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Aspects of human obesity

Evaluation of a clinical intervention program in overweight and obese children and studies in adults of underlying metabolic phenotypes of selected obesity genes

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Ph.D. thesis

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Abbreviations

^{1}H-MRS	proton magnet resonance spectroscopy
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BDNF	brain-derived neurotrophic factor
BMI	body mass index
CC	case control
Cm	centimetres
CT	computed tomography
CV	coefficient of variation
CVD	cardiovascular disease
DXA	dual-energy X-ray absorptiometry
FGIR	fasting glucose/insulin ratio
FTO	*fat mass and obesity associated*
GCKR	glucokinase regulator protein
GGT	gamma-glutamyl transpeptidase
GWAS	genome wide association study
HDL	high density lipoprotein
HOMA	homeostasis model assessment
HOXB5	homeobox A5
IR	insulin resistance
Kg	kilogram
L3	third lumbar vertebra
LD	linkage disequilibrium
LEP	leptin
LEPR	leptin receptor
LYPLAL1	lysophospholipase-like protein 1
M^2	meters square
MAF	transcription factor maf
MC4R	melanocortin 4 receptor
Mets	metabolic syndrome
MR	magnetic resonance

MSRA	methionine sulfoxide reductase A
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NCAN	neurocan
NRXN3	neurexin 3
OGTT	oral glucose tolerance test
OLFM4	olfactomedin 4
PC1	prohormone convertase 1
Ph.D	Doctor of Philosophy
PNPLA3	patatin-like phospholipase 3
POMC	proopiomelanocortin
QUICKI	quantitative insulin−sensitivity check index
SAT	subcutaneous adipose tissue
SD	standard deviation
SDC	Steno Diabetes Center
SDS	standard deviation score
T	tesla
T2DM	type 2 diabetes mellitus
TFAP2B	transcription factor activating enhancer-binding protein 2 beta
VAT	visceral adipose tissue
VOI	volume of interest
WC	waist circumference
WHR	waist/hip ratio
WHtR	waist/height ratio

Preface

I have submitted this thesis to obtain the degree of Doctor of Philosophy (Ph.D.) from The University of Copenhagen, Faculty of Health and Medical Sciences. The project has mainly been carried out at The Paediatric Department, Holbæk University Hospital, in The Children's Obesity Clinic, from January 2009 to October 2012 supervised by Associate Professor Jens-Christian Holm. The work has been performed in collaboration with the Novo Nordisk Foundation Center for Basic Metabolic Research, Section of Metabolic Genetics with Professor Oluf Pedersen and Professor Torben Hansen as supervisors. Furthermore, Professor Henrik S. Thomsen and Ph.D. Elizaveta Chabanova at The Department of Diagnostic Radiology at Herlev University Hospital have contributed with magnetic resonance examinations and have been very helpful and supportive.

The overall objective of this thesis is to examine childhood obesity with focus on non-alcoholic fatty liver disease and its treatment. Further, we aim to investigate the impact of genetic variation on obesity, especially abdominal obesity.

During the Ph.D. project I have contributed to nine papers [1-9].

The thesis is based on three papers representing clinical and experimental results obtained during the study. These three papers are enclosed in Appendix I and are the following:

1. Liver fat content investigated by magnetic resonance spectroscopy in obese children and youths included in multidisciplinary treatment. Bille D.S., Chabanova E., Gamborg M., Fonvig C. E., Nielsen T. R. H., Thisted E., Thomsen H. S., and Holm J-C. *Clinical Obesity* 2, 2012; pp 41-49.
2. Effect of a multidisciplinary intervention on ^{1}H-MRS measured hepatic steatosis in Danish overweight and obese children and youths. Bille D.S., Chabanova E., Gamborg M., Nielsen T. R. H., Fonvig C. E., Thisted E., Pedersen O., Hansen T., Thomsen H. S., and Holm J-C.
3. Implications of central obesity-related variants in *LYPLAL1, NRXN3, MSRA,* and *TFAP2B* on quantitative metabolic traits in adult Danes. Bille D.S., Banasik K., Justesen J.M., Sandholt C.H., Sandbæk A., Lauritzen T., Jørgensen T., Witte D.R., Holm J.-C., Hansen T., Pedersen O. *PLoS One*. 2011;6(6):e20640.

Contribution to the papers

Paper 1 and 2

Designed the studies together with Jens-Christian Holm. Responsible for protocol, approval from the ethical committee, data agency, and clinical trials. Responsible for recruiting and booking participants for the magnetic resonance examinations. Participated in the team performing the clinical examinations (measuring anthropometrics and drawing blood samples) and thus collecting data. Main responsible for data analyses and writing of the manuscripts.

Paper 3

Responsible for hypotheses, data-analyses and writing the manuscript.

Contents

1. Introduction

1.1 Epidemiology of childhood obesity

One of the greatest public health challenges of the 21st century is obesity and its co-morbidities. The number affected has increased at an alarming rate, particularly among children. According to the World Health Organization more than 1.4 billion adults were overweight in 2008. In 2010, more than 40 million children under the age of five were overweight. The most updated numbers in Denmark estimate that 16% and 12% of young girls and boys, respectively, are overweight or obese and among adolescents it is 25% of the girls and 19% of the boys, respectively [10]. Since the beginning of the new millennium the prevalence of obesity and overweight among schoolchildren has been stabilizing but a continued increase in prevalence remains among adolescents from high-income countries [11-14].

1.2 Defining obesity

Obesity is defined as an excess of body fat accumulation. In daily clinical practise it is difficult to measure the degree of obesity, but body mass index (BMI), has for practical reasons been used as a surrogate measure. However, it is recognized that the BMI association with mortality and morbidity risk may vary in different groups of ethnicity and age [15,16] and as growth and development influence BMI, the age and sex normalized percentiles or standard deviation (SD) of BMI are used in children and adolescents (Figure 1).

The limitations of BMI as a risk assessment tool have been recognized, and there is continued interest in identifying alternative or complementary indices linking obesity with disease risk. It is well established that abdominal visceral fat accumulation plays a central role in metabolic disorders associated with obesity [17]. Waist circumference (WC), waist-hip ratio (WHR), and waist-height ratio (WHtR) are often used as surrogate measures for the degree of intra-abdominal obesity [18-20].

The Expert committee recommendations in Pediatrics 2007:

Underweight: BMI <5th percentile

Healthy weight: BMI 5th–84th percentile

Overweight: BMI 85th–94th percentile

Obesity: BMI ≥95th percentile

Cut-offs supposed by the World Health Organization in 2007:

Overweight: BMI >+1SD (equivalent to BMI 25 kg/m^2 at 19 years)

Obesity: BMI >+2SD (equivalent to BMI 30 kg/m^2 at 19 years)

Thinness: BMI <-2SD

Severe thinness: BMI <-3SD

Figure 1 Definitions of childhood obesity according to BMI percentiles [21] and SD [22].

1.3 Aetiology of obesity

The aetiology of obesity is far from known in detail. The condition is characterized by excessive fat storage [18], causing fat depot size to increase beyond the normal range. Previously it was generally thought that the main reason, for developing obesity was a positive energy balance (energy intake exceeded energy expenditure). However, it seems increasingly clear that the interaction between calorie intake and calorie expenditure is in fact more complex. Obesity is a neuroendocrine disorder in which environmental risk factors and a genetic predisposition act in concerto. It develops as a result of interplay between a network of contributory components including social, cultural, psychological, metabolic, and genetic factors. Figure 2 illustrates a simplified model of some plausible factors influencing the risk of becoming obese.

Physical inactivity and intake of energy dense food are the most commonly accepted environmental factors driving the obesity epidemic and its co-morbidities. Yet, the accumulation of fat may also be a natural and beneficial process driven by the body, due to an anticipation of that may be a lack of energy at a later time, and that it is individually how much fat each person can accumulate before complications are manifested. Since some individuals accumulate more body fat and thus are more susceptible to become obese, great

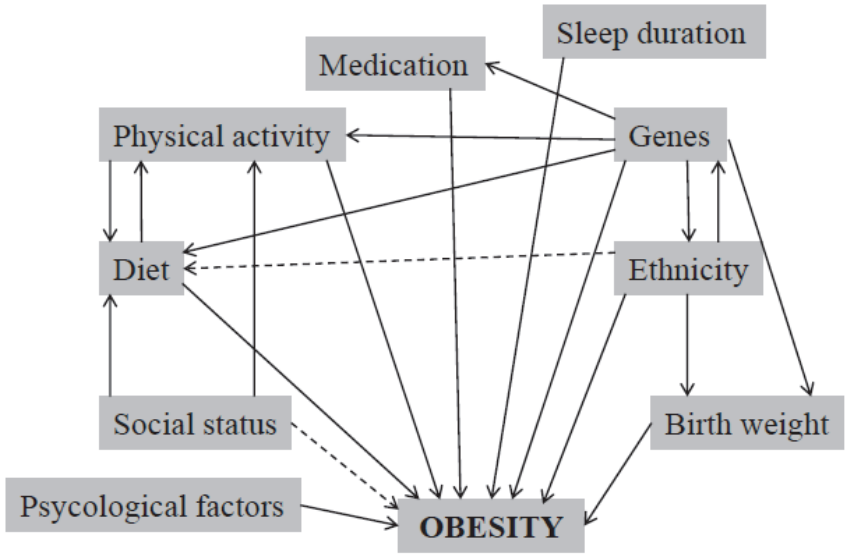

Figure 2 A simplified model of theoretical influencing factors on obesity.

efforts have been made to identify the predisposing genetic components. Through twin-, family-, and adoption studies it is estimated that 40-70% of the inter-individual variation in common obesity is caused by the proportion of phenotypic variance that can be attributed to genetic factors [23,24], and this genetic component appears to vary with age and may have a relative greater influence during childhood than adult life [25]. It is shown that the heritability estimates of BMI peak during childhood and especially at 7-8 years and 13-14 years of age [26], indicating that obesity during these periods of life are strongly influenced by genetic factors. The heritability of obesity as a function of age is illustrated in Figure 3.

1.4 Obesity and its co-morbidities

In obesity complex metabolic derangements occur often referred to as the metabolic syndrome (Mets). Several definitions of the Mets in childhood have been published [27-31] and in 2007 The International Diabetes Federation made a consensus report with the newest definition of Mets in children [32]. It is divided according to age-groups because of developmental challenges and is thus presented as age-related differences in children and

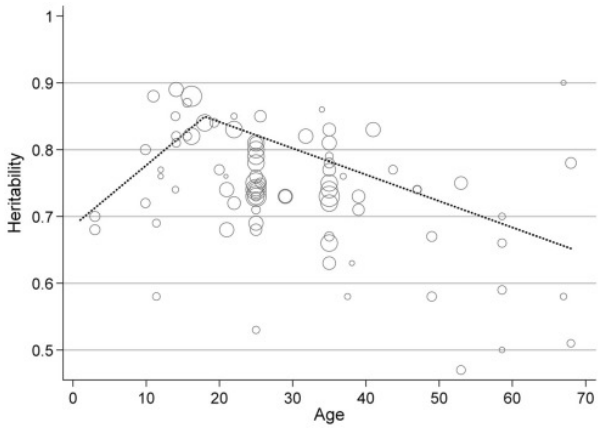

Figure 3 Predicted BMI heritability by age. The dotted line represents predicted BMI heritability by age, modeled using piecewise linear splines with a knot point at age 18 to separate childhood and adulthood. The figure shows that the relative contribution of genetic factors to variation in BMI increases over childhood before declining during adult life. Each circle represents an individual estimate of BMI heritability, and the size of the circle is proportional to the inverse of the SE of the heritability estimate. Age is based on the mean age of the study sample, or the mid-point of the age range where this was not reported. The figure is reprinted from Elks *et* al. 2012 [25].

adolescents. Furthermore, central obesity is the main variable of the Mets and the syndrome is diagnosed by increased WC and the presence of two or more of the following paraclinical/clinical features; elevated triglycerides, decreased high density lipoprotein (HDL)-cholesterol, high blood pressure, and increased plasma glucose.

Gaining weight in childhood drastically increases the risk of developing a number of obesity related complications such as mental disorders, underachievement in school and lower self-esteem, besides the Mets, cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), orthopaedic problems, sleep apnoea, and later in life increased risk of cancer [33]. Moreover, the inability to appropriately expand adipose tissue could be a possible explanation of the development of inflammation, insulin resistance (IR), and fat deposition in organs such as the liver and muscles.

1.4.1 Non-alcoholic fatty liver disease

The liver component of the Mets is non-alcoholic fatty liver disease (NAFLD) [34,35]. It is a clinicopathologic condition characterized by abnormal lipid deposition (accumulation of triglyceride) in hepatocytes in the abscence of excess alcohol intake. A combination of genetic and environmental factors is likely responsible for both the development of NAFLD and its progression from simple steatosis to non-alcoholic steatohepatitis (NASH). NAFLD represents a spectrum of diseases, from asymptomatic steatosis to cirrhosis (Figure 4) with complications such as liver failure and hepatocellular carcinoma [36].

NAFLD;	the more benign form of simple steatosis and mild inflammation or the summarizing term for the entire spectrum of the condition
NASH;	macrovesicular hepatocellular steatosis with portal inflammation, with or without portal fibrosis, in the absence of balloning degeneration and perisinusoidal fibrosis
Cirrhosis;	the most advanced stage of fibrosis Stage 3 = bridging fibrosis, stage 4 = cirrhosis

Figure 4 Definitions of the spectrum of clinic-pathological entities of non-alcoholic fatty liver disease

The first stage of NAFLD "simple benign steatosis" can be hypothesized as the capacity of adipose tissue in the liver being exceeded and in response to this, lipids being accumulated in the hepatocytes. This might lead to inflammation and in later stages, NASH and subsequent liver cell damage, fibrosis and scars in the tissue, which may ultimately lead to the development of hepatic cirrhosis [37,38].

The majority of lipids stored in the liver is triglycerides, however several other lipid types and metabolites also accumulate; e.g. free fatty acids, free and esterified cholesterol, and phospholipids [39,40]. The liver's ability to handle these excess amounts of lipids affects its function.

For children and adolescents age 2 to 19 years, the prevalence of fatty liver adjusted for age,

gender, race, and ethnicity is estimated to be 9.6%, but this increases to 38% among obese individuals [41]. Paralleling the increasing prevalence of obesity in the paediatric population, NAFLD is expected to become one of the most common causes of end-stage liver disease in both children and young adults. In a follow-up study spanning up to 20 years, it is shown that children with NAFLD have ~14-fold higher risk of dying or requiring liver transplantation than the general population of same age and sex distribution [42].

1.4.2 Diagnosing non-alcoholic fatty liver disease – tools and definition

In the diagnosis of NAFLD the fat content in the liver is evaluated. If more than 5% of the liver cells are infiltrated with micro- or macrovesicular fat, it is defined as simple steatosis. Early diagnosis of NAFLD in children may help prevent the development of liver disease during adulthood [43,44]. Invasive and non-invasive methods are used and their advantages and disadvantages are listed below.

Liver biopsy

The ultimate standard method diagnosing and staging NAFLD is liver biopsy [45]. Histological examination of biopsy samples can assess the presence of inflammation and fibrosis. Furthermore, it can differentiate between macro- and micro-vesicular steatosis. However, it is subject to sampling error due to histological heterogeneity and its ability to detect modest changes is limited [46]. Furthermore, it is expensive, invasive, and is unfortunately associated with the risks of morbidity and mortality [47].

Therefore non-invasive imaging techniques have been sought, developed, and used as surrogate measures of the liver fat content [48].

Proton magnetic resonance spectroscopy

Proton magnetic resonance spectroscopy (^{1}H-MRS) quantitatively measures intrahepatic triglycerides by differentiating between signals from lipids and water [2,49]. It is probably the most accurate method detecting fatty infiltration and it is safe and non-invasive, however, it is expensive [50]. Furthermore, ^{1}H-MRS is the best non-invasive method for repetitive measures of fat in the liver in the same individual The method has its limitations too, as it is not possible to define the degree of fibrosis in the liver.

Computed tomography

Computed tomography (CT) provides a semi-quantitative method for the evaluation of

intrahepatic triglycerides based on the change in image intensity between the liver and either the spleen, which stores no fat, or an external lipid standard [50]. An increase in liver:spleen ratio or liver density is indicative of reduced intrahepatic triglycerides. CT is not preferable in young individuals due to the exposure of radiation.

Ultrasonography

A liver ultrasound examination is useful for confirming steatosis. Fatty infiltration of the liver produces a diffuse increase in echogenicity and vascular blurring. Ultrasound exhibits considerable variability, it is operator dependent as a diagnostic method, limited in the ability of differentiating fibrosis from steatosis, and is consequently less precise in the quantification of fat accumulation in the liver [50,51].

By ultrasound elastography, the stiffness of the liver is measured and reflects the degree of fibrosis [52], unfortunately this method has its limitations too. In case of obesity the amount of subcutaneous adipose tissue (SAT) attenuates the ultrasound waves, and in general a considerable variation of stiffness in the liver is measured, influencing the accuracy of diagnosis of liver fibrosis [53].

Biochemical tests

In clinical practise elevated serum hepatobiliary enzymes (primarily alanine aminotransferase (ALT), aspartate aminotransferase (AST), AST/ALT ratio, and gamma-glutamyl transpeptidase (GGT)) are frequently used as screening tools for NAFLD. They are inexpensive and easily available analyses to perform. However, their sensitivities in regards to NAFLD are low [54].

IR is associated with NAFLD and a high serum insulin level could therefore indicate NAFLD. The measurement of insulin exhibits a higher sensitivity than the hepatobiliary enzymes but its specificity in regards to NAFLD is low [35].

1.4.3 Insulin resistance

In obese children and adolescents IR and glucose intolerance are frequently reported, and may lead to the development of T2DM and both conditions are highly associated with NAFLD [38]. It is discussed whether IR causes NAFLD or vice versa, but currently IR is considered as a consequence rather than the cause of NAFLD. IR occurs as a response to the physiological effects of insulin and is present in various tissues (liver, muscle, and fat). Factors such as

gender, age, ethnicity, pubertal stage, and degree of adiposity seem to influence IR. Various methods have been developed to determine insulin sensitivity and IR. Two models; the euglycemic hyperinsulinemic clamp or intravenous glucose tolerance test are considered to be gold standards for measurement of various aspects of whole body insulin sensitivity. However, they are complex and resource demanding invasive tests [55,56]. Surrogate measures of IR derived from an oral glucose tolerance test (OGTT) have been shown to be reasonably informative compared with a frequently sampled intravenous glucose tolerance test and a clamp test in determining insulin sensitivity [57,58]. However, the use of OGTT in large populations is limited. Therefore, methods such as fasting insulin level, fasting glucose/insulin ratio (FGIR), homeostasis model assessment (HOMA) for IR and quantitative insulin−sensitivity check index (QUICKI) are frequently used in population screening. HOMA−IR has been found to be more reliable than FGIR and QUICKI in determining IR in obese children [59].

1.5 Genetics of obesity

The use of genetic information in some metabolic disorders has been common practise for recent years. Yet, its application has, so far, been limited to diagnosis and prediction of mainly rare, often monogenic traits in some cases leading to a personalised treatment.

Monogenic or rare syndromic forms of obesity have been identified using family studies and have been demonstrated to be caused by mutations in leptin *(LEP),* leptin receptor *(LEPR),* proopiomelanocortin *(POMC)*, melanocortin 4 receptor (*MC4R)*, brain-derived neurotrophic factor *(BDNF)*, and prohormone convertase 1 *(PC1)*. Thus, most of the Mendelian obesity disorders (*LEP, LEPR, POMC*, and *MC4R*) cause abnormalities in the leptinergic-melanocortinergic system [60]. Besides these monogenic forms of obesity, the syndromic forms (single-gene-disorders), referring to a complex clinical syndrome in which obesity is only one of a constellation of physical and developmental anomalies (*e.g.* Prader Willi and Bardet-Biedl syndromes) also exit. The cases of non-monogenic or non-syndromic obesity are often a polygenic disorder in which the direct identification of the genetic background has been problematic. Yet, there is a constant development in methods to identify new genes, rare and common, predisposing to obesity.

1.5.1 The four waves of genome wide association studies in obesity

The extensive and rapid development of genetic technology has resulted in genome wide association studies (GWAS). The GWAS started in 2007 and investigated the genetic causes of many common diseases, including obesity [61-63]. Until now there have been four waves of obesity GWAS, using BMI as the adiposity phenotype, and most published GWAS have examined adult white European populations.

The first wave consisted of a study comprising ~5,000 individuals which identified the fat mass and obesity associated gene *(FTO)*. In realisation of the lack of statistical power meta-analyses of ~ 17,000 individuals were performed in the second wave and variants in or near *MC4R* were identified [64]. The third wave of obesity GWAS was dominated by a meta-analysis and an independent GWAS, both performed in ~32,000 individuals as well as a study of early-onset of extreme obesity [65-67] and ten loci associated with obesity were identified (Figure 5). In the fourth wave the meta-analyses were expanded to include a total of ~ 250,000 individuals [68], leading to identification of 18 new obesity loci, however, with decreasing effect sizes per adiposity risk allele as a consequence.

Parallel GWAS of additional measures of body adiposity, using WC and WHR, were performed. In 2009 the first wave of abdominal obesity GWAS was performed and four loci were demonstrated to associate with WC and WHR [69,70]. In the second wave ~ 77,000 individuals were included in the meta-analyses identifying 13 loci associating with WHR adjusted for BMI [71].

At present, a total of 32 loci have been found to predispose to overall adiposity (BMI) and 18 loci to central obesity (WC, WHR) (Figure 5). All of these loci associate with a p-value $<5x10^{-8}$, which is considered genome-wide-significant taking multiple comparisons into account.

1.5.2 Genetics of childhood obesity

Recently, a GWAS based on a meta-analysis (n~3,800) showed that nine loci identified to associate with BMI in adults also contributed to the determination of common obesity in children and adolescent [72], and another meta-analysis of 14 existing childhood obesity GWAS datasets (n~14,000) identified two new loci associating with early onset of obesity, rs9568856 in olfactomedin 4 *(OLFM4)* and rs9299 in homeobox A5 *(HOXB5)* [73]. Recently, 11 variants known to associate with adult BMI were investigated to explore their associations

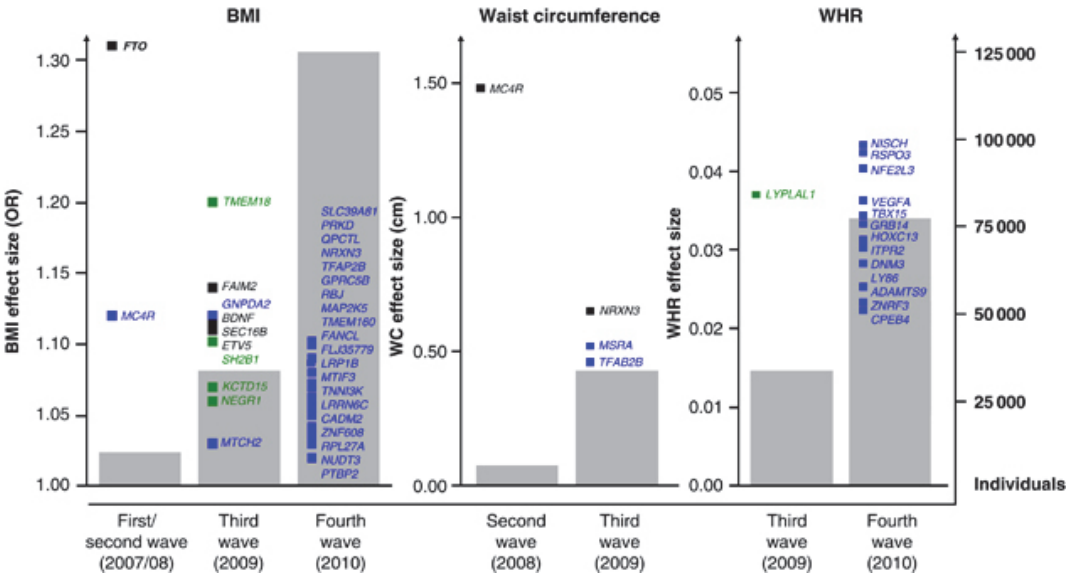

Figure 5 Development during the obesity GWAS waves. The progression of the four obesity GWAS waves (2007–2010); genome-wide significant associated loci associating with BMI, waist circumference and WHR, respectively, identified in individual GWAS (black), in both individual GWAS and meta-analysis (green) and in meta-analyses alone (blue). The number of identified genome-wide significant loci increases concurrently with an increase in individuals included in the studies (grey bars), having a decreasing effect size as a consequence (squares). Reprinted from Sandholt *et* al. 2012 [75].

with body size from birth to adulthood [74]. The strongest association of the variants with weight occurred at ages 11 and 20 years. These findings interestingly indicate that humans are being "programmed" during childhood to become susceptible to obesity or not in later adult life. More and larger study samples of children are needed to contribute to the identification of genetic variants causing childhood obesity. The EGG (Early Growth Genetics) consortium has been established. Such consortia including thousands of children should with meta-analysis of obesity phenotypes be able to add new knowledge of the metabolic impact of adiposity genes in the near future.

1.5.3 Genetic impact of weight loss

Multiple obesity-predisposing gene variants are known and might interact with weight change and the ability to maintain weight loss afterwards. Identifying variants that associate with

weight regain after a successful weight loss might help clinicians in targeting susceptible individuals who even though they have lost weight may require a long term follow-up to maintain their weight loss. Some studies have tested such hypothesis; e.g. if children carrying *MC4R* mutations exhibited a worse prognosis during lifestyle interventions [76,77] and similar hypotheses have been tested in adults [78]. In line with this, Reinehr *et* al. reported that children 5-16 years of age with functional *MC4R* mutations (n=9) were able to reduce their weight in a 1 year intervention to a similar degree as age and sex matched obese children without a *MC4R* mutation (n=46) [77]. However, 2 years after baseline, the children with *MC4R* mutations were unable to maintain their weight loss. Furthermore, exploratory evidence for sex specific effects of variants near *MC4R* has been observed for reduction in BMI SDS in a study of 889 children and adolescents [79].

1.6 Treatment of childhood obesity

It is important to acknowledge childhood obesity and its complications as major medical problems. In 2007 *The American Academy of Pediatrics*, *The American Medical Association*, and *The Centers of Disease Control and Prevention* established recommendations for prevention and treatment of childhood obesity [21,80-82]. At that time in Denmark; the paediatric departments had no national guidelines for the treatment of childhood obesity, several small projects were in progress at the municipal level focussing on treatment of childhood obesity [83], and financed by the revenue from public sale of Christmas seals, there was the well-established weight loss camps [84]. The weight loss camps offer a 10 weeks stay at one of the four Danish Christmas Seal Houses, where obese children 6-14 years of age recover from the stresses and strains that often result from troubles at home and school when being obese. During the stay the children are introduced to a new lifestyle where they exercise every day, eat healthy meals, but their parents are not directly involved in their new daily-life. Unfortunately, most of these individuals regain weight some time after they return to their daily life at home. In view of these aspects The Children's Obesity Clinic at the Paediatric Department, Holbæk Hospital was established in late 2007, providing a chronic care multidisciplinary intervention program for obese children and their families primarily in the Region of Zealand, but also for some children living other places in Denmark [5].

