Adult weight change and cardiometabolic disease: studies into underlying pathways

Verkouter, I.

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You can be anything you want to be
Freddie Mercury (1991)

Voor mijn lieve ouders

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CHAPTER 1

General introduction and outline of this thesis

ABSTRACT

Body weight gain during the life course is a well-established risk factor for type 2 diabetes mellitus and cardiovascular diseases. However, the mechanisms that underlie this relation between body weight gain and cardiometabolic diseases are still largely unknown. The main aim of this thesis was to study the cardiometabolic consequences of obesity and weight gain during the life course.

We aimed to investigate the association between body mass index (BMI) and cardiometabolic disease using Mendelian randomization in which we were particularly interested in whether the underlying causes of high BMI (e.g., different gene expression in brain or peripheral/adipose tissues) were differentially related to risk of diabetes or cardiovascular diseases. We identified 17 tissue-grouped gene sets, where BMI-associated genes were differentially expressed. However, in tissue-grouped Mendelian randomization analyses, all BMI-associated gene sets were similarly associated with increased risks of diabetes and coronary artery disease, and thus we argued that regardless of the cause of a high BMI, the risks of diseases as type 2 diabetes mellitus and coronary artery disease are similar.

We observed that abdominal adiposity in adolescence was associated with early changes in metabolomic measures indicative of an atherogenic profile already present at young adulthood, but this was only observed in young men. Also, when we investigated weight gain during adulthood, this was specifically related to an atherogenic metabolic profile, in addition to increased adipocyte size. Adult weight gain between age 20 years and middle age was associated with increased visceral fat and liver fat at middle age irrespective of total body fat. In addition, the association between adult weight gain and insulin resistance at middle age was partly mediated by the increased levels of visceral fat and liver fat at middle age. Lastly, not all individuals with obesity develop cardiometabolic disease. We observed that a favourable body fat distribution as well as metabolic profile are associated with a decreased risk of incident cardiometabolic disease in a population with obesity.

In conclusion, the results described in this thesis suggest that the cardiometabolic consequences of weight gain during both adolescence and adulthood are mediated by the amount of visceral and ectopic fat, and are reflected by a more atherogenic metabolomic profile and increased cardiometabolic risk. Once obese, preserving low glucose levels, non-smoking, and preventing abdominal obesity are important to prevent the onset of cardiometabolic disease. Overall, the results of this thesis emphasize the importance of maintaining a stable body weight during young adulthood throughout middle age. With increased attention being given to promoting a healthy lifestyle, there is potential for cardiometabolic disease prevention in promoting a healthy body weight during the life course.

GENERAL INTRODUCTION

The main objective of this thesis was to study the cardiometabolic consequences of weight gain during the life course. Body weight gain is a well-established risk factor for cardiometabolic disease, but the underlying pathways are still largely unexplored. This general introduction describes the epidemiology of overweight and obesity, the current knowledge about body weight gain during adulthood and how it may influence body fat distribution, the metabolomic profile, and risk of cardiometabolic disease. In addition, this introduction addresses the existing gaps in knowledge on this research topic and introduces the studies we conducted to address these knowledge gaps.

Overweight and obesity: a growing public health problem

Overweight and obesity are characterized by the accumulation of excess body fat, ultimately leading to chronic (cardiometabolic) disease. Body mass index (BMI), calculated as body weight in kilograms divided by the square of height in meters (kg/m^2) is commonly to classify overweight and obesity. According to the World Health Organization, a normal body weight in people from European descent is defined by a BMI between 18.5 and 24.9 kg/m^2, overweight is defined by a BMI between 25.0 and 29.9 kg/m^2, and obesity by a BMI above 30.0 kg/m^2 (1).

The worldwide prevalence of obesity doubled between 1980 and 2015 (2). In 2016, 39% of adults aged 18 years or older had overweight, and 13% of the total world's adult population were obese (3). This trend is also visible in the Netherlands: in 1990 one in three Dutch adults had overweight or obesity and this has increased to half of all Dutch adults in 2019 (4). In addition to overweight and obesity in the adult population, the global prevalence of obesity among children and adolescents has risen from 4% in 1975 to over 18% in 2016 (3). Childhood obesity is associated with insulin resistance, dyslipidaemia and the metabolic syndrome (5-7), as well as an increased risk of cardiometabolic disease and increased mortality later in life (8-10). Obesity during childhood and adolescence tends to track into adulthood (11).

Obesity in adulthood is a well-established and causal risk factor for type 2 diabetes, and weight loss interventions also show to decrease the risk of type 2 diabetes (12, 13). In addition, overweight and obesity are strong causal risk factors for cardiovascular disease (14) and its risk factors such as hypertension and dyslipidaemia (15). Obesity is also associated with increased mortality due to all cancers combined as well as cancer at specific sites, including the oesophagus, colon and rectum, liver, gallbladder, pancreas, and kidney (16). Lastly, from 1990 to 2017, the global deaths attributable to high BMI have more than doubled for both men and women (17). However, it is important to note that not everyone with overweight or obesity will develop cardiometabolic abnormalities or disease (18). Insights in additional risk factors that need to be averted to reduce the risk of future obesity-related disease are likely to yield particular targets for interventions which show the largest reduction in cardiometabolic-disease risk.

Body fat distribution and ectopic fat

BMI is a widely used, non-invasive measure of overall adiposity. However, BMI does not distinguish between body fat and lean body mass: a high BMI might be reflected by either a high total body fat percentage, high lean body mass or a combination of both. Furthermore, BMI does not yield information on whether body fat is stored centrally at the abdomen, or peripherally at the hips and thighs. Body fat distribution differs greatly between men and women as men tend to store body fat at the abdomen, whereas women are more likely to store body fat peripherally (19, 20).

Abdominal adiposity is characterized by an increased storage of excess fat in the abdominal subcutaneous (under the skin) and visceral (around the organs) adipose tissue depots. According to the 'lipid overflow hypothesis', adipose tissue becomes dysfunctional when the capacity of hypertrophic adipocytes to expand is exceeded as a result of body weight gain (21, 22). This in turn leads to 'lipid overflow': the accumulation of triglycerides in visceral adipose tissue and ectopic fat deposition in normally lean organs such as the heart, skeletal muscles, pancreas, and liver (21-23). Compared to subcutaneous adipocytes, adipocytes in the visceral depot have a high secretion rate of non-esterified fatty acids, very low-density lipoproteins and cytokines, such as IL-6 and TNF-alpha, thereby inducing a systemic low-grade inflammatory state and oxidative stress (23-26). Finally, intracellular non-esterified fatty acid accumulation in non-adipose tissues leads to impaired insulin signalling and insulin resistance (27).

Previous studies have shown that both excess visceral fat and liver fat are more strongly related to the metabolic syndrome than abdominal subcutaneous adipose tissue (28, 29). Additionally, excess visceral adipose tissue is strongly associated with an adverse metabolic profile, indicated by insulin resistance (30), increased blood pressure, triglycerides, hypertension and presence of the metabolic syndrome (31). Precise measurements, such as magnetic resonance imaging (MRI) to directly assess the amount of visceral adipose tissue, may be useful in characterization of cardiometabolic risk. Additionally, to examine the accumulation of fat deposition in organs, for example the measurement of liver fat content, proton magnetic resonance spectroscopy (^{1}H-MRS) can be used. As both MRI and ^{1}H-MRS are expensive and time-consuming methods, waist circumference can be used in addition to BMI to measure fat deposition in and around the abdomen.

Body weight gain during adulthood

Overweight and obesity at middle age are preceded by a gain in body weight during the life course. Weight gain during adulthood has been associated with a considerable increased risk of major chronic diseases in middle-aged individuals, including type 2 diabetes mellitus, cardiovascular disease, and obesity-related cancers, regardless of BMI (32-34). However, the mechanisms that underlie the relation between body weight gain and cardiometabolic disease are still largely unknown. During adult weight gain, excess adipose tissue is stored in different areas of the body, depending on various factors including genetic variation, sex, age, ethnicity and lifestyle (35-37). However, it remains unclear how weight gain during adulthood is associated with body fat distribution at middle age.

