. Contrast tests were used in mixed models to assess the effect of generation while controlling for dependency among family members with random effects. P-values were adjusted to control for the false discovery rate (FDR) using the method of Benjamini and Hochberg when comparing metabolites between the low and high HOMA-IR groups[53]. Pearson correlation analysis was used to determine the relationship between clinical characteristics, serum metabolites and adipose tissue gene expression. Statistical significance was set at $p < 0.05$.

References

1. DeFronzo, R. A. & Tripathy, D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes care* **32** Suppl 2, S157–163, doi: 10.2337/dc09-S302 (2009).
2. Muoio, D. M. & Newgard, C. B. Mechanisms of disease:Molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nature reviews. Molecular cell biology* **9,** 193–205, doi: 10.1038/nrm2327 (2008).
3. Abbasi, F., Brown, B. W., Jr., Lamendola, C., McLaughlin, T. & Reaven, G. M. Relationship between obesity, insulin resistance, and coronary heart disease risk. *Journal of the American College of Cardiology* **40,** 937–943 (2002).
4. Lee, J. *et al.* Should central obesity be an optional or essential component of the metabolic syndrome? Ischemic heart disease risk in the Singapore Cardiovascular Cohort Study. *Diabetes care* **30,** 343–347, doi: 10.2337/dc06-1866 (2007).
5. Hollenbeck, C. & Reaven, G. M. Variations in insulin-stimulated glucose uptake in healthy individuals with normal glucose tolerance. *The Journal of clinical endocrinology and metabolism* **64,** 1169–1173, doi: 10.1210/jcem-64-6-1169 (1987).
6. Karelis, A. D., St-Pierre, D. H., Conus, F., Rabasa-Lhoret, R. & Poehlman, E. T. Metabolic and body composition factors in subgroups of obesity: what do we know? *The Journal of clinical endocrinology and metabolism* **89,** 2569–2575, doi: 10.1210/jc.2004-0165 (2004).
7. Ferrannini, E. *et al.* Insulin resistance and hypersecretion in obesity. European Group for the Study of Insulin Resistance (EGIR). *The Journal of clinical investigation* **100,** 1166–1173, doi: 10.1172/JCI119628 (1997).
8. Boden, G. Obesity, insulin resistance and free fatty acids. *Current opinion in endocrinology, diabetes, and obesity* **18,** 139–143, doi: 10.1097/MED.0b013e3283444b09 (2011).
9. Lynch, C. J. & Adams, S. H. Branched-chain amino acids in metabolic signalling and insulin resistance. *Nature reviews. Endocrinology* **10,** 723–736, doi: 10.1038/nrendo.2014.171 (2014).
10. Wang, T. J. *et al.* Metabolite profiles and the risk of developing diabetes. *Nature medicine* **17,** 448–453, doi: 10.1038/nm.2307 (2011).
11. Wiklund, P. K. *et al.* Serum metabolic profiles in overweight and obese women with and without metabolic syndrome. *Diabetology & metabolic syndrome* **6,** 40, doi: 10.1186/1758-5996-6-40 (2014).
12. Guilherme, A., Virbasius, J. V., Puri, V. & Czech, M. P. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nature reviews. Molecular cell biology* **9,** 367–377, doi: 10.1038/nrm2391 (2008).
13. Kahn, S. E., Hull, R. L. & Utzschneider, K. M. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* **444,** 840–846, doi: 10.1038/nature05482 (2006).
14. Kabir, S. M., Lee, E. S. & Son, D. S. Chemokine network during adipogenesis in 3T3-L1 cells: Differential response between growth and proinflammatory factor in preadipocytes vs. adipocytes. *Adipocyte* **3,** 97–106, doi: 10.4161/adip.28110 (2014).
15. Soronen, J. *et al.* Adipose tissue gene expression analysis reveals changes in inflammatory, mitochondrial respiratory and lipid metabolic pathways in obese insulin-resistant subjects. *BMC medical genomics* **5,** 9, doi: 10.1186/1755-8794-5-9 (2012).
16. Hardy, O. T. *et al.* Body mass index-independent inflammation in omental adipose tissue associated with insulin resistance in morbid obesity. *Surgery for obesity and related diseases: official journal of the American Society for Bariatric Surgery* **7,** 60–67, doi: 10.1016/j.soard.2010.05.013 (2011).
17. Boden, G. & Shulman, G. I. Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction. *European journal of clinical investigation* **32** Suppl 3, 14–23 (2002).
18. Shulman, G. I. Ectopic fat in insulin resistance, dyslipidemia, and cardiometabolic disease. *The New England journal of medicine* **371,** 1131–1141, doi: 10.1056/NEJMra1011035 (2014).
19. Arner, P. & Ryden, M. Fatty Acids, Obesity and Insulin Resistance. *Obesity facts* **8,** 147–155, doi: 10.1159/000381224 (2015).
20. Karpe, F., Dickmann, J. R. & Frayn, K. N. Fatty acids, obesity, and insulin resistance: time for a reevaluation. *Diabetes* **60,** 2441–2449, doi: 10.2337/db11-0425 (2011).
21. Felig, P., Marliss, E. & Cahill, G. F., Jr. Plasma amino acid levels and insulin secretion in obesity. *The New England journal of medicine* **281,** 811–816, doi: 10.1056/NEJM196910092811503 (1969).
22. McCormack, S. E. *et al.* Circulating branched-chain amino acid concentrations are associated with obesity and future insulin resistance in children and adolescents. *Pediatric obesity* **8,** 52–61, doi: 10.1111/j.2047-6310.2012.00087.x (2013).
23. Newgard, C. B. *et al.* A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell metabolism* **9,** 311–326, doi: 10.1016/j.cmet.2009.02.002 (2009).
24. Sunny, N. E. *et al.* Cross-talk between branched-chain amino acids and hepatic mitochondria is compromised in nonalcoholic fatty liver disease. *American journal of physiology. Endocrinology and metabolism* **309,** E311–319, doi: 10.1152/ajpendo.00161.2015 (2015).

25. Krebs, M. *et al.* Mechanism of amino acid-induced skeletal muscle insulin resistance in humans. *Diabetes* **51,** 599–605 (2002).
26. Tremblay, F. *et al.* Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America* **104,** 14056–14061, doi: 10.1073/pnas.0706517104 (2007).
27. Gougeon, R. *et al.* Determinants of whole-body protein metabolism in subjects with and without type 2 diabetes. *Diabetes care* **31,** 128–133, doi: 10.2337/dc07-1268 (2008).
28. Bolea, S., Pertusa, J. A., Martin, F., Sanchez-Andres, J. V. & Soria, B. Regulation of pancreatic beta-cell electrical activity and insulin release by physiological amino acid concentrations. *Pflugers Archiv: European journal of physiology* **433,** 699–704 (1997).
29. Yang, J. *et al.* Leucine stimulates insulin secretion via down-regulation of surface expression of adrenergic alpha2A receptor through the mTOR (mammalian target of rapamycin) pathway: implication in new-onset diabetes in renal transplantation. *The Journal of biological chemistry* **287,** 24795–24806, doi: 10.1074/jbc.M112.344259 (2012).
30. She, P. *et al.* Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. *American journal of physiology. Endocrinology and metabolism* **293,** E1552–1563, doi: 10.1152/ajpendo.00134.2007 (2007).
31. Leskinen, T. *et al.* Differences in muscle and adipose tissue gene expression and cardio-metabolic risk factors in the members of physical activity discordant twin pairs. *PloS one* **5,** doi: 10.1371/journal.pone.0012609 (2010).
32. Masoodi, M., Kuda, O., Rossmeisl, M., Flachs, P. & Kopecky, J. Lipid signaling in adipose tissue: Connecting inflammation & metabolism. *Biochimica et biophysica acta* **1851,** 503–518, doi: 10.1016/j.bbalip.2014.09.023 (2015).
33. Kern, P. A., Di Gregorio, G. B., Lu, T., Rassouli, N. & Ranganathan, G. Adiponectin expression from human adipose tissue: relation to obesity, insulin resistance, and tumor necrosis factor-alpha expression. *Diabetes* **52,** 1779–1785 (2003).
34. Zimmet, P. Z. *et al.* Is there a relationship between leptin and insulin sensitivity independent of obesity? A population-based study in the Indian Ocean nation of Mauritius. Mauritius NCD Study Group. *International journal of obesity and related metabolic disorders: journal of the International Association for the Study of Obesity* **22,** 171–177 (1998).
35. Segal, K. R., Landt, M. & Klein, S. Relationship between insulin sensitivity and plasma leptin concentration in lean and obese men. *Diabetes* **45,** 988–991 (1996).
36. Tilg, H. & Moschen, A. R. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nature reviews. Immunology* **6,** 772–783, doi: 10.1038/nri1937 (2006).
37. Pietilainen, K. H. *et al.* Global transcript profiles of fat in monozygotic twins discordant for BMI: pathways behind acquired obesity. *PLos medicine* **5,** e51, doi: 10.1371/journal.pmed.0050051 (2008).
38. Weisberg, S. P. *et al.* Obesity is associated with macrophage accumulation in adipose tissue. *The Journal of clinical investigation* **112,** 1796–1808, doi: 10.1172/JCI19246 (2003).
39. Cinti, S. *et al.* Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *Journal of lipid research* **46,** 2347–2355, doi: 10.1194/jlr.M500294-JLR200 (2005).
40. Cline, G. W. *et al.* Impaired glucose transport as a cause of decreased insulin-stimulated muscle glycogen synthesis in type 2 diabetes. *The New England journal of medicine* **341,** 240–246, doi: 10.1056/NEJM199907223410404 (1999).
41. Krssak, M. *et al.* Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. *Diabetologia* **42,** 113–116, doi: 10.1007/s001250051123 (1999).
42. Patti, M. E. *et al.* Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. *Proceedings of the National Academy of Sciences of the United States of America* **100,** 8466–8471, doi: 10.1073/pnas.1032913100 (2003).
43. Rome, S. *et al.* Microarray profiling of human skeletal muscle reveals that insulin regulates approximately 800 genes during a hyperinsulinemic clamp. *The Journal of biological chemistry* **278,** 18063–18068, doi: 10.1074/jbc.M300293200 (2003).
44. Meugnier, E. *et al.* Acute hyperglycemia induces a global downregulation of gene expression in adipose tissue and skeletal muscle of healthy subjects. *Diabetes* **56,** 992–999, doi: 10.2337/db06-1242 (2007).
45. Wang, Q. *et al.* Is bone loss the reversal of bone accrual? Evidence from a cross-sectional study in daughter-mother-grandmother trios. *Journal of bone and mineral research: the official journal of the American Society for Bone and Mineral Research* **26,** 934–940, doi: 10.1002/jbmr.291 (2011).
46. Cheng, S. *et al.* Trait-specific tracking and determinants of body composition: a 7-year follow-up study of pubertal growth in girls. *BMC medicine* **7,** 5, doi: 10.1186/1741-7015-7-5 (2009).
47. Borra, R. J. *et al.* Nonalcoholic fatty liver disease: rapid evaluation of liver fat content with in-phase and out-of-phase MR imaging. *Radiology* **250,** 130–136, doi: 10.1148/radiol.2501071934 (2009).
48. Soininen, P. *et al.* High-throughput serum NMR metabonomics for cost-effective holistic studies on systemic metabolism. *The Analyst* **134,** 1781–1785, doi: 10.1039/b910205a (2009).
49. Matsuda, M. & DeFronzo, R. A. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes care* **22,** 1462–1470 (1999).
50. Bolstad, B. M., Irizarry, R. A., Astrand, M. & Speed, T. P. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **19,** 185–193 (2003).
51. Gentleman, R. C. *et al.* Bioconductor: open software development for computational biology and bioinformatics. *Genome biology* **5,** R80, doi: 10.1186/gb-2004-5-10-r80 (2004).
52. Gautier, L., Cope, L., Bolstad, B. M. & Irizarry, R. A. affy–analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* **20,** 307–315, doi: 10.1093/bioinformatics/btg405 (2004).
53. Benjamini Y, H. Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B* **57,** 289–300 (1995).