1.7 The Children's Obesity Clinic

The multidisciplinary children obesity team includes paediatricians, dieticians, nurses, psychologists, research technicians, social workers, and secretaries. The main aim of the clinic is to diminish the development of childhood obesity, to practise an obesity treatment for those with childhood obesity, and to ensure maintenance of compliance to anti-obesity therapy. To do so, the treatment regimens are based on a best-practice chronic care model, individualized programs, and involvement of the entire family. Furthermore, the clinic follows three criteria of success;

1. the patient shows up at appointments
2. the weight of the patient is not increasing but is stabilized
3. the patient's BMI is stabilizing or decreasing.

Furthermore, the Children's Obesity Clinic aims to establish a biobank for research of childhood obesity in order to optimise future childhood obesity treatment and prevention.

The patients in the Children's Obesity Clinic are referred from the general practitioners, other national paediatric departments, and school and municipality doctors. The criteria of inclusion are shown in Figure 6.

Criteria of inclusion

- Aged 2-18 years
- BMI > 90th percentile according to the Danish BMI charts (see Appendix III)
- Referred from general practitioners or health visitors

Figure 6 Criteria of inclusion for The Children's Obesity Clinic

1.7.1 Examinations in the Children's Obesity Clinic

A flow chart of the course of a patient within The Children's Obesity Clinic is illustrated in Figure 7. At the first visit, the patient is seen by a paediatrician for one hour resulting in a detailed medical history and a complete physical examination is performed. In an including dialogue involving the paediatrician, the patient, and his/her family a treatment plan is tailored comprising 10–20 treatment advices. The advices are proposed by the paediatrician and accepted by the patient and his/her family and consist of comprehensive oral and written

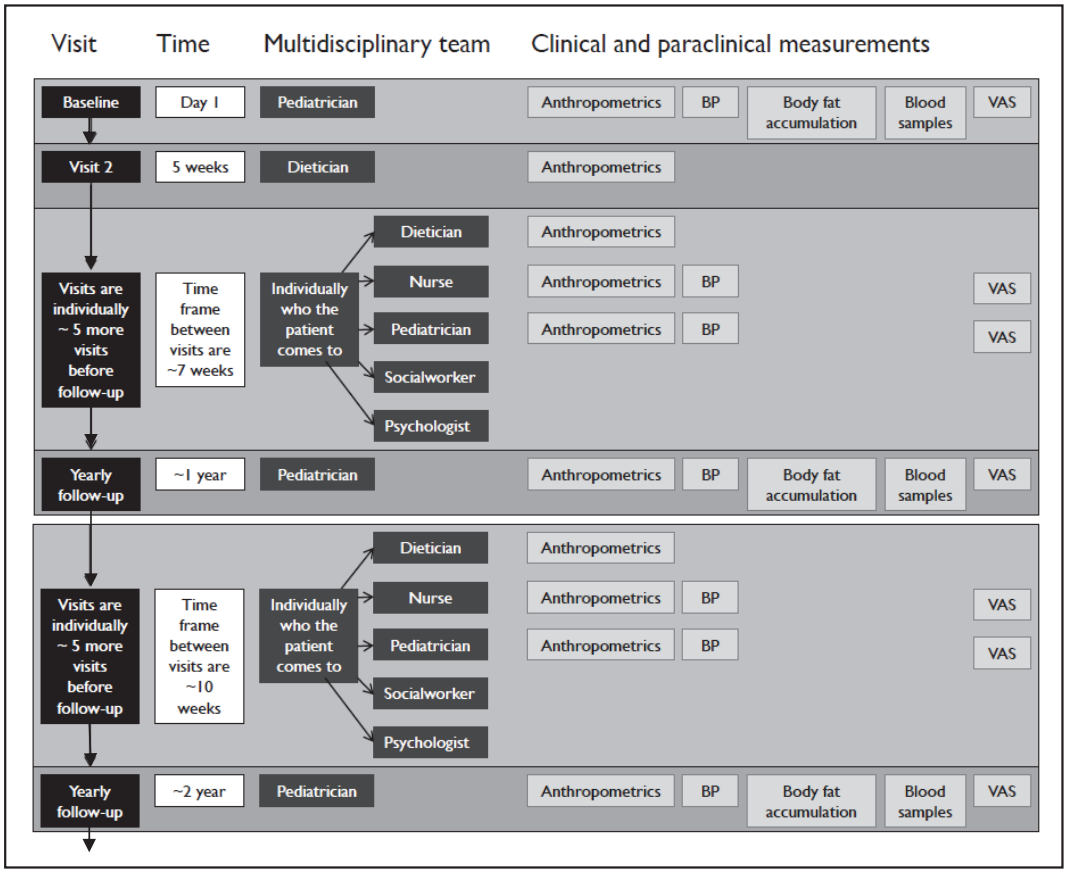

Figure 7 Flow chart showing the Children's Obesity Clinic's program. Anthropometrics include; height, weight, waist circumference, and hip circumference. Body fat accumulation include; magnetic resonance examination and dual-energy X-ray absorptiometry. Blood samples include measures of fasting thyroidal function, lipids, liver enzymes, haemoglobin, electrolytes, insulin, and glucose. Furthermore, buffy coat, serum and plasma is stored at -80 degrees Celsius in The Danish Childhood Obesity biobank. Visual analog score (VAS) includes that the patient scores itself according to the following variables: quality of life, mood, appetite, bulleting, self-esteem. This is performed by a VAS (illustrated by a line of 10 cm where there is a 0 at one end and a 10 at the other and the patient draw a X on the line, where they think, they are according the specific variable) at the visits at the paediatrician and the nurse. BP: Blood Pressure

advices on physical activity and inactivity, guidelines for sources of nutrition throughout the entire day, for weekends, and when eating outside of home, handling of eating disturbances including finicky eating, satiety training, and care of psychosocial functioning in cases of neglect [5]. The advice strategy is to fulfil as many of the advices as possible whereafter treatment is focused upon those changes that are most difficult to implement in the family.

Appointments for paraclinical tests, including fasting blood samples, dual-energy X-ray absorptiometry (DXA), and magnetic resonance (MR) assessments are made. After

approximately five weeks the patient and his/hers family are seen at a second visit in the clinic by a dietician, where the individualized treatment plan is modified. Advices primarily about food items are detailed. At the following visits, the plan is further modified according to the need and performance ability of the patient and family. In general, the focus of the visits is based on the hypothesis defined by the homeostatic endocrinological response to weight loss, which imposes reduced energy expenditure in complex physiological systems (i.e. growth, pubertal development, temperature, activity, immunology etc) with the aim to oppose weight loss and thus maintain a stable energy amount in the body. The following visits of the patients and the family to the clinic are upon individuals needs met by various members of the multidisciplinary team. Every child or adolescent has their "contact-doctor", meaning that one doctor is responsible for this patient with regards to treatment plan, paraclinically tests, and is the person of the team, who the patient always can contact.

1.7.2 Clinical and paraclinical measurements in the Children's Obesity Clinic

In the treatment of obesity, its co-morbidities are important to take into account as well. Therefore the Children's Obesity Clinic includes different clinical and paraclinical measurements in their protocol. These variables include anthropometrics, blood pressure, pubertal stage, biochemical measurements, and distribution of body fat. They are used in the monitoring of the patients. Furthermore, every child, adolescent, and their parents are asked if they want to be included in the research biobank, focusing on research in childhood obesity. If they give content the clinical and paraclinical measurements of the child are included in the Danish Childhood Obesity Biobank.

Anthropometrics

These measures are performed with the children or adolescents wearing no shoes and light indoor clothing. The Tanita Digital Medical scale (WB-100 MA, Tanita Corp., Tokyo, Japan) is used to measure body weight (in grams (g)) and a stadiometer for height (in centimeters (cm)). BMI is calculated as weight in kilograms (kg) divided by height in meters square (m^2). Weight standard deviation score (SDS), height SDS, and BMI SDS are calculated by the least mean square method [85]. WC is measured with a non-elastic anthropometric tape in cm, at the level of the umbilicus, at the end of a gentle expiration.

Blood pressure

Blood pressure is measured in the supine position after a rest of five minutes, using a size of cuff adjusted to the age and arm circumference of the patient. The measure is performed three times and the averages of the systolic blood pressure and the diastolic blood pressure are used.

Stages of puberty

Puberty stages (development of genitals and pubic hair in boys and development of breasts and pubic hair in girls) are assessed according to Marshall and Tanner [86].

Biochemical tests

After the first visit in the clinic the patient has a venous blood sample drawn from the antecubital vein after an overnight fast (eight hours). If required, a local anaesthetic cream is applied one hour before the blood sampling. Some biochemical analyses are performed immediately after venipuncture and include measures of thyroid function, lipids, liver enzymes, haemoglobin, electrolytes, insulin, and glucose. These analyses are performed at the Biochemical Departments at Holbæk and Ringsted Hospitals. In Appendix V the coefficient of variation (CV) for each variable is listed. If the patient has given consent, buffy coat, serum and plasma for the Danish Childhood Obesity Biobank are also drawn and stored at -80 degrees Celsius for future analysis.

Body fat accumulation

The excess of body fat accumulation defines obesity. Central obesity can be measured by WC, WHR, and/or WHtR, and general obesity is often expressed as BMI. Different compartments of fat depots are possible to assess by different methods, e.g. SAT, visceral adipose tissue (VAT), and liver fat content by MR and total body fat distribution by DXA investigations. We estimate the percentage of liver fat content, SAT, and VAT using an Achieva 3.0 tesla (T) MR imaging system (Philips Medical Systems, Best, the Netherlands) and a SENSE cardiac coil [2]. The MR examination is offered to the patients at their first visit in the clinic and is performed at the Diagnostic Department of Radiology, Herlev University Hospital, Denmark.

Yearly, the total body fat content is measured by DXA examination at Holbæk University Hospital.

2. Overall aim of the thesis

The establishment of The Children's Obesity Clinic made the present Ph.D. project possible. During my time as a Ph.D. student, I was a part of the team in the Children's Obesity Clinic and have followed 186 patients in the clinic for one year of treatment.

The overall objective of this thesis was to examine aspects of childhood obesity with focus on NAFLD and its treatment. Further, we aimed to contribute to studies of the genetics of obesity. The specific aims were to;

- describe the degree of NAFLD among overweight and obese Danish children (Study I).
- investigate the effect of a multidisciplinary clinically uncontrolled intervention for one year on liver fat content (Study II).
- analyze changes in liver fat content in relation to changes in levels of fasting blood variables to see if any of them could be used as a tool for monitoring hepatic steatosis in a clinical setting (Study II).
- investigate the association between selected genetic variants and measures of obesity in a Danish study sample (Study III).

2.1 Study populations

For the studies we used two study populations; a paediatric study sample (Study I and II) and an adult study sample (Study III).

2.1.1 The paediatric study sample

Individuals 6-20 years of age were recruited from the Children's Obesity Clinic. From February 2009 all new patients in the clinic were asked to participate in this project. Criteria of inclusions were:

- Age 6-20 years
- BMI above the 90th percentile for age and gender (~ +1.28 SDS) according to the Danish BMI charts

Criteria of exclusion were:

- Weight above 130 kg due to the space capacity of the MR machine

- Unable to remain calm in the MR machine during the examination

Figure 8 shows a flow chart for the inclusion of patients for Study I and Figure 9 shows a flow chart for the inclusion of patients for Study II.

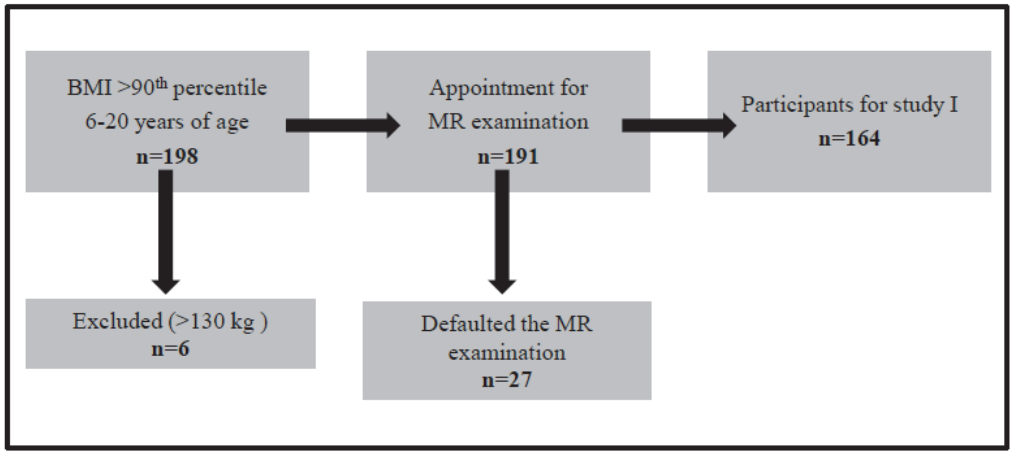

Figure 8 Flow chart for the inclusion of patients for study I

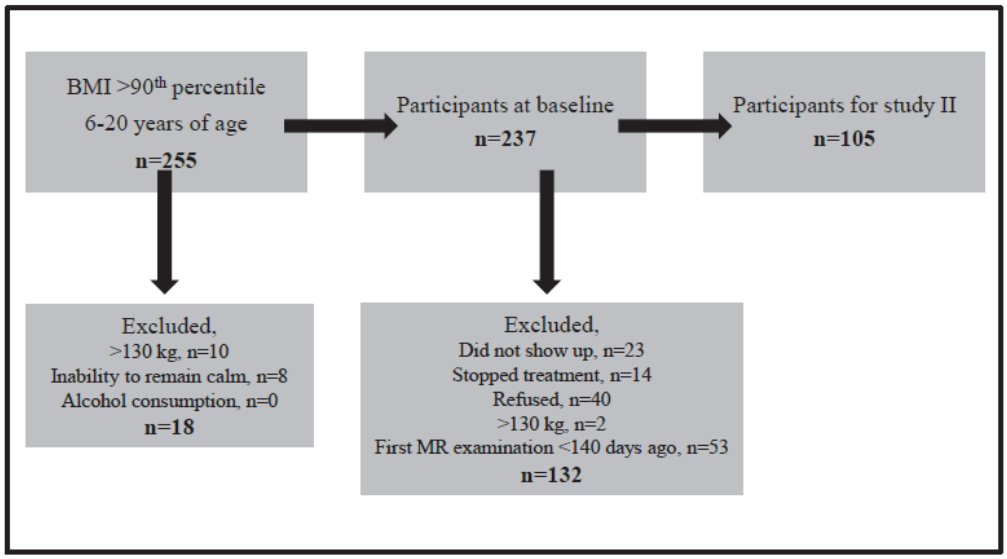

Figure 9 Flow chart for the inclusion of patients for Study II. The excluded 132 patients are the "lost-to-follow-up" group characterized in Table 1.

Informed written consent was obtained from all patients aged 18 years and older and from the parents of children younger than 18 years. The study was approved by the Danish Data Protection Agency and the Ethics Committee of the Region Zealand in Denmark (ID-no.: SJ-98) and is registered at ClinicalTrials.gov (ID-no.: NCT00823277). Simultaneously with this project the Children's Obesity Clinic recruited patients for the Danish Childhood Obesity Biobank (ID-no.: SJ-104 and ClinicalTrials.gov (ID-no.: NCT00928473) and some of these patients are also included in the studies included in the present thesis.

2.1.2 The adult study sample

Study III was performed in adult Danes. Four different study groups were used: 1) the Inter99 cohort from Center of Prevention and Health, Glostrup Hospital (n=6,162) [87], 2) a cohort comprising individuals with type 2 diabetes sampled at Steno Diabetes Center (SDC) (n=1,695), 3) a randomized, population-based group of unrelated middle-aged individuals examined at SDC, Copenhagen (n=730), and 4) The Danish ADDITION screening cohort (n=6,739) [88].

3. Study I

clinical obesity doi: 10.1111/j.1758-8111.2012.00038.x

Liver fat content investigated by magnetic resonance spectroscopy in obese children and youths included in multidisciplinary treatment

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The article is included in Appendix I, page 73.

3.1 Background and objective

Since obesity and NAFLD are strongly correlated we aimed to investigate the degree of hepatic steatosis in a cohort of overweight and obese, Danish children from the Children's Obesity Clinic. Further the associations with liver fat content and the amount of abdominal SAT and VAT, metabolic phenotypes, and biochemical measures of liver enzymes were analysed.

3.2 Methods and Materials

3.2.1 Participants

In total 164 patients were included in the present study (Figure 8). Anthropometric measures and blood samples obtained within 120 days from the date of the MR scanning were available in a subgroup of 124 of the participants. WHtR was calculated by dividing WC (cm) by height (cm).

3.2.2 MR examination

All MR measurements were performed by highly skilled MR-trained personnel from the Diagnostic Department of Radiology, Herlev University Hospital, Denmark, using an Achieva 3.0 T MR imaging system (Philips Medical Systems, Best, the Netherlands) and a

SENSE cardiac coil [2]. Based on paediatric studies hepatic steatosis was defined as a liver fat content above 5% (steatosis-5%) and 9% (steatosis-9%) [54,89-97].

During the examination the patient was placed in the supine position. Liver fat content was measured by ^{1}H-MRS. T2-weighted turbo spin echo coronal and axial slices taken through the abdomen were used to position the spectroscopy volumes of interest (VOI). The spectroscopy VOI was determined individually and was positioned over the middle of the right lobe of the liver. The MR scanner's software was used to fit the acquired spectrum to the relative content of water and lipid. Spectroscopic hepatic lipid content was expressed as lipid content relative to water and was calculated as: spectroscopic fat (%) = [fat metabolite area / (fat metabolite area + water metabolite area)] x 100, Figure 10.

Visceral and subcutaneous fat volumes were measured by MR scanning. A transverse slice of 10 mm thickness was acquired for all subjects in the middle of the third lumbar vertebra (L3). The volumes of subcutaneous and visceral fat at L3 were measured in cm^3 using 'segmentation tool' in 'volume analyses' on the Philips ViewForum workstation, Figure 11.

3.3 Results and discussion of Study I

Steatosis-5% and steatosis-9% were found in 45% and 27%, respectively of the 164 patients. Liver fat content associated with SAT, VAT, BMI SDS, WHtR, elevated levels of fasting serum ALT and GGT, and low SAT/VAT ratio.

3.3.1 Paediatric non-alcoholic fatty liver disease in Denmark

The histology definition of NAFLD is defined as 5% or above of hepatocytes containing macrovesicular fat. However, conflicting views of the definition of NAFLD diagnosed by non-invasive methods exist. Based on paediatric studies [54,89-97], we decided to use liver fat content of 5% and 9% as limits for hepatic steatosis. The degrees of hepatic steatosis of 45% and 27%, respectively, found in this sample of Danish overweight and obese children and adolescents were comparable with other studies of similar study groups of ethnicity and age, and were in concordance with our hypothesis. After this study was performed, another Danish study has recently shown that 43% of a study sample of 117 obese children had increased liver echogenity assessed by ultrasound [98], supporting our finding that a substantial proportion of obese Danish children suffers from NAFLD.

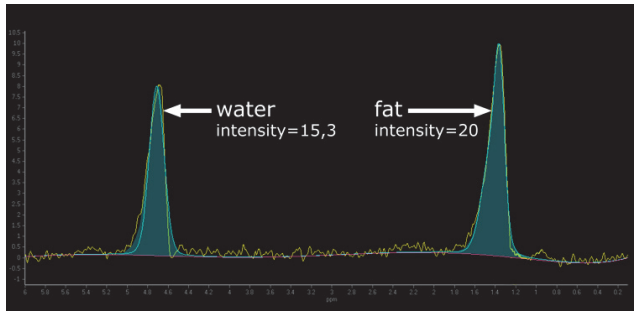

Figure 10 The 1H-MRS spectrum showing contents of water and fat in the liver

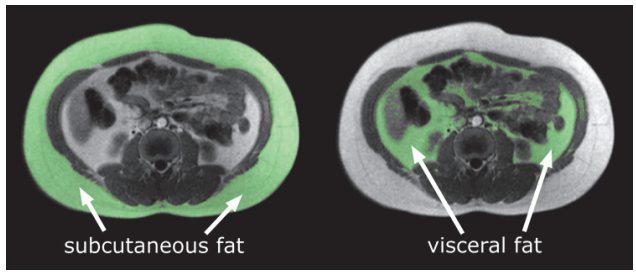

Figure 11 Abdominal subcutaneous and visceral adipose tissue obtained by MR examination

3.3.2 Central obesity and non-alcoholic fatty liver disease

Furthermore our results showed that the degree of hepatic steatosis was associated with measures of central obesity e.g. increased WHtR, high VAT, and low SAT/VAT. Our results strongly support the hypothesis that central obesity is highly associated with hepatic triglyceride content. We found that the amount of VAT was higher among those with steatosis-9% than those with steatosis-5%. In this study we also used WHtR as a surrogate measure of central obesity. When WC is measured in conjunction with height, it gives an index which may be a good measure for intraabdominal fat. In children this ratio is relevant as it can be used without adjustment for sex or age. The WHtR is a relative newly developed index, proposed to be superior to the BMI and WHR, mainly because of its relationship to

cardiovascular risks [18]. Our findings indicate that WHtR may be related to NAFLD underlining the usefulness of the index in clinical settings.

3.3.3 Biochemical measurements

We find significant differences in fasting serum ALT and GGT levels, ranging from 34%-63%, when comparing the non-steatosis and steatosis groups. The intra-individual CV of the biochemical analyses is 4% (Appendix IV) for each variable suggesting that our findings are reliable [99].

3.3.4 Strengths and limitations

Strengths of this cross-sectional descriptive study are the relative large number of included patients and that they were investigated by ^{1}H-MRS assessment, which is considered a superior non-invasive method for diagnosing NAFLD. However, there are also limitations. We had to exclude those patients with weight above 130 kg, since they could not enter the MR machine. However, this group is at risk of having the most pronounced degree of NAFLD which may have led to an underestimation of the prevalence of NAFLD among overweight and obese Danish children and adolescents included in obesity treatment. Due to this concern, we performed a study, where we compared hepatic fat fractions obtained using an open 1T system with assessment with 3T ^{1}H-MRS and found that an open 1T system is feasible in the assessment of liver fat content in obese individuals with a weight above 120 kg [3]. Even though, the paraclinical and clinical variables were scheduled to be measured at the first visit in the clinic, Figure 6, anthropometrics and blood samples were only available in 124 of the participants. This was due to logistic challenges as the patients had to go to Herlev (60 kilometres from the Children's Obesity Clinic) for the MR examination. Further, blood samples were drawn in the morning in order to obtain fasting conditions. In accordance to these procedures the patient and his/hers family had to come three times for different examinations for this study; 1) visit in the clinic, 2) blood samples, and 3) MR examination. Due to school, job and other practical and logistic consideration the time interval between these visits differs. For future treatment program and research the children and adolescents will have their blood samples drawn and the MR examination performed within 2 weeks from their first visit in the Children's Obesity Clinic.

This descriptive study concludes that the prevalence of NAFLD among overweight and obese

children and adolescents in Denmark is comparable with similarly studies of paediatric study samples. Forty-five % of the children had liver fat content above 5% measured by ^{1}H-MRS. As we had now investigated the prevalence of paediatric NAFLD at baseline, we also aimed to investigate if the Children's Obesity Clinic's obesity-treatment had effect on their degree of hepatic steatosis. In line of this, Study II was established.

4. Study II

Effect of a multidisciplinary intervention on ^{1}H-MRS measured hepatic steatosis in overweight and obese Danish children and youths

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The manuscript draft is included in Appendix I, page 85.

4.1 Background and objective

The evidence regarding treatment of children with fat deposition in the liver is limited. In view of the increasing prevalence of paediatric NAFLD, developement of appropriate treatments for this disorder should be an important priority in the health care system. The most efficient treatment of paediatric fatty liver disease has been suggested to be lifestyle changes and subsequent weight loss [43,98,100-106], and lifestyle interventions should always be the first choice in the treatment of this condition [107,108].

This study aimed to investigate the effect of an observational childhood-obesity-treatment program upon changes in liver fat content measured by ^{1}H-MRS and its associations with related metabolic traits.

4.2 Methods and Materials

4.2.1 Participants

Figure 9 shows a flow diagram for inclusion of participants for this study. In total 237 patients were included at the baseline. In Appendix IV baseline characteristics of these 237 patients are shown. At the time for follow-up, we had data on 105 patients and 132 patients were "lost-to-follow-up". In Figure 9 the reasons for these "lost-to-follow-up" individuals are listed. This group were elder ($p<0.001$), however, there were no significant differences in

their BMI SDS, WHtR, or liver fat content compared to the 105 participants included in the study (Table 1).

In a subset of 41 individuals we had biochemical measurements drawn within 3 months from the date of the MR scanning.

Table 1 Main characteristics of the group of included participants and the group of "lost-to-follow-up" individuals at baseline

	Included participants	"lost-to-follow-up"	p-value
N (boys/girls)	105 (45/60)	132 (61/71)	
Age (years)	12.2 (6.6-20.4)	14.0 (6.5-20.8)	<0.001
Weight SDS	4.08 (2.90-4.55)	3.99 (2.90-4.56)	0.04
Height SDS	0.80 (-1.52 -2.90)	0.83 (-2.12-3.25)	0.82
BMI SDS	2.93(1.32-5.20)	3.02 (1.42-4.39)	0.15
WHtR	0.62 (0.51-0.81)	0.63 (0.47-0.77)	0.13
Liverfat percentage (%)	4.2 (0.2-67.1)	4.5 (0.1-69.0)	0.59

Data are medians (ranges). P-values calculated by Mann-Whitney U test.
BMI; body mass index, SDS; standard deviation score, WHtR; waist/height ratio

4.3 Results and discussion of Study II

The present study showed that the treatment offered by the Children's Obesity Clinic is associated with diminished BMI SDS and also the degree of liver fat content measured by ^{1}H-MRS.

4.3.1 Paediatric intervention studies of non-alcoholic fatty liver disease

Some of the first studies investigating the effect of an intervention on paediatric NAFLD were in the 1990's by Vajro *et* al. and Franzese *et* al. [101,105]. By ultrasound and biochemical measurements they diagnosed NAFLD in 9 and 38 children, respectively and followed them with lifestyle intervention for 12 and 6 months, respectively. A positive effect was shown in one of the studies [105]; however it was only performed in 9 patients. The other study did not find any effect of lifestyle interventions on NAFLD [101]. Other studies have followed, with interventions of physical activity, diet advices, behaviour therapy, and during the last years the effects of pharmacological treatments have been tested as well. However, these studies

have demonstrated a variable effect in treatment of paediatric NAFLD (Table 2). In the studies summarized in Table 2 the intervention periods differ from one month to two years. The diagnostic tools used were mostly ultrasound [98,100-106]. Unfortunately, less precise quantification of fat accumulation in the liver is recognized to be exhibited by ultrasound, making the comparison with our study more difficult. Three of the studies had liver biopsy [89,109,110], in which it was possible to measure the development and degree of steatosis and fibrosis. We were not able to perform this differentiation in our study as we used ^{1}H-MRS as a diagnostic tool. However, our study contributes to the knowledge of how the content of liver fat in paediatric obese patients can be reduced and evaluated in a clinical setting focusing on changes of lifestyle elements.

4.3.2 Serum AST and serum ALT

Interestingly, we found that the serum AST/ALT ratio might be useful in monitoring paediatric patients with suspected or verified hepatic steatosis. The AST/ALT ratio was first described as a clinical screening tool for identification of cirrhosis in chronic hepatitis C patients [111,112] and have ever since been used in different groups of patients susceptible to cirrhosis. Separately, serum levels of ALT and AST are recognized to associate with NAFLD and hepatic steatosis but with low sensitivities. Nevertheless, these serum variables are still included as markers in a recent published guideline for treatment of fatty liver in the paediatric clinic [99]. Yet, our results indicate that it would be more appropriate to use their ratio. Still, caution is much needed since our data are based on our subgroup of 41 individuals why replication studies preferable with much more statistical power are needed.