It was previously suggested that the number of adipocytes remained constant in both lean and overweight individuals after age 20 years (38). This suggests that adult weight gain after the age of 20 years might result in hypertrophy of adipocytes, and storage of excess lipids in the visceral compartment or deposition at ectopic sites after the storage capacity of subcutaneous adipocytes is reached. Therefore, we hypothesized that the accumulation of visceral fat and ectopic fat as a consequence of body weight gain may explain the link between body weight gain and increased risk of type 2 diabetes later in life.

Metabolomic measures as intermediates of weight gain and cardiometabolic disease

High throughput metabolomic measures have emerged during recent years as important intermediates between risk factors, including obesity and lifestyle exposures, and cardiometabolic disease outcomes. Metabolomic measures include small molecule substrates, intermediates or products of metabolism. Examples of metabolomic measures are lipoproteins, amino acids and fatty acids. Previous studies on the metabolomic profile associated with high BMI have helped to further elucidate underlying pathways to cardiometabolic disease. In a cross-sectional study of 12,664 adolescents, it was observed that BMI was strongly associated with the concentrations of very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) and inversely associated with high-density lipoprotein (HDL) (39). Additionally, a high BMI has been associated with higher concentrations of monounsaturated fatty acids and saturated fatty acids, as well as branched-chain amino acids (39). In turn, these adiposity-related metabolomic measures were linked to cardiometabolic disease: in an observational study, NMR-based measures of circulating cholesterol and triglycerides in VLDL and LDL particles, Apolipoprotein B and glucose were strongly associated with the risk of myocardial infarction (40). Mendelian randomization analyses showed that both LDL cholesterol, triglycerides, and Apolipoprotein B play a causal role in the development of coronary heart disease (41, 42).

Despite the fact that these metabolic alterations may link adult weight gain with the onset of cardiometabolic disease, the metabolomic profile associated with body weight gain in different stages of the life course has not been clearly defined to date. Additionally, longitudinal studies of changes in metabolomic measures are lacking, whereas these studies could aid to identify adiposity-related changes in the metabolomic profile indicative of early atherogenic progression in young adults (39).

Novel approaches to using Mendelian randomization

Results from observational studies on obesity and cardiometabolic disease may be influenced (residual) confounding and reverse causation, thereby impairing causal inference of the exposure-outcome relationship. As randomized controlled trials for overweight or obesity are usually not feasible or ethical for long study periods, alternative approaches such as Mendelian randomization can provide information on the causality of obesity-outcomes relations. In Mendelian randomization studies, genetic variants associated with the exposure of interest, assigned randomly at conception, are used as instrumental variables. These instrumental variables are then related to the outcome thereby mimicking randomized controlled trials (43). Results from Mendelian randomization studies that fulfil all necessary assumptions do not suffer from confounding or reverse causation and can therefore

be causally interpreted. To date, genetic variants associated with body weight gain during adulthood still need to be elucidated. Previous studies identified many genetic variants associated with an increased risk of developing obesity and a high body mass index (44-46) that influence different molecular mechanisms and are expressed in different tissues, and could be used as instrumental variables in Mendelian randomization studies. The most recent Genome-Wide Association Study (GWAS) identified 656 independent genetic variants associated with BMI, which collectively explained up to 6% of the total population variation in BMI (46). By using the BMI-associated genetic variants as instrumental variables, Mendelian randomization studies strengthened evidence for a causal effect of both overall obesity and abdominal obesity on coronary heart disease, stroke and type 2 diabetes (47).

In an earlier MR study on the association between BMI and anxiety, SNPs were categorized based on three mechanistic domains through which they were likely to influence BMI: appetite, adipogenesis and cardiopulmonary function, derived from an earlier GWAS on BMI (48). Based on this categorisation, we hypothesized that the characterization of genetic variants that are associated with BMI, and the genes corresponding to them, can provide insights into the heterogenous biological causes of a high BMI, which may be differentially related to disease risk. For example, BMI genes related to adipogenesis may be differentially related to cardiovascular risk than BMI genes related to cardiopulmonary function. A more detailed investigation of the differential causes of complex traits such as obesity will allow a comprehensive overview of potential specific targets for (personalized) interventions.

OUTLINE OF THIS THESIS

Previous studies have investigated the consequences of body weight gain during (young) adulthood; however, significant gaps in the scientific knowledge remain. To date, the molecular and metabolomic pathways underlying the relationship between adult weight gain and cardiometabolic disease are still largely unknown. Therefore, the objective of this thesis was to study the cardiometabolic consequences of obesity and weight gain during the life course.

Genome-wide associations studies on BMI already identified many genetic variants associated with an increased risk of developing a high BMI that influence different molecular mechanisms and are expressed in different tissues. In **Chapter 2**, we investigated the association between BMI and cardiometabolic disease using Mendelian randomization, in which we were particularly interested in whether the underlying genetic causes of high BMI (e.g., different gene expression in brain or peripheral/adipose tissues) are differentially related to risk of diabetes or cardiovascular diseases.

As childhood obesity is often carried over into adulthood, it is important to study changes in the metabolomic profile associated with obesity in young adulthood, which might provide opportunities to prevent the cardiometabolic consequences of obesity already during childhood. In **Chapter 3**, we therefore aimed to investigate the relations between trunk fat and total fat mass with changes in the concentrations of metabolomic measures during young adulthood in men and women.

Body weight gain during adulthood is associated with an increased risk of cardiometabolic disease. We hypothesized that specific metabolomic alterations as a consequence of adult weight gain indicate the onset of cardiometabolic disease. In **Chapter 4**, we investigated which metabolomic measures are specifically associated with adult weight gain, as opposed to those associated with BMI at age 20 years or BMI at middle age. We also examined the relation between adult weight gain and its identified specific metabolomic measures with adipocyte volume.

We hypothesized that the accumulation of visceral fat and ectopic fat as a consequence of body weight gain may explain the link between body weight gain and increased risk of type 2 diabetes later in life. Therefore, in **Chapter 5**, we investigated the associations of adult weight change with visceral adipose tissue and hepatic triglyceride content at middle age, taking into account total body fat at middle age. In **Chapter 6**, we further elaborated on the work described in **Chapter 5** by investigating to what extent the association of adult weight gain with insulin resistance was mediated by the amounts of visceral fat and liver fat at middle age.

Once adult weight gain has resulted in obesity, there is substantive heterogeneity in the onset of cardiometabolic disease. Not all individuals with obesity develop cardiometabolic diseases. Insights in factors may prevent the risk of cardiometabolic disease in individuals with obesity may yield particular targets for interventions. In **Chapter 7**, we investigated

which potential risk factors, including measures of body fat distribution, metabolic factors, and lifestyle factors, are needed for obesity to result in cardiometabolic disease. Finally, in **Chapter 8** we provide an overview of the main findings from the studies described in this thesis, discuss the strengths and limitations and interpretation of the results, and provide implications and future research directions.