Acknowledgements

We thank Ms. Shu Mei Cheng, Arja Lyytikainen, Eveliina Munukka, and other researchers for their technical support and help with the data collection, Riitta Lahesmaa and Omid Rasool for help with the microarray analysis, and Juha Hulmi for his expertise in western blot analysis. The study was supported by the Academy of Finland SKID-KIDS program (No. 135038) and an Academy of Finland post-doc grant (No. 267719), the Shanghai Jiao Tong University Zhiyuan Foundation (CP2014013) and the China State Sport General Administration (2013B040), and EVO research grants 2012/2013 from Oulu University Hospital.

Author Contributions

The authors' contributions are as follows: P.W. participated in the data collection, analyses and interpretation, and drafted the manuscript. X.Z., participated in data analysis, interpretation of data and writing the manuscript. S.P., R.A., L.K. and Y.Y. participated in the data collection, analyses and interpretation, and editing of the manuscript. S.K.-K. participated in the data analyses and interpretation, and editing of the manuscript. M.A. served as the study physician and participated in the planning of the study, data collection, analyses and interpretation, editing of the manuscript, and doctoral student supervision. S.C. was the principal investigator (PI). She designed the

study and oversaw the implementation of the project, trained the researchers, supervised the doctoral students, and participated in the data collection, analyses and interpretation, and editing of the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Wiklund, P. *et al.* Insulin resistance is associated with altered amino acid metabolism and adipose tissue dysfunction in normoglycemic women. *Sci. Rep.* **6**, 24540; doi: 10.1038/srep24540 (2016).

IV

NORMAL WEIGHT OBESITY AND CARDIO-METABOLIC RISK IN FEMALES A 7-YEAR LONGITUDINAL STUDY FROM PRE-PUBERTY TO ADULTHOOD

by

Wiklund P, Törmäkangas T, Shi Y, Wu N, Vainionpää A , Alen M, and Cheng S

(submitted for publication)

Normal weight obesity and cardio-metabolic risk: A 7 year longitudinal study in girls from pre-puberty to early adulthood

Petri Wiklund[1,2], Timo Törmäkangas[2,1], Yi Shi[3], Na Wu[1,], Aki Vainionpää[4] Markku Alen[4,5], and Sulin Cheng[1,2]

[1]Exercise, Health and Technology Center, Shanghai Jiao Tong University, Shanghai, China

[2]Department of Health Sciences, University of Jyväskylä, Jyväskylä, Finland

[3] Shanghai Center for Systems Biomedicine, Shanghai Jiao Tong University, Shanghai, China

[4]Department of Medical Rehabilitation, Oulu University Hospital, Oulu, Finland

[5]Center for Life Course Health Research, University of Oulu, Oulu, Finland

Short title: normal weight obesity

Key terms: normal weight obesity, cardiometabolic risk,longitudinal study, females

Corresponding author and requests for reprints to: Prof. Sulin Cheng, Exercise, Health and Technology Center, Department of Sport and Physical Education, Shanghai Jiao Tong University, Shanghai, China and Department of Health Sciences, FIN-40014 University of Jyväskylä, Finland. Tel: +358 14 2602091 Fax: +358 14 2602011

E-mail: shulin.cheng@jyu.fi

Disclosure statement: The authors have nothing to disclose.

Abstract

Background

Normal weight obesity, defined as the combination of normal body mass index (BMI) and high body fat content appears to be highly prevalent in children and adolescents, but the long-term consequences for health are still unclear.

Methods

This was an 8 year longitudinal study of 236 girls followed from pre-puberty to early adulthood. Body composition was assessed by DXA and cardiometabolic risk by calculating continuous clustered risk score using abdominal fat mass, glucose, triacylglycerols, HDL-cholesterol and blood pressure. Growth chart was obtained from birth to 18 years. Subjects were categorized based on body weight status at the age 18 as overweight or obese (BMI >25 with fat% >30), Normal Weight Obese (BMI; 18.5-24.9 with fat% >30) and Normal Weight lean (BMI; 18.5-24.9 with fat% <30). The association of body composition and cardiometabolic risk was examined retrospectively for these groups.

Results

Continuous cardio-metabolic risk was higher already at the age of 11 years in subjects who were overweight or obese at the age of 18 compared with normal weight subjects ($p<0.001$ for all). The cardio-metabolic risk score was also higher in the normal weight obese subjets compared with their normal weight lean peers in childhood and this difference peristed into early adulthood ($p<0.001$ for all).

Conclusion

children and adolescent with normal BMI and elevated body fat percentage may be at increased risk for cardiometabolic morbidity in adulthood. Screening for adiposity in children and adolescent with a normal BMI could better identify those at higher risk for cardiometabolic morbidities.

Introduction

Obesity in childhood is associated with increased risk for coronary heart disease later in life[1]. Timely recognition of excess adiposity in childhood is therefore highly important for prevention of the adverse health consequences. Clinical guidelines for diagnosis of childhood overweight and obesity recommend measuring height and weight, calculate the body mass index (BMI) and determine the weight status of children with based on national reference data[2].

Although BMI has many advantages as a surrogate of body fatness such as simplicity and reproducibility[3], its diagnostic performance is not optimal to identify excessive adiposity because it cannot distinguish between fat mass and fat-free mass[4], both of which contribute to BMI. Indeed, recent studies suggest that over a quarter of children and adolescent with high percent body fat may be misclassified as normal weight when using only BMI to diagnose obesity[5]. However, whether children with normal body weight but high percent body fat are at increased risk for cardio-metabolic disease in adulthood is not known. The purpose of this study was to assess the relationship between adiposity and cardio-metabolic risk among peripubertal girls with different body weight status using a longitudinal study design from childhood to early adulthood.

Methods

Study population

A total of 396 girls (mean age 11.2 years at baseline) participated in a longitudinal study for an average of 7.5 years. Detailed information regarding the participants and study design has been reported previously[6-8]. Briefly, the subjects were first contacted via class teachers teaching grades 4 to 6 (age 9 to 13 years old) in 61 schools in the city of Jyväskylä and its surroundings in Central Finland (96% of all the schools in these areas). Of the eligible samples, a subgroup (n=236) had total body composition assessments and serum metabolomics analysis at the age of 18 years and were included in this report. The participants provided their written consent in accordance with the guidelines laid down by the ethical committees.

Growth chart and define relative weight groups

Growth charts of each participant were obtained from Finnish School Health Care System from birth to 17-20 years of age (10-41 tests per subjects) in 61 schools from the city of Jyväskylä and its surroundings in Central Finland (96% of the total schools in these areas). To be able to compare the growth at the certain time points, the Weight % and height z-score was extrapolated from the growth chart using the form which was created by the Finnish Paediatric Research Association and accepted by Finnish National Health Administration (Form No 7466:92). On the basis of their growth chart data, participants were classified into under weight (relative weight to height from growth chart under -10%), normal weight (relative weight between -10 to +20%)，and overweight+obese (relative weight between > +20%).

In addition, on the basis of their body composition assessment by dual X-ray densitometry (DXA) of fat mass % (above or below 30% as obese or normal weight)[9] and combined with growth chart defination, the subjects are classified into 3 groups: 1) underweight (UW, relative weight to height from growth chart under -10% and fat <30% ,n=22), 2) normal weight (NW, relative weight between -10 to +20% and fat % <30%, n=87), 3) normal weight obese (NWO, relative weight between -10 to +20% and fat % >30%, n=92), and over weight+obese (OWOB, relative weight between > +20% and fat % >30%, n=35). The study protocol was approved by the ethical committee of the University of Jyvaskyla and the Central Hospital of Central Finland (memo 22/8/2008).

Background information assessment

Medical history were collected via validated self-administered questionnaire. Subjects under 15 years of age filled in the questionnaire with their guardians' assistance, and all the questionnaires were checked by a study nurse. Body weight was measured using an electronic scale and height using stadiometer with subjects wearing light clothes and without shoes. Body mass index was calculated by dividing body weight in kilograms by the square of the body height in meters. Blood pressure (BP) in the right arm was recorded using automated oscillometric device in a sitting position in the morning after 10 minute rest. Two consecutive measurements were performed, and the mean of the measurements were used. Waist circumference was measured on bare skin with a tape measure, midway between the top of the iliac crest and the bottom of the rib cage. Two independent measurements were performed and

the mean value was used. The menarche age of girls (the first onset of menstrual bleeding) were collected by questionnaire and retrospective by phone call as well as during follow-up visits.