4.3.3 Insulin resistance and non-alcoholic fatty liver disease

Obesity and T2DM are associated with hyperinsulinemia and IR, which may inhibit fatty acid oxidation and increase the presentation of fatty acids to the liver [113]. Hyperinsulinemia has been suggested to contribute to the development of fatty liver in childhood obesity [114] and the severity of hyperinsulinemia is predictive of the degree of steatosis, inflammation and fibrosis. Hence, IR is likely a principal part of the chain of events leading to paediatric NAFLD. Because of the metabolic and endocrine changes of growth spurts during infancy and puberty, insulin at high concentrations at various developmental windows may be physiological rather than pathological [115,116]. Such as HOMA−IR cut−off values are

Table 2 Clinical intervention studies in paediatric NAFLD

Description			Treatment	Diagnostic tool				Effect^	Reference
Study design	*Duration*	*Group n (age)*		*Biop-sy*	*MR*	*Ultra-sound*	*Blood variables*		
Prospective study	12 months	9 (4-11 years)	Intervention (P.A., diets)			X	X	+	Vajro P., 1994 [105]
Prospective study	6 months	72[$] (4-15 years)	Intervention (P.A., diets)			X	X	0	Franzese A., 1997 [101]
Prospective study	12 weeks	73 (15-19 years)	Intervention (P.A., diets)			X		+	Tock L., 2006 [104]
Prospective study	12 weeks	43* (15-19 years)	Intervention (P.A., diets, b.t.)			X		+	De Piano A., 2007 [100]
Randomized, control trial	1 month	76 (10-17 years)	Intervention (P.A., diets) vs vitamin E			X		0	Wang C.L., 2008 [106]
Prospective, CC-study	Intervention 1year, fol.up 2 years	Cases= 109, controls= 51 (6-16 years)	Intervention (P.A., diets, b.t.) vs controls			X		+	Reinehr T., 2009 [103]
Prospective study	6 months	144 (mean 14 years)	Intervention (P.A., diets, b.t.)			X		+	Koot, B.G.P., 2011 [102]
Prospective study	Intervention 10 weeks, fol.up 1 year	117 (mean 12 years)	Intervention (P.A., diets)			X		+	Grønbæk H., 2012 [98]
Prospective CC-study	2 years	Cases=30, controls= 30 (9-18 years)	Intervention (P.A., diets) vs metformin	X				+	Nobili V., 2008 [110]
Double-blind randomized study	6 months	50 (12-18 years)	Intervention (diets) vs metformin			X		+ ~	Nadeau K.J., 2009 [117]
Double-blind randomized trial	96 weeks	173 (8-17 years)	Metformin or vitamin E vs placebo	X			X	0	Lavine J.E., 2011 [109]
Prospective study	12 months	25 (7-16 years)	Intervention (P.A., diets)	X	X	X	X	+	Pacifico L., 2011[89]

P.A.= physical activity, b.t.= behaviour therapy, cc=case control, MR=magnetic resonance

[$] 38 of the children had NAFLD

*13 of the children had NAFLD

^ +=decrease in NAFLD, 0=no change in NAFLD, ~ significantly better in the group received metformin

expected to be different in pre-pubertal and pubertal children [118]. Taking this into account we adjusted for pubertal stages in the analyses. However, we were not able to find a significant improvement in the serum insulin levels at the end of the intervention. At the baseline IR (predefined as HOMA-IR > 4.39) was found in 30% of the children and adolescents and in 24% at follow-up. Yet, the cut-off used in this study is high compared to other studies, indicating that the 30% of patients we found exhibiting IR might be underestimated.

4.3.4 Strengths and limitations

Strengths of the present study include the use of ^{1}H-MRS for measuring liver fat content and the relative large number of individuals (n=105). As the study population was recruited in an on-going treatment clinic the follow-up period of median one year which is strength as well as a limitation. The range was from 147 to 520 days. This is due to the challenges occurring in a clinical setting where patients cancel their appointments; the dates are changed or dismissed etc. However, this is "real life" conditions and the results reflect a treatment program in the Danish health care system. In addition, another limitation of the present prospective study was that we did not have blood samples from all of our participants at the same time as the MR examinations were performed. Furthermore, the ^{1}H-MRS measurements of liver fat content were not able to differentiate between the histology stages of NAFLD, e.g. fibrosis and cirrhosis. In the present study we also identified patients, who did not reduce their liver fat content, and this group should be offered an even more intensified and individualized treatment regimen. Recently, it has been demonstrated that children with NAFLD have an increased sensitivity of dietary fructose [119] and that the consumption of sucrose-sweetened soft drinks is highly associated with fat content in the liver [120], suggesting that fructose and the consumption of sucrose-sweetened soft drinks should be evaluated in the treatment in obese children.

Furthermore, the study was not designed as a randomized case-control study. To evaluate a clinical set up like the treatment in the Children's Obesity Clinic, it would have been optimal with a randomized clinically controlled study. Yet, we find it ethically incorrect to ask obese individuals and their families if they want to participate in a study where they might be in the group without treatment for more than a year. The patients in our clinic have waited for up to 8 months to attain their first appointment. The lack of a control group of normal weighted

children is another limitation of the protocol. The Children's Obesity Clinic now recruits schoolchildren from the Holbæk area to establish a control study group. The control group is featured by anthropometrics, bio-impedance for total body fat content, fasting biochemical variables, and MR examination.

In conclusion, the treatment offered by the Children's Obesity Clinic not only reduces average BMI SDS but also reduces the degree of liver fat content measured by ^{1}H-MRS in a substantial subset of the overweight and obese children and adolescents. Development of improved and sustained long-term therapeutic care of these at-risk children will require clinically controlled interventions which likely will be based on various multifactorial treatment approaches combining individually tailored family behaviour modification and maybe drug intervention.

5. Study III

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PLoS one

Implications of Central Obesity-Related Variants in *LYPLAL1, NRXN3, MSRA,* and *TFAP2B* on Quantitative Metabolic Traits in Adult Danes

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The article is included in Appendix I, page 109.

5.1 Background and objective

To provide new insights into the genetic background and ideally also the pathophysiology of obesity, GWAS of various adiposity measures have been performed. When GWAS were first introduced, it was widely used to validate a susceptibility locus in independent study samples since no consensus regarding significance level did exist producing some non-replicable findings. The genome-wide-significant threshold ($p>5x10^{-8}$) was first introduced in the third and fourth wave of the obesity GWAS due to the challenges finding independent large enough study samples.

The present study was performed to replicate susceptible loci from the first GWAS wave of central obesity [69,70]. Further, we aimed to elucidate potential underlying phenotypes for these variants and investigate whether the suggested variants associated with: 1) quantitative metabolic traits, 2) anthropometric measures (WC, WHR, and BMI), or 3) T2DM.

5.2 Methods and Materials

We genotyped rs2605100 near lysophospholipase-like protein 1 (*LYPLAL1)*, rs10146997 in neurexin 3 *(NRXN3)*, rs545854 (former rs7826222) near methionine sulfoxide reductase A *(MSRA)*, and rs987237 near transcription factor activating enhancer-binding protein 2 beta (*TFAP2B)* in a total of 15,326 individuals. The risk-allele frequencies were 70% (rs2605100 G-allele near *LYPLAL1*), 21% (rs10146997 G-allele in *NRXN3*), 15% (rs545854 G-allele near *MSRA*), and 17% (rs987237 G-allele near *TFAP2B*) and all genotyped variants obeyed Hardy-Weinberg equilibrium ($p>0.05$),

5.3 Results and discussion of Study III

Interestingly, rs2605100 near *LYPLAL1* associated with elevated concentrations of fasting serum triglycerides and fasting serum insulin and with estimates of central obesity. The association with fasting serum triglycerides was restricted to men. rs10146997 in *NRXN3* associated with increased WC among women. Neither the rs545854 near *MSRA* nor the rs987237 near *TFAP2B* showed any significant associations in our study sample. The inability to validate the findings of the GWAS likely reflects the lack of statistical power as a consequence of the relatively low effect sizes exerted by the variants and our relatively small study sample compared with the discovery sample [69,70]. At present no reports are available about attempts to verify our findings of obesity gene variants to intermediary phenotypes. However, after publication of our study, *TFAP2B* rs987237 has been found to associate with childhood BMI in a replication study of 658 children and adolescents [121]. Furthermore, in a meta-analysis of 2,258 individuals *MSRA* was found to associate with early onset of obesity [122].

5.3.1 Associations with other obesity related phenotypes than WC or WHR

The four loci investigated in the present study are now all validated obesity associated loci. After the publication of this paper the metabolic impact of the loci has been elucidated in different studies. Variants near *LYPLAL1* have been investigated in association with different

metabolic risk factors e.g. an association with an increased risk of T2DM of 9% was found [123], an association with VAT/SAT has been published [124], and another variant near *LYPLAL1* (rs4846567) has achieved genome-wide significance for WHR and associates with increased levels of fasting serum triglycerides, fasting serum insulin, and HOMA-IR [71,125]. These findings are in line with previous knowledge that a higher fat-mass causes increased lipogenesis, resulting in higher levels of circulating triglycerides and free fatty acids, which are major contributor to IR [126]. The association found with *LYPLAL1* and increased levels of serum triglycerides may result from an increased expression of the lipase gene, which then facilitates higher triglyceride lipase activity in the adipose tissue. These mechanisms are also involved in the development of NAFLD where the main component of fat in the hepatocytes is triglycerides. rs12137855 near *LYPLAL1* has been reported to associate with histological NAFLD [127]. In summary *LYPLAL1* has now been found to associate with central obesity (increased WC and VAT/SAT) and the related metabolic abnormalities as T2DM, IR, and NAFLD.

A study of whether or not sleep deprivation can affect body weight modified by variants has been performed. Variants of *NRXN3* have been investigated in such interaction in children. rs10146997 associated nominally with shorter sleep duration as well as with BMI, WC, VAT, and HOMA-IR [128].

5.3.2 Influence of obesity loci on weight loss

Genetic studies can also contribute to the knowledge of targeting susceptible individuals for individual weight loss intervention. Recently, *TFAP2B* rs987237 was investigated in a study of weight loss in 580 obese men and women on a 10-weeks energy restricted diet low or high in fat to carbohydrate content [129]. The variant clearly modified the effect of a high versus low-fat diet on weight loss. Whilst non-carriers of the obesity risk-allele lost more weight on the low-fat diet (1.0 kg (95% CI, 0.4; 1.6)), the opposite was shown among homozygotes of the obesity risk-allele (2.6 kg (1.1; 4.1)) (interaction p-value; p=0.00007), indicating that *TFAP2B* may influence the effect of dietary fat on weight loss under energy restriction. If confirmed in independent studies, this finding might be one of the first examples of how knowledge of some aspects of the genetics of common forms of obesity may be useful in tailoring individual weight loss strategies.

5.3.3 Strengths and limitations

One strength of the present study is the relatively well characterised phenotypes of study participants. The study did not only aim to replicate the findings from a GWAS but also to elucidate underlying intermediary phenotypes. Even though our study samples included ~15,000 individuals our statistical power was not sufficient for some of the analyses due to the relatively low effect sizes of the selected gene variants.

6. Perspectives

The Children's Obesity Clinic is now an established unit at the Paediatric Department, Holbæk Hospital, and is continuously recruiting patients for childhood obesity treatment and the Danish Childhood Obesity Biobank. To further optimize treatment of childhood obesity it is important to evaluate the effect of the given intervention. Recently, we have optimized the time between visits, meaning that the child or adolescent are now getting their blood samples taken at the same day as the first visit and the MR assessment is performed within 14 days of the first visit. Furthermore, paediatricians are more aware of the fact that NAFLD is a co-morbidity to childhood obesity that deserves special attention as well.

6.1 Measuring body fat composition

BMI and WC, WHtR, and WHR have been the applied measures of general obesity and central obesity, respectively, used in the clinical and genetic studies Direct quantification of of the adipose tissue is, however, of major relevance since it directly associates with various metabolic phenotypes and co-morbidities of obesity e.g. NAFLD, as shown in the present thesis. By MR it is possible to measure SAT and VAT. However, MR examinations are often not doable in large-scale studies. Bio-impedance in combination with WC, WHR, and WHtR might be a realistic alternative which can be used in the clinic and for research. Interesting, in the field of genetics, GWAS of the obesity measures of SAT and VAT distributions have now been published [124]. Also it may be envisioned that in future studies of obesity genetics, functional imaging studies of hypothalamic regions will be novel obesity traits of major interest.

6.2 Identification of factors predicting weight loss

Preliminary analyses have been made to investigate whether it would be possible to predict which obese children and adolescents who would benefit the most from the multidisciplinary therapeutic package as implemented at the Children's Obesity Clinic. Hyperinsulinaemia is strongly associated with obesity, especially central obesity and NAFLD and it is relative easy to measure fasting serum insulin. In preliminary analyses of 314 individuals we found that a

high fasting serum insulin concentration at the baseline might be a predictor for the inability to reduce BMI SDS. The highest tertile of fasting serum insulin was associated with less BMI SDS reduction (-0.06 BMI SDS) compared with the lowest tertile (-0.31 BMI SDS) (p=0.001) of fasting serum insulin concentration. In total there is a significant difference between the change in BMI SDS and the baseline tertile of serum insulin (p=0.04). These preliminary data suggest that fasting serum insulin levels may be a useful tool to predict who will benefit from a behaviour modification as outlined in this thesis.

Obvious goals for future studies include elucidation of which genetics components determine short-term and long-term body adiposity responses to clinical interventions.

6.3 Genetics of obesity

GWAS have revolutionised the discovery of genes for common diseases, including obesity-related traits. During the recent 5 years 32 loci have been reported to predispose to overall adiposity (BMI) and 18 loci to central obesity (WC, WHR) [61,63-71,73,122,130-133]. These loci have, however, only small effects on obesity-susceptibility and explain just few percent of the total variance of body adiposity. As such, their accuracy to predict obesity is poor and not competitive with the predictive ability of conventional risk markers. It is also of concern that some of these loci already are being used in commercially available consumer genetics tests to estimate individuals' lifetime risk of obesity even though they do not have the ability to differentiate with any clinically useful accuracy between high-risk and low-risk individuals. The progress within the field of genetics of complex disorders including common metabolic traits is, however, rapid and promising and hopefully ongoing large-scale genotyping studies with imputation of millions of low-frequency and rare variants together with whole-genome sequencing studies will provide much new information about a number of novel and various types of genetic variation increasing the risk of common forms of obesity.

6.4 Genetics and NAFLD

NAFLD susceptible loci have also been identified by GWAS [127,134]. First a variant near patatin-like phospholipase 3 (*PNPLA3*) was shown to associate with MR measured hepatic

steatosis ($p=5.4x10^{-10}$) [127] and recently, this variant, rs738409 ($p=4.3x10^{-34}$), and rs2228603 near neurocan (*NCAN*) ($p=5.29x10^{-5}$), rs780094 near glucokinase regulator protein (*GCKR*) ($p=2.59x10^{-8}$), and rs12137855 near *LYPLAL1* ($p=4.12x10^{-5}$) were reported to associate with histological NAFLD. In the near future it would be of great interest to investigate the study sample described in this thesis (study I and II) to explore associations between these variants, liver fat content and eventually their intervention response.

Finally, individuals included in the Children's Obesity Clinic are representing a tail of the BMI distribution and together with the detailed phenotype measures of these patients; they might be a possible study sample for a GWAS of extreme phenotypes with BMI as a binary trait, adding new knowledge to our understanding of function of the identified BMI loci.

7. Concluding Remarks

In conclusion, we have investigated the prevalence of paediatric NAFLD and conducted a feasibility study of a multidisciplinary treatment program for overweight and obese children and adolescents. We showed that NAFLD is present in 45% of obese Danish children and adolescents, serum ALT and GGT levels are elevated among patients with NAFLD at baseline, and that serum AST/ALT-ratio might be a clinical tool for monitoring of paediatric NAFLD. Furthermore, The Children's Obesity Clinic's treatment program in general is associated with diminished BMI SDS and liver fat content. Also by performing studies of selected obesity gene variants we demonstrated associations to underlying intermediary metabolic phenotypes.

During the past decades, numerous scientists and organizations around the world have worked tirelessly to answer the question "how to reduce the increasing prevalence of obesity and its co-morbidities?" An enormous progress in trying to understand the pathophysiology of obesity, from the genetic part to energy metabolism and fat distribution to social and individual behaviours and the role of the environmental factors have been made. Continuing efforts are improving and standardizing our diagnostic and management tools. Importantly there is an increasing emphasis on reducing obesity risks as early in life as possible. This thesis and the Children's Obesity Clinic are only minor steps in this progress; however, we are confident that we are on the right track.

8. Summary in English

Obesity and its co-morbidities are major public health challenges of the 21st century. The number of those affected has been rising at an alarming rate, especially among children. The prevalence of obesity and overweight among schoolchildren is stabilizing but a continuing increase in prevalence remains among adolescents. Obesity is a complex disorder with both a genetic component and an environmental component that drive the epidemic of obesity and its co-morbidities.

The overall objective of this thesis was to examine childhood obesity with focus on non-alcoholic fatty liver disease (NAFLD) and its treatment. Further, we aimed to investigate the impact of genetic variation on obesity, especially abdominal obesity. Three studies are included in the thesis, representing the clinical and analytical work performed during the Ph.D. study.

In a descriptive study, we examined the prevalence of paediatric NAFLD, defined as liver fat content> 5% among 164 overweight and obese Danish children and adolescents. By magnetic resonance (MR) spectroscopy we measured liver fat content. NAFLD was identified in 45% of the patients.

In a prospective follow-up study including 105 overweight and obese children and adolescents, we investigated the effect of an observational childhood-obesity intervention on body mass index (BMI) standard deviation score (SDS) and NAFLD, measured by MR spectroscopy. Anthropometry, MR scannings, and fasting biochemical variables were measured at baseline and after a median of 358 days. The intervention was associated with a decrease of BMI SDS, waist-height ratio, liver fat content, and abdominal visceral adipose tissue. Furthermore, changes in liver fat associated with changes in abdominal subcutaneous adipose tissue and changes in serum aminotransferase ratio.

In 6.038 Danish adults, we analyzed four genetic variants (rs2605100 near *LYPLAL1*, rs10146997 in *NRXN3*, rs545854 near *MSRA*, and rs987237 near *TFAP2B*) previously identified by genome wide association studies to associate with increased waist circumference (WC) and/or waist-hip ratio. Our major finding was that the risk-allele of rs2605100 associated with elevated concentrations of fasting serum triglycerides and fasting serum insulin and with estimates of central obesity. The risk-allele of rs10146997 associated with increased WC among women. Neither rs545854 nor rs987237 showed clear associations with obesity in our study sample.

We found that NAFLD was present in 45% of obese Danish children and adolescents that aminotransferases might be a clinical tool for monitoring of paediatric NAFLD, and that The Children's Obesity Clinic's treatment program associated with reduced values of BMI SDS and liver fat content. By performing variant association studies we showed that the risk-alleles in rs2605100 and rs10146997 associated with estimates of central obesity. This knowledge might be used in future treatment of obesity and its co-morbidities.

9. Summary in Danish

Fedme og dens ko-morbiditeter er en af de største udfordringer for folkesundheden i det 21. århundrede. Antallet af berørte har været stigende i et alarmerende tempo, især blandt børn. Forekomsten af fedme og overvægt blandt skolebørn er stabiliseret omkring 15% men der ses fortsat en stigning i forekomsten blandt unge. Fedme er en kompleks sygdom, der skyldes et samspil mellem forskellige disponerende genvarianter og miljøfaktorer, der driver fedmeepidemien og dens ko-morbiditeter.

Det overordnede formål med denne afhandling var at undersøge børnefedme med fokus på ikke-alkoholisk fedtlever sygdom (NAFLD) og behandlingen heraf samt undersøge genetiske varianters indflydelse på fedme, især abdominal fedme. Der er inkluderet tre studier i afhandlingen, som repræsenterer det kliniske og analyserende arbejde, der er udført under ph.d. studiet.

I et deskriptivt studie undersøgte vi prævalensen af NAFLD, defineret som lever fedtindhold >5% blandt 164 danske overvægtige børn og unge. Ved magnetisk resonans (MR) spektroskopi målte vi leverens fedtindhold. NAFLD blev identificeret i 45% af patienterne.

Effekten af en livsstilssintervention på BMI SDS og fedtlever målt ved MR spektroskopi blev undersøgt i et prospektivt follow-up studie inkluderende 105 overvægtige børn og unge. Antropometri, MR skanninger, og blodprøver blev målt ved baseline og efter median 358 dage. Interventionen reducerede BMI SDS, talje-højde-forhold, lever fedtindhold, og abdominalt visceralt fedtvæv. Endvidere associerede ændringen i leverens fedtindhold med ændringer i abdominalt subkutant fedtvæv og ændringer i serum aminotransferase-ratioen.

I 6.038 voksne danskere analyserede vi 4 genetiske varianter (rs2605100 nær *LYPLAL1*, rs10146997 i *NRXN3*, rs545854 nær *MSRA*, og rs987237 nær *TFAP2B*) tidligere identificeret i helgenoms associations studier til at associere med øget taljemål og / eller talje-hofte ratio. Her observerede vi, at risiko-allelet for rs2605100 associerede med forhøjede koncentrationer af faste serum triglycerider, faste serum insulin og med surrogat mål for central fedme. Risiko allelet for rs10146997 associerede med øget taljemål blandt kvinder. Hverken rs545854 eller rs987237 viste tydelige associationer til fedme.

Sammenfattende har vi undersøgt prævalensen af pædiatrisk NAFLD samt evalueret et behandlings tilbud for overvægtige børn og unge i Danmark. Ved at udføre variant-associations studie har vi analyseret 4 varianters association til abdominal fedme. Vi kan konkludere at NAFLD findes hos 45% af overvægtige danske børn og unge, at

aminotransferaser kan være et klinisk redskab til monitorering af NAFLD samt at ”Enheden for overvægtige børn og unge”s behandlingstilbud reducerede BMI SDS og leverfedtindholdet. Studierne i denne afhandling bidrager til vores viden om fedme samt til at forbedre fremtidig behandling af fedme og dens ko-morbiditeter.

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"I denne verden er du måske kun et menneske,
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Appendix I

Papers

Study I

Liver fat content investigated by magnetic resonance spectroscopy in obese children and youths included in multidisciplinary treatment.

By Dorthe Sadowa Bille, Elizaveta Chabanova, Michael Gamborg, Cilius Esmann Fonvig, Tenna Ruest Haarmark Nielsen, Ebbe Thisted, Henrik S. Thomsen, Jens-Christian Holm

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Liver fat content investigated by magnetic resonance spectroscopy in obese children and youths included in multidisciplinary treatment

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What is already known about this subject

- Investigations of non-alcoholic fatty liver disease (NAFLD) by non-invasive imaging procedures have limited evidence.
- Thirty percent of obese children are estimated to have NAFLD and implications for future morbidity are uncertain.

What this study adds

- Many obese children and youths exhibit a high liver fat content as examined by magnetic resonance spectroscopy.
- Associations between liver fat content, anthropometry, abdominal adipose tissue distribution and liver enzymes are illustrated.

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Summary

The study aims to investigate the degree of hepatic steatosis and associations with the amount of abdominal subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT), anthropometric data and biochemical measures of liver enzymes in children and youths included in obesity treatment. The study included 164 patients, aged 6–20 years, with a body mass index (BMI) above the 90th percentile for sex and age. Liver fat content was measured by magnetic resonance spectroscopy (MRS). SAT and VAT were measured by magnetic resonance imaging. Hepatic steatosis was defined as liver fat content >5% (steatosis-5%) and 9% (steatosis-9%), respectively. Data on waist circumference (WC) and blood samples were available in 124 patients. Steatosis-5% and steatosis-9% were identified in 45% and 27% of the patients, respectively. These patients had increased SAT, VAT, BMI standard deviation score, WC/height ratio, alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT) levels. GGT, ALT and VAT were found to be independent risk factors of hepatic steatosis. In this study, a substantial proportion of obese children and youths have hepatic steatosis. Therefore, it is important to examine these subjects for the degree of fat in their liver. Future studies focusing on hepatic steatosis should consider the use of MRS in addition to blood samples.

Keywords: Child, hepatic steatosis, magnetic resonance spectroscopy, obesity.

Abbreviations: ALT, alanine aminotransferase; AP, alkaline phosphatase; BMI, body mass index; SDS, standard deviation score; TE, echo time; FOV, field of view; GGT, gamma-glutamyl transferase; LDH, lactate dehydrogenase; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; TR, repetition time; SAT, subcutaneous adipose tissue; L3, third lumbar vertebra; TSE, turbo spin echo; U L^{-1}, units per litre; VAT, visceral adipose tissue; VOI, volumes of interest; WC, waist circumference

Introduction

Paediatric fatty liver disease developing in the absence of alcohol intake, termed non-alcoholic fatty liver disease (NAFLD), is being recognized with the increasing prevalence of childhood obesity (1). Data from an American histology-based autopsy study suggest that 9.6% of the normal-weight population aged 2–19 years are affected by NAFLD, defined as above or equal to 5% of hepatocytes containing macrovesicular fat (2). The prevalence of NAFLD increases with age and increasing degree of obesity (2). In a study of 44 obese Italian children aged 6–16 years examined by magnetic resonance imaging (MRI), 14 children (31.8%) had NAFLD, where NAFLD was defined as a liver fat content above 9% (3). However, there are conflicting views of the definition of NAFLD in children; i.e. the amount of liver fat content to be considered NAFLD is suggested to be in the range of 5–10% (4–9). The clinical diagnosis is difficult because NAFLD encompasses a wide spectrum of conditions with elevated amounts of fat in the liver, from asymptomatic steatosis with normal or elevated concentrations of aminotransferases to steatohepatitis, which may progress into fibrosis and cirrhosis (10).

The gold standard for the diagnosis of NAFLD is liver biopsy, which provides information about the degree of fat infiltration, inflammation and scarring (11–13). Non-invasive techniques such as ultrasound and MR measurements have been developed (11). However, ultrasound exhibits considerable variability, is operator dependent as a diagnostic method (14) and is consequently less precise in the quantification of fat accumulation in the liver (15). By ultrasound elastography, also known as Fibroscan®, the liver's stiffness is measured and reflects the degree of fibrosis (16); unfortunately, this method has its limitations, too. In case of obesity, the amount of subcutaneous adipose tissue (SAT) attenuates the waves, and in general a considerable variation of stiffness in the liver is measured, influencing the diagnosis of liver fibrosis (17). Furthermore, the method is not recommended for patients under the age of 18 years (http://www.fibroscan.co.uk). MR techniques are appealing because they are non-invasive, precise, less operator dependent and do not expose the patient to ionization; however, they are expensive. Proton MR spectroscopy (MRS) measures the triglyceride content in liver cells, which can be used to estimate the amount of fat in the liver (18,19), and is more able to detect even very low levels of liver fat content compared to MRI and ultrasound (11,20,21). By using MRI, it is possible to estimate the volumes of abdominal visceral adipose tissue (VAT) and SAT as well (9). Recently, the amount of VAT has been shown to be associated with NAFLD (22) which indicates a higher cardiovascular risk among adolescents (23). Hepatic fat accumulation is associated with obesity, an adverse cardiovascular risk profile in children (24) and insulin resistance (25). However, it is unknown which imaging techniques are optimal and efficacious in estimating the fat content in the livers of obese children and adolescents (26).