Study designs and populations used in this thesis

For the study described in **Chapter 2**, we used summary-level data from several publicly available Genome-Wide Associations studies (body mass index [GIANT], type 2 diabetes mellitus [DIAGRAM], coronary artery disease [CARDIoGRAMplusC4D], waist circumference [GIANT] and total body fat [the Neale Lab]) (46, 49-52). The study described in **Chapter 3** has been performed in the Avon Longitudinal Study of Parents and Children (ALSPAC), a population-based birth cohort study from the United Kingdom (53) ALSPAC included 13,988 children in 1991, who have been followed since. Most studies described in this thesis (**Chapter 4, 5, 6 and 7**) were performed in the Netherlands Epidemiology of Obesity (NEO) study (54). This is a population-based cohort of 6671 individuals aged 45 to 65 years, with an oversampling of individuals with BMI≥27 kg/m^2, living in the West of the Netherlands. Participants of the NEO study were recruited between 2008 and 2012 and have been followed since. Next to data from the NEO study, for the study described in **Chapter 4** we used data from the Oxford Biobank, which is a population-based cohort study from Oxfordshire, United Kingdom (55). The Oxford Biobank recruitment began in 1999 and included 7640 participants in 2016.

CHAPTER 2

The contribution of tissue-grouped BMI-associated gene sets to cardiometabolic-disease risk: a Mendelian randomization study

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ABSTRACT

Background: Body mass index (BMI)-associated loci are used to explore the effects of obesity using Mendelian Randomization (MR), but contribution of individual tissues to risks remain unknown. We aimed to identify tissue-grouped pathways of BMI-associated loci and relate these to cardiometabolic disease using MR analyses.

Methods: Using Genotype-Tissue Expression (GTEx) data, we performed overrepresentation tests to identify tissue-grouped gene sets based on mRNA expression profiles from 634 previously published BMI-associated loci. We conducted two-sample MR with inverse-variance weighted methods, to examine associations between tissue-grouped BMI-associated genetic instruments and type 2 diabetes mellitus (T2DM) and coronary artery disease (CAD), with use of summary-level data from published genome-wide association studies (T2DM: 74,124 cases, 824,006 controls; CAD: 60,801 cases, 123,504 controls). Additionally, we performed MR analyses on T2DM and CAD using randomly sampled sets of 100 or 200 BMI-associated genetic variants.

Results: We identified 17 partly overlapping tissue-grouped gene sets, of which 12 were brain areas, where BMI-associated genes were differentially expressed. In tissue-grouped MR analyses, all gene sets were similarly associated with increased risks of T2DM and CAD. MR analyses with randomly sampled genetic variants on T2DM and CAD resulted in a distribution of effect estimates similar to tissue-grouped gene sets.

Conclusion: Overrepresentation tests revealed differential expression of BMI-associated genes in 17 different tissues. However, with our biology-based approach using tissue-grouped MR analyses we did not identify different risks of T2DM or CAD for the BMI-associated gene sets, which was reflected by similar effect estimates obtained by randomly sampled gene sets.

INTRODUCTION

The prevalence of obesity and obesity-related diseases is increasing worldwide (1). Previous studies identified many genetic variants associated with an increased risk of developing obesity and a high body mass index (BMI) (2-4). A Genome-Wide Association Study (GWAS) by Yengo et al. (2018) identified 656 independent genetic variants associated with BMI, which collectively explained up to 6% of the total variation in BMI (4). High BMI is a well-known risk factor for cardiometabolic disease, such as type 2 diabetes mellitus and cardiovascular disease (5-8), which has been confirmed in Mendelian Randomization (MR) analyses, in which genetic variants associated with an exposure are used as instrumental variables (9).

Characterization of genetic variants that are associated with BMI, and the genes corresponding to these single nucleotide polymorphisms (SNPs), can provide insight in the biological causes of a high BMI. In an earlier MR study on the association between BMI and anxiety (10), SNPs were categorized based on three mechanistic domains through which they were likely to influence BMI: appetite, adipogenesis and cardiopulmonary function, derived from an earlier GWAS on BMI (3). As an alternative and complementary approach to gene categorization based on mechanistic domains, tissue-grouped gene sets of BMI-associated genes can be identified by analyzing the differential expression of BMI-associated genes in tissues: genes which expression is significantly up- or down-regulated in a given tissue compared with other tissues (11).

Enrichment of BMI-associated genes has been observed in tissues of the central nervous system, notably in the hypothalamus, pituitary gland, hippocampus and limbic system (2), which are brain areas involved in appetite regulation, cognition, and emotion, and thereby collectively highlighted the potential biological processes involved in the regulation of body composition. Additionally, a recent study combined genes for BMI, waist-hip ratio (WHR) and WHR adjusted for BMI (WHRadjBMI), which are all proxies of body composition, to identify enrichment of these genes on a tissue level (12). In this study, differential expression of these body composition-related genes was observed in the central nervous system, adipocyte-related tissues, and the digestive and urogenital system (12). Additionally, BMI-associated genes that show differential expression in specific tissues, thereby reflecting potential tissue-grouped causes of a high BMI, may have distinct effects on disease risk as well (13-16). A more detailed investigation of these biological causes will allow a comprehensive overview of potential targets for interventions.

For this reason, we aimed to group BMI-associated genetic instruments based on tissue expression profiles. With these newly identified combinations of BMI-associated genetic instruments, we aimed to conduct two-sample Mendelian Randomization (MR) analyses on cardiometabolic diseases and measures of abdominal adiposity, in order to unravel the underlying pathways of tissue-grouped causes of a high BMI leading to cardiometabolic disease risk.

METHODS

Selection of genetic variants and gene annotation for BMI
We selected single nucleotide polymorphisms (SNPs) for all genetic loci independently associated with BMI, which were identified in the GWAS meta-analysis by Yengo et al (4).

For the meta-analysis, results of the GWAS on BMI in the UK Biobank were combined with publicly available summary statistics of an earlier GWAS on BMI in the Genetic Investigation of ANthropometric Traits (GIANT) consortium, comprising around 700,000 individuals of mainly European ancestry in total. After meta-analysis, 656 independent SNPs associated with BMI were identified in their main genome-wide significance association (p-value $<5\times10^{-8}$) (see **Supplementary Table 1**).

The 656 SNPs were annotated and mapped to genes based on several lines of evidence using the SNP2GENE function within the Functional Mapping and Annotation (FUMA) v1.3.5e web application (date of accession: 12-11-2019) (17). First, we uploaded a file with the GWAS summary statistics, including the chromosome and position, reference SNP (rs) ID, p-value, effect allele, non-effect allele, beta, and standard deviation for each SNP, and the sample size of the GWAS.

We did not predefine lead SNPs of a genomic region, but instead used the following default parameters: minimum p-value of lead SNPs $<5e^{-8}$, maximum p-value cut-off <0.05, r^2 threshold to define independent significant SNPs ≥0.6, second r^2 threshold to define lead SNPs ≥0.1, reference panel population 1000G Phase3 EUR, minimum Minor Allele Frequency (MAF)≥0, and maximum distance between LD blocks to merge into a locus <250 kb. We included variants in the reference 1000G reference panel (non-GWAS tagged SNPs in LD with an independent significant SNP) (18). Gene annotation was based on positional mapping obtained from Annotate Variation (ANNOVAR) with a maximum distance to genes of 10 kilobases (19). For the gene types, we used Ensembl v92 and selected protein coding gene types. A set of prioritized genes is derived from the combined mapping strategies, based on the provided GWAS summary statistics and filter settings. The output from FUMA comprised genome-wide plots, a summary of the results, and result tables, from which we selected the table with independently significant lead SNPs and the table with mapped genes, and merged these to obtain a list with lead SNPs and their mapped genes. Due to the use of a filter to identify independent SNPs, 22 lead SNPs were omitted, resulting in 634 mapped genes of interest which were used to identify the tissue-grouped gene sets. All exact parameters used for gene annotation and mapping can be found in the **Supplementary Methods**.