Body Composition Assessments

Lean tissue mass (LM), and fat mass (FM) of the whole body, android and gynoid region were assessed using DXA (Prodigy GE Lunar Corp., Madison, WI USA). The precision of the results of the repeated measurements in this study were expressed by the percentage coefficient of variation (CV%) which was on average, 1.0% for LM, and 2.2% for FM.

Biochemical analyses

Blood samples were collected in the morning between 7:00 and 9:00 am after overnight fasting. The samples were collected on 2 to 5 days after menstruation among those girls with regular menses. Plasma glucose, high-density lipoprotein cholesterol (HDL-C) and triglycerides was assessed by KONELAB 20XTi analyzer (Thermo Fischer Scientific inc.Waltham, MA, USA).

Cardiometabolic risk assessment

To assess cardiometabolic risk we constructed a standardised, continuously distributed variable for clustered metabolic risk similarly to previously published scores[10,11]. The risk score was calculated by standardising and then summing the following continuously distributed metabolic traits to create a z score: mean arterial pressure ([(2 x diastolic blood pressure)+systolic blood pressure]/3); abdominal fat mass; fasting plasma glucose; serum HDL cholesterol x -1; and fasting serum

triglyceride z score. A higher score indicates a less favorable cardiometabolic risk profile. The purpose of using such continuously distributed risk score was to maximise statistical power[12] because average differences in metabolic traits are relatively small in children and adolescents.

Statistical analyses

Continuous data were checked for normality by Shapiro-Wilk's test before each analysis in the SPSS for Windows statistical software package version 18 (SPSS Inc., Chicago, IL, USA). If data were not normally distributed, natural logarithm transformations were used. ANOVA with the Least Significant Difference post hoc test was used to compare differences between OWOB, NWO, NW and UW groups. Statistical significance was set at $p < 0.05$.

Results

Longitudinal change of body weight

Longitudinal change of body weight collected from growth charts from birth to age of 20 are shown in **Figure 1**. It can be seen that the relative body weight was higher already at the age of four years in subjects who were OWOB at the age of 18 compared with those who were NWO, NW, or UW. However, the differences in relative body weight between the NWO and NW were not significant during growth.

Insert Figure 1 here

Longitudinal change in body composition:

Total and regional adiposity increased throughout growth in all groups from age of 11 to the age of 18 (**Figure 2)**. At the age of 11, there was about 10kg difference in total FM between the OWOB and NW group ($p<0.001$) and about 7kg between the NWO and NW group ($p<0.001$), respectively. The most rapid gain in FM was between the age of 11 and 14 in all groups. The average increase of total FM was 13kg in the OWOB group, whereas the average increase in NWO and NW groups was about 6kg from prepubertal to early adulthood. In terms of FM distribution, the increase of FM was greatest in gynoid (lower body) region in all groups. Noticeably, in the OWOB group FM in the android region (abdominal area) increased significantly compared to the other weight groups ($p<0.001$ for all). The increase in LM was also greatest between the age of 11 and 14, the relative accrual being similar in all groups (**Figure 2**).

Insert Figure 2 here

Cardio-metabolic risk:

The OWOB group had higher MetS score compared with all other weight groups throughout growth ($p<0.001$ for all, **Figure 3**). The MetS score was also higher in the NWO group compared with the NW and UW groups at all time points ($p<0.001$ for all), but no difference was found between the NW and UW groups.

Insert Figure here 3

Discussion

This longitudinal study showed that subjects who were overweight or obese in adulthood had higher relative body weight to height already at the age of four compared with those who were normal weight. Moreover, overweight and obese subjects had a worse cardio-metabolic risk profile than normal weight subjects in childhood, and this difference persisted through puberty into early adulthood. We also showed that children with normal body weight but high body fat percent had signficantly higher cardio-metabolic risk compared with normal weight children with low body fat percent, but no difference in relative body weight was observed throughout childhood and adolesence. These findings suggest that excess adiposity starts to develop early in life and it is a significant risk factor for cardio-metabolic disease in adulthood regardless of body weight status.

Higher BMI in childhood and adolescence is associated with an increased risk of cardiometabolic morbidity and mortality in adulthood[13-16]. Prevention remains the primary goal in the management of obesity, and therefore pediatricians are advised to measure BMI regularly and prescribe and support lifestyle modifications to the patient and their family[17,18]. Although BMI is considered a useful tool in assessing the weight status[19], recent evidence suggest that signifant percentage of children might be at risk being misdiagnosed as lean if obesity is defined solely based on BMI[5]. Indeed, our study showed children who had normal body weight but high body fat percent are

virtually indistinguishable from their normal weight lean peers in terms of relative body weight in childhood and adolescence. Moreover, we showed that these normal weight but obese children have signifcantly higher cardio-metabolic risk compared with their normal weight lean or underweight subjects in childhod and this persisted into early adulthood. These results suggest that simply maintaining a normal body weight in childhood does not necessarily protect against cardio-metabolic abnormalities later in life.

Direct comparison of our results with earlier studies is difficult, because normal weight obesity has not been studied longitudinally before in children and adolescents. Overall, data on normal weight obesity children is sparse. There are few reports that describe cross-sectional data on children and adolescents with cardio-metabolic risk factors who are not obese according to BMI, but in these studies body composition was not assessed and thus it remains unclear whether these children had low or high body fat percent [20]. Moreover, most of the children with metabolic abnormalities in these studies had a family history of hypertension, atherogenic serum lipid profile or type II diabetes, suggesting that family history of cardio-metabolic disease is a signficant cardiovascular risk factor in non-obese children. In our study, normal weight obese children did not have history of cardio-metabolic disease in their immediate family, therefore it seems likely that the increased cardio-metabolic risk in these children is attributable to their relatively high body fat content. In supporting our findings, studies in adults have shown that normal weight obesity is associated

with higher cardiovascular risk factors[21], cardio-metabolic dysregulation[22], coronary heart disease[23] and cardiovascular mortality[24,25]. Taken together, our results suggest that the cumulative burden of excessive body fat begins at an early age and this is irrespective of body weight.

Our results provide and important message for clinicians and public health officers as well as the individual child and their families. Measurements of body composition should be included in the screening for cardiovascular risk because evidently BMI does not recognize a substential number of children who are at increased risk for cardio-metabolic disease later in life. Failure to recognize excess adiposity in childhood may translate into missed opportunities to prescribe appropriate lifestyle modification to prevent future cardio-metabolic morbidity. These results should also encourage research in the field to identify and validate definition of normal weight obesity in children because currently there is no universally accepted defintion of a normal value for percent body fat.

Our study is not without limitations. The sample size for obese and normal weight obese subjects was relatively small. Also, the study subjects included only females, so the results may not applied to males. Strengths of this study include research-quality measures of body composition and cardiometabolic biomarkers, and the data obtained from growth charts.

Conclusion

The results of our study suggest that children and adolescent with normal BMI and elevated body fat percentage may be at increased risk for cardiometabolic morbidity in adulthood. Screening for adiposity in children and adolescent with a normal BMI could better identify those at higher risk for cardiometabolic morbidities.

References

1. Baker JL, Olsen LW, Sorensen TI. Childhood body-mass index and the risk of coronary heart disease in adulthood. The New England journal of medicine 2007;357:2329-37.
2. Richardson L, Paulis WD, van Middelkoop M, Koes BW. An overview of national clinical guidelines for the management of childhood obesity in primary care. Preventive medicine 2013;57:448-55.
3. Reilly JJ, Kelly J, Wilson DC. Accuracy of simple clinical and epidemiological definitions of childhood obesity: systematic review and evidence appraisal. Obesity reviews : an official journal of the International Association for the Study of Obesity 2010;11:645-55.
4. Prentice AM, Jebb SA. Beyond body mass index. Obesity reviews : an official journal of the International Association for the Study of Obesity 2001;2:141-7.
5. Javed A, Jumean M, Murad MH, et al. Diagnostic performance of body mass index to identify obesity as defined by body adiposity in children and adolescents: a systematic review and meta-analysis. Pediatric obesity 2015;10:234-44.
6. Xu L, Nicholson P, Wang Q, Alen M, Cheng S. Bone and muscle development during puberty in girls: a seven-year longitudinal study. J Bone Miner Res 2009;24:1693-8.
7. Cheng S, Volgyi E, Tylavsky FA, et al. Trait-specific tracking and determinants of body composition: a 7-year follow-up study of pubertal growth in girls. BMC medicine 2009;7:5.
8. Volgyi E, Alen M, Xu L, et al. Effect of long-term leisure time physical activity on lean mass and fat mass in girls during adolescence. J Appl Physiol (1985) 2011;110:1211-8.
9. De Lorenzo A, Martinoli R, Vaia F, Di Renzo L. Normal weight obese (NWO) women: an evaluation of a candidate new syndrome. Nutrition, metabolism, and cardiovascular diseases : NMCD 2006;16:513-23.
10. Ekelund U, Anderssen SA, Froberg K, Sardinha LB, Andersen LB, Brage S. Independent associations of physical activity and cardiorespiratory fitness with metabolic risk factors in children: the European youth heart study. Diabetologia 2007;50:1832-40.
11. Viitasalo A, Lakka TA, Laaksonen DE, et al. Validation of metabolic syndrome score by confirmatory factor analysis in children and adults and prediction of cardiometabolic outcomes in adults. Diabetologia 2014;57:940-9.
12. Ragland DR. Dichotomizing continuous outcome variables: dependence of the magnitude of association and statistical power on the cutpoint. Epidemiology 1992;3:434-40.
13. Franks PW, Hanson RL, Knowler WC, Sievers ML, Bennett PH, Looker HC. Childhood obesity, other cardiovascular risk factors, and premature death. The New England journal of medicine 2010;362:485-93.
14. Juonala M, Magnussen CG, Berenson GS, et al. Childhood adiposity, adult adiposity, and cardiovascular risk factors. The New England journal of medicine 2011;365:1876-85.
15. Raitakari OT, Juonala M, Kahonen M, et al. Cardiovascular risk factors in childhood and carotid artery intima-media thickness in adulthood: the Cardiovascular Risk in Young Finns Study. Jama 2003;290:2277-83.
16. Tirosh A, Shai I, Afek A, et al. Adolescent BMI trajectory and risk of diabetes versus coronary disease. The New England journal of medicine 2011;364:1315-25.