We have recently published data showing efficient treatment results with an acceptable rate of retention irrespective of baseline body mass index (BMI) standard deviation score (SDS), age and social class in obese children and adolescents without any prior eligibility criteria (27). In the present study, we investigated the degree of fat deposition in the livers in a group of obese children and youths included in treatment. Hepatic steatosis and the volumes of SAT and VAT in the abdomen were examined using MRS and MRI. The associations between liver fat content, abdominal fat distribution, anthropometric data and biochemical measures were investigated.

Materials and methods

Subjects

In total, 198 children and youth were enrolled prospectively from August 2009 to August 2010 at The Children's Obesity Clinic, Department of Pediatrics, Copenhagen University Hospital, Holbæk, Denmark; The Children's Obesity Clinic has previously been described (27). The criteria for inclusion were 6–20 years of age and a BMI above the 90th percentile for age and sex (28). The exclusion criteria were body weight above 130 kg, which was the maximum capacity of the MR equipment, or if the patient was not able to remain calm in the MR machine for 45 min. Information on alcohol consumption was obtained from all patients. Six of the patients were known consumers of alcohol, but the amounts were <20 g d^{-1}, which is the amount thought to be required for the development of alcohol-dependent fatty liver disease (4). One hundred ninety-one children and youths agreed to be enrolled in the study; however, 27 defaulted the MRI and MRS assessments, leaving 164 patients for study. Ninety-four percent of them were Caucasians and 6% were from the Middle East area. All participants provided a detailed medical history and underwent a complete physical examination. Pubertal stages were assessed according to the methods of Tanner (29). Anthropometric measures and blood samples were available in 124 patients and were obtained within 120 d from the date of the MR scans. There were no significant differences in mean BMI SDS or age between the 124 patients with MR, anthropometric and biochemical measurements and the 40 patients who were investigated by MR only. Table 1 shows the baseline characteristics of the patients. The study sample was divided into groups according to the amount of liver fat. Cut-off values of 9 and 5%, respectively, were established for content of liver fat based on previous studies (2,3,30). Steatosis-5% was

Table 1 Baseline characteristics in obese children and youths included in the study

	Total	Patients with height, weight, WC and blood samples included
N	164	124
N boys/girls	74/90	57/67
Pre-pubertal (%)*	30	32
Pubertal (%)*	49	47
Post-pubertal (%)*	21	21
Age (years)	13.3 (6.6–20.4)	13.0 (7.8–20.4)
BMI SDS	3.03 (1.3–5.4)	3.01 (1.6–5.4)
WC/height		0.6 (0.5–0.8)

*Puberty is defined according to Tanner stages. Stage 1 = pre-pubertal, stage 2–4 = pubertal, stage 5 = post-pubertal. Data are expressed as percentages.
Data are stratified into two groups: those with measurements obtained by MRI only versus those with measurements obtained by MRI, waist circumference and blood samples.
Data are expressed as unadjusted means with ranges.
BMI, body mass index; SDS, standard deviation score; WC, waist circumference.

defined as a liver fat content above 5%, and non-steatosis-5% as a liver fat content equal to or below 5%. Similarly, steatosis-9% was defined as having a liver fat content above 9%, and non-steatosis-9% was defined as having a liver fat content equal to or below 9%. The non-steatosis-9% group included 90 patients from the non-steatosis-5% group and 29 patients from the steatosis-5% group.

None of the 164 children or youths had their HBsAg and anti-HCV serology checked. However, the prevalence for both hepatitis B and C is low among individuals in Denmark, i.e. a 0.01% prevalence for hepatitis B (http://www.ssi.dk/Service/Sygdomsleksikon/H/Hepatitis%20B.aspx.) (date: 09.02.2012) and 0.2% for hepatitis C, respectively (http://www.ssi.dk/Service/Sygdomsleksikon/H/Hepatitis%20C.aspx) (date: 09.02.2012).

Informed written consent was obtained from all patients aged 18 years and older and from the parents of children aged younger than 18 years. The study was approved by the Danish Data Protection Agency and the Ethics Committee of the Region Zealand in Denmark (ID-no.: SJ-98 and SJ-104) and is registered at ClinicalTrials.gov (ID-no.: NCT00823277 and NCT00928473). The study was performed in accordance with the Helsinki Declaration.

MR examination

All MR measurements were performed by highly skilled MR-trained personnel using an Achieva 3.0 T MR imaging system (Philips Medical Systems, Best, the Netherlands) and a SENSE cardiac coil (31). Patients were examined in the supine position.

Liver fat content was measured by MRS. T2-weighted turbo spin echo (TSE) coronal and axial slices taken through the abdomen were used to position the spectroscopy volumes of interest (VOI). The parameters for the TSE sequence were: TSE factor = 93, repetition time (TR) = 2182 ms, echo time (TE) = 80 ms, field of view (FOV) = 420 mm. The spectroscopy VOI (11 mm × 11 mm × 11 mm) was positioned over the middle of the right lobe of the liver. This position was determined individually to avoid major intrahepatic blood vessels and bile ducts. A single voxel spectrum was recorded using PRESS sequence, with TE = 75 ms, TR = 2000 ms and 48 averages. The MR scanner's software was used to fit the acquired spectrum to the relative content of water and lipid. Spectroscopic hepatic lipid content was expressed as lipid content relative to water and was calculated as: spectroscopic fat (%) = [fat metabolite area / {fat metabolite area + water metabolite area}] × 100.

Visceral and subcutaneous fat volumes were measured by MRI. A fast T1-weighted turbo field echo (TFE) MR sequence in the transverse plane was used to obtain images for estimating the adipose tissue volumes (TFE sequence, TFE factor = 136, TR = 10 ms, TE = 2.3 ms, FOV = 480 mm, and a respiratory trigger compensation with trigger delay of 1000 ms). A transverse slice of 10 mm thickness was acquired for all subjects in the middle of the third lumbar vertebra (L3). The volumes of subcutaneous and visceral fat at L3 were measured in cm^3 using 'segmentation tool' in 'volume analysis' on the Philips ViewForum workstation.

Anthropometry

Anthropometric measurements were obtained in light indoor clothes with empty pockets and without shoes. Body weight was measured to the nearest 0.1 kg on a Tanita Digital Medical scale (WB-100 MA, Tanita Corporation, Tokyo, Japan). Height was measured on a stadiometer to the nearest 1 mm. BMI was calculated as weight in kg divided by height in m^2. The BMI SDS was calculated by the least mean square method, which transforms BMI into normal distributions for each age and sex using the median, coefficient of variation and a measure of the skewness based on the Box–Cox power according to the Danish BMI charts (28). Waist circumference (WC) was measured with an anthropometric tape in centimetres (cm), at the level of the umbilicus, at the end of a gentle expiration.

Blood sampling

Venous blood samples were drawn from the antecubital vein in the fasting condition. If needed, an anaesthetic cream (lidocain/prilocain mixture, Emla®, AstraZeneca, Stockholm, Sweden) was applied 1 h before venipuncture.

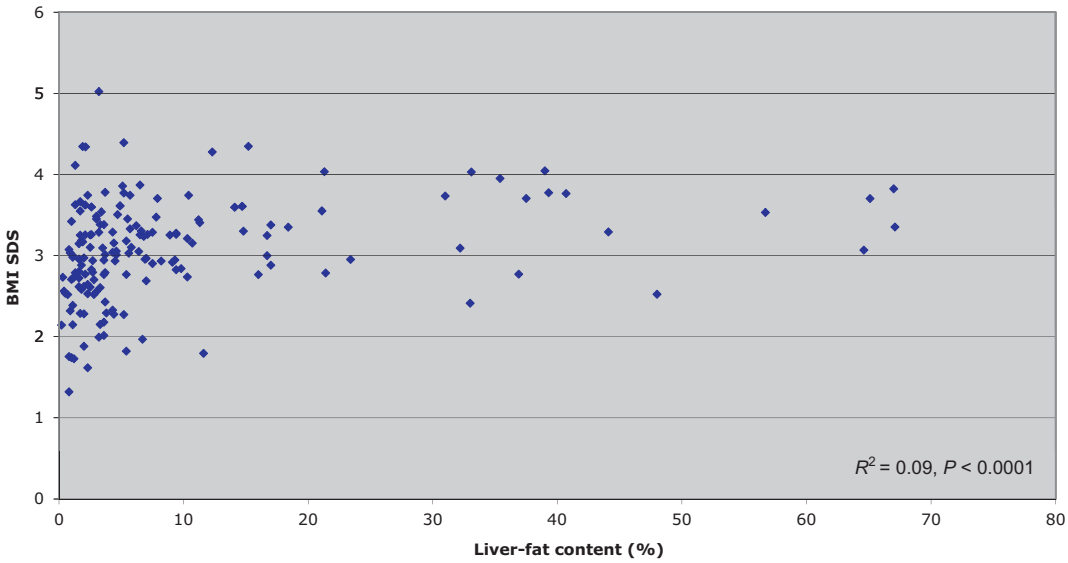

Figure 1 Association between liver fat content and BMI SDS in 164 obese children and youths. BMI, body mass index; SDS, standard deviation score.

The biochemical analyses were performed immediately after venipuncture and included measurement of the levels of alanine aminotransferase (ALT), alkaline phosphatase (AP), lactate dehydrogenase (LDH) and gamma-glutamyl transferase (GGT). ALT level was measured by the absorbance reduction at 340 nm. AP level was measured by the increase in absorbance at 409 per 450 nm. LDH level was measured by the increase in absorbance at 340 nm. GGT level was measured by the increase in absorbance at 409 per 415 nm. All enzyme levels were measured on a Cobas 6000® and are expressed as units per litre (U L^{-1}). An ALT level above 40 U L^{-1} and a GGT level above 80 U L^{-1} were defined as increased.

Data analysis

The data between the hepatic steatosis with either 5 or 9% fat in their livers were compared with a non-steatosis group using *t*-tests. The values for SAT and VAT, and the levels of ALT, AP, LDH and GGT, had non-normally distributed residuals and were logarithmically transformed before the statistical analyses. The differences in SAT and VAT, and the levels of ALT, AP, LDH and GGT between patients with and without steatosis, are presented as percentages and with 95% confidence intervals (CI). The differences in BMI SDS, WC and WC/height are given as actual values with 95% CIs. The associations between steatosis and sex, BMI SDS, ALT and GGT levels were assessed by chi-square tests. Multiple logistic regression analysis was used to investigate if the risk of hepatic steatosis was associated with the degree of obesity. The analyses were adjusted for gender, age and Tanner stage. Further, we elaborated our analyses with multiple logistic regression analyses on those risk factors that associated with steatosis-5% and steatosis-9% in the 124 patients in order to identify independent risk factors. These analyses were also adjusted for age, gender and Tanner stages. Simple linear regression was used for Fig. 1. A *P*-value <0.05 was considered statistically significant. The statistical analyses were performed using SAS, version 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

MRS and MRI were performed on 164 obese children and youths aged 6–20 years. Seventy-four patients (39 boys and 35 girls) were in the steatosis-5% group and 45 patients (26 boys and 19 girls) were in the steatosis-9% group. Liver fat content ranged from 0.2% to 67.1%. In the different groups of liver fat content, the medians and ranges were: non steatosis-5% median 2.1% (range 0.2–4.9%), steatosis-5% median 10.3% (5.1–67.1%), non-steatosis-9% median 2.8% (0.2–8.9%) and steatosis-9% median 17% (9.1–67.1%). The ranges of SAT and VAT across all four groups of hepatic steatosis were 104 cm^3 to 752 cm^3 and 30 cm^3 to 258 cm^3, respectively. Figure 1 shows the relationship between liver fat content and BMI SDS for all 164 patients. Steatosis-5% and steatosis-9% were associated with increased BMI SDS, SAT and VAT (Tables 2 and 3). Patients with a BMI SDS > 3.0 had a higher frequency of steatosis-5% ($P = 0.003$) and

Table 2 Abdominal adipose tissue distribution in 164 obese children and youths stratified by the degree of liver fat content investigated by MRI and MRS

	Steatosis-5%	Non-steatosis-5%	*P* value	Difference	95% CI
N	74	90			
Boys/girls	39/35	35/55			
Age (years)	13.9	12.9	0.022	1.0	0.2; 1.9
BMI SDS	3.25	2.84	<0.0001	0.4	0.2; 0.6
VAT (cm^3)	107.3	75.8	<0.0001	42%	24%; 61%
SAT (cm^3)	359	279	<0.0001	28%	14%; 45%
SAT/VAT	3.3	3.7	0.092	–10%	–23% 2%
	Steatosis-9%	Non-steatosis-9%	*P* value	Difference	95% CI
N	45	119			
Boys/girls	26/19	48/71			
Age (years)	13.9	13.1	0.205	0.8	–0.4; 1.9
BMI SDS	3.32	2.84	0.0001	0.4	0.2; 0.6
VAT (cm^3)	118.3	79.5	<0.0001	49%	23%; 72%
SAT (cm^3)	358.5	297.5	0.008	21%	5%; 38%
SAT/VAT	3.0	3.7	0.018	–21%	–3%; –42%

Data are expressed as the unadjusted means for age and BMI SDS, and as the geometrical means for VAT, SAT and SAT/VAT. Before the analysis, the differences between the SAT and VAT values were logarithmically transformed. The *P* values, differences and CIs were analysed by *t*-tests. For age and BMI SDS, the differences between the steatosis and non-steatosis groups are presented as actual values. For VAT and SAT, the differences between the steatosis and non-steatosis groups are presented as percentages. Further, VAT, SAT and SAT/VAT analyses were also performed using Mann–Whitney analyses. Results are not shown as the *P* values were almost similar.
BMI, body mass index; CI, confidence interval; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; SAT, subcutaneous adipose tissue; SDS, standard deviation score; VAT, visceral adipose tissue.

steatosis-9% ($P = 0.004$); the risks were 56% and 33%, respectively. Patients with a BMI SDS < 3.0 had a low frequency of hepatic steatosis, and the risks for steatosis-5% and steatosis-9% declined to 30% and 11%, respectively. Logistic regression analyses showed that the risk of steatosis-5% and steatosis-9% were significantly associated with BMI SDS; steatosis-5% [OR = 5.35 per BMI SDS, 95% CI {2.03; 14.12}, $P = 0.0015$] and steatosis-9% [OR = 15.17 per BMI SDS {3.82; 60.18}, $P < 0.001$], respectively. Patients in the steatosis-9% group had a lower SAT/VAT ratio than the patients in the non-steatosis-9% group [difference = 21%, 95% CI {3; 42}, $P = 0.02$].

The patients in the steatosis-5% group were older than those in the non-steatosis-5% group [difference = 1.0 year {0.2; 1.9}, $P = 0.02$]. In patients with steatosis-9%, boys were more affected than girls ($P = 0.045$). WC/height ratio was higher in the patients with steatosis-5% and steatosis-9% than the patients in the non-steatosis-5% [difference = 0.03 {0.01; 0.05}, $P = 0.004$] and non-steatosis-9% groups [difference = 0.03 {0.01; 0.06}, $P = 0.009$] (Table 3).

Patients with steatosis-5% and steatosis-9% had high levels of ALT and GGT (Table 3). The range of the ALT levels were 12–93 U L^{-1}, and the range of the GGT levels were 8–134 U L^{-1}. There were 23 of the 124 patients who exhibited elevated levels of ALT (>40 U L^{-1}); 16 of these patients had steatosis-5% and 13 had steatosis-9%. When ALT levels were elevated, the risks of having steatosis-5% and steatosis-9% were 70% and 57%, respectively (steatosis-5%, $P < 0.005$ and steatosis-9%, $P < 0.0001$). Similarly, the frequencies of having steatosis-5% and steatosis-9% decreased to 37% and 15%, respectively, when the level of ALT was low. GGT showed a similar tendency (steatosis-5%, $P = 0.04$ and steatosis-9%, $P = 0.001$); i.e. a high GGT level was associated with a 100% risk of having steatosis-5% or steatosis-9%. A low GGT level was associated with a low risk of having steatosis-5% (42%) or steatosis-9% (21%). In total, three patients had an elevated GGT level above reference levels (>80 U L^{-1}).

Multiple logistic regression analyses showed that the GGT level was the only independent risk factor for steatosis-5% ($P < 0.0001$). For steatosis-9%, we found the following independent risk factors: ALT and VAT ($P < 0.0001$).

Discussion

A substantial proportion of the 164 obese Danish children and youths included in the present study had MRS-verified hepatic statosis, defined as liver fat content above 5%

Table 3 Anthropometric and biochemical characteristics of 124 obese children and youths stratified by the degree of liver fat content investigated by MRI and MRS

	Steatosis-5%	Non-steatosis-5%	*P* value	Difference	95% CI
N	53	71			
Boys/girls	30/23	27/44			
BMI SDS	3.22	2.85	0.0007	0.4	0.2; 0.6
WC/height	0.7	0.6	0.004	0.03	0.01; 0.05
VAT (cm^3)	105.4	76.2	0.01	31%	21%; 45%
SAT (cm^3)	354	275	0.002	33%	11%; 46%
SAT/VAT	3.2	3.4	0.74	–6%	–31%; 70%
ALT (U L^{-1})	31.3	23.5	0.0007	34%	13%; 57%
AP (U L^{-1})	193.3	199.2	0.74	–7%	–63%; –42%
LDH (U L^{-1})	219.1	210.4	0.32	5%	4%; 13%
GGT (U L^{-1})	22.7	15.9	<0.0001	43%	22%; 68%
	Steatosis-9%	Non-steatosis-9%	*P* value	Difference	95% CI
N	28	96			
Boys/girls	19/9	38/58			
BMI SDS	3.40	2.89	<0.0001	0.5	0.3; 0.8
WC/height	0.7	0.6	0.009	0.03	0.01; 0.06
VAT (cm^3)	122.4	77.4	<0.0001	62%	24%; 63%
SAT (cm^3)	364.8	296.3	0.008	34%	13%; 42%
SAT/VAT	3.0	3.8	0.03	–34%	–6%; –46%
ALT (U L^{-1})	38.1	23.9	<0.0001	59%	32%; 92%
AP (U L^{-1})	202	195.1	0.75	4%	20%; 28%
LDH (U L^{-1})	222.8	211.7	0.28	5%	4%; 16%
GGT (U L^{-1})	27	16.6	<0.0001	63%	35%; 96%

Data are expressed as the unadjusted means for age and BMI SDS, and as the geometrical means for VAT, SAT and SAT/VAT. Before the analysis, the differences between the SAT and VAT values were logarithmically transformed. The *P* values, differences and CIs were analysed by *t*-tests. For age and BMI SDS, the differences between the steatosis and non-steatosis groups are presented as actual values. For VAT and SAT, the differences between the steatosis and non-steatosis groups are presented as percentages. Further, VAT, SAT and SAT/VAT analyses were also performed using Mann–Whitney analyses. Results are not shown as the *P* values were almost similar.
ALT, alanine aminotransferase; AP, alkaline phosphatase; BMI, body mass index; CI, confidence interval; GGT, gamma-glutamyl transferase; LDH, lactate dehydrogenase; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; SAT, subcutaneous adipose tissue; SDS, standard deviation score; U L^{-1}, units per litre; VAT, visceral adipose tissue; WC, waist circumference.

(45%) or 9% (27%). Hepatic steatosis was associated with high volumes of SAT and VAT, low SAT/VAT ratio, high BMI SDS, high WC/height ratio, and elevated levels of ALT and GGT. The present study suggests that high levels of GGT and ALT indicate hepatic steatosis in the clinical setting; however, low levels do not exclude hepatic steatosis.

The quantitative definition of hepatic steatosis by fat-sensitive imaging methods has varied between 1.8% and 10% (3–9,30,32,33), most applying, however, a cut-off of 5% or 9%. In a study by Kim *et al*., liver fat content was measured by MRS and MRI in order to compare the methods (7). They applied a 3.6% cut-off value for normal liver fat content which yielded the best measures of sensitivity and specificity in these MR techniques. Other studies applying MRI and ultrasound (5,9) have used a 9% liver fat content cut-off value. Recently, it was demonstrated that the presence of hepatic steatosis was defined as greater than 1.8% measured by MRS and that the presence of substantial – moderate to severe – hepatic steatosis was defined as more than 7.7% of liver fat content (33). Pacifico *et al*. performed a case-control study in which cases were 25 obese children with biopsy-proven NAFLD. Controls were 25 obese children matched for age and gender, without NAFLD at ultrasonography and with normal levels of aminotransferases and insulin. At the diagnostic threshold of around 5% for hepatic fat fraction, MRI had 95.8% sensitivity for diagnosing any degree of hepatic steatosis (at histologic analysis as the reference standard). A threshold of 9% for hepatic fat fraction was the best cut-off for the diagnosis of moderate to severe steatosis (sensitivity 100%) (8). Based on these studies that all (3–9,30,32,33) except the study by Pacifico *et al*. (8) have used cut-offs previously validated in adult populations, we decided to include 5% and 9% as limits for hepatic steatosis in order to examine whether a higher or lower degree of hepatic steatosis was important in the relationships established with tentative risk factors.

Obesity, measured by BMI SDS, was related to an increased risk of having hepatic steatosis and the risk of having hepatic steatosis was lower in those with a lower BMI SDS. In the logistic regression, the OR of having steatosis-5% or steatosis-9% was 5.35 and 15.17 per unit increase in BMI SDS, respectively, which is comparable with findings from other studies (5,6,8). However, information on Tanner stage was not available in all patients, which resulted in relative wide CI in our results. In a study by Pacifico *et al*., no correlation between BMI and the amount of liver fat content was demonstrated (9). They investigated a group of 50 obese children; however, their mean BMI SDS of 2.48 was lower than in the present study. Further, the inclusion criteria were either hepatomegaly or elevated levels of liver transaminases while the clinical condition of these children may have been a different from that of the children in the present study, which might explain the difference in our findings. In an adult case-control study with biopsy-proven hepatic steatosis and controls without liver disease measured by ultrasound (34), the authors compared five individuals with steatosis, five individuals with NAFLD and five healthy individuals, and showed that the BMI of the healthy volunteers were lower than in subjects with steatosis ($P < 0.05$) or NAFLD ($P < 0.05$). This supports our findings that BMI and hepatic steatosis are associated conditions.

We analysed the relationship between abdominal adipose tissue distribution and hepatic steatosis, and found associations. The SAT/VAT ratio was lower in the steatosis-9% group than in the non-steatosis-9% group, but this difference was not observed between the groups. Taksali *et al.* showed an increased VAT in obese children, and that the SAT amount was decreasing as the proportion of VAT was increasing across tertiles (22). These findings suggest that, in addition to obesity in general, the visceral distribution of adipose tissue might be associated with the amount of fat in the liver. However, the SAT/VAT ratio is biased because the proportion of VAT inherently increases during the development of hepatic steatosis, and this decreases the SAT/VAT ratio. Furthermore, it is well known that VAT and intrahepatic fat are related to each other and that both are linked to the same metabolic abnormalities (6,22,25,32).

The WC/height ratio was higher in patients with steatosis-5% and steatosis-9% compared with the respective non-steatosis groups. This suggests that both general obesity, measured by BMI SDS, and abdominal obesity, measured by the WC/height ratio, are informative about the development of hepatic steatosis in children and youths. This tendency was also seen in a study of 591 healthy Korean adults showing that the WC/height ratio is a useful tool for identifying men and women with NAFLD diagnosed by computerized tomography scanning (35).

Relative increased levels of ALT and GGT yet still below upper reference levels were associated with an increased liver fat content, and patients with increased levels of ALT and GGT also had an increased risk of hepatic steatosis. However, we only had three patients with levels of GGT above reference limits. This finding implicates that relative high GGT levels within the reference levels are associated with the development of hepatic steatosis. These findings are in line with results from previous studies which established associations between the concentrations of liver enzymes and liver fat content. However, normal levels of liver enzymes do not exclude NAFLD or steatosis, and the sensitivity of ALT levels are low in sensitivity in detecting NAFLD and steatosis (4–6,8,9,32,36). Furthermore, these studies also found associations between the degree of hepatic steatosis and blood variables, e.g. triglycerides and insulin. Unfortunately, we did not collect these data in the present study. LDH levels were high in most patients in the present study but were not associated with the development of hepatic steatosis. However, because LDH is an acute phase reactant, it is likely that an elevated LDH level may reflect general inflammation *per se* in obese patients and thus not specifically associated with the degree of steatosis or the degree of inflammation in the liver in the patients.

The strengths of the present study are that a relatively large number of patients were included and the liver fat content and abdominal adipose tissue distribution were investigated by MRS and MRI, respectively, which are techniques that are recognized to be good non-invasive alternatives to liver biopsy (9,11,21,37); however, there are concerns about their costs.

There are several limitations. First, the findings from the present study may have been biased as patients were selected from obese children and youths referred to a childhood obesity treatment programme. On the other hand, it is intriguing that the present study reports data on obese children and youths included in clinical obesity treatment without any prior eligibility criteria. As a consequence, our results actually do reflect 'real life' circumstances as patients with pre-existing paediatric conditions besides obesity that are known to complicate childhood obesity treatment are included. Second, patients weighing above 130 kg were excluded from the MR investigations, which may have led to an underestimation of the degree of fatty liver disease, as these very obese patients harbour a higher risk of developing hepatic steatosis as our results indicate. Third, the data are not complete because 164 patients had MR measures of their liver fat content, whereas only 124 had MR measures of fat content plus the WC/height ratio and biochemical measures. However, the established associations in the present study did not seem to be unilaterally affected by either the degree of obesity, sex or age in these groups, suggesting that our findings are relatively robust. Fourth, liver fat content was measured in a single voxel. Hepatic steatosis can be heterogeneous, why multiple voxel measurements should be preferred. However, the amount

of liver tissue measured by MRS is larger than the amount taken by biopsy; therefore, this sampling error should be comparable to this reference standard. Last, MR investigations are not informative of the histological alterations in the liver, including the degree of inflammation and scarring (e.g. fibrosis and cirrhosis), which need to be investigated separately in suspected high-risk patients.

In conclusion, it seems important to examine obese children and youths for the degree of fat in their livers. Future studies focusing on hepatic steatosis should consider the use of MRS in addition to blood samples.

Conflict of Interest Statement

All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research project.

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DSB drafted the manuscript, and contributed in data collection, treatment of patients, literature searches and data analyses. EC carried out the MR examinations. MG provided statistical analyses. CEF, ET and TRHN contributed to literature searches and data collection. HST contributed to the MR examinations. J-CH established The Childhood Obesity Clinic and The Danish Childhood Obesity Biobank and contributed to the design of the study, treatment of patients, statistical analyses and interpretation of data. All authors were involved in writing the paper and had final approval of the submitted and published version.

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Study II

Effect of a multidisciplinary intervention on ^{1}H-MRS measured hepatic steatosis in overweight and obese Danish children and youths

By Dorthe Sadowa Bille, Elizaveta Chabanova, Michael Gamborg, Tenna Ruest Haarmark Nielsen, Cilius Esmann Fonvig, Ebbe Thisted, Oluf Pedersen, Torben Hansen, Henrik S. Thomsen, Jens-Christian Holm

Submitted manuscript

Effect of a multidisciplinary intervention on ^{1}H-MRS measured hepatic steatosis in overweight and obese Danish children and youths

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Running title: Hepatic steatosis in paediatric obesity treatment

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Authors contributions:

DSB collected data, conducted data cleansing and statistical analyses, literature searches, interpretation of data and drafting of manuscript; EC conducted MR examinations, interpretation of data, revisions of the manuscript; MG conducted data cleansing and statistical analyses; TRHN contributed with collection of data, revisions of manuscript; CEF contributed with data collection, revisions of manuscript; ET contributed with data collection, revisions of manuscript; OBP contributed with conception and design of study, revisions of manuscript; TH contributed with conception and design of study, revisions of manuscript; HST contributed with MR examinations, design of the study, revisions of manuscript; JCH contributed with conception and design of the study, interpretation of data, revisions of manuscript. All authors have approved this manuscript to be published.