Tissue-grouped gene sets
Normalized gene expressions (reads per kilo base per million, RPKM) of 54 non-diseased tissue types were available in GTEx version 8 (11), of which we excluded five tissue types because no eQTL analysis was available (bladder, cervix-ectocervix and -endocervix, fallopian tube and kidney medulla). Tissue types included different brain areas, adipose tissue depots, skin, blood, spleen, kidney cortex, and tissues involved in digestion (e.g. esophagus, stomach, small intestine, pancreas and colon) or reproduction (e.g. ovaries, uterus, vagina

and testis). In the GENE2FUNC in FUMA, the average of the normalized expression (zero mean of log2(RPKM +1)) per tissue per gene was shown as expression value, to allow for comparison of expression across tissue types. By this means, we identified differentially expressed gene sets (genes of which the expression is significantly up- or downregulated in a given tissue compared with other tissues) for each of the 54 tissue types available in GTEx v8. Two-sided Student's t-tests with Bonferroni correction were performed per gene per tissue against all other tissues to identify differentially expressed genes in a given tissue. In GENE2FUNC in FUMA, genes are tested against those differentially expressed gene sets by hypergeometric tests to evaluate whether the prioritized genes (genes of interest) are over-represented in differentially expressed gene sets in the 54 specific tissue types. We entered the 634 BMI-associated genes (our mapped genes of interest based on the independent lead SNPs) in the GENE2FUNC function and selected 'All' background genes, Ensembl version v92 and the GTEx v8 54 tissue type (date of accession: 12-11-2019). We selected the tissues that showed significant differential gene expression in GTEx v8 after adjusting for multiple testing using a false discovery rate (FDR) threshold of 5%. Using the summary data retrieved from FUMA, we identified the specific genes that were differentially expressed in each tissue to establish the gene sets used in the tissue-grouped Mendelian Randomization analyses. All tissue-grouped gene sets were considered strong instruments for BMI based on their F-statistics. All exact parameters used to generate the tissue-grouped gene sets can be found in the **Supplementary Methods**.

Outcome data on type 2 diabetes and coronary heart disease

As study outcomes for the MR analyses, we used publicly available meta-analysis summary statistics of GWAS on type 2 diabetes mellitus and coronary heart disease by the DIAbetes Genetics Replication and Meta-analysis (DIAGRAM) and the Coronary Artery Disease Genome wide Replication and Meta-analysis plus The Coronary Artery Disease Genetics (CARDIoGRAMplusC4D) consortia, respectively. These datasets, based on the largest number of participants to date, contain the per-allele beta-estimates of all investigated SNPs on the outcomes, accompanying standard errors, and the effect alleles.

The report of the DIAGRAM consortium was a meta-analysis of 32 different cohort studies of European ancestry (including the UK Biobank), consisting of 74,124 cases of T2DM and 824,006 controls (20). T2DM was defined as a fasting glucose concentration higher than 6.9 mmol/L or 2-hour plasma glucose of ≥11.1 mmol/L, treatment with glucose-lowering agents, diagnosis by a general practitioner or medical specialist, or self-report.

Within the CARDIoGRAMC4D consortium, GWAS data of 48 studies were meta-analysed, with 60,801 cases and 123,504 controls (21). Coronary artery disease was defined by a history of myocardial infarction, acute coronary syndrome, chronic stable angina or >50% coronary stenosis.

Outcome data on anthropometric traits

The underlying mechanism of the different effects of the tissue-grouped gene sets on disease risk could be via the effect of the tissue-grouped gene sets on body fat distribution measured by waist circumference, or total body fat. By this means, we aimed to give insight

in the underlying mechanisms of tissue-grouped causes of a high BMI and their effect on disease risk. We used publicly available meta-analysis summary statistics of anthropometric traits conducted by the Genetic Investigation of Anthropometric Traits (GIANT) consortium and by the Neale Lab. The data of the GIANT consortium were reported as meta-analysis of 57 multi-ancestry cohorts, with oversampling of cohorts of European-ancestry, genotyped by genome-wide SNP arrays and of 44 cohorts genotyped with the Metabochip. In the present analysis we used waist circumference (in centimetres, unadjusted for BMI) in up to 224,459 individuals (22).

The GWAS on total body fat (as a percentage) was based on data from the UK Biobank, in up to 331,117 individuals of white British genetic ancestry (23). Closely related individuals, individuals with sex chromosome aneuploidies, and individuals who had withdrawn consent from the UK Biobank study had been removed from the analysis.

Mendelian Randomization analysis

Methods for MR analyses of summary-level data based on two study samples have been described in detail previously (24, 25). We estimated the associations between all genetic instruments for BMI, the tissue-grouped gene sets and the outcome measures (coronary artery disease, type 2 diabetes mellitus and anthropometric traits). We allowed for use of LD proxies ($R^2 > 0.8$) and we allowed palindromic SNPs with a MAF threshold of 0.3. Using inverse-variance weighted (IVW) analyses, we combined the effects of the individual genetic instruments to obtain a genetically determined association between exposure and outcome under the assumption of absence of horizontal pleiotropy. The IVW analyses resulted in a weighted mean estimate of a genetically determined increase of 1 standard deviation (SD) of BMI (kg/m^2) on the odds ratio of type 2 diabetes mellitus and coronary artery disease, or on waist circumference (SD, in centimetres), and on total body fat (SD, percentage). In the present study, an increase of 1 SD of BMI (kg/m^2) corresponded to an increase in BMI of 4.8 kg/m^2.

MR analyses could suffer from bias due to pleiotropic effects, when a genetic variant also affects other phenotypes, and thereby influences the outcome via alternative pathways other than through the exposure. Therefore, as sensitivity analyses, we performed MR Egger regression (26) and weighted median estimator (WME) analyses (27). MR Egger accounts for potential pleiotropy and tests for the presence of directional pleiotropy, whereas WME estimates a weighted median effect instead of a weighted mean effect. Similarity of the IVW and MR Egger and WME effect estimates indicates that the results of the MR analysis are robust. In addition to MR Egger and WME, we used the recently described Weighted Mode to detect causal effects, a method that is consistent when the largest number of similar individual causal effect estimates comes from valid instruments, even if the majority of instruments is invalid (28).

For MR analyses, we removed the genetic instrument mapped to TCF7L2 (rs7903146) from the gene sets (and thereby all MR analyses) given its previously described complex and pleiotropic effect (29). Discrepancies between the total numbers of genetic variants in the tissue-grouped gene sets and the numbers of genetic variants in the tissue-grouped gene

sets which were used in the MR analyses are due to the removal of palindromic genetic instruments with intermediate allele frequencies.

The combined effects of the genetic variants in the overall and sensitivity analyses were estimated using the R-based package "TwoSampleMR" (30). The R script for the Mendelian Randomization analyses can be found in the **Supplementary Methods**.

Mendelian randomization analyses using random samples of genetic variants
To gain insight in whether GTEx-based subsets of genetic instruments are the result of chance, we additionally determined the effects estimates for CAD and T2DM by randomly selecting genetic variants. We randomly sampled 1000 gene sets, consisting of 100 or 200 genetic variants from the 633 BMI-associated genetic variants and performed MR analyses (IVW) on CAD and T2DM with these random gene sets. The R script for the Mendelian Randomization analyses using random samples of genetic variants can be found in the **Supplementary Methods**.

RESULTS

Identification of tissue-grouped expression gene sets
Figure 1 shows the analysis procedure we followed to obtain the tissue-grouped BMI-associated gene sets from 656 BMI-associated SNPs. Based on the analysis with GTEx data, we identified 17 tissues in which genes associated with BMI were differentially expressed (**Figure 2A**), of which 12 derived from brain areas. Other tissues were derived from the arteries, digestive system, spleen, or kidney cortex. Differentially expressed genes in each tissue were identified to establish the gene sets (see **Figure 2A**). There was substantial overlap in the genes that were differentially expressed in the brain tissues, but none had a completely overlapping expression profile. Gene symbols and their expression in the different tissue-grouped gene sets are provided in **Supplementary Table 1**. Clustering of the BMI-associated genes in the tissue-grouped gene sets is shown in **Figure 2B**. It should be noted that a substantial number of genetic instruments was not differentially expressed in any of the identified tissues.