17. Brown CL, Halvorson EE, Cohen GM, Lazorick S, Skelton JA. Addressing Childhood Obesity: Opportunities for Prevention. Pediatric clinics of North America 2015;62:1241-61.
18. August GP, Caprio S, Fennoy I, et al. Prevention and treatment of pediatric obesity: an endocrine society clinical practice guideline based on expert opinion. The Journal of clinical endocrinology and metabolism 2008;93:4576-99.
19. Flegal KM, Ogden CL. Childhood obesity: are we all speaking the same language? Adv Nutr 2011;2:159S-66S.
20. Guerrero-Romero F, Rodriguez-Moran M. Metabolically obese normal-weight children. World journal of clinical pediatrics 2012;1:37-9.
21. Marques-Vidal P, Pecoud A, Hayoz D, et al. Normal weight obesity: relationship with lipids, glycaemic status, liver enzymes and inflammation. Nutrition, metabolism, and cardiovascular diseases : NMCD 2010;20:669-75.
22. Shea JL, King MT, Yi Y, Gulliver W, Sun G. Body fat percentage is associated with cardiometabolic dysregulation in BMI-defined normal weight subjects. Nutrition, metabolism, and cardiovascular diseases : NMCD 2012;22:741-7.
23. Romero-Corral A, Somers VK, Sierra-Johnson J, et al. Diagnostic performance of body mass index to detect obesity in patients with coronary artery disease. European heart journal 2007;28:2087-93.
24. Coutinho T, Goel K, Correa de Sa D, et al. Combining body mass index with measures of central obesity in the assessment of mortality in subjects with coronary disease: role of "normal weight central obesity". Journal of the American College of Cardiology 2013;61:553-60.
25. Sahakyan KR, Somers VK, Rodriguez-Escudero JP, et al. Normal-Weight Central Obesity: Implications for Total and Cardiovascular Mortality. Annals of internal medicine 2015;163:827-35.

Figure Legends

Figure 1. Longitudinal changes of relative body weight from birth to age of 18 years. Bodyweight groups are defined by BMI and fat % at the age of 18 years and the comparison between groups were done retrospectively.

Figure 2. Longitudinal change in body composition from age of 11 to age of 18. OWOB = overweight and obese (BMI>25 and fat % >30), NWO = normal weight obese (BMI 18.5 - 24.9 and fat % >30), NW = normal weight lean (BMI 18.5 - 25 and fat% <30), UW = underweight (BMI<18.5).

Figure 3. Longitudinal change in continuous cardio-metabolic risk (Mets) score from age of 11 to age of 18. Higher score indicates greater risk. OWOB = overweight and obese (BMI>25 and fat % >30), NWO = normal weight obese (BMI 18.5 - 24.9 and fat % >30), NW = normal weight lean (BMI 18.5 - 25 and fat% <30), UW = underweight (BMI<18.5).

Figure 1.

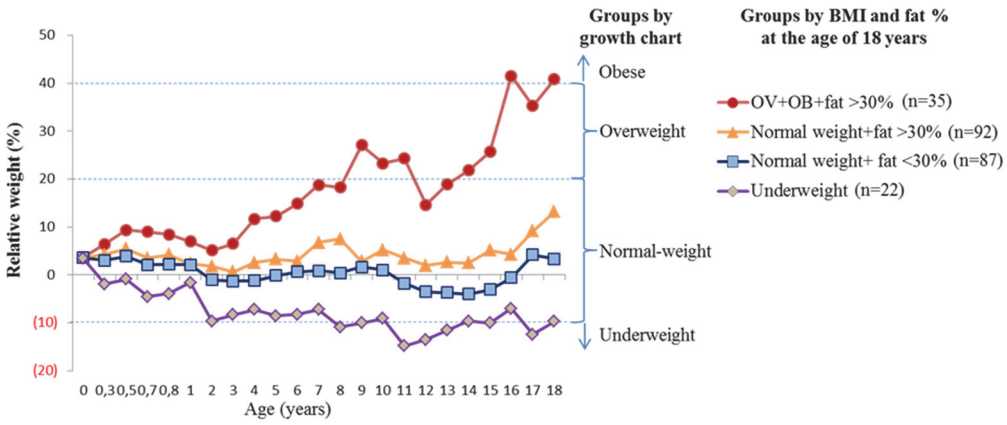

Groups by growth chart
Groups by BMI and fat % at the age of 18 years
Obese
Overweight
Normal-weight
Underweight
OV+OB+fat >30% (n=35)
Normal weight+fat >30% (n=92)
Normal weight+ fat <30% (n=87)
Underweight (n=22)
Relative weight (%)
50
40
30
20
10
0
(10)
(20)
0 0,30,50,70,8 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Age (years)

Figure 2.

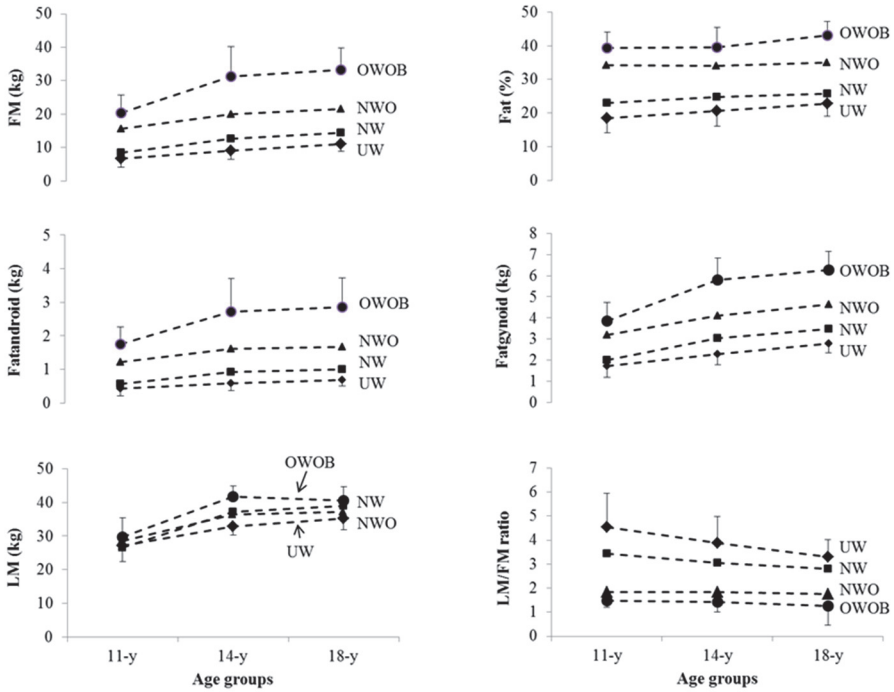
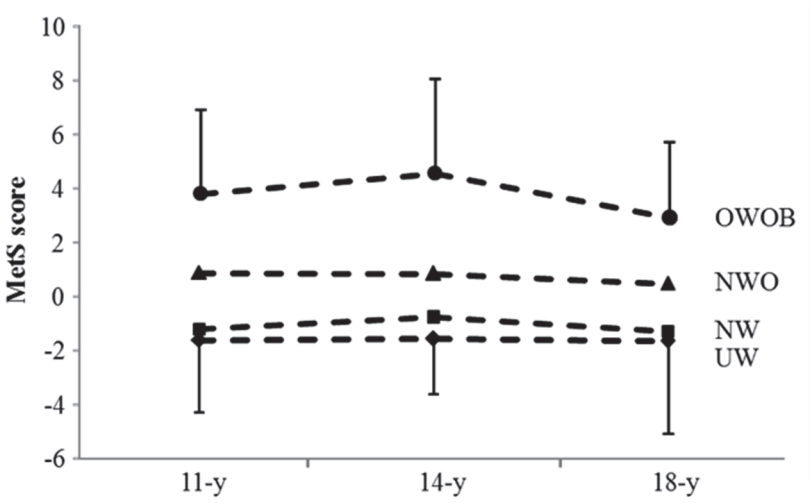

FM (kg)
Fat (%)
Fatandroid (kg)
Fatgynoid (kg)
LM (kg)
LM/FM ratio
OWOB
NWO
NW
UW
11-y
14-y
18-y
Age groups

Figure 3.



V

SERUM AMINO ACID PROFILES IN CHILDHOOD PREDICT TRIGLYCERIDE LEVEL IN ADULTHOOD: A 7-YEAR LONGITUDINAL STUDY IN GIRLS

by

Wiklund P, Zhang X, Tan X, Keinänen-Kiukaanniemi S, Alen M, Cheng S

J Clin Endocrinol Metab 2016 May; 101(5):2047-55

Serum Amino Acid Profiles in Childhood Predict Triglyceride Level in Adulthood: A 7-Year Longitudinal Study in Girls

Petri Wiklund,* Xiaobo Zhang,* Xiao Tan, Sirkka Keinänen-Kiukaanniemi, Markku Alen, and Sulin Cheng

Department of Health Sciences (P.W., X.T., S.C.), University of Jyväskylä, 40014 Jyväskylä, Finland; School of Life Sciences and Biotechnology (X.Z.), Shanghai Jiao Tong University, Shanghai 200240, China; Center for Life Course Health Research (S.K.-K., M.A.), University of Oulu, 90014 Oulu, Finland; Unit of Primary Health Care and Medical Research Center (S.K.-K.), and Department of Medical Rehabilitation (M.A.), Oulu University Hospital, 90220 Oulu, Finland; and Exercise, Health, and Technology Center (S.C.), Shanghai Jiao Tong University, Shanghai 200240, China

Context: Branched-chain and aromatic amino acids are associated with high risk of developing dyslipidemia and type II diabetes in adults.

Objective: This study aimed to examine whether serum amino acid profiles associate with triglyceride concentrations during pubertal growth and predict hypertriglyceridemia in early adulthood.

Design: This was a 7.5-year longitudinal study.

Setting: The study was conducted at the Health Science Laboratory, University of Jyväskylä.

Participants: A total of 396 nondiabetic Finnish girls aged 11.2 ± 0.8 years at the baseline participated in the study.

Main Outcome Measures: Body composition was assessed by dual-energy x-ray absorptiometry; serum concentrations of glucose, insulin, and triglyceride by enzymatic photometric methods; and amino acids by nuclear magnetic resonance spectroscopy.