Conflict of interest:

All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research project.

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Children, magnetic resonance spectroscopy, obesity, hepatic steatosis, intervention

Abbreviations

Alanine aminotransferase (ALT)

Aspartate aminotransferase (AST)

Cardiovascular diseases (CVD)

Centimetres (cm)

Coefficient of variation (CV)

Gamma-glutamyl transferase (GGT)

High-density lipoprotein cholesterol (HDL)

Homeostasis model assessment (HOMA)

Insulin resistance (IR)

Low-density lipoprotein cholesterol (LDL)

Magnetic resonance (MR)

MR imaging (MRI)

Non-alcoholic fatty liver disease (NAFLD)

Proton magnetic resonance spectroscopy (^{1}H-MRS)

Standard deviation score (SDS)

Subcutaneous adipose tissue (SAT)

Visceral adipose tissue (VAT)

Waist circumference (WC)

Waist-height ratio (WHt)

ABSTRACT

Background: Hepatic steatosis is prevalent in obese children. In this study the effect of an observational childhood-obesity-treatment program upon changes in liver fat content and its associations with related metabolic traits were investigated prospectively.

Design and methods: The study included 105 individuals (45 boys) with a median age of 12.1 years (range 6.6-20.4) and a median body mass index (BMI) standard deviation score (SDS) of 2.92 (range 1.32-5.20). By proton magnetic resonance (MR) spectroscopy liver fat content was quantified. Adipose tissue; subcutaneous (SAT) and visceral (VAT) were estimated by MR imaging. In a subset of 41 individuals fasting blood variables were obtained. MR examinations, anthropometry and blood samples were measured at baseline and after a median of 358 days of intervention. Hepatic steatosis was defined as liver fat content ≥5%.

Results: The intervention improved BMI SDS ($p<0.001$), waist-height ratio ($p=0.02$), liver fat content ($p=0.003$), and SAT/VAT ($p<0.001$). At baseline steatosis was found in 39%, who had higher BMI SDS ($p=0.002$), waist circumference ($p=0.02$), SAT ($p=0.01$), and VAT ($p<0.001$) than patients without steatosis. Degree of hepatic steatosis at baseline could not predict changes in anthropometrics. Furthermore, change in steatosis was associated with changes in SAT ($p=0.02$) and changes in the serum aspartate aminotransferase (AST)/ alanine aminotransferase (ALT) ratio ($p=0.01$).

Conclusions: This childhood obesity intervention resulted in a decrease in hepatic steatosis. Reduction in liver fat content associated with reductions in SAT and serum AST/ALT ratio, indicating that serum AST/ALT ratio might be a clinical tool for monitoring paediatric patients with hepatic steatosis.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a clinico-pathological condition with increasing prevalence in the general population including children and young people. In the United States it is estimated that 9.6% of children aged 2-19 years are affected by NAFLD [1,2]. Mortality among adult patients with NAFLD is higher than in the general population, mainly due to cardiovascular diseases (CVD) and liver dysfunction [3-6]. Recently, a longitudinal follow-up study over 20 years concluded that children with NAFLD have a shorter long-term survival, and that NAFLD is a progressive disease with the potential need for liver transplants in selected cases [7].

At present, very few evidence-based guidelines for treatment of NAFLD exist and no pharmacological agents have been approved for treatment of paediatric NAFLD [8-10]. Lifestyle modifications remain the recommended therapeutic management for obese patients with NAFLD [9,11]. In general, NAFLD intervention programs in obese children exhibit variable results with relative short treatment durations and are most often performed in selected study groups with stringent eligibility criteria leaving many clinically relevant obese children and youths out of treatment protocols [12-18]. The conclusions in these studies are similar, suggesting that an increased surveillance as well as early treatment of co-morbidities, such as obesity, dyslipidaemia, CVD, and type 2 diabetes, is important.

The present prospective study investigates the effect of a long-term (median 358 days) multidisciplinary childhood obesity treatment program upon changes in liver fat content and anthropometrics in 105 obese children and youths. Furthermore, in a subset of 41 individuals changes in liver fat content were analysed in relation to changes in fasting blood variables of serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum gamma-glutamyl transferase (GGT), plasma glucose, serum insulin, total serum cholesterol, serum low-density lipoprotein cholesterol (LDL), serum high-density lipoprotein cholesterol (HDL), and serum triglycerides to see if any of them could be used as a clinical tool for monitoring hepatic steatosis.

DESIGN AND METHODS

Study sample

Two hundred and thirty-seven children and youths were included at baseline in a chronic care multidisciplinary intervention program at The Children's Obesity Clinic, Department of Paediatrics, Copenhagen University Hospital, Holbæk, Denmark [19] and followed prospectively from August 2009 to December 2011. The criteria for inclusion were 6–20 years of age and a BMI above the 90th percentile for age and sex according to the Danish BMI charts [20,21] and cut-offs supposed by an expert committee in Pediatrics in 2007 [22]. The exclusion criteria for participating in this study were 1) a body weight above 130 kg (which was the maximum capacity of the magnetic resonance (MR) equipment), 2) inability to remain calm in the MR machine for 45 minutes, and 3) alcohol consumption >140 g/week. One hundred and thirty-two individuals did not have the follow-up MR examination performed after approximately 1 year, due to the following circumstances; 1) did not

show up for the assessment (n=23), 2) had stopped their obesity treatment, due to success, moved to another part of the country, or simply because they did not want to continue treatment in our clinic (n=14), 3) did not want to have the MR scan repeated (n=40), 4) had become too large for the MR equipment (n=2), and 5) had their baseline MR examination performed closely (within 140 days) to the last day of inclusion (n=53). Of the 40 individuals who did not want to have the MR examination repeated and the 23 who did not show up, 57 had a liver fat content <5% which may explain why they chose not to repeat the MR investigation. Only 16 individuals with NAFLD did not have their MR investigation repeated. Consequently, 105 patients were included in the study. In table 1 the main characteristics of the 105 patients included in the study and the 132 individuals "lost-to-follow-up" are summarized.

None of the children or youths had their HBsAg and anti-HCV serology checked. However, the prevalence for both hepatitis B and C is low in Denmark i.e. 0.04% prevalence for hepatitis B, and 0.4% for hepatitis C, respectively [23], and assumed even lower amongst children. Informed written consent was obtained from all patients aged 18 years and older and from the parents of children younger than 18 years of age. The study was approved by the Danish Data Protection Agency and the Ethics Committee of the Region Zealand in Denmark (ID-no.: SJ-98 and SJ-104) and is registered at ClinicalTrials.gov (ID-no.: NCT00823277 and NCT00928473).

Baseline phenotyping and intervention

The clinic encompasses a multidisciplinary team including paediatricians, nurses, dieticians, psychologists, research technicians, social workers, and secretaries [19]. At baseline, the patient was seen by a paediatrician and provided a detailed medical history and had a complete physical examination performed. The patient and his/her family received an individually tailored treatment plan comprising 10–20 advances from the paediatrician [19]. This plan consisted of comprehensive oral and written advices on physical activity and inactivity, guidelines for sources of nutrition throughout the entire day, for weekends, and when eating outside of home, handling of eating disturbances including finicky eating, satiety training, and care of psychosocial functioning in cases of neglect [19]. Appointments for fasting blood samples, and MR assessments were made. During the intervention, the plan was modified according to the needs of, and in collaboration with, the individual child or youth and his/her family [19]. Anthropometry was continuously measured and after a median of one year of treatment, initial clinical and paraclinical investigations were repeated. Figure 1 illustrates a flow-diagram of the intervention.

Magnetic resonance examination

The liver fat content was quantified by proton magnetic resonance spectroscopy (^{1}H-MRS). MR imaging (MRI) sequence in transverse plane (a slice with 10 mm thickness in the middle of the third lumbar vertebra) was used for obtaining images for estimating abdominal visceral adipose tissue (VAT) and abdominal subcutaneous adipose tissue (SAT). An Achieva 3.0 T MR imaging

system (Philips Medical Systems, Best, The Netherlands) with a SENSE cardiac coil was applied. The procedure has previously been described in earlier studies [21,24,25]. Steatosis was defined as a liver fat percentage equal to or above 5% [21].

Anthropometric measurements

Anthropometric measurements were performed in the children and youths wearing light indoor clothing and without shoes. We used a Tanita Digital Medical scale (WB-100 MA, Tanita Corp., Tokyo, Japan) to measure body weight (in kg) and a stadiometer for height (in cm). BMI was calculated as weight in kg divided by height in m^2. Weight standard deviation score (SDS), height SDS, and BMI SDS were calculated by the least mean square method [20]. Waist circumference (WC) was measured with a non-elastic anthropometric tape in cm, at the level of the umbilicus, at the end of a gentle expiration. Puberty stages (development of genitals and pubic hair in boys and development of breasts and pubic hair in girls) were assessed according to Marshall and Tanner [26].

Biochemical measurements

In a subset of 41 individuals we had viable blood samples drawn within 3 months from the date of the MR scan. Venous blood samples were drawn from the antecubital vein after an overnight fast (12 hours). If required, a local anaesthetic cream (lidocain/prilocain mixture, Emla®, AstraZeneca, Stockholm, Sweden) was applied 1 hour before the blood sampling. The biochemical analyses were performed immediately after venipuncture and included the measurements of concentrations of serum ALT, AST, serum GGT, plasma glucose, serum insulin, total serum cholesterol, serum LDL, serum HDL, and serum triglycerides. ALT and AST levels were measured by their catalytic effect and defined by the absorbance reduction at 340 nm. The coefficient of variation (CV) according to the analyses was 4% for each variable. GGT level was measured by an enzymatic colorimetric method and its concentration was defined as the increase in absorbance at 409 per 415 nm, CV was 4%. Glucose was analyzed by an enzymatic reference method with hexokinase and measured by the absorbance increasing at 340 nm, CV was 3%. Total cholesterol, HDL, and triglycerides were measured by enzymatic colorimetric methods and their concentrations were measured by absorbance increasing at 340 nm. Their CV was 3%, 4%, and 4%, respectively. All measurements were performed on a Cobas 6000® and are expressed as units per litre (U L-1) or mmol/l. LDL levels were calculated (LDL= total cholesterol – ((triglycerides * 0.45) + HDL))). By autoDELFIA insulin kit; time delayed fluoroimmunoassay, insulin levels were analyzed. CV for insulin was 2%. Homeostasis model assessment (HOMA) was used to estimate insulin resistance (IR); HOMA-IR = ((fasting plasma glucose (mmol/l) * (fasting serum insulin (pmol/l) /6)) / 22.5) [27]. For the present study IR was defined as HOMA-IR greater than 4.39 [28].

Data analysis

Prior to analyses, values of weight, height, BMI, WC, waist-height ratio (WHt), liver fat content, SAT, VAT, SAT/VAT, and all concentrations of various serum and plasma variables were logarithmically transformed in order to approximate a normal distribution. Analyses of anthropometrics and fat distribution were performed in the 105 individuals. In a subset of 41 individuals, analyses including blood samples were performed. Changes in continuous variables were analysed using paired *t*-test. Analysing the effect of the intervention on those with hepatic steatosis at baseline compared to those without hepatic steatosis, a *t*-test was used. Further, the data were also analysed using Mann-Whitney analyses. These results are not shown as the p-values were almost similar.

The relationships between concomitant changes in anthropometry, various fasting serum and plasma variables, and liver fat content were analysed using a general linear model of the changes in the logarithmically transformed liver fat content on the changes in the logarithmically transformed anthropometric measures and blood variables, adjusted for the logarithmically transformed baseline liver fat content, gender, baseline age, and baseline pubertal stage.

A p-value <0.05 was considered significant. The statistical analyses were performed using the statistical program R, version 2.13.2.

RESULTS

The characteristics of the included 105 obese children and youths are listed in table 2.

The intervention-period was median 358 days (range 147-580). Using paired *t*-tests we found a significant improvement in following variables during intervention; BMI SDS ($p<0.001$), weight SDS ($p=0.002$), height SDS ($p=0.04$), WHt ($p=0.02$), SAT/VAT ($p<0.001$), liver fat content ($p=0.003$), and VAT ($p=0.04$). During the intervention 60 individuals (57%) reduced their liver fat percentage with a median -2% (range -0.1- -52.1), and 36 individuals (35%) increased their liver fat percentage with a median 2% (range 0.1-32.1). Unchanged liver fat content, defined as the percentage of liver fat content at follow-up minus the percentage of liver fat content at baseline equal to 0, was found in 9 individuals (8%).

At the baseline 41 individuals had hepatic steatosis, 50% of them reduced their liver fat content (figure 2), 9% increased their liver fat content and 41% exhibited unchanged liver fat percentage. Furthermore, those with hepatic steatosis at baseline were taller (height SDS, $p=0.04$), had higher BMI SDS ($p=0.002$), WC ($p=0.02$), SAT ($p=0.01$), and VAT ($p<0.001$) than those without steatosis. After intervention, the group of individuals having hepatic steatosis at baseline were still taller (height SDS, $p=0.03$), and had higher BMI SDS ($p<0.001$), WC ($p=0.03$), SAT ($p<0.001$), VAT ($p<0.001$), and SAT/VAT ($p=0.03$) compared to those with liver fat contents below 5%. However the relative changes in the anthropometrics between those with hepatic steatosis at

baseline and those without did not differ. Table 3 shows differences in measurements between the patients stratified by hepatic steatosis.

In a subset of 41 individuals who had viable fasting blood samples we analysed the improvements of serum liver enzymes, plasma glucose, serum insulin and serum lipids during the intervention using paired *t-test* (data not shown). However, we did not find any significant changes. Nor did we find any significant differences in the blood variables between the group of individuals having hepatic steatosis at baseline and the individuals with a liver fat content <5% (table 4). IR defined as HOMA-IR greater than 4.39 was found in 30% of the patients at baseline and in 24% at follow-up.

A simple linear model was used to describe how the change in liver fat percentage was associated with changes in measures of anthropometrics (n=105) and blood variables (n=41) (table 5). E.g. a change of 10% in SAT was associated with a change of 11% in liver fat content (p=0.02), similar a change of 10% in serum AST/ALT ratio was associated with a change in liver fat content of 44% (p=0.01), and a change in HDL of 10% was non-significantly associated with a change in the liver fat content by 30% (p=0.25).

DISCUSSION

The present prospective study demonstrated beneficial effects of a multidisciplinary childhood obesity intervention on the changes in liver fat content and anthropometrical measures in a cohort of 105 overweight and obese children and youths. Overall, 57% of all the included children and youths and 50% of the patients having hepatic steatosis at baseline reduced their liver fat percentage during intervention. Furthermore, in a subset of 41 individuals with fasting biochemical tests, change of the serum AST/ALT ratio exhibited a strong association with change in liver fat content achieved by the multidisciplinary intervention.

The aim of the present childhood obesity intervention program was instituted to treat childhood obesity as measured by BMI SDS [19]. However, the present study shows that the degree of hepatic steatosis is improved as well. Other paediatric studies have shown beneficial effects of lifestyle interventions including exercise, diet and behaviour modifications in the reduction of hepatic steatosis [12-18,29,30]. Most of these studies have measured the degree of liver fat content by ultrasound; a measurement that is believed to be operator dependent as a diagnostic method with considerable variability [31] , and thus less precise in the quantification of the liver fat content [32]. Yet, Nobili *et al* investigated 84 obese children with elevated serum transaminases concentrations and NAFLD confirmed by liver biopsy [13]. These children received an individually tailored 12 months diet and exercise program where measures of insulin sensitivity, liver enzymes, and liver echogenicity on ultrasonography were improved. A similar positive effect of multidisciplinary intervention on hepatic steatosis and associated indices was observed in the present study. However, we used ^{1}H-MRS both at baseline and at follow-up as a diagnostic tool for hepatic steatosis. These methodological constraints make the comparison between the studies more difficult.

Studies including pharmacological approaches have been introduced in the paediatric field of NAFLD treatment, yet no additional effect has been found [33-35]. E.g. the effects of a 24 months lifestyle change and weight loss treatment with or without the use of vitamin C and E in 53 children with biopsy-proven NAFLD were investigated [36]. The authors showed that a mean weight loss of around 4.7 kg produced significant histological improvement in the degree of steatosis, lobular inflammation and ballooning degeneration in hepatocytes as determined by a liver biopsy [36]. The study showed no additional effect of vitamin C or E on improvement in steatosis or weight loss alone [36].
The present study also showed that reductions of the serum AST/ALT ratio are associated with reductions in liver fat content. However, individually the serum transaminase concentrations did not show the same tendency. Previously, we have found that high serum ALT levels at baseline associated with increased content of liver fat [21] as also seen in other studies [37,38]. However, in a study by Burgert *et. al* only 48% of the individuals with NAFLD had increased levels of serum ALT [37], indicating that serum ALT is not a precise clinical tool for the identification of NAFLD. However, serum levels of ALT and AST are recognized to associate with NAFLD (or hepatic steatosis) and in a recent publication they are included as such markers in the flow chart for treatment of fatty liver in the paediatric clinic [10]. The findings in the present study may indicate that the serum AST/ALT ratio might be more appropriate in the clinical setting and thus may be useful in monitoring paediatric patients with suspected or verified hepatic steatosis. Yet, our results are based on a subgroup of 41 individuals, and replication in larger study samples is needed.

As obese children with NAFLD are also reported to exhibit components of the metabolic syndrome such as dyslipidaemia, hypertension and altered glucose metabolism, we investigated some of these relationships, but found no association with steatosis. Neither did we identify any individuals with type 1 nor 2 diabetes mellitus. IR at the predefined HOMA-IR threshold was found in 30 % of the children and youths. During the intervention the HOMA-IR level decreased. This finding, however, was not significant, nor did our data support the potential of a decreased HOMA-IR to predict a reduction in liver fat content. It is recognized that a transient IR develops in children during puberty and this IR is accepted as a physiological condition rather than a pathologic one [39,40]. Studies have shown that the development of IR during puberty is as follows; with the onset of puberty IR increases, peaking at Tanner stage 3, and recedes to pre-pubertal levels at the end of puberty. These changes in insulin activation and secretion are not clear. However, a theory is that the changes in insulin secretion during puberty are related to a mechanism enhancing the anabolic effect of insulin and growth hormone during rapid somatic growth [41]. This argument might explain our finding of the change in HOMA-IR. Yet, the cut-off used in the present study is high compared to others indicating that the 30% of the patients we found exhibiting IR are relevant cases of IR regardless of which cut off value is used. Furthermore, we showed that patients with hepatic steatosis had a significantly higher WC and WHt, both at baseline and at follow-up, than those without steatosis at baseline. The WC and, especially the WHt are indirect measures of abdominal obesity and are parts of some definitions of the metabolic syndrome [42]. However, we did not establish an association between changes of these two measures and changes in liver fat content. A case-control study by Schwimmer *et al* including 300 obese adolescents showed that children with metabolic syndrome

had an odds of 5.0 of NAFLD compared to overweight and obese children without metabolic syndrome [43]. In view of these findings one should bear in mind the increased risk of cardiovascular disease when identifying an obese child with NAFLD and thus the need to initiate obesity treatment. Such interventions should incorporate comprehensive considerations to factors that may elicit or worsen childhood obesity. In previous studies, we have shown consistent beneficial effects of intervention. Thus, after one and two year(s) of a multidisciplinary anti-obesity intervention 68.7% and 62.5% of the patients, respectively, reduced their BMI SDS [19] with accompanying improved serum lipid profiles (Nielsen *et* al., unpublished data).

The strengths of the present study was the relative large number of individuals included and that liver fat content was measured by ^{1}H-MRS, which is recognized to be a good non-invasive alternative to biopsy [44-50]. Furthermore, we evaluated the patients after a median of approximately 1 year. As the study population was recruited from an on-going clinic this strength according to a long follow-up period can also be seen as a limitation due to the range in time. The limitations of the present observational study were that we did not have blood samples from all of our participants at the same time as MR examinations were performed, why these results might be biased and underpowered. The ^{1}H-MRS measurements of liver fat content were not able to differentiate between the histological stages of NAFLD, e.g. fibrosis and cirrhosis. In the present study we also identified patients, who did not reduce their liver fat content, and this group should be offered an even more intensified and individualized treatment regimen. Furthermore, the study design included each patient as his/hers own control and thus investigated the effects of the long-term multidisciplinary childhood obesity treatment program upon changes in liver fat content and anthropometrics. This design can be discussed as a randomized case-control study might be another opportunity in this scientific manner. However, our study provides important information to the knowledge of the output of paediatric intervention studies.

In conclusion, the treatment offered by this chronic care multidisciplinary childhood obesity intervention not only reduces BMI SDS but also reduces the degree of liver fat content measured by ^{1}H-MRS in a substantial group of these obese children and adolescents. Further, the treatment results in improvements in central obesity, measured by WHt, and VAT. Reduction in liver fat content was associated with reductions in SAT and serum AST/ALT ratio, indicating that the serum AST/ALT ratio might be a clinical tool for monitoring paediatric patients with hepatic steatosis.

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Table 1
Main characteristics of the included patients and the "lost-to-follow-up" individuals

	Included participants	"lost-to-follow-up"	p-value
N (boys/girls)	105 (45/60)	132 (61/71)	
Age (years)	12.2 (6.6-20.4)	14.0 (6.5-20.8)	0.0006
Weight SDS	4.08 (2.90-4.55)	3.99 (2.90-4.56)	0.04
Height SDS	0.80 (-1.52 -2.90)	0.83 (-2.12-3.25)	0.82
BMI SDS	2.93(1.32-5.20)	3.02 (1.42-4.39)	0.15
WC-height ratio	0.62 (0.51-0.81)	0.63 (0.47-0.77)	0.13
Liverfat percentage (%)	4.2 (0.2-67.1)	4.5 (0.1-69.0)	0.59

Data are medians (ranges). P-values calculated by Mann-Whitney U test.
BMI: Body mass index. SDS: standard deviation score. WC: Waist circumference

Table 2.

Characteristics of 105 obese children and youths before and after median 358 days of intervention

	Baseline	**Follow-up**	**Changes**	**p-value*** **
Total N (boys/girls)	105 (45/60)	105 (45/60)	105 (45/60)	
Age (years) (median with ranges)	12.1 (6.6-20.4)	13.1 (7.5-21.4)		
Anthropometrics				
Weight (kg)	68.1(37.9-127.7)	71.9 (43.5-121.5)	6%	<0.001
Weight SDS*	3.94 (2.90-4.55)	3.89 (2.91-4.54)	-0.05	0.002
Height (cm)	156 (131-188)	161 (136-191)	3%	<0.001
Height SDS*	0.83 (-1.52-2.90)	0.84 (-1.72-2.98)	0.05	0.04
BMI (kg/m^2)	28.3 (19.8-41.8)	28.2 (19.7-42.4)	-0.1	0.60
BMI SDS*	2.91 (1.32-5.20)	2.71 (0.82-4.64)	-0.19	<0.001
Puberty stage**				
Pre-pubertal (%)	39	22		
Pubertal (%)	46	56		
Post-pubertal (%)	15	22		
Waist circumference (cm)	97 (79-128)	97 (71-129)	0.1%	0.91
Waist-height ratio	0.62 (0.51-0.81)	0.60 (0.45-0.79)	-2%	0.02
MR measured fat distributions				
Liver fat content (%)	4.2 (0.2-67.1)	3.2 (0.1-65.1)	-22%	0.003
SAT (cm^3)	278 (104-665)	274 (74-711)	-2%	0.52
VAT (cm^3)	80 (30-234)	73 (21-484)	-8%	0.04
SAT/VAT ratio	3.46 (1.6-10.3)	3.91 (1.9-9.2)	12%	<0.001

Data (weight, height, BMI, waist circumference, waist-height ratio, liver fat content, SAT, VAT, and SAT/VAT) are log transformed prior to analyses to become normal distributed and presented as geometric means with ranges
*Data are means with ranges
**Puberty stages based on Tanner stages; stage 1=pre-pubertal, stage 2-4=pubertal, and stage 5=post-pubertal
*** Baseline values are compared with follow-up value analysed by paired *t*-test. Further, data were also analysed using Mann-Whitney analyses. Results are not shown as the p-values were almost similar
Changes are the differences (follow-up – baseline) presented as percentages
BMI; body mass index, MR; magnetic resonance, SAT; subcutaneous adipose tissue, SDS; standard deviation score, VAT; visceral adipose tissue

Table 3.