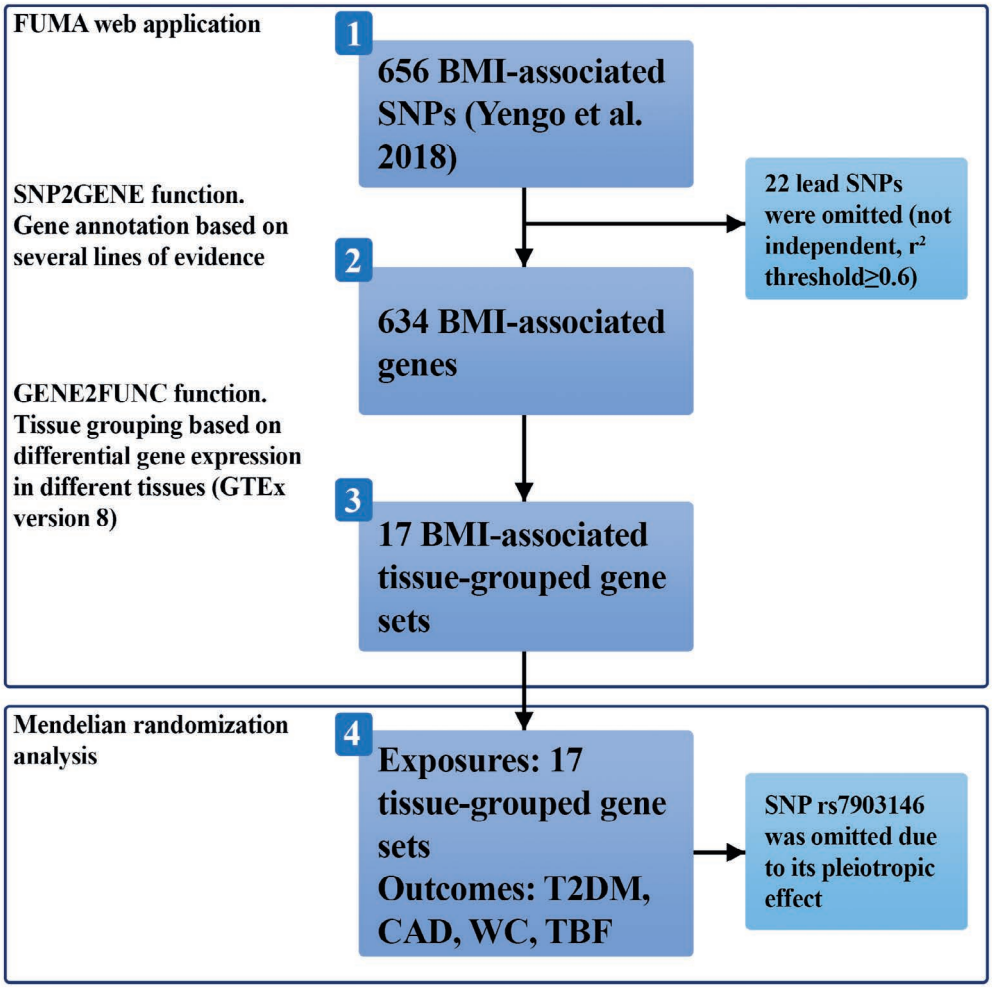

Figure 1. The analysis procedure. 1) We selected 656 single nucleotide polymorphisms (SNPs) independently associated with BMI (4), 2) Using the SNP2GENE function in the FUMA web application, gene annotation was performed for the selected SNPs based on positional mapping obtained from Annotate Variation (ANNOVAR) (19). In this step, 22 SNPs were omitted based on a r^2 threshold of ≥0.6. 3) The 634 BMI associated genes were grouped into 17 tissue-grouped gene sets using the GENE2FUNC function in Functional Mapping and Annotation (FUMA). These gene sets were based on differential gene expression in different tissues with the use of GTEx version 8. 4) The 17 tissue-grouped gene sets were used as exposures in Mendelian Randomization (MR) analyses, with the outcomes type 2 diabetes mellitus (T2DM), coronary artery disease (CAD), waist circumference (WC) and total body fat (TBF). The genetic instrument rs7903146 was removed from the gene sets (and thereby all MR analyses) given its pleiotropic effect.

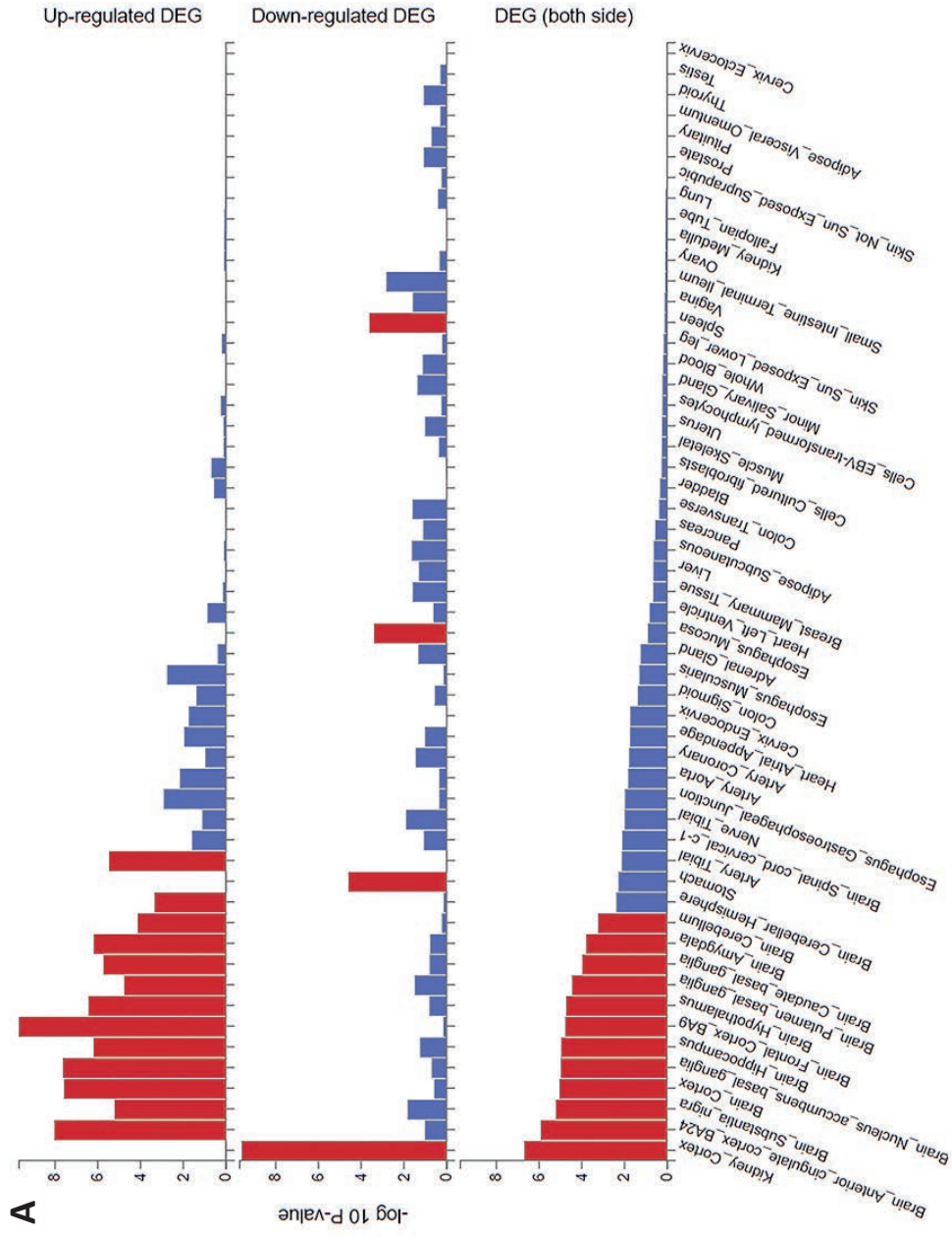

Figure 2. A) Based on the analyses using GTEx v8 54 tissue types data, we identified 17 tissues in which body mass index (BMI)-associated genes were differentially expressed, indicated by red bars. Figure 2A was directly taken from the online tool Functional Mapping and Annotation (FUMA). B) Clustering of the BMI-associated genes in the tissue-grouped gene sets. Differentially expressed genes in a given tissue are shown in red, blue indicates no differential expression in a given tissue.