Results: Serum leucine and isoleucine correlated significantly with future triglyceride, independent of baseline triglyceride level ($P < .05$ for all). In early adulthood (at the age of 18 years), these amino acids were significantly associated with hypertriglyceridemia, whereas fat mass and homeostasis model assessment of insulin resistance were not. Leucine was the strongest determinant discriminating subjects with hypertriglyceridemia from those with normal triglyceride level (area under the curve, 0.822; 95% confidence interval, 0.740–0.903; $P = .000001$).

Conclusions: Serum leucine and isoleucine were associated with future serum triglyceride levels in girls during pubertal growth and predicted hypertriglyceridemia in early adulthood. Therefore, these amino acid indices may serve as biomarkers to identify individuals at high risk for developing hypertriglyceridemia and cardiovascular disease later in life. Further studies are needed to elucidate the role these amino acids play in the lipid metabolism. **(*J Clin Endocrinol Metab* 101: 2047–2055, 2016)**

Dyslipidemia is a well-established risk factor for cardiovascular disease in adults (1–3). This atherogenic disorder of lipid and lipoprotein metabolism is characterized with elevated levels of serum triglyceride and small low-density lipoprotein cholesterol, with low levels of high-density lipoprotein cholesterol (4). A recent meta-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received January 8, 2016. Accepted March 8, 2016.
First Published Online March 11, 2016

* P.W. and X.Z. contributed equally to this manuscript.

Abbreviations: AUC, area under the curve; BMI, body mass index; CI, confidence interval; CV, coefficient of variation; HOMA-IR, homeostasis model assessment of insulin resistance; LTPA, leisure-time physical activity; NMR, nuclear magnetic resonance.

analysis demonstrated that elevated triglyceride levels, in particular, are associated with increased risk of coronary heart disease (5). Although dyslipidemia is generally not associated with significant negative health outcomes in childhood, studies have shown that unfavorable structural (fatty streaks and fibrous plaque lesions) and functional changes of the vasculature start to develop early in life. These vascular findings are associated with disordered serum lipid and lipoprotein profiles (6). A follow-up study in Finnish children revealed that dyslipidemia in childhood predicted increased carotid artery intima-media thickness in adulthood (7). Moreover, the Princeton follow-up study showed that elevated triglyceride concentrations in childhood predicted clinical cardiovascular events several decades later (8).

Hypertriglyceridemia can be due to hereditary factors (ie, familial hypertriglyceridemia) or result from secondary causes such as diet, sedentary lifestyle, medical conditions (eg, hypothyroidism, insulin resistance, and type II diabetes) and the use of certain medications, or a combination of the above (9). For many years, studies have focused on searching for biomarkers that would allow for early prediction of hypertriglyceridemia. Recent genome-wide association studies have found numerous loci associated with triglyceride levels in adults (10). However, the predictive value of these gene variants is limited because they can only explain around 10% of the variation in lipid levels within the population (11). Further understanding of disordered lipid metabolism could be provided by metabolomics, which can reveal a global overview on subtle metabolic changes in the body. Metabolomics in clinical and epidemiological research have revealed alterations in several amino acid concentrations in subjects with dyslipidemia (12–14). Moreover, a recent longitudinal study in middle-aged and elderly men and women showed that increased levels of amino acids at baseline, including branched-chain amino acids (isoleucine, leucine, and valine), were associated with an increased risk of hypertriglyceridemia after 7-year follow-up (15). However, little is known about how these amino acids associate with serum triglyceride during pubertal growth. Considering future prediction of developing dyslipidemia and cardiovascular disease, it is important to assess whether the associations between amino acids and serum triglyceride in adults are already evident in children transitioning from prepuberty to early adulthood.

Therefore, we explored the patterns of longitudinal changes of serum amino acids and triglyceride across pubertal growth in girls in a 7.5-year prospective study using hierarchical models.

Subjects and Methods

Subjects

A total of 396 girls (mean age, 11.2 years at baseline) participated in a longitudinal study (main aim was to study the determinants of body composition during growth) for an average of 7.5 years. Detailed information regarding the participants has been reported previously (16). Briefly, for the purpose of this report, we included only nondiabetic (type I or type II) girls with no diagnosed diseases affecting lipid metabolism and who had valid data for body composition and serum amino acid, glucose, insulin, and triglyceride concentrations. There were 13 girls who reported the use of oral contraceptives at the age of 18 years. Because oral contraceptives can affect lipid metabolism (17), as well as circulating amino acid concentrations (18), these girls were excluded from the final analysis. The total number of subjects was 230 at baseline, 213 at the 2-year follow-up, and 220 at the 7.5-year follow-up assessments.

Anthropometric measurement

All information was collected and laboratory tests were performed within a 2-week period during the same month throughout the 7.5-year follow-up to avoid seasonal effects. Lifestyle and behavioral characteristics as well as medical history were collected via a validated self-administered questionnaire. Girls under 15 years of age filled in the questionnaire with their guardians' assistance, and all the questionnaires were checked by a study nurse. The age at menarche was defined as the first onset of menstrual bleeding as reported by questionnaire or phone call during the follow-up. Dietary information was obtained from a food-intake diary recorded for 3 days as described elsewhere (19). Leisure-time physical activity (LTPA) of hours per week (participating in exercise such as walking, jogging, running, gym fitness, ball games, swimming, etc) was evaluated using a validated self-administrated physical activity questionnaire described previously (20). All measurements were performed after overnight fasting. Participants' weight was determined within 0.1 kg for each subject using an electronic scale and was calibrated before each measurement session. Height was determined using a fixed wall-scale measuring device to the nearest 0.1 cm. Body mass index (BMI) was calculated as weight (kilograms) per height (meters)^2. Dual-energy x-ray absorptiometry) (Prodigy, with software version 9.3; GE Lunar Corp) was used to measure whole body fat mass. Precision of the repeated measurements expressed as a coefficient of variation (CV) was 2.2% for fat mass.

Serum amino acid, triglyceride, glucose, and insulin assessments

Blood samples from the antecubital vein were collected between 7 and 9 AM after an overnight fast. The samples were collected 2 to 5 days after menstruation among those girls with regular menses. Serum and plasma were extracted from blood by centrifugation and stored immediately at −80°C until analyzed.

Circulating amino acid concentrations were analyzed using a high-throughput serum nuclear magnetic resonance (NMR) metabolomics platform. The experimental protocols, including sample preparation and NMR spectroscopy, have been described in detail elsewhere (21). This NMR platform has recently been applied in various extensive epidemiological studies (22). For amino acids assessment, a proton NMR spectrum was ob-

tained where spectral signals from the macromolecules and lipoprotein lipids were suppressed to enhance detection of the amino acid signals. The current NMR methodology allows accurate quantification of nine amino acids (alanine, glutamine, glycine, histidine, isoleucine, leucine, phenylalanine, tyrosine, and valine) expressed in millimoles per liter. Concentrations of other common amino acids in the serum are below detection level of the current methodology and therefore were not assessed in this study. CV for alanine, glutamine, glycine, histidine, valine, leucine, isoleucine, phenylalanine, and tyrosine were 2.6, 3.2, 3.5, 4.1, 2.7, 3.6, 4.8, 3.9, and 5.3%, respectively. To address how well leucine was resolved from its isomer isoleucine, leucine signal was located at 0.974 ppm (ie, 487.3 Hz at 500 MHz NMR spectrometer). Isoleucine signal was located at 1.022 ppm (ie, 511.3 Hz). Typical line width at half height for leucine and isoleucine signals is ca. 1.5 Hz. Thus, isoleucine and leucine signals were clearly resolved.

Serum triglyceride and glucose were analyzed using the KONELAB 20XTi analyzer (Thermo Fisher Scientific Inc). Insulin was determined by immunofluorescence using the IMMULITE Analyzer (Diagnostic Products Corporation). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as follows: (fasting insulin concentration × fasting glucose concentration)/22.5. The inter- and intra-assay CVs were 3.4 and 2.9% for triglyceride, 2.0 and 3.7% for glucose, and 11 and 3.4% for insulin, respectively.

Ethics

Written informed consent was obtained from all participants before the study. If the participant was underage, a written informed consent was obtained from parents or a legal guardian on behalf of the child. The study protocol was approved by the ethical committee of the Central Finland Health Care District.

Statistical analysis

Continuous data were checked for normality by Shapiro-Wilk's test before each analysis. If data were not normally distributed, their natural logarithms were used in all analyses. Descriptive statistics were presented as means and 95% confidence interval (CI) for the mean at the follow-ups. Data for the different time points were compared with each other using the general linear model. A hierarchical (multilevel) nonlinear model with random effects (MLwin2.20 software, Multiple Project; Institute of Education, University of London) was used to explore the patterns of longitudinal changes of amino acids, triglyceride, and HOMA-IR from prepuberty to early adulthood. The hierarchical model allows inclusion of the data from every subject despite irregularity of temporally spaced follow-up or missing data (23). Time relative to menarche was entered as the explanatory variable in the form of polynomial spline functions to explain the change of target variables over time, as described in detail elsewhere (16). The best model was determined by three criteria: the largest reduction in deviance test (2log likelihood by iterative generalized least squares), the lowest within-individual variance, and the necessary parsimony of the model.