Differences of anthropometric and biochemical characteristics in105 obese children and youths stratified by MR spectroscopy investigated hepatic steatosis

	Steatosis at baseline (n=41)			**Non-steatosis at baseline (n=64)**			**p-value (A vs D)^**	**p-value (B vs E)^**	**p-value (C vs F)^**
	Baseline (A)	**Follow-up (B)**	**Change (C)****	**Baseline (D)**	**Follow-up (E)**	**Change (F)****			
Anthropometrics									
Weight SDS^^	3.90 (2.92-4.55)	3.87 (2.91-4.54)	-0.01	3.96 (2.90-4.51)	3.91 (2.91-4.51)	-0.08	0.53	0.76	0.05
Height SDS^^	1.07 (-0.88-2.78)	1.12 (-0.34-2.97)	0.05	0.69 (-1.52-2.89)	0.69 (-1.72-2.98)	-0.02	0.04	0.03	0.41
BMI SDS^^	3.13 (1.84-4.04)	2.99 (1.85-3.79)	-0.15	2.77 (1.32-5.20)	2.55 (0.83-4.64)	-0.21	0.002	<0.001	0.51
Waist circumference (cm)*	101 (79-127)	102 (79-129)	1%	95 (79.5-128)	94 (71-123)	-0.1%	0.02	0.03	0.69
Waist-height ratio*	0.63 (0.55-0.74)	0.62 (0.48-0.77)	-2%	0.62 (0.51-0.81)	0.60 (0.45-0.79)	-3%	0.16	0.19	0.86
MR measured fat distributions									
Liver fat content (%)*	13.8 (5.1-67.1)	9.0 (1.0-65.1)	-35%	2.0 (0.2-4.7)	1.7 (0.1-9.1)	-13%			0.08
≥5% (n)*	41	31		0	7				
<5% (n)*	0	10		64	57				
SAT (cm^3)*	319 (156-665)	324 (154-672)	1%	255 (104-647)	246 (74-711)	-3%	0.01	<0.001	0.34
VAT (cm^3)*	99 (38-234)	94 (36-484)	-4%	70 (30-168)	62 (21-396)	-10%	<0.001	<0.001	0.35
SAT/VAT ratio*	3.23 (2.00-8.40))	3.58 (1.9-6.8)	10%	3.62 (1.6-10.3)	4.14 (1.9-9.2)	14%	0.12	0.03	0.46

*Data are log transformed prior to analyses to become normal distributed and presented as the geometric means with ranges
**Changes are the differences (follow-up – baseline) presented as percentages
^ Data are analysed by *t*-test
^^Data are presented as means with ranges
Steatosis is defined as a liver fat content ≥5%
BMI; body mass index, MR; magnetic resonance, SAT; subcutaneous adipose tissue, SDS; standard deviation score, VAT; visceral adipose tissue

Table 4. Differences of biochemical characteristics in 41 of the 105 obese children and youths stratified by MR spectroscopy investigated hepatic steatosis

	Steatosis at baseline (n=14)			**Non-steatosis at baseline (n=27)**			**p-value (A vs D)^**	**p-value (B vs E)^**	**p-value (C vs F)^**
	Baseline (A)	**Follow-up (B)**	**Change (C)****	**Baseline (D)**	**Follow-up (E)**	**Change (F)****			
Anthropometrics									
Weight SDS^^	4.11 (2.96-4.55)	4.12 (2.93-4.47)	0.004	3.86 (2.90-4.43)	3.75 (2.91-4.36)	-0.11	0.09	0.02	0.07
Height SDS^^	1.06 (0.02-2.31)	1.00 (-0.34-2.49)	-0.06	0.78 (-0.72-2.90)	0.80 (-0.68-2.98)	-0.02	0.36	0.43	0.75
BMI SDS^^	3.12 (2.27-3.82)	2.89 (2.29-3.75)	-0.23	2.77 (1.34-5.20)	2.60 (1.15-4.64)	-0.17	0.05	0.13	0.69
Waist circumference (cm)*	98 (85-123)	97 (80-128)	-1%	97 (80-128)	93 (70.7-122.0)	-2%	0.80	0.44	0.94
Waist-height ratio*	0.63 (0.55-0.74)	0.61 (0.48-0.77)	-35%	0.63 (0.55-0.81)	0.62 (0.47-0.77)	-34%	0.96	0.50	0.95
MR measured fat distributions									
Liver fat content (%)*	10.8 (5.2-67)	6.0 (1-32)	-44%	2.0 (0.2-4.3)	1.9 (0.5-8.9)	-2%			0.03
≥5% (n)*	14	8		0	3				
<5% (n)*	0	6		27	24				
SAT (cm^3)*	274 (156-556)	281 (175-577)	2%	263 (108-647)	245 (74-711)	-8%	0.77	0.32	0.32
VAT (cm^3)*	85 (40-186)	74 (36-183)	-15%	70 (33-127)	64 (32-396)	-8%	0.17	0.39	0.68
SAT/VAT ratio*	3.18 (2.0-5.7)	3.78 (2.4-6.6)	17%	3.71 (2.0-6.8)	4.26 (2.5-7.1)	13%	0.15	0.22	0.71
Fasting blood variables									
Serum ALT (U/l)*	32.0 (12-87)	31.4 (13-127)	-6%	23.7 (12-129)	22.1 (10-69)	-7%	0.09	0.13	0.58
Serum AST (U/l)*	29.9 (12.6-40.6)	28.1 (16.3-53.8)	-3%	25.1 (15.3-85.5)	22.9 (14.3-36.7)	-2%	0.18	0.24	0.98
Serum AST/ALT ratio*	0.96 (0.58-1.71)	0.90 (0.42-1.30)	-6%	1.05 (0.41-1.75)	0.98 (0.37-1.6)	-1%	0.47	0.62	0.67
Serum GGT (U/l)*	20.5 (9-134)	16.2 (11-29)	-6%	16.1 (9-61)	15.0 (4-42)	-7%	0.24	0.52	0.88
Plasmaglucose (mM)*	5.0 (4.3-6.0)	5.1 (4.8-5.5)	3%	5.1 (4.2-5.9)	5.2 (4.5-5.8)	2%	0.50	0.27	0.75
Serum insulin (mU/l)*	96.8 (44-216)	82.7 (43-144)	-15%	76.0 (21-174)	71.9 (16-154)	-12%	0.16	0.46	0.88
HOMA-IR*	3.57 (1.60-8.32)	3.15 (1.62-5.48)	-13%	2.91 (0.72-7.35)	2.75 (0.53-6.04)	-10%	0.26	0.54	0.87
Total serum cholesterol (mM)*	4.5 (2.6-7.3)	4.3 (2.9-5.6)	-5%	4.2 (2.6-7.3)	4.2 (2.9-9.4)	-0.3%	0.39	0.93	0.41
Serum LDL (mM)*	2.6 (1.1-4.8)	2.4 (0.9-3.7)	-4%	2.5 (1.2-5.9)	2.5 (1.6-7.9)	-3%	0.84	0.91	0.85
Serum HDL (mM)*	1.3 (0.8-2.2)	1.2 (0.9-1.9)	-3%	1.2 (0.6-1.6)	1.2 (0.5-1.8)	3%	0.31	0.73	0.35
Serum triglycerides (mM)*	1.12 (0.5-3.2)	1.05 (0.6-2.1)	-4%	0.97 (0.5-2.0)	0.99 (0.4-2.2)	2%	0.35	0.67	0.69

*Data are log transformed prior to analyses to become normal distributed and presented as the geometric means with ranges
**Changes are the differences (follow-up – baseline) presented as percentages
^ Data are analysed by *t*-test
^^Data are presented as means with ranges
Steatosis is defined as a liver fat content ≥5%
ALT; alanine aminotransferase, AST; aspartate aminotransferase, BMIGGT; gamma-glutamyl transferase, HDL; high density lipoprotein, HOMA-IR; homeostasis model assessment insulin resistance, LDL; low density lipoprotein; MR; magnetic resonance, SAT; subcutaneous adipose tissue, SDS; standard deviation score, VAT; visceral adipose tissue

Table 5.

Prediction of improvement in ^{1}H-MR spectroscopy measured liver fat content in obese children and youths

10% change in variables*	Change in liver fat content (%)*	p-value
Anthropometrics (n=105)		
weight SDS*	-8%	0.51
height SDS*	3%	0.57
BMI SDS*	8%	0.10
waist circumference	58%	0.18
waist-height ratio	51%	0.22
MR measured fat distributions (n=105)		
SAT	11%	0.02
VAT	5%	0.19
SAT/VAT ratio	-8%	0.10
Fasting blood variables (n=41)		
serum ALT	9%	0.36
serum AST	31%	0.47
serum AST/ALT ratio	44%	0.01
serum GGT	40%	0.25
plasma glucose	-17%	0.77
serum insulin	0.3%	0.98
HOMA-IR	2%	0.91
total serum cholesterol	39%	0.40
serum LDL	38%	0.22
serum HDL	-30%	0.25
serum triglycerides	14%	0.34

Data are analysed using a general linear model adjusted for baseline liver fat content (logarithmic transformed), age at baseline, pubertal stage at baseline, and gender.

*Changes are the differences between the follow-up value and the baseline value presented as percentages. The changes are calculated as the logarithmic transformed follow-up value minus the logarithmic transformed baseline value for each person, however weight SDS, height SDS, and BMI SDS are not logarithmic transformed

ALT; alanine aminotransferase, AST; aspartate aminotransferase, BMI; body mass index, GGT; gamma-glutamyl transferase, HDL; high density lipoprotein, HOMA-IR; homeostasis model assessment insulin resistance, LDL; low density lipoprotein; MR; magnetic resonance, SAT; subcutaneous adipose tissue, SDS; standard deviation score, VAT; visceral adipose tissue

Figure legend:

Figure 1 Flow-chart illustrating the treatment regimen.

Figure 2 ^{1}H MRS spectra from the hepatic volume (marked by white arrow on MR images) at the baseline MR examination and at the follow-up MR examination one year later. Liver fat was reduced from 11% to 3%.

Figure 1

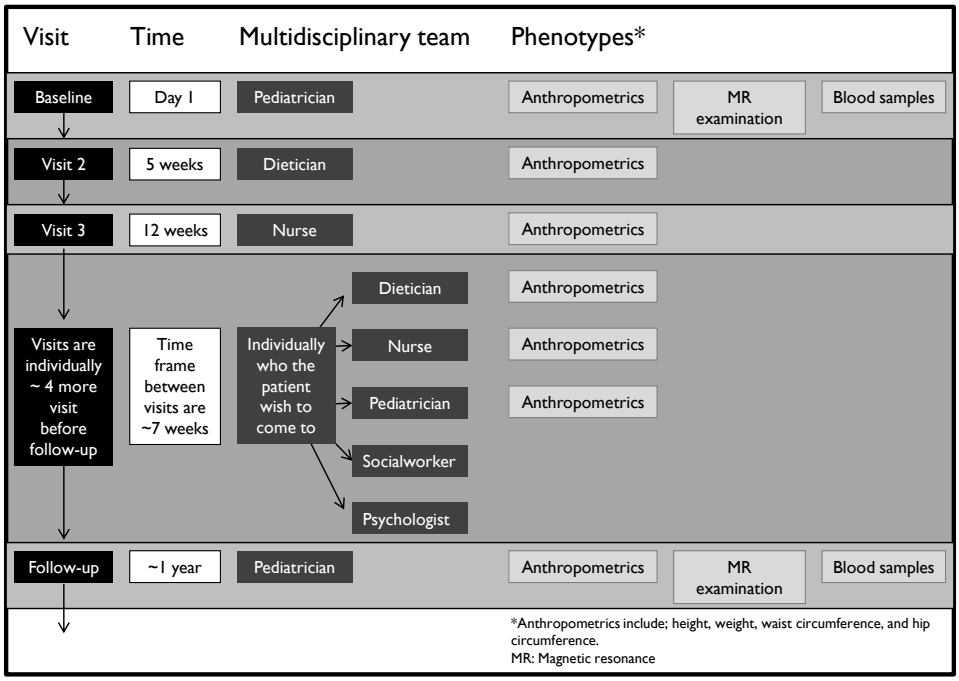
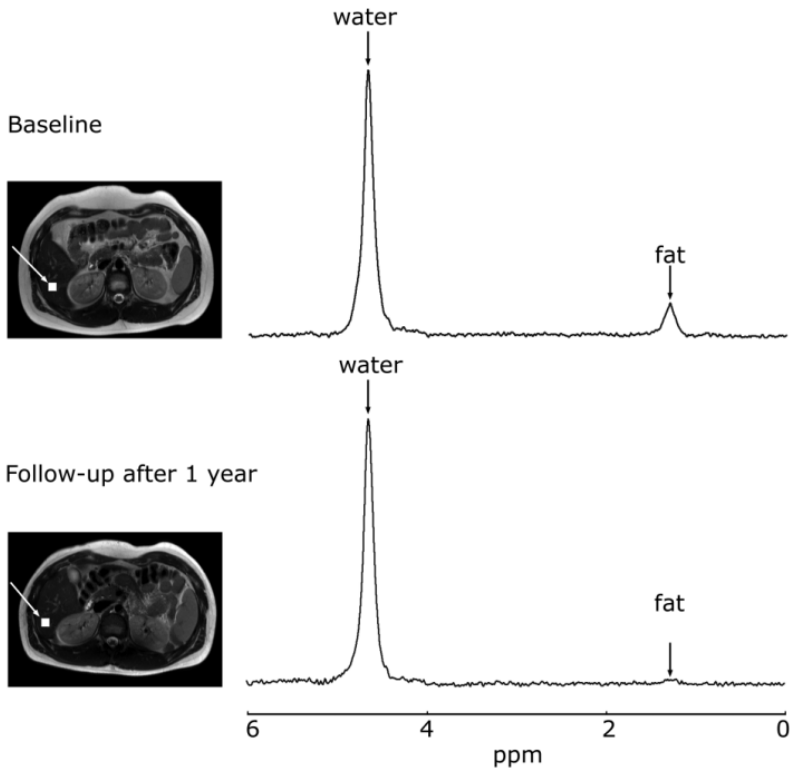

Visit
Time
Multidisciplinary team
Phenotypes*
Baseline
Day 1
Pediatrician
Anthropometrics
MR examination
Blood samples
Visit 2
5 weeks
Dietician
Anthropometrics
Visit 3
12 weeks
Nurse
Anthropometrics
Visits are individually ~ 4 more visit before follow-up
Time frame between visits are ~7 weeks
Individually who the patient wish to come to
Dietician
Nurse
Pediatrician
Socialworker
Psychologist
Anthropometrics
Anthropometrics
Anthropometrics
Follow-up
~1 year
Pediatrician
Anthropometrics
MR examination
Blood samples
*Anthropometrics include; height, weight, waist circumference, and hip circumference.
MR: Magnetic resonance

Figure 2



Study III

Implications of central obesity-related variants in *LYPLAL1, NRXN3, MSRA*, and *TFAP2B* on quantitative metabolic traits in adult Danes

By Dorthe Sadowa Bille, Karina Banasik, Johanne Marie Justesen, Camilla Helene Sandholt, Annelli Sandbæk, Torsten Lauritzen, Torben Jørgensen, Daniel R. Witte, Jens-Christian Holm, Torben Hansen, Oluf Pedersen

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Implications of Central Obesity-Related Variants in *LYPLAL1, NRXN3, MSRA,* and *TFAP2B* on Quantitative Metabolic Traits in Adult Danes

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Abstract

Background: Two meta-analyses of genome-wide association studies (GWAS) have suggested that four variants: rs2605100 in lysophospholipase-like 1 *(LYPLAL1),* rs10146997 in neuroxin 3 *(NRXN3),* rs545854 in methionine sulfoxide reductase A *(MSRA),* and rs987237 in transcription factor activating enhancer-binding protein 2 beta (*TFAP2B)* associate with measures of central obesity.To elucidate potential underlying phenotypes we aimed to investigate whether these variants associated with: 1) quantitative metabolic traits, 2) anthropometric measures (waist circumference (WC), waist-hip ratio, and BMI), or 3) type 2 diabetes, and central and general overweight and obesity.

Methodology/Principal Findings: The four variants were genotyped in Danish individuals using KASPar®. Quantitative metabolic traits were examined in a population-based sample ($n = 6{,}038$) and WC and BMI were furthermore analyzed in a combined study sample ($n = 13{,}507$). Case-control studies of diabetes and adiposity included 15,326 individuals. The major G-allele of *LYPLAL1* rs2605100 associated with increased fasting serum triglyceride concentrations (per allele effect (β) = 3%(1;5(95%CI)), $p_{additive} = 2.7 \times 10^{-3}$), an association driven by the male gender ($p_{interaction} = 0.02$). The same allele associated with increased fasting serum insulin concentrations ($\beta = 3\%(1;5)$, $p_{additive} = 2.5 \times 10^{-3}$) and increased insulin resistance (HOMA-IR) ($\beta = 4\%(1;6)$, $p_{additive} = 1.5 \times 10^{-3}$). The minor G-allele of rs10146997 in *NRXN3* associated with increased WC among women ($\beta = 0.55$cm (0.20;0.89), $p_{additive} = 1.7 \times 10^{-3}$, $p_{interaction} = 1.0 \times 10^{-3}$), but showed no associations with obesity related metabolic traits. The *MSRA* rs545854 and *TFAP2B* rs987237 showed nominal associations with central obesity; however, no underlying metabolic phenotypes became obvious, when investigating quantitative metabolic traits. None of the variants influenced the prevalence of type 2 diabetes.

Conclusion/Significance: We demonstrate that several of the central obesity-associated variants in *LYPLAL1, NRXN3, MSRA,* and *TFAP2B* associate with metabolic and anthropometric traits in Danish adults. However, analyses were made without adjusting for multiple testing, and further studies are needed to confirm the putative role of *LYPLAL1*, *NRXN3, MSRA*, and *TFAP2B* in the pathophysiology of obesity.

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Introduction

Obesity is a major health problem with increasing prevalence in Western societies, and obesity, as well as the often associated insulin resistance (IR), are important risk factors for type 2 diabetes, cardiovascular diseases, hypertension, and several other chronic diseases. To provide new insight into the pathophysiology of obesity, genome-wide association studies (GWAS) have in the last few years

been performed with elaborating results [1,2,3,4,5,6,7] identifying variants associated with body mass index (BMI) – an indirect measure of general adiposity. Following, two meta-analyses demonstrated four loci to associate with measures of central adiposity (waist circumference (WC) and waist–hip-ratio (WHR)): 1) a meta-analysis of 16 GWAS ($n = 38,580$) with follow-up studies in a maximum of 118,691 individuals of European origin, identified variants near lysophospholipase-like 1 *(LYPLAL1),* methionine sulfoxide reductase A *(MSRA),* and transcription factor activating enhancer-binding protein 2 beta *(TFAP2B),* to strongly associate with measures of central adiposity using information about adult WC and WHR [8], and 2) a large-scale meta-analysis of GWAS from the CHARGE Consortium involving 70,014 Caucasian individuals identified neuroxin 3 (*NRXN3)* as a novel locus for central adiposity [9].

Lysophospholipase-like 1 protein, encoded by *LYPLAL1,* is thought to act as a triglyceride lipase and reported to be up-regulated in subcutaneous adipose tissue of obese individuals [10]. In the meta-analysis of 16 GWAS, the major G-allele of rs2605100 in *LYPLAL1* associated with increased WHR ($p = 2.6\times10^{-8}$) only among women [8]. *NRXN3* encodes the neuroxines protein family that functions in the vertebrate nervous system as cell adhesion molecules and receptors. In patients with schizophrenia, *NRXN3* has been demonstrated to associate with alcohol dependence and the degree of nicotine dependence [11,12]. The minor G-allele of rs10146997 in *NRXN3* associated with WC ($p = 6.4\times10^{-7}$) and BMI ($p = 7.4\times10^{-6}$) in the CHARGE GWAS; however, the association with WC attenuated ($p = 0.32$), when adjusted for BMI [9]. *MSRA* encodes an antioxidant enzyme that repairs proteins inactivated by oxidative damage, but the biological connections between the *MSRA* locus and adiposity are unclear [8]. In the meta-analysis of 16 GWAS, a strong association was found with the minor G-allele of rs545854 in *MSRA* and central obesity measured by WC ($p = 8.9\times10^{-9}$) (mixed-gender analysis) [8]. The association diminished ($p = 0.11$), when adjusted for BMI.

The binding-protein encoded by *TFAP2B* is expressed in adipose tissue [13]. Studies have shown an association between *TFAP2B* and type 2 diabetes, and over-expression of *TFAP2B* in adipocytes leads to IR and accumulation of triglycerides inside the adipocytes [13,14,15]. In a study of 1,176 adolescents, Nordquist *et al.* found that men carrying the 4 repeat allele of intron 2 polymorphism (intronic variable number tandem repeat) of *TFAP2B* had higher insulin sensitivity and central obesity (measured by skin folds) [16]. Another study, comprising 81 individuals suggested that *TFAP2B* might be a novel candidate gene for development of the metabolic syndrome, as it was shown to regulate the expression of various adipokines [17]. In the meta-analysis of 16 GWAS, the minor G-allele of rs987237 in *TFAP2B* associated significantly with central obesity measured by WC ($p = 1.9\times10^{-11}$) and general obesity measured by BMI ($p = 7.0\times10^{-12}$ (stage 2 alone)) in the mixed-gender analysis [8]. Although these genes are considered obvious candidates for obesity, the causal variants and their role in the pathogenesis of obesity remain to be elucidated.

The aim of the present study was to investigate central obesity-associated variants in *LYPLAL1, NRXN3, MSRA,* and *TFAP2B* for associations with quantitative metabolic traits in a population-based sample of 6,038 adult Danes (The Inter99 study sample). In addition, we examined associations between these variants and anthropometric measures (WC, WHR, and BMI) in a larger group of individuals. Also, we conducted large case-control studies involving a total of 15,326 Danes, investigating associations between these variants and type 2 diabetes, as well as both central and general overweight and obesity. Since three of the variants (*LYPLAL1, MSRA,* and *TFAP2B*) in previous studies have been investigated using sex-stratified analyses, we performed all analyses stratified according to sex.

Results

In the Inter99 population, the major G-allele of rs2605100 in *LYPLAL1* associated with fasting serum triglyceride concentrations (per allele effect(β) = 3%, ((95% confidence interval(CI))1;5), $p_{\text{additive (add)}} = 2.7\times10^{-3}$), fasting serum insulin concentrations (β = 3% (1;5), $p_{\text{add}} = 2.5\times10^{-3}$), and homeostasis model assessment-insulin resistance (HOMA-IR) (β = 4% (1;6), $p_{\text{add}} = 1.5\times10^{-3}$) (table 1). When stratifying according to sex the association with fasting serum triglycerides was restricted to men (β = 6% (3;9), $p_{\text{add}} = 2.4\times10^{-4}$) (table 1). Indeed, the interaction analysis revealed an interaction between sex and genotype for triglyceride levels ($p_{int} = 0.02$) (Table S2). Furthermore, we found a borderline association between rs2605100 and decreased BMI (β = −0.18 kg/m^2 (−0.36;−1×10^{-3}), $p_{\text{add}} = 0.05$), and decreased WC (β = −0.08 cm (−0.29;0.13), $p_{\text{add}} = 0.04$) (table 1). However, the association with WC diminished, when adjusted for BMI ($p_{\text{add}} = 0.47$) (Table S2). The combined quantitative trait (QT) analysis showed associations with decreased WC (β = −0.48 cm (−0.81;0.16), $p_{\text{add}} = 0.004$) and BMI (β = −0.16 kg/m^2 (−0.28; −0.04), $p_{\text{add}} = 0.01$), but the associations diminished, when data were adjusted for BMI ($p_{\text{add}} = 0.12$) and WC ($p_{\text{add}} = 0.87$), respectively (Table S3 and Table S4). In the case-control study the variant associated with central obesity, measured by WC (Odds Ratio(OR)$_{\text{add}}$ = 0.92 (0.86–0.98(95% CI)), $p_{\text{add}} = 0.01$) (table 2), and the association strengthened, when WC was adjusted for BMI ($p_{\text{add}} = 0.004$) (Table S3). No other significant associations were found for central or general obesity (table 2).

The minor G-allele rs10146997 of *NRXN3* associated with increased WC among women (β = 0.55 cm (0.20;0.89), $p_{\text{add}} = 1.7\times10^{-3}$) (table 2) in the Inter99, which was underpinned by the interaction analysis ($p_{\text{int}} = 1.0\times10^{-3}$) (Table S5). The significance level attenuated, when adjusted for sex and age only (Table S5). This association was not found in the larger combined QT analysis (Table S3). Neither were we able to show any associations with other obesity-related metabolic phenotypes. In the case-control analyses the variant showed no association with general adiposity, measured by BMI ($OR_{\text{add}} = 1.02$ (0.94–1.11), $p_{\text{add}} = 0.66$) (Table S4). No interaction between sex and genotype was found in these analyses.

The rs545854 minor G-allele in *MSRA* associated with decreased fasting serum insulin concentrations (β = −4% (−8;−1), $p_{\text{add}} = 0.02$), and HOMA-IR (β = −5% (−9;−1), $p_{\text{add}} = 0.02$) among men (table 1). However, the interaction analysis did not show any interaction between sex and genotype for the two traits ($p_{int} = 0.08$) (Table S6). The variant was not associated with other quantitative metabolic or anthropometric traits (Table S3, S4, and S6). When comparing the genotype distribution between lean and obese individuals a borderline association between rs545854 and central obesity was observed ($OR_{\text{add}} = 1.08$ (1.00–1.18), $p_{\text{add}} = 0.05$) (table 2), and when adjusted for BMI, this association was strengthened ($p_{\text{add}} = 0.02$) (Table S3).

The minor G-allele rs987237 in *TFAP2B* did not associate with any metabolic traits in the QT analysis (Table S3, S4, and S7). However, the variant was borderline associated with central obesity ($OR_{\text{add}} = 1.08$ (1.0–1.17), $p_{\text{add}} = 0.06$) in the case-control analysis (Table S3). This borderline association became nominal significant, when stratified to women ($OR_{\text{add}} = 1.17$ (1.03–1.32), $p_{\text{add}} = 0.01$; $p_{\text{int}} = 0.04$). When adjusted for BMI, the association became stronger among women ($p_{\text{add}} = 0.001$) and it also became evident among men ($p_{\text{add}} = 0.01$) (Table S3).

Table 1. Variants showing statistical significant associations with quantitative metabolic traits ($n = 6{,}038$).

	Quantitative metabolic traits						
Variant	*BMI (kg/m2)*	*WC (cm)*	*WHR*	*Fasting serum triglyceride*	*Fasting serum insulin*	*Fasting plasma glucose*	*Insulin resistance, HOMA-IR*
LYPLAL1 rs2605100							
All	−0.18 (−0.36; -1×10^{-3}), 0.05	−0.08 (−0.29; 0.13), 0.04		3% (1;5), 2.7×10^{-3}	3% (1;5), 2.5×10^{-3}		4% (1;6), 1.5×10^{-3}
Men				6% (3;9), 2.4×10^{-4}			
Women					4% (1;6), 0.01	1% (0;1), 0.04	4% (1;7), 6.0×10^{-3}
NRXN3 rs10146997							
Women		0.55 (0.20; 0.89), 0.02	4×10^{-3} (2×10^{-4};0.01), 0.02				
MSRA rs545854							
Men					−4% (−8;−1), 0.02		−5% (−9;−1), 0.02

The table includes effect sizes and p-values (β (95% CI), p-value) for significant associations only.
Values of fasting serum triglycerides, serum insulin, plasma glucose, and HOMA-IR were logarithmically transformed prior to statistical analyses, and their effect sizes (β) are presented as the increase/decrease in percent. *P*-values were calculated assuming an additive model (p_{add}). BMI, waist circumference and waist-hip ratio were adjusted for age and sex.
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No heterogeneity between the study groups was observed for the investigated variants, except for *TFAP2B* rs987237 in the combined QT analysis in relation to general obesity (BMI) (Fixed effect, I^2: 78%[41%–92%], $p = 0.003$) (Table S4).

None of the variants associated with central and general overweight, or type 2 diabetes in the case-control analyses (data not shown).

Table 2. Variants showing statistical significant associations with BMI and/or WC in the case-control analyses ($n = 15{,}326$).

	BMI	*WC*
	OR (95%CI), p-value	*OR (95%CI), p-value*
LYPLAL1 rs2605100		
All		0.92(0.86–0.98), 0.004
Men		0.89(0.82–0.97), 0.0004
NRXN3 rs10146997		
All	1.02 (0.94–1.11), 0.04	
Women	1.04 (0.93–1.17), 0.04	
MSRA rs545854		
All		1.08(1.00–1.18), 0.02
Women		1.09(0.96–1.23), 0.04
TFAP2B rs987237		
Men		1.01 (0.91–1.13), 0.01
Women		1.17 (1.03–1.32), 0.001

The effect is the odds ratio (OR) presented as the increase/decrease and 95% confidence interval (CI). Effect and *p*-values shown are for an additive genetic model (p_{add}) and are adjusted for age, sex, and diabetes treatment for the obese cases. BMI, body mass index; WC, waist circumference.
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Discussion

In this study we found several associations with quantitative metabolic and anthropometric traits for the central obesity-associated variants in *LYPLAL1, NRXN3, MSRA*, and *TFAP2B*.

As a major finding the G-allele (risk-allele) of rs2605100 in *LYPLAL1* associated with elevated concentrations of fasting serum triglycerides and fasting serum insulin and with estimates of central obesity. In a recent meta-analysis of 32 GWAS for WHR, a variant in *LYPLAL1* (rs4846567) in high linkage disequilibrium ($r^2 = 0.64$, D' = 0.84, HapMap CEU population) with rs2605100 achieved genome-wide significance, and the subsequent association analyses with related metabolic traits in 77,167 participants (follow-up comprising up to 113,636 subjects) showed that this variant associated with increased levels of serum triglycerides ($p = 0.018$), fasting insulin ($p = 1.1\times10^{-5}$), and HOMA-IR ($p = 9.8\times10^{-6}$) [18]. These previously reported findings strengthen the present observations in our somewhat smaller study population. Moreover, we additionally investigated sex-specific effects of rs2605100 and found that the association with fasting serum triglycerides was restricted to men. This association has not previously been reported.