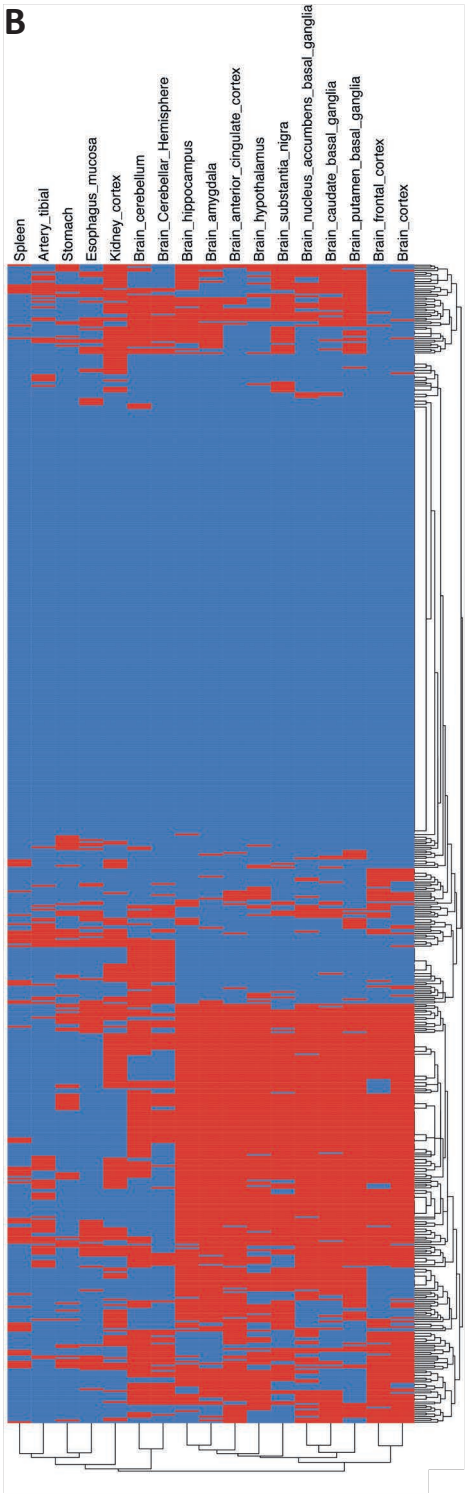

Figure 2. Continued.

Tissue-grouped Mendelian Randomization analyses

Type 2 diabetes mellitus and coronary artery disease
We performed Mendelian Randomization with the tissue-grouped gene sets on T2DM and CAD (**Table 1**). Considering all BMI genetic instruments, an increase in BMI was associated with an increased risk of both T2DM (per SD BMI OR 2.71, 95% CI 2.49; 2.94) and CAD (per SD BMI, OR 1.48, 1.37; 1.59).

In the tissue-grouped MR analyses, all BMI-associated tissue-grouped gene sets were associated with an increased risk of T2DM (**Table 1**), with ORs ranging from 2.42 (2.04; 2.89) for the brain caudate basal ganglia to 2.94 (2.49; 3.47) for the brain cerebellar hemisphere. For CAD, the ORs were ranging from 1.32 (1.02; 1.70) for the spleen, to 1.58 (1.40; 1.78) for the brain putamen basal ganglia. The sensitivity analyses (MR Egger, WME and Weighted Mode) provided similar effects of the tissue-grouped gene sets on T2DM and CAD, although effect estimates were slightly larger compared with the IVW results (see **Supplementary Table 2**).

Table 1. Association between the BMI-overall and BMI-associated tissue-grouped gene sets and risk of type 2 diabetes and coronary heart disease using (tissue-grouped) inverse-variance weighted analyses.

	Type 2 diabetes			Coronary artery disease		
	n_{genes}	Beta (SE)	Odds ratio (95% CI)	n_{genes}	Beta (SE)	Odds ratio (95% CI)
BMI – all genetic variants	616	1.00 (0.04)	2.71 (2.49; 2.94)	613	0.39 (0.04)	1.48 (1.37; 1.59)
Artery tibial	86	0.93 (0.13)	2.54 (1.99; 3.50)	86	0.43 (0.10)	1.53 (1.26; 1.87)
Brain amygdala	231	0.90 (0.08)	2.45 (2.08; 2.89)	230	0.41 (0.07)	1.51 (1.32; 1.72)
Brain anterior cingulate cortex	232	0.93 (0.08)	2.54 (2.19; 2.94)	231	0.43 (0.06)	1.54 (1.37; 1.73)
Brain caudate basal ganglia	222	0.89 (0.09)	2.42 (2.04; 2.89)	221	0.40 (0.07)	1.49 (1.30; 1.69)
Brain cerebellar hemisphere	160	1.08 (0.09)	2.94 (2.49; 3.47)	159	0.34 (0.08)	1.41 (1.22; 1.63)
Brain cerebellum	214	1.00 (0.08)	2.72 (2.33; 3.19)	213	0.37 (0.07)	1.45 (1.28; 1.65)
Brain cortex	213	0.95 (0.08)	2.59 (2.22; 3.03)	212	0.43 (0.06)	1.53 (1.36; 1.73)
Brain frontal cortex	207	0.97 (0.08)	2.63 (2.25; 3.07)	206	0.43 (0.06)	1.54 (1.37; 1.73)
Brain hippocampus	237	0.96 (0.08)	2.60 (2.22; 3.05)	237	0.44 (0.06)	1.55 (1.37; 1.75)
Brain hypothalamus	214	0.97 (0.08)	2.65 (2.27; 3.09)	213	0.40 (0.06)	1.49 (1.33; 1.68)
Brain nucleus accumbens basal ganglia	224	0.99 (0.08)	2.70 (2.31; 3.16)	223	0.40 (0.06)	1.48 (1.31; 1.68)
Brain putamen basal ganglia	237	0.92 (0.08)	2.52 (2.16; 2.95)	237	0.46 (0.06)	1.58 (1.40; 1.78)
Brain substantia nigra	226	0.95 (0.08)	2.58 (2.20; 3.03)	225	0.44 (0.06)	1.55 (1.37; 1.75)
Esophagus mucosa	99	1.00 (0.13)	2.72 (2.12; 3.50)	99	0.33 (0.09)	1.39 (1.17; 1.65)
Kidney cortex	191	1.00 (0.09)	2.72 (2.29; 3,22)	190	0.36 (0.07)	1.44 (1.25; 1.65)
Spleen	63	1.00 (0.12)	2.72 (2.17; 3,42)	63	0.28 (0.13)	1.32 (1.02; 1.70)
Stomach	81	0.94 (0.12)	2.55 (2.01; 3.24)	81	0.41 (0.09)	1.51 (1.25; 1.82)

Betas and odds ratios are obtained using Mendelian Randomization analyses. The odds ratio can be interpreted per increase of 1 standard deviation of BMI (kg/m^2) which is equivalent to an increase of 4.8 kg/m^2. n_{genes} indicates the number of genes in each gene set. Abbreviations: BMI, body mass index; SE, standard error; CI, confidence interval.