To determine the associations with longitudinal changes of triglyceride with fat mass, HOMA-IR, and amino acids before and after menarche, we used hierarchical models in which the outcome variable was triglyceride and the independent variables were amino acids. The time of menarche is selected as a shift knot for the model, which means that the coefficients of independent variables could be different before and after menarche. Thus, the associations between amino acids and triglyceride can be assessed by regression coefficients before and after menarche, respectively. A t test was used to assess whether the β coefficients were statistically different from zero. Furthermore, we divided subjects into quartiles based on their triglyceride levels at baseline and at the 2- and 7 year follow-ups and compared amino acid levels adjusting for fat mass, HOMA-IR, and protein intake. Finally, we used receiver operating characteristics curve analyses to determine the predictive effect of variables to identify hypertriglyceridemia in early adulthood. The area under the curve (AUC) is considered a measure of the use of the predictor variable and represents the trade-off between the correct identification of

Table 1. General Characteristics at Different Measurement Time Points in Adolescent Girls

	Baseline (n = 230)		2-Year Follow-Up (n = 213)		7-Year Follow-Up (n = 220)	
	Mean	95% CI	Mean	95% CI	Mean	95% CI
Age, y	11.2	11.1–11.5	13.3	13.0–13.5	18.1	17.8–18.4
Height, cm	146.3[a,b]	145.0–147.6	158.0[b]	156.9–159.1	165.1	163.7–166.5
Weight, kg	39.7[a,b]	38.4–41.1	50.1[b]	48.5–51.6	60.3	59.0–61.6
BMI, kg/m^2	18.4[a,b]	18.0–18.9	20.0[b]	19.5–20.5	21.9	21.5–22.3
Fat mass, kg	10.7[a,b]	10.0–11.4	13.8[b]	12.8–14.8	19.5	18.4 − 20.7
Fat-free mass, kg	26.9[a,b]	26.4–27.4	33.8[b]	33.2–33.4	38.1	37.5–38.6
Glucose, mmol/L	5.5[a,b]	5.4–5.5	5.3	5.2–5.4	5.2	5.1–5.3
Insulin, IU/mL	9.1[a,b]	7.4–10.8	11.7[b]	10.5–12.8	8.4	7.4–9.1
Energy intake, kcal/d	1564[a,b]	1517–1610	1739	1675–1803	1780	1717–1843
Protein, E%	15.5[b]	15.1–16.0	15.5[b]	15.0–16.1	17.3	16.8–17.8
Fat, E%	33.5[b]	32.7–34.2	32.5	31.7–33.4	31.7	30.7–32.7
Carbohydrates, E%	51.0	50.0–51.9	51.9	50.9–53.0	50.2	49.1–51.3
LTPA, h/wk	2.7	2.12–3.28	3.15	2.53–3.78	3.52	2.84–4.20

Abbreviation: E, energy. Natural logarithm transformation data were used for the comparison of different time points. The Sidak method was used for multiple comparisons.

[a] $P < .05$ compared with 2-year follow-up.

[b] $P < .05$ compared with 7-year follow-up.

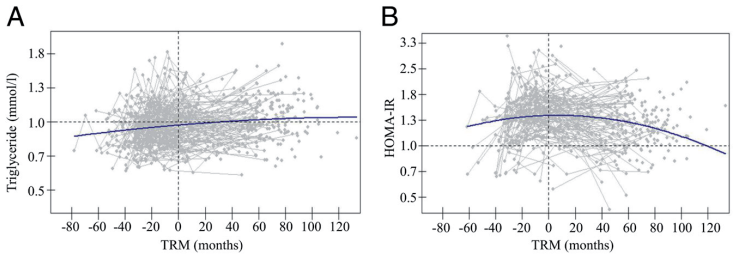


Figure 1. Change patterns of serum triglyceride (A) and HOMA-IR (B). Data for triglyceride and HOMA-IR are plotted against time relative to menarche (TRM). Gray lines represent longitudinal change of each individual, and the black lines are the best fitting lines derived from hierarchical models. The values on y-axis are back-transformed from log triglyceride and log HOMA-IR, respectively.

individuals with hypertriglyceridemia (sensitivity) and the correct identification of normolipidemic individuals (specificity). Hypertriglyceridemia was classified using a cutoff of 1.70 mmol/L, as recommended by the American Heart Association (24). Statistical significance was set at $P < .05$.

Results

General characteristics measured at different time points are shown in Table 1. The mean age at menarche was 12.9 years. Body height, weight, BMI, fat mass, and fat-free mass increased significantly over time, respectively ($P < .05$ for all). Although glucose was higher at baseline compared with 2- and 7-year follow-up, insulin was higher at the 2-year follow-up compared with baseline or 7-year follow-up ($P < .05$ for all). Dietary energy and protein intake increased, and fat intake decreased over time ($P < .05$ for all). Time (hours per week) spent in LTPA did not change significantly over time.

Longitudinal change patterns of serum triglyceride, HOMA-IR, and amino acids are shown in Figures 1 and 2. Although triglyceride increased steadily throughout the follow-up period, HOMA-IR increased before and around menarche and then decreased steadily until early adulthood ($P < .05$ for both). In general, amino acid concentrations were increased before menarche and peaked around menarche, except alanine, for which increments

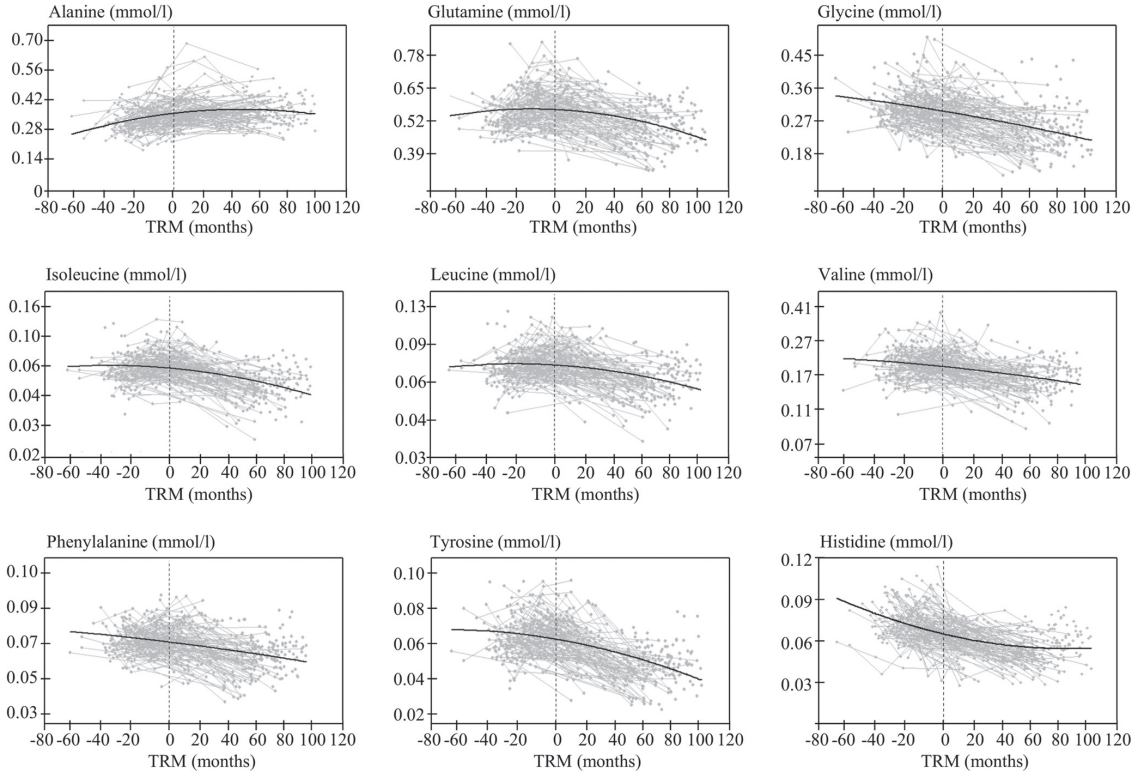


Figure 2. Longitudinal change patterns of amino acids. Data for amino acids are plotted against time relative to menarche (TRM). Gray lines represent longitudinal change of each individual, and the black lines are the best fitting lines derived from hierarchical models. The concentrations of amino acids on y-axis are back-transformed from natural log values, respectively.

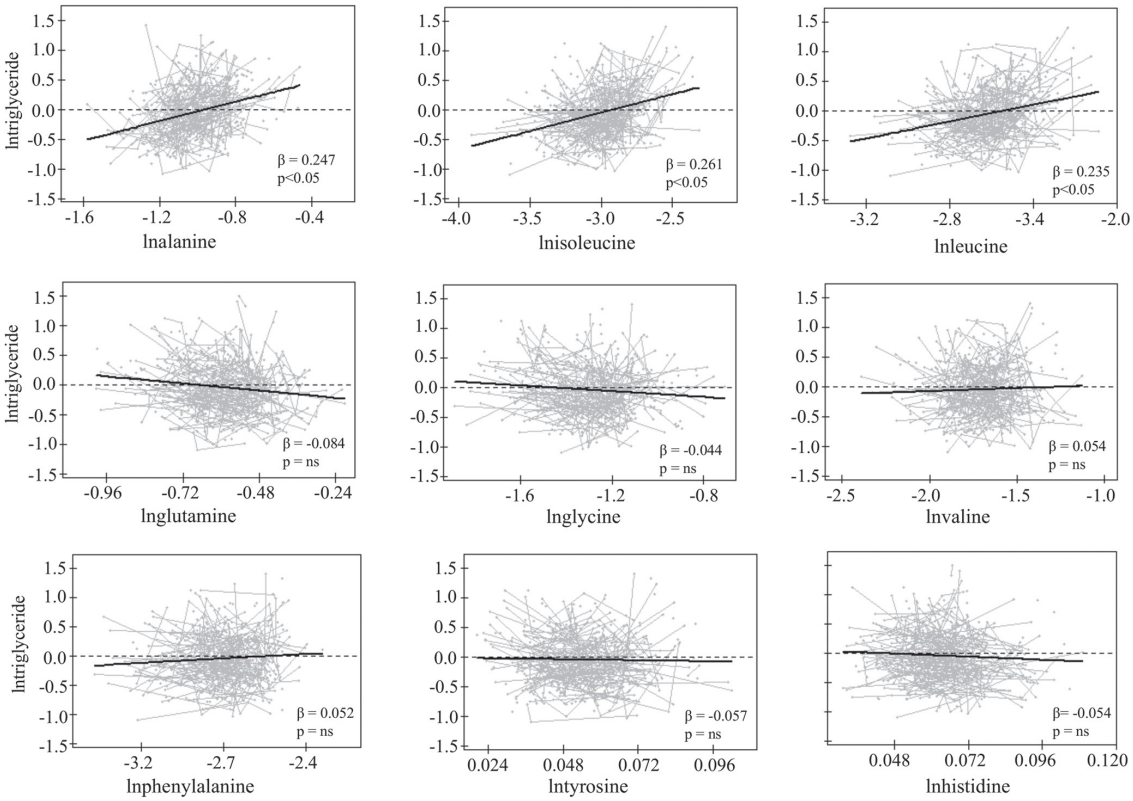


Figure 3. Longitudinal correlations between serum triglyceride and amino acids. Gray lines represent longitudinal change of each individual's values, and the black lines are slopes obtained from the hierarchical regression models. Values on y- and x-axes are natural log-transformed values. β is the regression coefficient, and ns indicates nonsignificant association.

were ceased around 30 months after menarche (15 years old). Glutamine, isoleucine, and leucine levels remained relatively constant before menarche and decreased gradually after menarche, whereas glycine, valine, phenylalanine, tyrosine, and histidine decreased steadily from prepuberty until early adulthood ($P < .05$ for all).