Our findings for rs2605100 in *LYPLAL1* are in line with previous observations that a higher fat-mass causes increased lipogenesis, resulting in higher levels of circulating triglycerides and IR [19,20,21]. In mice, it has been demonstrated that insulin inhibits the breakdown of fat in adipose tissue by inhibiting the intracellular triglyceride lipase in the beta-cell [10,21]. The elevated triglyceride concentrations may result from an increased expression of the lipase gene, which then facilitates higher triglyceride lipase activity in the adipose tissue. Furthermore, obesity is associated with an increased number and/or size of adipose cells. These cells respond more poorly to insulin, as they have impaired insulin signaling, resulting in increased activity of the lipoprotein lipases, including the triglyceride lipase. The increased lipase activity and increased mass of adipose tissue lead

to an increase in circulating FFAs, which is a major contributor to the development of IR [22]. Thus, this underlying insulin resistant phenotype might add to explain the associations found between the *LYPLAL1* variant and obesity.

The minor G-allele rs10146997 in *NRXN3* associated with increased WC among women, but we found no clear association with BMI as reported in a recent meta-analysis of 249,796 individuals [23]. Neither did we observe an association between rs10146997 and fasting serum insulin levels nor IR (HOMA-IR) as reported for rs10150332 in *NRXN3* ($r^2 = 1.00$, D' = 1.00, HapMap CEU population) in the meta-analysis [23]. The reason for these inconsistencies may be 1) lack of statistical power in our study due to the relatively low minor allele frequency, or 2) the association observed in the meta-analysis may reflect a random correlation between IR and BMI.

In this study, the *MSRA* rs545854 minor G-allele showed associations with decreased fasting levels of serum insulin and decreased HOMA-IR among men, but no association with obesity was evident. The latter finding is consistent with the recent meta-analyses that failed to demonstrate genome-wide significant evidence of associations between this variant and BMI or WHR [18,23]. Also, analyses of rs987237 in *TFAP2B* showed borderline association with central obesity among women. The GWAS showed no evidence of sex-specific associations. However, *TFAP2B* was confirmed as an obesity locus in the recent meta-analysis, where this variant associated with BMI at a genome-wide significant level [23].

It is well known that substantial gender-specific differences in fat distribution exist [18]. These have been shown to reflect genetic influences i.e. on WC, and hip circumference, and for WHR the genetic variance is almost twice as large in women than in men [24]. The underlying molecular mechanisms are not clearly understood. In this study, the observed effect sizes did not provide sufficient statistical power to account for the gender stratification. Therefore, we cannot exclude that the observed gender-specific results reflect spurious findings. Further studies are needed to investigate a putative influence of gender on the associations observed for these variants.

Whether variation in the examined four genes is causal in relation to obesity remain unclear. *NRXN3* is expressed in the brain, as many of the other recently discovered obesity loci, and variants in *NRXN3* may provide changes of the brain function and behavior [11]. Eating disorders have been ascribed to the hypothalamic part of the brain [25], and recently, *NRXN3* has been investigated in relation to addiction of alcohol and smoking [11,12]. *MSRA* is thought to be involved in the oxidative damage in cells but the molecular mechanisms are not yet elucidated. Another candidate gene located near *MSRA*, the TRF1-interacting ankyrin-related ADP-ribose polymerase (*TNKS*), has been proposed as the causal gene for the association with central obesity [8]. Previously, *TFAP2B* has been investigated for associations with several phenotypes correlated to diabetes and obesity [13,14,15,16,17], and has been proposed to be a new gene for the metabolic syndrome. We showed an association between central obesity and rs987237 *TFAP2B* minor G-allele, however, we were not able to determine the underlying phenotype for this association in the quantitative trait analysis of obesity-related measures, nor did we show any association with type 2 diabetes.

In conclusion, we found that several of the central obesity-associated variants in *LYPLAL1, NRXN3, MSRA*, and *TFAP2B* associated with metabolic and anthropometric traits among adult Danes. However, analyses were made without adjusting for multiple testing, and further studies are needed to elucidate the involvement of *LYPLAL1, NRXN3, MSRA*, and *TFAP2B* in the pathophysiology of obesity.

Materials and Methods

The studies were approved by the appropriate Regional Ethics Committees, and were in accordance with the principles of the Helsinki Declaration.

Study samples

This study comprises 15,326 Danish individuals ascertained from four different study groups (Table S1): 1) the Inter99 cohort, which is a population-based, randomized, non-pharmacological intervention study of middle-aged individuals for the prevention of ischemic heart disease ($n = 6,162$ individuals, aged 30–61 years) from the Research Centre for Prevention and Health in Glostrup, Copenhagen (ClinicalTrials.gov ID-no.: NCT00289237) [26], 2) a cohort comprising individuals with type 2 diabetes ($n = 1,695$) sampled at Steno Diabetes Center (SDC), 3) a randomized, population-based group of unrelated middle-aged individuals ($n = 730$) examined at SDC, Copenhagen, and 4) The Danish ADDITION screening cohort ($n = 6,739$), which is part of the Anglo-Danish-Dutch study of Intensive Treatment in People with Screen-detected Diabetes in Primary Care (ClinicalTrials.gov ID-no.: NCT00237548) [27].

A standandardized oral glucose tolerance test was undertaken in volunteers of Inter99. In this cohort QT analyses were performed in treatment-naive individuals involving individuals with normal glucose tolerance, impaired fasting glucose and impaired glucose tolerance. The exact numbers are given in tables. Patients with known type 2 diabetes ($n = 124$) were excluded from the QT analyses.

To increase statistical power, QT analyses for BMI, WC, and WHR were additionally performed including individuals from all study populations with the respective phenotypes available. Again, individuals with known type 2 diabetes were excluded. The combined study sample comprised 13,507 individuals.

The case-control studies of type 2 diabetes included all unrelated type 2 diabetic case patients and all glucose-tolerant control individuals from study group 1–4. All control individuals had normal fasting glycaemia and were glucose tolerant following an oral glucose tolerance test. Individuals from study group 4 with BMI$<$25 kg/m^2 were excluded. Diabetes, impaired fasting glucose, and impaired glucose tolerance were defined in accordance with World Health Organization 1999 criteria [28].

In the case-control studies for overweight and obesity, central obesity was estimated using WC and general obesity was estimated using BMI. Overweight was defined as BMI$\geq$25 kg/m^2 and $<$30 kg/m^2. Obesity was defined as BMI$\geq$30 kg/m^2. A lean control individual was defined as BMI$<$25 kg/m^2. For WC the definition of overweight and obesity was sex-specific. For women overweight was defined as WC$\geq$80 cm and $<$88 cm, and obesity as WC$\geq$88 cm. The definitions for men were a WC$\geq$94 cm and $<$102 cm for overweight, and a WC$\geq$102 cm for obesity. A lean control individual was defined as WC$<$80 cm, for women, and WC$<$94 cm for men.

All participants were Danes by self-report. Before participation informed written consent was obtained from all subjects.

Anthropometrical and biochemical measurements

The anthropometrical measurements of weight, height, WC, and hip were performed in light indoor clothes without shoes. BMI was calculated as weight in kg divided by height in m^2. WC was measured midway between the iliac crest and the lower costal

margin with the participants in standing position. Hip was measured at the level of trochante major. WC and hip were calculated in cm. The methods used to obtain biochemical measurements have been described previously [26,27]. HOMA-IR was calculated as: (fasting plasma glucose (mmol/l) × fasting serum insulin (pmol/l))/22.5 [29].

Genotyping

The rs2605100 in *LYPLAL1,* rs10146997 in *NRXN3,* rs545854 (former rs7826222) in *MSRA,* and rs987237 in *TFAP2B* were genotyped using KASPar® with success rates >97% and error rates = 0%. The risk-allele frequencies were 70% (rs2605100 G-allele in *LYPLAL1*), 21% (rs10146997 G-allele in *NRXN3*), 15% (rs545854 G-allele in *MSRA*), and 17% (rs987237 G-allele in *TFAP2B*), which are in accordance with HapMap, and obeyed Hardy-Weinberg equilibrium ($p>0.05$).

Statistical analyses

The four variants were investigated for associations with quantitative metabolic traits. The quantitative variables were tested for differences between genotyped groups using linear regression, assuming an additive model. All analyses were adjusted for sex, age, and BMI, when appropriate. BMI was additionally adjusted for WC. *P*-values given in parentheses were only adjusted for age and sex. Values of serum triglyceride, serum insulin, HOMA-IR, and plasma glucose had non-normally distributed residuals and were logarithmically transformed prior to statistical analyses. Their effect sizes (β) are presented as an increase/decrease in percent with 95% CI. Values without transformation (BMI, WC, and WHR) are given as actual values with 95% CI. The case-control studies were analyzed using logistic regression, assuming an additive model. Here we included type 2 diabetes patients, why these analyses were adjusted for sex, age and diabetes treatment, and with or without BMI or WC, respectively. Heterogeneity was assessed with the generic inverse variance meta-analysis method (R package: meta), which describes the proportion of variation in the effects that is attributable to genuine differences across the study groups rather than to random error.

To investigate whether the effect of the alleles differed between genders, we included an interaction term between sex and the genotype of interest in the linear model. In this model, we assumed an additive effect for the genotypes and sex as a binary vector. All statistical analyses were performed using R version 2.9.2. *P*-values were not adjusted for multiple hypothesis testing and $p<0.05$ was considered nominally significant.

Statistical power calculations in the case-control analyses were done using CaTS, power calculations for large genetic association studies, available at http://www.sph.umich.edu/csg/abecasis/cats/. Depending on the type of analysis, as well as the risk allele frequency (RAF) of the analyzed variant, we had between 72% and 100% power to detect genetic effects in this study ($p<0.05$) (Table S2). The lowest and the highest RAF of the examined SNPs were 15% and 70%, respectively. Using the population-based Inter99 cohort as reference, the prevalence of central overweight and obesity in the Danish population were estimated to 23% and 22%, respectively, the prevalence of general overweight and obesity in the Danish population were estimated to 39% and 17%, respectively, and the prevalence of type 2 diabetes in the examined Danish population was 5%. Our power calculations estimated a statistical power of 99% and 100% to detect associations with central overweight and obesity, respectively, for a variant with a RAF of 15% with a relative risk of 1.15 (Table S2). The statistical power estimates for quantitative traits were estimated in R using 1,000 simulations, $n = 6,162$, and with a significance threshold of 0.05 (Table S2).

Supporting Information

Table S1 Characteristics for individuals included in the analyses stratified according to study group. Data are means ± standard deviation. SDC, Steno diabetes center. WC, waist circumference.
(DOCX)

Table S2 Quantitative metabolic traits in 5,769 treatment-naïve middle-aged Danes from the population-based Inter99 cohort according to *LYPLAL1* rs2605100 genotype. Data are unadjusted means ± standard deviation or medians (interquartile range). Values of fasting serum triglycerides, serum insulin, plasma glucose, and HOMA-IR were logarithmically transformed prior to statistical analyses, and their effect sizes (β) are presented as the increase/decrease in percent. *P*-values were calculated assuming an additive model (p_{add}). Interaction analysis (p_{int}) was performed to test whether the effect of the alleles differed between men and women. All analyses were adjusted for age, sex, and BMI. Furthermore, waist circumference and waist-hip ratio were adjusted for age and sex, and the *p*-values are shown in parentheses.
(DOCX)

Table S3 *LYPLAL1* rs2605100, *NRXN3* rs10146997, *MSRA* rs545854, and *TFAP2B* rs987237 in relation to central obesity. Data are number of individuals, divided into genotype groups. The effect is either the odds ratio (OR) or the per allele effect size presented as the increase/decrease and 95%CI. Effect and *p*-values shown are for an additive genetic model (p_{add}) and are adjusted for age, sex and diabetes treatment (without/ with BMI) for the obese cases, and QT analyses are adjusted for age and sex (without/with BMI). QT, quantitative trait; WC, waist circumference; WHR, waist-hip ratio.
(DOCX)

Table S4 *LYPLAL1* rs2605100, *NRXN3* rs10146997, *MSRA* rs545854, and *TFAP2B* rs987237 in relation to general obesity. Data are number of individuals, divided into genotype groups. The effect is either the odds ratio (OR) or the per allele effect size presented as the increase/decrease and 95%CI. Effect and *p*-values shown are for an additive genetic model (p_{add}) and are adjusted for age, sex and diabetes treatment (without/ with WC) for the obese cases, and QT analyses are adjusted for age and sex (without/with WC). QT, quantitative trait.
(DOCX)

Table S5 Quantitative metabolic traits in 5,789 treatment-naïve middle-aged Danes from the population-based Inter99 cohort according to *NRXN3* rs10146997 genotype. Data are unadjusted means ± standard deviation or medians (interquartile range). Values of fasting serum triglycerides, serum insulin, plasma glucose, and HOMA-IR were logarithmically transformed prior to statistical analyses, and their effect sizes (β) are presented as the increase/decrease in percent. *P*-values were calculated assuming an additive model (p_{add}). Interaction analysis (p_{int}) was performed to test whether the effect of the alleles differed between men and women. All analyses were adjusted for age, sex, and BMI. Furthermore, waist circumference and waist-hip ratio were adjusted for age and sex, and the *p*-values are shown in parentheses.
(DOCX)

Table S6 Quantitative metabolic traits in 5,804 treatment-naïve middle-aged Danes from the population-based Inter99 cohort according to *MSRA* rs545854 genotype. Data are unadjusted means ± standard deviation or medians (interquartile range). Values of fasting serum triglycerides, serum insulin, plasma glucose, and HOMA-IR were logarithmically transformed prior to statistical analyses, and their effect sizes (β) are presented as the increase/decrease in percent. *P*-values were calculated assuming an additive model (p_{add}). Interaction analysis (p_{int}) was performed to test whether the effect of the alleles differed between men and women. All analyses were adjusted for age, sex, and BMI. Furthermore, waist circumference and waist-hip ratio were adjusted for age and sex, and the *p*-values are shown in parentheses.
(DOCX)

Table S7 Quantitative metabolic traits in 5,764 treatment-naïve middle-aged Danes from the population-based Inter99 cohort according to *TFAP2B* rs987237 genotype. Data are unadjusted means ± standard deviation or medians (interquartile range). Values of fasting serum triglycerides, serum insulin, plasma glucose, and HOMA-IR were logarithmically transformed prior to statistical analyses, and their effect sizes (β) are presented as the increase/decrease in percent. *P*-values were calculated assuming an additive model (p_{add}). Interaction analysis (p_{int}) was performed to test whether the effect of the alleles differed between men and women. All analyses were adjusted for age, sex, and BMI. Furthermore, waist circumference and waist-hip ratio were adjusted for age and sex, and the *p*-values are shown in parentheses.
(DOCX)

Table S8 Statistical power estimates. *Central overweight:* ~4,500 controls and ~3,700 overweight individuals. Prevalence = 0.23 (Men with waist circumference ≥94 cm and <102 cm and women with waist circumference ≥80 cm and <88 cm from the Inter99). *General overweight:* ~3,200 controls and ~7,000 overweight individuals. Prevalence = 0.39 (Individuals with BMI≥25 kg/m^2 and BMI<30 kg/m^2 from the Inter99). *Central obesity:* ~4,500 controls and ~7,000 obese individuals. Prevalence = 0.22 (men with waist circumference ≥102 cm and women with waist circumference ≥88cm from the Inter99). *General obesity:* ~3,200 controls and ~4,800 obese individuals. Prevalence = 0.17 (Individuals with BMI≥30 kg/m^2 from the Inter99). *Type 2 diabetes:* ~ 4,900 controls and ~ 3,500 type 2 diabetics. Prevalence = 0.05 (Individuals with screen detected or known type 2 diabetes from the Inter99). The statistical power calculations for quantitative traits were estimated in R using 1,000 simulations and a significance threshold of 0.05. The statistical power calculations in the case-control analyses were done using CaTS, power calculations for large genetic association studies, available at http://www.sph.umich.edu/csg/abecasis/cats/. RAF = risk allele frequency.
(DOCX)

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Author Contributions

Conceived and designed the experiments: AS TL TJ DRW J-CH TH OP. Performed the experiments: AS TL TJ DRW TH OP. Analyzed the data: DSB KB JMJ CHS TH OP. Contributed reagents/materials/analysis tools: AS TL TJ DRW TH OP. Wrote the paper: DSB KB JMJ CHS TH OP.

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Supplementary table 1

Characteristics for individuals included in the analyses stratified according to study group.

	The population-based Inter99 study sample	The SDC type 2 diabetic patients	The SDC population-based study group	The ADDITION study cohort
n [men/women]	6,162 [3,070/3,092]	1,695 [1,045/650]	730 [332/398]	6,739 [3,862/2,877]
Age (years) [men/women]	46.2±7.9 [46.6±7.8/45.9±8.0]	62.4±11.3 [61.3±10.8/64.3±11.9]	57.8±8.6 [58.9±8.4/56.9±8.6]	59.7±6.8 [59.4±6.8/60.2±6.8]
Weight (kg) [men/women]	78.3±16.1 [85.7±14.1/70.9±14.3]	89.7±18.9 [95.0±17.7/81.5±17.8]	74.5±13.5 [81.8±12.0/68.4±11.5]	88.3±14.3 [92.6±13.2/82.6±13.1]
Height (cm) [men/women]	172.3±9.2 [178.7±6.9/165.9±6.3]	171.7±9.9 [177.5±7.0/162.7±6.5]	169.3±9.0 [176.1±6.7/163.7±6.4]	170.9±9.2 [176.5±6.6/163.3±6.2]
WC (cm) [men/women]	86.7±13.3 [93.2±11.0/80.3±12.2]	104.8±14.8 [107.6±13.7/100.4±15.5]	86.9±11.5 [93.4±9.3/81.6±10.3]	101.2±11.5 [104.4±10.4/96.8±11.6]
BMI (kg/m^2) [men/women]	26.3±4.6 [26.8±4.0/25.8±5.0]	30.3±5.6 [30.1±5.1/30.8±6.3]	25.9±3.8 [26.4±3.3/25.5±4.2]	30.2±4.2 [29.7±3.7/30.9±4.7]

Data are means ± standard deviation. SDC, Steno diabetes center. WC, waist circumference.

Supplementary table 2

Quantitative metabolic traits in 5,769 treatment-naïve middle-aged Danes from the population-based Inter99 cohort according to *LYPLAL1* rs2605100 genotype.

LYPLAL1 rs2605100	AA (HO)	GA (HE)	GG (WT)	β (95% CI)	p_{add}	p_{int}
n (all)	505	2458	2806			
n (men)	265	1190	1414			
n (women)	240	1268	1392			
Age (all), years	46.6±7.9	46.1±7.9	46.1±7.9			
BMI (kg/m^2)						
All	26.7+/-5.1	26.2+/-4.5	26.1+/-4.4	-0.18 (-0.36;-1x10^{-3})	0.05	0.81
Men	27.1+/-4.4	26.7+/-4	26.7+/-3.9	-0.13 (-0.36;0.09)	0.25	
Women	26.2+/-5.8	25.6+/-4.8	25.6+/-4.8	-0.23 (-0.51;0.05)	0.10	
Waist circumference (cm)						
All	87.6+/-14.3	86.4+/-13.1	86.2+/-13.0	-0.08 (-0.29;0.13)	0.47 (0.04)	0.53 (0.96)
Men	93.8+/-12.4	93.1+/-11	92.6+/-10.7	-0.15 (-0.43;0.13)	0.30 (0.13)	
Women	80.8+/-13.1	80+/-11.7	79.8+/-11.8	0.02 (-0.29;0.33)	0.90 (0.18)	
Waist-hip ratio						
All	0.9+/-0.1	0.9+/-0.1	0.9+/-0.1	-5x10^{-4} (-3x10^{-3};2x10^{-3})	0.64 (0.19)	0.35 (0.48)
Men	0.9+/-0.1	0.9+/-0.1	0.9+/-0.1	-3x10^{-4} (-3x10^{-3};3x10^{-3})	0.84 (0.41)	
Women	0.8+/-0.1	0.8+/-0.1	0.8+/-0.1	-4x10^{-4} (-4x10^{-3};3x10^{-3})	0.80 (0.41)	
Fasting serum triglycerides (mmol/l)						
All	1.1 (0.8-1.6)	1.0 (0.8-1.5)	1.1 (0.8-1.5)	3 % (1;5)	2.7 x 10^{-3}	0.02
Men	1.2 (0.8-1.6)	1.2 (0.8-1.7)	1.2 (0.9-1.8)	6 % (3;9)	2.4 x 10^{-4}	
Women	1.0 (0.7-1.4)	0.9 (0.7-1.3)	1.0 (0.7-1.3)	0.1 % (-2.4;2.6)	0.94	
Fasting serum insulin (pmol/l)						
All	33 (24-52)	33 (23-49)	35 (24-52)	3 % (1;5)	2.5 x 10^{-3}	0.09
Men	34 (23-50)	36 (24-55)	37 (25-56)	3 % (-0.1;6)	0.06	
Women	32 (24-54)	31 (23-45)	33 (23-49)	4 % (1;6)	0.01	
Fasting plasma glucose (mmol/l)						
All	5.5 (5.1-5.9)	5.4 (5.1-5.8)	5.5 (5.1-5.8)	0.3 % (-0.1;0.8)	0.11	0.54
Men	5.6 (5.3-6.0)	5.6 (5.3-6.0)	5.6 (5.3-6.0)	0.1 % (-0.5;0.8)	0.69	
Women	5.2 (5.0-5.6)	5.3 (5.0-5.6)	5.3 (5.0-5.6)	1 % (0;1)	0.04	
Insulin resistance, HOMA-IR						
All	8.0 (5.7-12.7)	8.1 (5.5-12.4)	8.6 (5.7-13.2)	4 % (1;6)	1.5 x 10^{-3}	0.10
Men	9.0 (5.8-13.0)	9.1 (6.0-14.2)	9.2 (6.0-14.4)	3 % (-0.2;6)	0.07	
Women	7.6 (5.4-12.4)	7.5 (5.2-10.8)	8.0 (5.3-11.9)	4 % (1;7)	6.0 x 10^{-3}	

Data are unadjusted means ± standard deviation or medians (interquartile range). Values of fasting serum triglycerides, serum insulin, plasma glucose, and HOMA-IR were logarithmically transformed prior to statistical analyses, and their effect sizes (β) are presented as the increase/decrease in percent. *P*-values were calculated assuming an additive model (p_{add}). Interaction analysis (p_{int}) was performed to test whether the effect of the alleles differed between men and women. All analyses were adjusted for age, sex, and BMI. Furthermore, waist circumference and waist-hip ratio were adjusted for age and sex, and the *p*-values are shown in parentheses.

Supplementary table 3

LYPLAL1 rs2605100, *NRXN3* rs10146997, *MSRA* rs545854, and *TFAP2B* rs987237 in relation to central obesity.

LYPLAL1 rs2605100	*n*	Genotype distribution *n* AA/GA/GG	Effect (95% CI)	p_{add}	p_{int}	*Heterogeneity* (I^2, *p*)
Controls						
All	4350	373/1831/2146				
Men	2412	207/992/1213				
Women	1938	166/839/933				
Obese case					0.37/0.13	
All	6949	673/2979/3297	0.92(0.86-0.98)	0.01/0.004		
Men	3420	336/1491/1593	0.89(0.82-0.97)	0.01/0.0004		
Women	3529	337/1488/1704	0.94(0.86-1.04)	0.25/0.83		
QT – WC (cm)					0.06/0.15	0% [0%; 56%], 0.27
All	13011	1213/5573/6225	-0.48 (-0.81;0.16)	0.004/0.12		
Men	6973	647/2988/3338	-0.58 (-0.99;0.17)	0.005/0.04		
Women	6038	566/2585/2887	-0.39 (-0.90;0.13)	0.14/0.91		
QT – WHR					0.44/0.40	0% [0%; 81%], 0.98
All	13011	1213/5573/6225	$-1x10^{-3}$ ($-4x10^{-3}$;$1x10^{-3}$)	0.36/0.86		
Men	6973	647/2988/3338	$-1x10^{-3}$ ($-4x10^{-3}$;$2x10^{-3}$)	0.52/0.88		
Women	6038	566/2585/2887	$-1x10^{-3}$ ($-5x10^{-3}$;$3x10^{-3}$)	0.59/1.00		
*NRXN3 r*s10146997	*n*	Genotype distribution *n* AA/GA/GG	Effect (95% CI)	p_{add}	p_{int}	
Controls						
All	4332	2711/1430/191				
Men	2332	1477/811/106				
Women	1938	1234/619/85				
Obese cases					0.35/0.43	
All	6757	4062/2374/321	1.06(0.99-1.14)	0.11/0.31		
Men	3310	1982/1188/140	1.03 (0.93-1.14)	0.53/0.52		
Women	3447	2080/1186/181	1.09(0.98-1.22)	0.11/0.37		
QT – WC					0.36/0.32	37% [0%; 78%], 0.80 (fixed)
All	12728	7845/4292/591	0.15 (-0.22;0.52)	0.43/0.58		
Men	6796	4187/2295/314	-0.10 (-0.57;0.37)	0.67/0.12		
Women	5932	3658/1997/277	0.40 (-0.18;0.98)	0.17/0.44		
QT – WHR					0.15/0.09	0% [0%; 70%], 0.53
All	12728	7845/4292/591	$1x10^{-3}$ ($-2x10^{-3}$;$4x10^{-3}$)	0.44/0.75		
Men	6796	4187/2295/314	$-1x10^{-3}$ ($-5x10^{-3}$;$2x10^{-3}$)	0.40/0.10		
Women	5932	3658/1997/277	$4x10^{-3}$ ($-2x10^{-4}$;$8x10^{-3}$)	0.07/0.08		
MSRA rs545854	*n*	Genotype distribution *n* CC/GC/GG	Effect (95% CI)	p_{add}	p_{int}	

Controls						
All	4364	3133/1131/100				
Men	2427	1748/616/63				
Women	1937	1385/515/37				
Obese cases					0.07/0.19	
All	6956	4889/1880/187	1.08(1.00-1.18)	0.05/0.02		
Men	3418	2396/942/80	1.08(0.97-1.21)	0.17/0.22		
Women	3538	2493/938/107	1.09(0.96-1.23)	0.18/0.04		
QT – WC					0.002/0.09	47% [0%; 82%], 0.91 (fixed)
All	13031	9291/3404/336	0.16 (-0.25;0.56)	0.46/0.85		
Men	6974	4987/1815/172	0.25 (-0.27;0.77)	0.35/0.50		
Women	6057	4304/1589/164	0.05 (-0.60;0.69)	0.89/0.37		
QT – WHR					0.22/0.34	45% [0%; 84%], 0.74
All	13031	9291/3404/336	$1x10^{-3}$ ($-2x10^{-3}$;$4x10^{-3}$)	0.38/0.49		
Men	6974	4987/1815/172	$3x10^{-3}$ ($-1x10^{-3}$;$7x10^{-3}$)	0.18/0.13		
Women	6057	4304/1589/164	$1x10^{-4}$ ($-5x10^{-3}$;$4x10^{-3}$)	0.96/0.76		
TFAP2B rs987237	*n*	Genotype distribution *n* AA/GA/GG	Effect (95% CI)	p_{add}	p_{int}	
Controls						
All	4341	3011/1217/113				
Men	2400	1643/685/72				
Women	1941	1368/532/41				
Obese cases					0.04/0.0002	
All	6921	4681/2022/218	1.08 (1.00-1.17)	0.06/0.81		
Men	3404	2322/983/99	1.01 (0.91-1.13)	0.83/0.01		
Women	3517	2359/1039/119	1.17 (1.03-1.32)	0.01/0.001		
QT – WC					0.10/0.13	50% [0%; 84%], 0.97
All	12956	8899/3697/360	0.44 (0.04;0.84)	0.03/0.96		
Men	6934	4758/1987/189	0.18 (-0.33;0.68)	0.49/0.19		
Women	6022	4141/1710/171	0.73 (0.10;1.36)	0.02/0.20		
QT – WHR					0.80/0.98	0% [0%; 87%], 0.46
All	12956	8899/3697/360	$-1x10^{-3}$ ($-4x10^{-3}$;$2x10^{-3}$)	0.65/0.40		
Men	6934	4758/1987/189	$-1x10^{-3}$ ($-5x10^{-3}$;$3x10^{-3}$)	0.64/0.53		
Women	6022	4141/1710/171	$-2x10^{-4}$ ($-5x10^{-3}$;$4x10^{-3}$)	0.91/0.66		

Data are number of individuals, divided into genotype groups. The effect is either the odds ratio (OR) or the per allele effect size presented as the increase/decrease and 95%CI. Effect and *p*-values shown are for an additive genetic model (p_{add}) and are adjusted for age, sex and diabetes treatment (without/ with BMI) for the obese cases, and QT analyses are adjusted for age and sex (without/with BMI). QT, quantitative trait; WC, waist circumference; WHR, waist-hip ratio.