Waist circumference and total body fat

We examined the associations of the tissue-grouped gene sets with the anthropometric traits waist circumference and total body fat as outcomes to explore potential underlying mechanisms of cardiometabolic disease (see **Table 2**). Considering all BMI genetic instruments, an increase of 1 SD of BMI was associated with an increase in waist circumference of 0.83 cm (95% CI 0.80; 0.85) and total body fat of 0.61% (95% CI 0.60; 0.63). All BMI-associated tissue-grouped gene sets had a positive effect on waist circumference and total body fat. Overall, the alternative MR methods MR-Egger, WME and Weighted Mode analyses indicated a positive effect of the tissue-grouped gene sets on waist circumference and total body fat (see **Supplementary Table 3**).

Table 2. Association between the BMI-overall and BMI-associated tissue-grouped gene sets and waist circumference and total body fat using (tissue-grouped) inverse-variance weighted analyses.

	Waist circumference (cm)		Total body fat (%)	
	n_{genes}	beta (95% CI)	n_{genes}	beta (95% CI)
BMI – all genetic variants	610	0.83 (0.80; 0.85)	615	0.61 (0.60; 0.63)
Artery tibial	84	0.84 (0.78; 0.90)	86	0.61 (0.57; 0.65)
Brain amygdala	229	0.84 (0.80; 0.88)	231	0.64 (0.61; 0.67)
Brain anterior cingulate cortex	230	0.85 (0.82; 0.89)	232	0.65 (0.63; 0.68)
Brain caudate basal ganglia	219	0.86 (0.82; 0.90)	221	0.64 (0.61; 0.67)
Brain cerebellar hemisphere	156	0.85 (0.81; 0.90)	160	0.61 (0.58; 0.64)
Brain cerebellum	210	0.85 (0.81; 0.89)	214	0.64 (0.61; 0.67)
Brain cortex	212	0.86 (0.82; 0.90)	213	0.65 (0.62; 0.68)
Brain frontal cortex	205	0.85 (0.82; 0.89)	206	0.66 (0.63; 0.69)
Brain hippocampus	236	0.84 (0.80; 0.88)	237	0.64 (0.61; 0.67)
Brain hypothalamus	212	0.84 (0.81; 0.88)	214	0.64 (0.61; 0.66)
Brain nucleus accumbens basal ganglia	222	0.86 (0.82; 0.90)	214	0.65 (0.62; 0.68)
Brain putamen basal ganglia	236	0.83 (0.79; 0.86)	237	0.65 (0.62; 0.67)
Brain substantia nigra	224	0.84 (0.80; 0.87)	226	0.63 (0.60; 0.66)
Esophagus mucosa	97	0.84 (0.78; 0.89)	99	0.61 (0.56; 0.65)
Kidney cortex	188	0.82 (0.78; 0.86)	191	0.63 (0.60; 0.66)
Spleen	62	0.85 (0.78; 0.91)	63	0.59 (0.54; 0.64)
Stomach	80	0.82 (0.75; 0.88)	81	0.61 (0.56; 0.66)

Betas are obtained using Mendelian Randomization analyses. The betas can be interpreted per increase of 1 standard deviation of BMI (kg/m^2). n_{genes} indicates the number of genes in each gene set. Abbreviations: BMI, body mass index; CI, confidence interval.

Mendelian randomization analyses using random samples of genetic variants

The distribution of ORs obtained by random sampling of 100 or 200 genetic variants (from the 633 BMI-associated genetic variants) was similar to the results of the tissue-grouped MR (IVW method), as presented in the histograms in **Figure 3**. For T2DM, the ORs ranged from 1.9 to 3.8 for the sampled gene sets of 100 variants, and from 1.7 to 3.4 for the sampled gene sets of 200 variants, with the most frequent OR around 2.7, which was similar to the estimates that were observed in the tissue-grouped MR analyses. Similar to the tissue-grouped results, the ORs of the randomly sampled gene sets for the outcome CAD were smaller than for T2DM, ranging from 1.2 to 2.1, with the most frequent OR around 1.4.

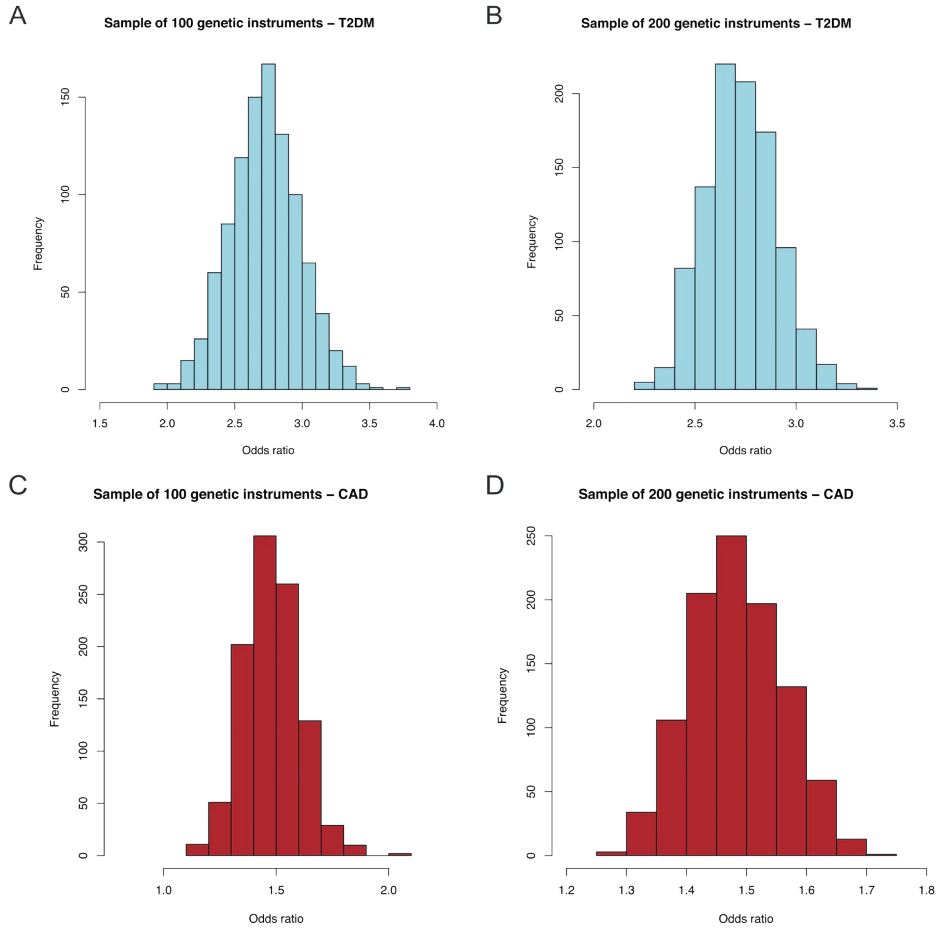

Figure 3. Histograms displaying the distribution of odds ratios (from inverse-variance weighted [IVW] analyses) for the association between randomly sampled sets of 100 or 200 genetic variants and type 2 diabetes mellitus (T2DM) and coronary artery disease (CAD). A) random sample of 100 genetic variants – T2DM, B) random sample of 200 genetic variants – T2DM, C) random sample of 100 genetic variants – CAD, D) random sample of 200 genetic variants – CAD.

DISCUSSION

The aims of the present study were to identify tissue-grouped pathways of BMI-associated loci and their relation to cardiometabolic disease and anthropometric measures using MR analyses. The results of our study imply that genetic variants associated with BMI show enrichment in different tissues. In earlier studies, enrichment of BMI-associated genes was observed in the central nervous system (2), in accordance with the results of the present study. Expression of genetic variants which were associated with a decreased WHR was enriched in the digestive and urogenital system (12). We observed enrichment of BMI-grouped genes in the esophagus mucosa, stomach, spleen, and kidney cortex.