We further applied longitudinal models to assess the associations between triglyceride and amino acids (Figure 3). We found that alanine, isoleucine, and leucine correlated significantly with triglyceride ($P < .05$ for all), but no significant associations for other amino acids with triglyceride were found. Triglyceride level at baseline predicted triglyceride level at the 2-year follow-up (r = 0.386) and the 7-year follow-up (r = 0.703; $P < .001$ for both). However, after adjusting for baseline leucine and isoleucine level, the earlier triglyceride level no longer predicted subsequent triglyceride levels. We further assessed whether the early level of leucine and isoleucine predicted future triglyceride level, independent of earlier triglyceride level. We found that baseline isoleucine predicted 7-year triglyceride (r = 0.278; $P = .026$), and baseline leucine predicted 2-year triglyceride (r = 0.279; $P = .01$), even after adjusting for baseline triglyceride level.

Triglyceride was also correlated significantly with fat mass and HOMA-IR ($P < .05$ for both; Figure 4). To assess whether amino acids were associated with elevated triglyceride independent of fat mass and HOMA-IR, we divided subjects into quartiles based on their triglyceride concentrations and compared amino acid levels, adjusting for these variables (Figure 5). We found that isoleucine, leucine, and alanine were significantly higher in the highest quartile compared to the lowest quartile at each time point ($P < .05$ for all).

When examining whether the amino acids may serve as biomarkers for hypertriglyceridemia in early adulthood, we found that at the end of the follow-up period when the girls had reached the age of 18 years, 29 (13%) of them presented with hypertriglyceridemia (>1.7 mmol/L). The AUC values for predicting hypertriglyceridemia for the significantly associated amino acids were as follows: alanine, 0.683 (95% CI, 0.577–0.789); isoleucine, 0.774 (95% CI, 0.665–0.883) ($P < .01$ for both). The strongest determinant was leucine

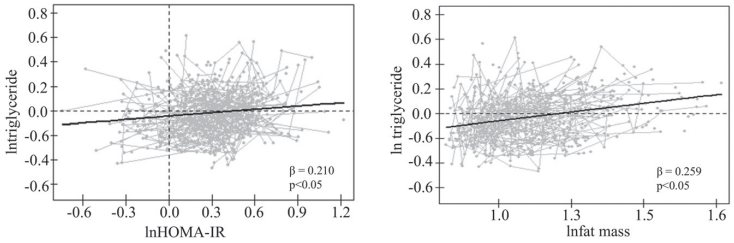


Figure 4. Longitudinal correlations between serum triglyceride, HOMA-IR, and fat mass. Gray lines represent longitudinal change of each individual's values, and the black lines are slopes obtained from the hierarchical regression models. Values on y- and x-axes are natural log transformed values. β is the regression coefficient, and ns indicates nonsignificant association.

(0.822; 95% CI, 0.740–0.903; $P = .000001$). By comparison, the AUC values for predicting hypertriglyceridemia were 0.528 for fat mass, 0.536 for fs-insulin, and 0.542 for HOMA-IR (all $P > .05$), respectively.

Discussion

This 7.5-year longitudinal study with pubertal girls demonstrates changes in serum amino acids and triglyceride concentrations during growth. We found that hydrophobic amino acids alanine, leucine, and isoleucine were associated with elevated triglyceride concentrations and were significant predictors of hypertriglyceridemia in early adulthood, independent of fat mass and HOMA-IR.

Amino acids play central roles in protein homeostasis and metabolism, promoting normal growth and development in children (25). In our study, the average level of most amino acids was highest before menarche and then decreased after menarche until early adulthood (except alanine, which increased from before to after menarche). The decrease in amino acid concentrations after menarche likely reflects somatic growth during puberty and adolescence, as well as changes in proteolysis and protein oxidation, which are lower during puberty compared to prepuberty in normal-weight children and adolescents (26). Nonetheless, earlier reports have suggested that plasma amino acid levels tend to increase throughout

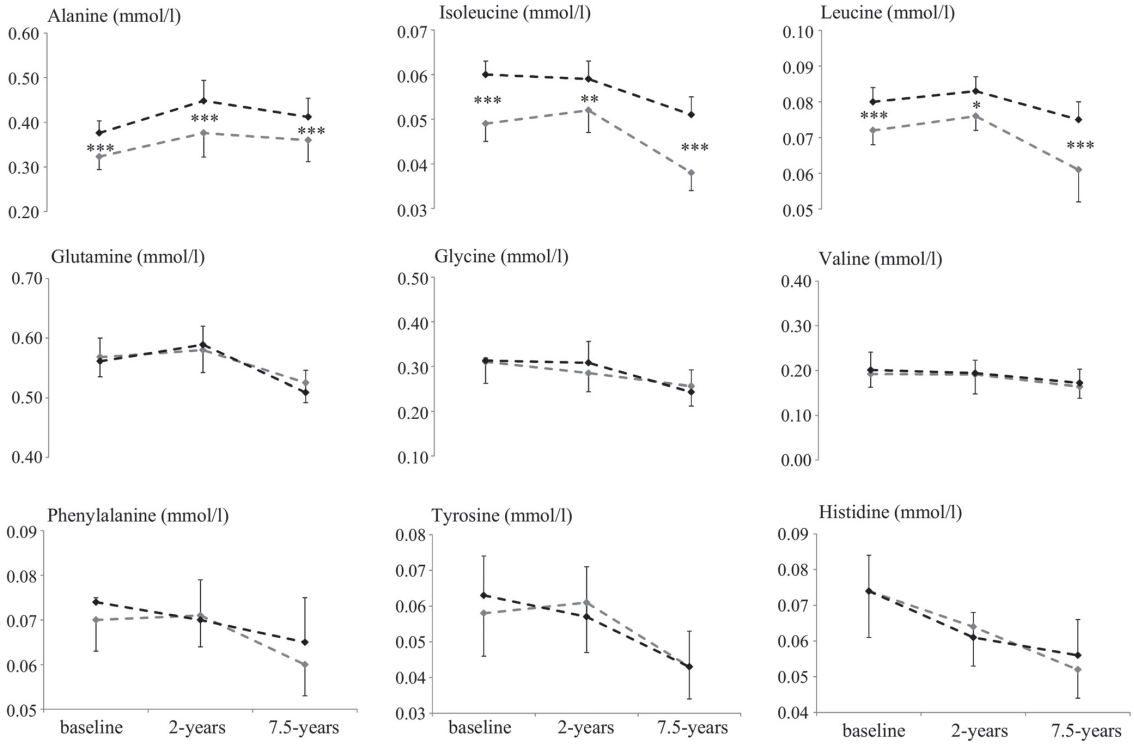


Figure 5. Comparison of amino acids between groups defined by triglyceride (highest quartile against lowest quartile) at baseline, 2-year follow-up, and 7-year follow-up adjusted for fat mass, HOMA-IR, and protein intake. The y-axis represents corresponding variables of estimated marginal means with standard error. Black lines indicate highest quartile, and gray lines indicate lowest triglyceride quartile. *, $P < .05$; **, $P < .01$; ***, and $P < .001$ indicate significant difference between highest and lowest triglyceride quartiles within each time point.

childhood and adolescence (27–30). There are several possible reasons for such divergent observations, but we believe that they may primarily be due to the study designs because our study used longitudinal data, whereas other studies were cross-sectional with relatively small sample sizes. Interindividual variation and wider range of age in these studies, as well as differences in methodology, such as sample preparation and analytical procedures, may also account for the discrepant results. Furthermore, the phase of the menstrual cycle, which has been demonstrated to affect plasma amino acid levels (31), has not been considered in the earlier studies, whereas in our study measurements were done at standard phases of the menstrual cycle in girls with regular cycles.

Recent studies have implicated branched-chain amino acids and other hydrophobic amino acids (eg, alanine and phenylalanine) with obesity (32), insulin resistance (33), type II diabetes (34), and dyslipidemia (14) in adults. These same amino acids have also been associated with adiposity and insulin resistance in obese children (35, 36). Our longitudinal study in children and adolescents demonstrates that isoleucine, leucine, and alanine are associated not only with obesity and insulin resistance, but also with serum triglyceride both before and after menarche. Further analysis showed that these amino acids were elevated in subjects with high triglyceride throughout the follow-up period, even after adjusting for fat mass and HOMA-IR. This finding is consistent with an earlier longitudinal study in nondiabetic middle-aged and elderly men and women, which showed associations of isoleucine and leucine with the risk of developing hypertriglyceridemia after 7-year follow-up, although the adjustment for BMI in that study did decrease the effect size to some extent (15). A recent longitudinal study in elderly Japanese men and women also demonstrated that branched-chained amino acids and alanine predicted development of dyslipidemia after 4 years even after controlling for BMI and HOMA-IR (13). Furthermore, branched-chained amino acids and related metabolites have been shown to associate independently with coronary artery disease (37) and to predict cardiovascular events in individuals with cardiovascular disease (38). Thus, our findings substantiate the idea that amino acids are associated with the development of dyslipidemia and cardiovascular disease and indicate that this relationship may exist already in childhood and adolescence. Therefore, these amino acid indices could be considered as biomarkers to identify young individuals at high risk for developing hypertriglyceridemia and cardiovascular diseases later in life.

It is unclear why branched-chain and other hydrophobic amino acids are elevated in subjects with elevated serum triglyceride level. Differences in adiposity, physical activity, and insulin sensitivity can affect amino acid levels in the circulation. In this study, physical activity did not change significantly over time, and although fat mass and HOMA-IR varied during the follow-up period, they did not confound the associations between the amino acids and triglyceride level. We previously demonstrated that branched-chain and aromatic amino acids were auspicious biomarkers determining metabolic health independent of obesity and physical activity in middle-aged women (39). We also reported that these amino acids were elevated in men and women with nonalcoholic fatty liver disease (40), and this was associated with significant down-regulation of branched-chain amino acid catabolism in sc adipose tissue, but not in the skeletal muscle. Thus, it could be that differences in enzyme activities involved in branched-chain amino acid catabolism in the adipose tissue may have contributed to elevated amino acid levels in subjects with high triglyceride levels also in the present study. However, whether elevated amino acids are functionally involved in the development of hypertriglyceridemia has not been established in humans, and further functional studies are needed to elucidate the underlying mechanisms that link amino acids and the risk of hypertriglyceridemia.