Supplementary table 4

LYPLAL1 rs2605100, *NRXN3* rs10146997, *MSRA* rs545854, and *TFAP2B* rs987237 in relation to general obesity.

LYPLAL1 rs2605100	*n*	Genotype distribution *n* AA/GA/GG	Effect (95% CI)	p_{add}	p_{int}	*Heterogeneity* (I^2, *p*)
Controls						
All	3171	269/1354/1548				
Men	1286	116/531/639				
Women	1885	153/823/909				
Obese cases					0.26/0.06	
All	4814	449/2046/2319	0.97(0.90-1.05)	0.44/0.32		
Men	2495	229/1086/1180	0.98 (0.87-1.09)	0.68/0.45		
Women	2319	220/960/1139	0.96 (0.87-1.07)	0.47/0.54		
QT – BMI (kg/m^2)					0.16/0.38	0% [0%;
All	13011	1213/5573/6225	-0.16 (-0.28;-0.04)	0.01/0.87		81%], 0.10
Men	6973	647/2988/3338	-0.15 (-0.30;-0.01)	0.04/0.44		
Women	6038	566/2585/2887	-0.18 (-0.38;0.03)	0.09/0.50		
NRXN3 rs10146997	*n*	Genotype distribution *n* AA/GA/GG	Effect (95% CI)	p_{add}	p_{int}	
Controls						
All	3179	1988/1040/151				
Men	1287	780/444/63				
Women	1892	1208/596/88				
Obese cases					0.77/0.27	
All	4686	2821/1651/214	1.02 (0.94-1.11)	0.66/0.04		
Men	2422	1444/868/110	0.99 (0.88-1.12)	0.90/0.48		
Women	2264	1377/783/104	1.04 (0.93-1.17)	0.46/0.04		
QT – BMI					0.76/0.62	0% [0%;
All	12728	7845/4292/591	0.08 (0.05;0.22)	0.24/0.28		71%], 0.19
Men	6796	4187/2295/314	0.04 (-0.13;0.20)	0.66/0.12		
Women	5932	3658/1997/277	0.12 (-0.11;0.35)	0.30/0.91		
MSRA rs545854	*n*	Genotype distribution *n* CC/GC/GG	Effect (95% CI)	p_{add}	p_{int}	
Controls						
All	3184	2289/826/69				
Men	1294	937/323/34				
Women	1890	1352/503/35				
Obese cases					0.16/0.56	
All	4827	3390/1301/136	1.11 (1.00-1.20)	0.06/0.11		
Men	2501	1753/680/68	1.11 (0.96-1.27)	0.16/0.88		
Women	2326	1637/621/68	1.08 (0.95-1.24)	0.22/0.08		
QT – BMI					0.01/0.50	0% [0%;
All	13031	9291/3404/336	0.08 (-0.07;0.23)	0.30/0.52		67%], 0.28
Men	6974	4987/1815/172	0.07 (-0.11;0.25)	0.46/0.80		
Women	6057	4304/1589/164	0.09 (-0.16;0.34)	0.48/0.27		
TFAP2B rs987237	*n*	Genotype distribution *n* AA/GA/GG	Effect (95% CI)	p_{add}	p_{int}	

Controls						
All	3180	2209/887/84				
Men	1287	901/350/36				
Women	1893	1308/537/48				
Obese cases					0.24/0.36	
All	4797	3226/1417/154	1.10 (1.01-1.21)	0.04/0.68		
Men	2487	1682/735/70	1.09 (0.95-1.25)	0.22/0.24		
Women	2310	1544/682/84	1.12 (0.98-1.26)	0.09/0.54		
QT – BMI					0.03/0.04	78% [41%; 92%], 0.003 (fixed)
All	12956	8899/3697/360	0.25 (0.11;0.39)	0.02/0.25		
Men	6934	4758/1987/189	0.20 (0.03;0.37)	0.13/0.06		
Women	6022	4141/1710/171	0.30 (0.07;0.53)	0.07/0.83		

Data are number of individuals, divided into genotype groups. The effect is either the odds ratio (OR) or the per allele effect size presented as the increase/decrease and 95%CI. Effect and *p*-values shown are for an additive genetic model (p_{add}) and are adjusted for age, sex and diabetes treatment (without/ with WC) for the obese cases, and QT analyses are adjusted for age and sex (without/with WC). QT, quantitative trait.

Supplementary table 5

Quantitative metabolic traits in 5,789 treatment-naive middle-aged Danes from the population-based Inter99 cohort according to *NRXN3* rs10146997 genotype.

NRXN3 rs10146997	AA (WT)	GA (HE)	GG (HO)	β (95% CI)	p_{add}	p_{int}
n (all)	3602	1915	272			
n (men)	1766	969	143			
n (women)	1836	947	129			
Age (all)	46.1±8.0	46.3±7.8	46.1±7.4			
BMI (kg/m^2)						
All	26.1±4.5	26.3±4.5	26.4±4.4	0.11 (-0.09;0.31)	0.29	0.95
Men	26.7±4.0	26.8±4.0	26.9±3.7	0.08 (-0.16;0.33)	0.51	
Women	25.6±4.9	25.7±4.9	25.9±5.0	0.13 (-0.17;0.44)	0.40	
Waist circumference (cm)						
All	86.1±13.2	86.9±13.2	87.2±12.5	0.15 (-0.08;0.38)	0.20 (0.12)	$1.0x10^{-3}$ (0.17)
Men	93.0±11.1	92.9±11	93.2±9.7	-0.24 (-0.55;0.07)	0.14 (0.93)	
Women	79.6±11.6	80.7±12.4	80.5±12.0	0.55 (0.20;0.89)	$1.7x10^{-3}$ (0.02)	
Waist-hip ratio						
All	0.9±0.1	0.9±0.1	0.9±0.1	$9x10^{-4}$ ($-1x10^{-3}$;$3x10^{-3}$)	0.47 (0.25)	0.09 (0.13)
Men	0.9±0.1	0.9±0.1	0.9±0.1	$-2x10^{-3}$ ($-5x10^{-3}$;$1x10^{-3}$)	0.23 (0.55)	
Women	0.8±0.1	0.8±0.1	0.8±0.1	$4x10^{-3}$ ($2x10^{-4}$;0.01)	0.04 (0.02)	
Fasting serum triglycerides (mmol/l)						
All	1.1 (0.8-1.5)	1.1 (0.8-1.6)	1.1 (0.8-1.6)	0.8 % (-1.4;2.9)	0.48	0.76
Men	1.2 (0.9-1.8)	1.2 (0.9-1.8)	1.3 (0.9-1.7)	0.2 % (-3.1;3.5)	0.90	
Women	1.0 (0.7-1.3)	1.0 (0.7-1.3)	1.0 (0.7-1.3)	1 % (-1;4)	0.31	
Fasting serum insulin (pmol/l)						
All	34 (24-50)	35 (23-52)	35 (24-53)	0.6 % (-1.7;2.8)	0.62	0.07
Men	36 (25-56)	36 (23-54)	39 (26-56)	-1 % (-4;2)	0.52	
Women	32 (23-46)	34 (23-51)	32 (23-50)	2 % (-1;5)	0.17	
Fasting plasma glucose (mmol/l)						
All	5.4 (5.1-5.8)	5.5 (5.1-5.8)	5.5 (5.2-5.8)	0.1 %(-0.4 ;0.6)	0.70	0.13
Men	5.6 (5.3-6.0)	5.6 (5.3-6.0)	5.7 (5.4-6.0)	-0.4 % (–1.1;0.4)	0.34	
Women	5.3 (5.0-5.6)	5.3 (5.0-5.7)	5.3 (5.0-5.7)	0.5 % (-0.1;1.2)	0.08	
Insulin resistance, HOMA-IR						
All	8.2 (5.7-12.8)	8.5 (5.5-13.2)	8.6 (5.9-12.7)	1 % (-2;3)	0.57	0.09
Men	9.1 (6.0-14.4)	9.1 (5.7-13.9)	9.8 (6.7-14.7)	-1 % (-5;2)	0.46	
Women	7.6 (5.2-11.1)	7.9 (5.3-12.3)	7.6 (5.5-12.2)	3 % (-1;6)	0.11	

Data are unadjusted means ± standard deviation or medians (interquartile range). Values of fasting serum triglycerides, serum insulin, plasma glucose, and HOMA-IR were logarithmically transformed prior to statistical analyses, and their effect sizes (β) are presented as the increase/decrease in percent. *P*-values were calculated assuming an additive model (p_{add}). Interaction analysis (p_{int}) was performed to test whether the effect of the alleles differed between men and women. All analyses were adjusted for age, sex, and BMI. Furthermore, waist circumference and waist-hip ratio were adjusted for age and sex, and the *p*-values are shown in parentheses.

Supplementary table 6

Quantitative metabolic traits in 5,804 treatment-naïve middle-aged Danes from the population-based Inter99 cohort according to *MSRA* rs545854 genotype.

MSRA rs545854	CC (WT)	GC (HE)	GG (HO)	β (95% CI)	p_{add}	p_{int}
n (all)	4161	1498	145			
n (men)	2082	732	76			
n (women)	2079	766	69			
Age (all)	46.1±7.8	46.1±8.1	46.3±7.8			
BMI (kg/m^2)						
All	26.2±4.5	26.2±4.5	26.5±4.7	0.10 (-0.13;0.32)	0.40	0.19
Men	26.7±4.0	26.8±4.0	26.4±4.0	-0.03 (-0.32;0.25)	0.82	
Women	25.6±4.9	25.7±5.0	26.7±5.5	0.23 (-0.12;0.58)	0.20	
Waist circumference (cm)						
All	86.5±13.1	86.4±13.5	86.8±12.8	-0.19 (-0.46;0.07)	0.15 (0.90)	0.23 (0.10)
Men	93.0±10.8	93.1±11.5	91.1±10.7	-0.14 (-0.49;0.22)	0.45 (0.58)	
Women	80.0±11.9	79.9±12.0	82.1±13.4	-0.22 (-0.61;0.16)	0.26 (0.50)	
Waist-hip ratio						
All	0.9±0.1	0.9±0.1	0.9±0.1	$-6x10^{-5}$ ($-3x10^{-3}$;$2x10^{-3}$)	0.96 (0.71)	0.06 (0.15)
Men	0.9±0.1	0.9±0.1	0.9±0.1	$3\ x10^{-3}$ (-0.001;0.007)	0.10 (0.22)	
Women	0.8±0.1	0.8±0.1	0.8±0.1	$-3\ x10^{-3}$ (-0.007;0.001)	0.16 (0.46)	
Fasting serum triglycerides (mmol/l)						
All	1.1 (0.8-1.5)	1.1 (0.8-1.5)	1.1 (0.8-1.7)	-1 % (-3;2)	0.66	0.98
Men	1.2 (0.9-1.8)	1.2 (0.9-1.7)	1.2 (0.9-1.9)	-1 % (-4;3)	0.72	
Women	1.0 (0.7-1.3)	0.9 (0.7-1.3)	1.0 (0.8-1.4)	-0.2 % (-3.4;2.9)	0.89	
Fasting serum insulin (pmol/l)						
All	34 (24-51)	34 (24-51)	32 (23-48)	-2 % (-4;1)	0.24	0.08
Men	37 (25-56)	36 (24-55)	30 (22-45)	-4 % (-8;-1)	0.02	
Women	33 (23-47)	33 (23-48)	38 (25-54)	1 % (-2;5)	0.41	
Fasting plasma glucose (mmol/l)						
All	5.4 (5.1-5.8)	5.4 (5.1-5.8)	5.5 (5.2-5.9)	-0.2 % (-0.7;0.4)	0.51	0.52
Men	5.6 (5.3-6.0)	5.6 (5.3-6.0)	5.6 (5.3-6.0)	-0.3 % (-1.2;0.4)	0.34	
Women	5.3 (5.0-5.6)	5.3 (5.0-5.6)	5.4 (5.1-5.8)	0.1 % (-0.6;0.8)	0.88	
Insulin resistance, HOMA-IR						
All	8.3 (5.7-12.9)	8.3 (5.5-12.9)	7.9 (5.2-12.6)	-2 % (-4;1)	0.24	0.08
Men	9.3 (6.0-14.4)	8.9 (5.8-14.1)	7.2 (5.0-11.3)	-5 % (-9;-1)	0.02	
Women	7.6 (5.3-11.4)	7.6 (5.3-11.6)	9.0 (5.7-13.2)	2 % (-2;5)	0.43	

Data are unadjusted means ± standard deviation or medians (interquartile range). Values of fasting serum triglycerides, serum insulin, plasma glucose, and HOMA-IR were logarithmically transformed prior to statistical analyses, and their effect sizes (β) are presented as the increase/decrease in percent. *P*-values were calculated assuming an additive model (p_{add}). Interaction analysis (p_{int}) was performed to test whether the effect of the alleles differed between men and women. All analyses were adjusted for age, sex, and BMI. Furthermore, waist circumference and waist-hip ratio were adjusted for age and sex, and the *p*-values are shown in parentheses.

Supplementary table 7

Quantitative metabolic traits in 5,764 treatment-naïve middle-aged Danes from the population-based Inter99 cohort according to *TFAP2B* rs987237 genotype.

TFAP2B rs987237	AA (WT)	AG (HE)	GG (HO)	β (95% CI)	p_{add}	p_{int}
n (all)	3994	1631	139			
n (men)	1993	805	65			
n (women)	2001	826	74			
Age (all)	46.1±7.9	46.2±7.8	45.3±7.6			
BMI (kg/m^2)						
All	26.2±4.6	26.2±4.5	25.8±4.6	-0.02 (-0.24;-0.20)	0.87	0.26
Men	26.7±4.1	26.8±3.9	25.8±3.2	-0.04 (-0.33;0.24)	0.77	
Women	25.7±4.9	25.6±5.0	25.9±5.5	$3x10^{-3}$ (-0.39;0.35)	0.98	
Waist circumference (cm)						
All	86.5±13.4	86.5±12.9	84.1±11.7	-0.16 (-0.42;-0.1)	0.22 (0.45)	0.20 (0.54)
Men	93.0±11.2	93.0±10.7	89.8±8.5	-0.30 (-0.65;0.06)	0.10 (0.31)	
Women	80.0±12.1	80.1±11.7	79.1±11.8	-0.01 (-0.39;0.36)	0.94 (0.94)	
Waist-hip ratio						
All	0.9±0.1	0.9±0.1	0.8±0.1	$1x10^{-3}$ ($-3x10^{-3}$;$1x10^{-3}$)	0.46 (0.46)	0.56 (0.99)
Men	0.9±0.1	0.9±0.1	0.9±0.1	$-7.4x10^{-4}$ ($-4x10^{-3}$;$3x10^{-3}$)	0.69 (0.62)	
Women	0.8±0.1	0.8±0.1	0.8±0.1	$-1x10^{-3}$ ($-5x10^{-3}$;$3x10^{-3}$)	0.61 (0.64)	
Fasting serum triglycerides (mmol/l)						
All	1.1 (0.8-1.5)	1.1 (0.8-1.6)	0.9 (0.7-1.3)	-1.2 % (-4;1)	0.35	0.95
Men	1.2 (0.9-1.8)	1.2 (0.9-1.7)	1.0 (0.8-1.4)	-1.0 % (-5;3)	0.59	
Women	1.0 (0.7-1.3)	1.0 (0.7-1.3)	0.8 (0.6-1.1)	-1.3 % (-4;2)	0.40	
Fasting serum insulin (pmol/l)						
All	34 (24-51)	35 (24-52)	31 (23-41)	-0.6 % (-3;2)	0.64	0.57
Men	36 (24-55)	38 (25-57)	34 (24-45)	1.0 % (-3;5)	0.61	
Women	33 (23-48)	33 (23-48)	29 (23-37)	-2.0 % (-5;1)	0.26	
Fasting plasma glucose (mmol/l)						
All	5.4 (5.1-5.8)	5.4 (5.1-5.8)	5.4 (5.1-5.8)	-0.4 % (-0.9;0.1)	0.13	0.96
Men	5.6 (5.3-6.0)	5.6 (5.3-6.0)	5.6 (5.1-6.1)	-0.3 % (-1.2;0.5)	0.40	
Women	5.3 (5.0-5.6)	5.3 (5.0-5.6)	5.3 (5.1-5.6)	-0.5 % (-1.2;0.2)	0.18	
Insulin resistance, HOMA-IR						
All	8.3 (10.6-12.9)	8.3 (10.7-13.2)	7.3 (8.6-10.0)	-1.1 % (-4;2)	0.44	0.60
Men	9.1 (5.9-14.2)	9.4 (6.0-14.6)	8.1 (5.7-11.5)	0.5 % (-3;5)	0.79	
Women	7.7 (5.3-11.7)	7.7 (5.3-11.6)	6.8 (5.2-9.0)	-2.4 % (-6;1)	0.19	

Data are unadjusted means ± standard deviation or medians (interquartile range). Values of fasting serum triglycerides, serum insulin, plasma glucose, and HOMA-IR were logarithmically transformed prior to statistical analyses, and their effect sizes (β) are presented as the increase/decrease in percent. *P*-values were calculated assuming an additive model (p_{add}). Interaction analysis (p_{int}) was performed to test whether the effect of the alleles differed between men and women. All analyses were adjusted for age, sex, and BMI. Furthermore, waist circumference and waist-hip ratio were adjusted for age and sex, and the *p*-values are shown in parentheses.

Supplementary table 8

Statistical power estimates.

		Power		
	RAF	15	20	70
Quantitative trait analyses	0.35 change in BMI units	85%	93%	97%
	0.85 cm change in waist circumference	72%	80%	90%
	0.007 change in waist-hip ratio	83%	91%	97%
	5% change in fasting serum triglyceride	95%	98%	99%
	1% change in fasting plasma glucose	91%	95%	98%
	5% change in fasting serum insulin	89%	93%	97%
Case-control analyses				
Central overweight	OR of 1.10	81%	88%	92%
	OR of 1.15	99%	100%	
General overweight	OR of 1.10	96%	99%	99%
	OR of 1.15	100%		
Central obesity	OR of 1.10	90%	95%	97%
	OR of 1.15	100%		
General obesity	OR of 1.10	73%	81%	86%
	OR of 1.15	96%	99%	99%
	OR of 1.20	100%		
Type 2 diabetes	OR of 1.10	63%	72%	78%
	OR of 1.15	92%	96%	97%
	OR of 1.20	99%	100%	

Central overweight: ~4,500 controls and ~3,700 overweight individuals. Prevalence = 0.23 (Men with waist circumference ≥94 cm and <102 cm and women with waist circumference ≥80 cm and <88 cm from the Inter99). General overweight: ~3,200 controls and ~7,000 overweight individuals. Prevalence = 0.39 (Individuals with BMI≥25 kg/m2 and BMI<30 kg/m2 from the Inter99). Central obesity: ~4,500 controls and ~7,000 obese individuals. Prevalence = 0.22 (men with waist circumference ≥102 cm and women with waist circumference ≥88cm from the Inter99). General obesity: ~3,200 controls and ~4,800 obese individuals. Prevalence = 0.17 (Individuals with BMI≥30 kg/m2 from the Inter99). Type 2 diabetes: ~ 4,900 controls and ~ 3,500 type 2 diabetics. Prevalence = 0.05 (Individuals with screen detected or known type 2 diabetes from the Inter99). The statistical power calculations for quantitative traits were estimated in R using 1,000 simulations and a significance threshold of 0.05. The statistical power calculations in the case-control analyses were done using CaTS, power calculations for large genetic association studies, available at http://www.sph.umich.edu/csg/abecasis/cats/. RAF = risk allele frequency.

Appendix II

Statistical analyses

This part describes the statistical methods used in each study. Most of this is described in the papers; however some aspects of the data will be described more in details.
The statistical analyses were performed using R version 2.9.2 and SAS version 9.2.

Study I

All included values were tested for normal distribution by histograms and QQ-plots. If they were not normally distributed, the values were logarithmically transformed prior to the analyses. Due to this the results are presented as means and geometrical means in the paper. Furthermore, the differences are presented as the actual value for the non-logarithmically data and the percentage for the logarithmically data.
In this study we had two independent groups of observations, we aimed to compare. In the paper we present results analysed as the mean difference between the two groups (*t*-test). However, as we had non-normal distributed values we also analysed data by non-parametric comparison Mann-Whitney analyses. Yet the results were almost similar, while we chose to present the results from the *t*-test in the paper.
To investigate if the risk of hepatic steatosis associated with obesity (BMI SDS) multiple regression analyses were performed. These analyses were adjusted for sex, age and pubertal stage (Tanner) due to deleting the influence of these variables on the output.

Study II

The data included in this study are all paired data, as they are from the same individual at baseline and at the time of follow-up. We aimed to investigate if there was a difference in these two measures and used paired *t*-test.
Furthermore we aimed to investigate if the changes in liver fat content were predictable by changes in any of the measured variables of anthropometrics, blood variables, or body fat depots. We used a general linear model and adjusted for logarithmically transformed baseline liver fat content, gender, baseline age, and baseline pubertal stage, due to deleting the influence of these variables on the output.

We only had blood samples from 41 individuals. This provided us a power problem. Our results based on the blood variables can only be interpreted as trends.

Study III

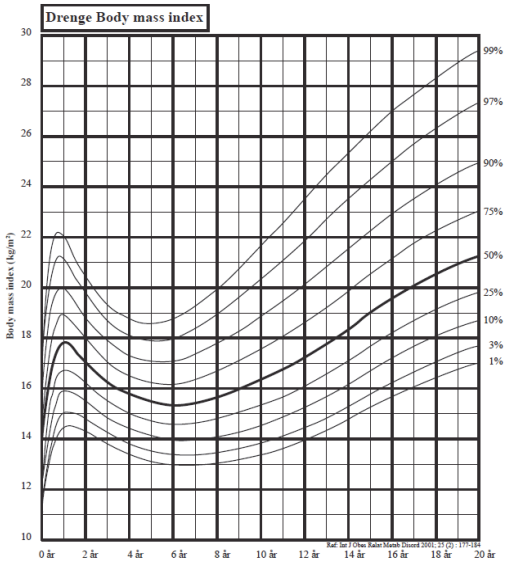
The quantitative variables were tested for differences between genotyped groups using a linear nonparametric regression, assuming an additive model. We used an additive model as it combines the effect of the explanatory variables (and their interaction) so they are equal to the sum of their separate effects. All analyses were adjusted for sex, age, and BMI, when appropriate. BMI was additionally adjusted for WC. These adjustments were performed due to deleting the influence of age, BMI, WC, and sex on the output. Prior to statistical analyses some values were logarithmically transformed, due to non-normally distributed residuals. Heterogeneity was assessed with the generic inverse variance meta-analysis method and performed by K. Banasik.

The case-control studies were analyzed using logistic regression, assuming an additive model. Making associations and replications studies it is important to have statistical power. If the studies are performed in too few individuals the results will be unreliable. For the quantitative variables statistical power estimates were estimated. The population-based Inter99 cohort was used as reference for estimation the prevalence of central overweight and obesity in the Danish population. P-values were not adjusted for multiple hypotheses testing which means that our results might just be random association attributable to multiple testing because of typing of a large number of alleles.

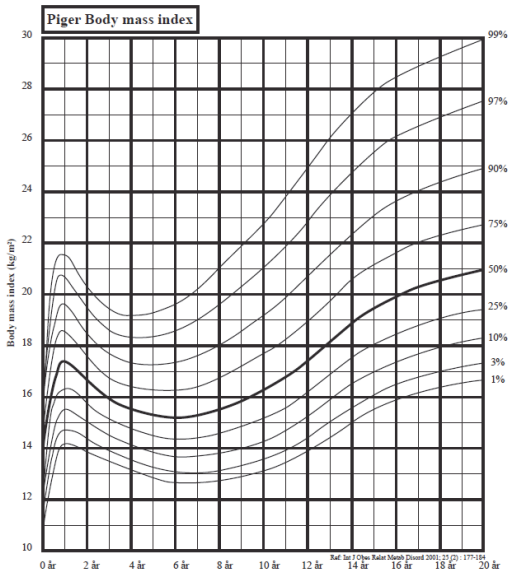
Appendix III

Danish BMI chart for boys and girls

Boys (drenge)



Girls (piger)



Appendix IV

Characteristics for the 237 individuals included at the baseline in study II

	Number	Medians with ranges
Total (boys/girls)	237 (106/131)	
Age (years)		13.5 (6.6-20.4)
Weight SDS		4.01 (2.89-4.56)
Height SDS		0.79 (-2.12-3.25)
BMI SDS		2.96 (1.32-5.20)
Puberty stage*		
Pre-pubertal (%)	31	
Pubertal (%)	48	
Post-pubertal (%)	21	
WC (cm)		100 (73.5-137)
WC/height ratio		0.62 (0.47-0.81)
Liver fat content (%)		3.7 (0.1-69)
NAFLD (≥5%)	102	11.3 (5-69)
Non-NAFLD (<5%)	135	2 (0.1-4.9)
SAT		304 (54-752)
VAT		88 (22-468)
SAT/VAT		3.5 (1.6-10.3)
ALT (U/l)		25 (10-150)
AST (U/l)		25.9 (12.6-91)
AP (U/l)		228 (50-774)
GGT (U/l)		17 (8-329)
glucose (mM)		5.1 (3.4-6.6)
insulin (mU/l)		15.67 (3.5-125.3)
total cholesterol (mM)		4.2 (2.2-7.3)
LDL (mM)		2.5 (0.9-5.9)
HDL (mM)		1.2 (0.6-2.2)
Triglycerides (mM)		1.1 (0.3-3.4)
HOMA-IR		3.48 (0.72-28.97)

data are medians with ranges

*Puberty stages based on Tanner stages; stage 1=pre-pubertal, stage 2-4=pubertal, and stage 5=post-pubertal

Appendix V

Biochemical measurements

The blood samples from the Children's Obesity Clinic are analysed at the Biochemical Departments at Holbæk and Ringsted Hospital. The measurements were performed on Cobas.

The coefficient of variation (CV) is defined as the spread divided by the mean. The CV for the blood variables are listed below and are given by the departments.

Variable	**CV (%)**
Albumine	4
ALT	4
Bilirubine	4-6
Cholesterol	3
Creatinine	5
GGT	4
HDL	4
LDH	4
Triglyceride	4
Urea	4
Urat	4
Potassium	3
Natrium	3
Thyroid stimulating hormone	7
Glucose	3
AST	4
Ferritin	10
Insulin	2
HgbA1C	2
Haemoglobin	1-2
Leucocytes	5
Thrombocytes	1.7-3.6

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