This study was subject to some limitations. First, only girls were included in this study; thus, caution should be taken if seeking to generalize from our results to boys during pubertal growth. Second, the girls in our study were normoglycemic, and hence the observations may not be applied to a diabetic population. However, considering that the subjects in this study are all Finnish girls, we believe that this cohort is appropriate for studying the relationships between amino acids and triglyceride metabolism during growth from childhood to early adulthood. The strength of this study is also the rigor exhibited in collecting blood samples in a strictly defined period of the menstrual cycle in girls with regular cycles.

In conclusion, this study demonstrated that serum leucine and isoleucine was associated with future serum triglyceride level in girls during pubertal growth, independent of baseline triglyceride level, and predicted hypertriglyceridemia in early adulthood. Therefore, these amino acid indices may serve as biomarkers to identify individuals at high risk for developing hypertriglyceridemia and cardiovascular disease later in life. Further studies are needed to elucidate the role that these amino acids play in the lipid metabolism and pathogenesis of the metabolic disorders.

Acknowledgments

We thank Ms Shu Mei Cheng for her valuable work and technical assistance on this project.

Address all correspondence and requests for reprints to: Prof Sulin Cheng, Department of Sport and Physical Education, Shanghai Jiao Tong University, Shanghai, China, and Department of Health Sciences, University of Jyväskylä, FIN-40014 Jyväskylä, Finland. E-mail: shulin.cheng@jyu.fi.

This work was supported financially by the Academy of Finland, the Ministry of Education of Finland, the University of Jyväskylä, the China State Sport General Administration (Grant 2013B040), the National Nature Science Foundation of China (Grant 31571219), the Shanghai Jiao Tong University Zhiyuan Foundation (Grant CP2014013), and EVO research grants 2012/2013 from Oulu University Hospital.

Author Contributions: P.W. and X.Z. participated in data collection, analyzed data, and wrote the manuscript. X.T. participated in writing and editing the manuscript. S.K.-K. and M.A. participated in study design, data collection, and writing and editing the manuscript. S.C. designed the study and participated in data collection and analysis and the writing and editing of the manuscript. All authors were involved in writing the paper and had final approval of the submitted version.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Bass KM, Newschaffer CJ, Klag MJ, Bush TL.** Plasma lipoprotein levels as predictors of cardiovascular death in women. *Arch Intern Med*. 1993;153:2209–2216.
2. **Hokanson JE, Austin MA.** Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. *J Cardiovasc Risk*. 1996;3:213–219.
3. **Rana JS, Visser ME, Arsenault BJ, et al.** Metabolic dyslipidemia and risk of future coronary heart disease in apparently healthy men and women: the EPIC-Norfolk prospective population study. *Int J Cardiol*. 2010;143:399–404.
4. **Musunuru K.** Atherogenic dyslipidemia: cardiovascular risk and dietary intervention. *Lipids*. 2010;45:907–914.
5. **Sarwar N, Danesh J, Eiriksdottir G, et al.** Triglycerides and the risk of coronary heart disease: 10,158 incident cases among 262,525 participants in 29 Western prospective studies. *Circulation*. 2007; 115:450–458.
6. **McGill HC Jr, McMahan CA, Herderick EE, Malcom GT, Tracy RE, Strong JP.** Origin of atherosclerosis in childhood and adolescence. *Am J Clin Nutr*. 2000;72:1307S–1315S.
7. **Juonala M, Viikari JS, Rönnemaa T, et al.** Associations of dyslipidemias from childhood to adulthood with carotid intima-media thickness, elasticity, and brachial flow-mediated dilatation in adulthood: the Cardiovascular Risk in Young Finns Study. *Arterioscler Thromb Vasc Biol*. 2008;28:1012–1017.
8. **Morrison JA, Glueck CJ, Horn PS, Yeramaneni S, Wang P.** Pediatric triglycerides predict cardiovascular disease events in the fourth to fifth decade of life. *Metabolism*. 2009;58:1277–1284.
9. **Vodnala D, Rubenfire M, Brook RD.** Secondary causes of dyslipidemia. *Am J Cardiol*. 2012;110:823–825.
10. **Kathiresan S, Willer CJ, Peloso GM, et al.** Common variants at 30 loci contribute to polygenic dyslipidemia. *Nat Genet*. 2009;41: 56–65.
11. **Johansen CT, Kathiresan S, Hegele RA.** Genetic determinants of plasma triglycerides. *J Lipid Res*. 2011;52:189–206.
12. **Skeie B, Kvetan V, Gil KM, Rothkopf MM, Newsholme EA, Askanazi J.** Branch-chain amino acids: their metabolism and clinical utility. *Crit Care Med*. 1990;18:549–571.
13. **Yamakado M, Nagao K, Imaizumi A, et al.** Plasma free amino acid profiles predict four-year risk of developing diabetes, metabolic syndrome, dyslipidemia, and hypertension in Japanese population. *Sci Rep*. 2015;5:11918.
14. **Cheng S, Rhee EP, Larson MG, et al.** Metabolite profiling identifies pathways associated with metabolic risk in humans. *Circulation*. 2012;125:2222–2231.
15. **Mook-Kanamori DO, Römisch-Margl W, Kastenmüller G, et al.** Increased amino acids levels and the risk of developing of hypertriglyceridemia in a 7-year follow-up. *J Endocrinol Invest*. 2014;37: 369–374.
16. **Cheng S, Volgyi E, Tylavsky FA, et al.** Trait-specific tracking and determinants of body composition: a 7-year follow-up study of pubertal growth in girls. *BMC Med*. 2009;7:5.
17. **Sitruk-Ware R, Nath A.** Metabolic effects of contraceptive steroids. *Rev Endocr Metab Disord*. 2011;12:63–75.
18. **Moller SE, Moller BM, Olesen M, Fjalland B.** Effects of oral contraceptives on plasma neutral amino acids and cholesterol during a menstrual cycle. *Eur J Clin Pharmacol*. 1996;50:179–184.
19. **Lyytikäinen A, Lamberg-Allardt C, Kannas L, Cheng S.** Food consumption and nutrient intakes with a special focus on milk product consumption in early pubertal girls in Central Finland. *Public health nutrition*. 2005;8:284–289.
20. **Völgyi E, Alén M, Xu L, et al.** Effect of long-term leisure time physical activity on lean mass and fat mass in girls during adolescence. *J Appl Physiol*. 2011;110:1211–1218.
21. **Soininen P, Kangas AJ, Würtz P, et al.** High-throughput serum NMR metabonomics for cost-effective holistic studies on systemic metabolism. *Analyst*. 2009;134:1781–1785.
22. **Soininen P, Kangas AJ, Würtz P, Suna T, Ala-Korpela M.** Quantitative serum nuclear magnetic resonance metabolomics in cardiovascular epidemiology and genetics. *Circ Cardiovasc Genet*. 2015; 8:192–206.
23. **Goldstein H.** Efficient statistical modelling of longitudinal data. *Ann Hum Biol*. 1986;13:129–141.
24. **Miller M, Stone NJ, Ballantyne C, et al.** Triglycerides and cardiovascular disease: a scientific statement from the American Heart Association. *Circulation*. 2011;123:2292–2333.
25. **Imura K, Okada A.** Amino acid metabolism in pediatric patients. *Nutrition*. 1998;14:143–148.
26. **Arslanian SA, Kalhan SC.** Protein turnover during puberty in normal children. *Am J Physiol*. 1996;270:E79–E84.
27. **Lepage N, McDonald N, Dallaire L, Lambert M.** Age-specific distribution of plasma amino acid concentrations in a healthy pediatric population. *Clin Chem*. 1997;43:2397–2402.
28. **Gregory DM, Sovetts D, Clow CL, Scriver CR.** Plasma free amino acid values in normal children and adolescents. *Metabolism*. 1986; 35:967–969.
29. **Hammarqvist F, Angsten G, Meurling S, Andersson K, Wernerman J.** Age-related changes of muscle and plasma amino acids in healthy children. *Amino Acids*. 2010;39:359–366.
30. **Teodoro-Morrison T, Kyriakopoulou L, Chen YK, et al.** Dynamic biological changes in metabolic disease biomarkers in childhood and adolescence: a CALIPER study of healthy community children. *Clin Biochem*. 2015;48:828–836.
31. **Wallace M, Hashim YZ, Wingfield M, et al.** Effects of menstrual cycle phase on metabolomic profiles in premenopausal women. *Hum Reprod*. 2010;25:949–956.
32. **Newgard CB, An J, Bain JR, et al.** A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab*. 2009;9:311–326.
33. **Würtz P, Soininen P, Kangas AJ, et al.** Branched-chain and aromatic amino acids are predictors of insulin resistance in young adults. *Diabetes Care*. 2013;36:648–655.
34. **Wang TJ, Larson MG, Vasan RS, et al.** Metabolite profiles and the risk of developing diabetes. *Nat Med*. 2011;17:448–453.

35. **Perng W, Gillman MW, Fleisch AF, et al.** Metabolomic profiles and childhood obesity. *Obesity (Silver Spring)*. 2014;22:2570–2578.
36. **McCormack SE, Shaham O, McCarthy MA, et al.** Circulating branched-chain amino acid concentrations are associated with obesity and future insulin resistance in children and adolescents. *Pediatr Obes*. 2013;8:52–61.
37. **Bhattacharya S, Granger CB, Craig D, et al.** Validation of the association between a branched chain amino acid metabolite profile and extremes of coronary artery disease in patients referred for cardiac catheterization. *Atherosclerosis*. 2014;232:191–196.
38. **Shah SH, Bain JR, Muehlbauer MJ, et al.** Association of a peripheral blood metabolic profile with coronary artery disease and risk of subsequent cardiovascular events. *Circ Cardiovasc Genet*. 2010;3:207–214.
39. **Wiklund PK, Pekkala S, Autio R, et al.** Serum metabolic profiles in overweight and obese women with and without metabolic syndrome. *Diabetol Metab Syndr*. 2014;6:40.
40. **Cheng S, Wiklund P, Autio R, et al.** Adipose tissue dysfunction and altered systemic amino acid metabolism are associated with non-alcoholic fatty liver disease. *PLoS One*. 2015;10:e0138889.