Instead of performing MR using all genetic instruments for a given exposure, we examined the contribution of associated tissues to risk of cardiometabolic disease, in order to unravel the role of specific tissues in the genetic risk of cardiometabolic disease due to overweight or obesity. Using MR, the associations between BMI and T2DM and CHD have been well established (5-8). Although the use of tissue-grouped gene sets of genetic instruments would mean a reduction in statistical power to identify effects of BMI, we were still able to identify effect estimates similar to the overall MR analysis comprising all genetic instruments, which was also reflected in the F-statistics of the tissue-grouped gene sets (31). Therefore, the increased availability of large GWAS datasets allows to examine the individual contributions of grouped genetic variants in more detail.

The results of the present study imply that genetic variants associated with BMI have similar effects on type 2 diabetes and coronary artery disease risk, regardless of the tissue in which the genes show differential expression, although there was some variation in the observed effect estimates. All BMI-associated gene sets were shown to have an effect on waist circumference and total body fat, which are both known to be associated with increased risk of T2DM and CAD. These results are supported by findings of additional analyses, in which we randomly selected 100 or 200 genetic instruments from the 633 BMI-associated genetic variants. After we repeatedly performed Mendelian randomization analyses on T2DM and CAD with randomly sampled BMI-associated gene sets, the distribution of the effect estimates was similar to the results of the tissue-grouped MR analyses. This suggests that the effect estimates of the BMI-associated gene sets based on tissue expression profiles were similar to BMI-associated gene sets obtained by random sampling of genetic instruments.

The identification of groups of genetic variants that have differential effects on an outcome measure, representing different mechanisms of disease, can be approached from various perspectives. In the present study, we explored the added value of clustering genetic instruments based on a biological perspective. Importantly, in previous research a statistical approach has been proposed (32). The authors were able to group high-density lipoprotein (HDL) cholesterol variants according to their effect on the risk of coronary heart disease and thus to identify groups of genetic variants showing different effects on disease. We therefore hypothesize that grouping the BMI-associated genetic variants by a statistical approach might identify groups of BMI-associated genetic variants that have different effects on disease risk.

A potential limitation of our study is the methodology of the clustering of the genetic instruments in the tissue-grouped gene sets. Currently, the GTEx analyses are performed using a standard overrepresentation test which does not take into account the effect size of the genetic instrument on the exposure. Genetic variants with large effect sizes therefore have the same weight in the clustering as genetic variants with much smaller effect sizes. As the GWAS by Yengo et al. identified many genetic variants for BMI with small effect sizes, this likely will have affected the clustering. Additionally, tissue-grouped gene sets identified by the enrichment analyses by GTEx are influenced by the number of tissue samples available for the different tissues. Subsequently, this has implications for the results of the MR analyses.

Another limitation of our study is the overlap between the genes within the tissue-grouped gene sets, which made it more difficult to mutually compare the tissue-grouped gene sets. Due to the overlap, the gene sets might be less specific to distinguish between the different tissues, however, they are specific for investigating the relation with cardiometabolic disease.

To conclude, this study explored whether we can add insight in causal disease biology when analysing tissue-grouped gene sets and by examining the effects of these gene sets on different outcome measures using MR analyses. In the context of BMI-associated genetic variants, our novel approach does not provide additional insight in the role of specific tissues in the genetic risk for specific cardiometabolic disease due to overweight or obesity.

CONFLICT OF INTEREST STATEMENT

All authors declare to have no conflict of interest.

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SUPPLEMENTARY MATERIAL

The supplementary material can be found at
https://academic.oup.com/ije/article/49/4/1246/5851556#supplementary-data.

CHAPTER 3

Abdominal adiposity in adolescence and early changes in atherogenic metabolites into young adulthood

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*Equal contributions

In preparation

ABSTRACT

Introduction: Obesity in adolescence tends to track into adulthood and increases the risk of cardiovascular disease later in life. It is unclear whether excess abdominal adiposity in adolescence is associated with changes in circulating metabolites indicative of an emerging atherogenic profile into young adulthood.

Methods: In first-generation offspring of the Avon Longitudinal Study of Parents and Children (ALSPAC), total and trunk fat mass were measured using dual-energy X-ray absorptiometry scans at age 15y. Targeted nuclear magnetic resonance-based metabolomics was used to quantify 145 metabolites (mostly subclasses of lipoproteins) at age 15y, 18y and 24y. Using linear spline multilevel models, we examined sex-specific associations of total and trunk fat mass at 15y with trajectories of metabolites from 15y to 24y.

Results: Analyses included 3851 participants, 53% female. At age 15y, median (interquartile range) total fat mass was 13.7 (8.3-20.1) kg and 6.1 (3.6-9.4) kg for trunk fat mass. Higher trunk fat at 15y was associated with an increase in concentrations of multiple metabolites, including non-high-density lipoproteins, more so in males than in females. Specifically, each SD ln higher trunk fat mass (corresponding to 0.69 kg in males and 0.48 kg in females) was associated with increasing cholesterol in large very-low-density lipoprotein (2.05 [95% confidence interval 0.54–3.57] SD, total serum triglycerides (2.10 [0.77–3.44] SD and apolipoprotein B (1.90 [1.13–2.66] SD in males, but not in females (0.05 [-0.67–0.77] SD, 0.23 [-0.64-1.10] SD and 0.29 [-0.54–1.13] SD, respectively). Results were similar for total fat mass.

Conclusion: Excess abdominal fat in adolescence was associated with an increasingly atherogenic lipid profile in males only. Adolescence may therefore be a critical period for the early prevention of adiposity-induced atherosclerosis in males.

INTRODUCTION

The prevalence of childhood and adolescent obesity is increasing worldwide, contributing to obesity-related disease burdens and associated healthcare costs (1). Obesity in childhood and adolescence, which typically persists into adulthood (2), is associated with an increased risk of cardiometabolic diseases later in life, including type 2 diabetes and coronary heart disease (CHD) (3, 4), as well as increased mortality in middle age (5). A recent Mendelian randomization (MR) analysis further suggested that higher body size in childhood, via body size in adulthood, increases risk of CHD (16). In addition, children and adolescents with obesity already show insulin resistance, dyslipidaemia, and increased prevalence of metabolic risk factor clustering (6-8). The increased cardiometabolic risk conferred by total body fat is likely driven by an adverse fat distribution, particularly by fat stored in and around the abdomen (9-11), which was also demonstrated in children and adolescents (12).

Circulating metabolites may be important intermediates of adiposity and cardiometabolic disease. In a cross-sectional study of 12,664 adolescents, higher body mass index (BMI) was strongly associated with unfavourable concentrations of very low-density lipoproteins (VLDLs) and low-density lipoproteins (LDLs), and high-density lipoprotein (HDLs), which was supported by MR analyses in young adults (13). Additionally, higher BMI was associated with higher concentrations of monounsaturated fatty acids and saturated fatty acids, as well as of branched-chain amino acids (13, 14). In a study of 7821 children and adolescents, higher body fat percentage was associated with adverse total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides (15). MR analyses further support the potential for higher body size in childhood (via body size in adulthood) to alter concentrations of numerous metabolites including apolipoprotein B-containing lipoproteins, and inflammatory glycoprotein acetyls (16), all indicative of a more atherogenic profile.

Taken together, there may be an early opportunity to reduce the atherogenic consequences of adiposity during childhood and adolescence, as adiposity often persists into adulthood (2). However, longitudinal studies in the field of metabolomics are lacking and it is unknown how directly measured body fat distribution during adolescence is associated with early subsequent changes in circulating metabolites. Given that men tend to store more fat in and around the abdomen, whereas women tend to store more fat subcutaneously in the hips and legs, we might also expect sex differences in effects of adiposity on metabolites (17).

In this study, we used a British birth cohort data to investigate the sex-specific associations of directly measured total and abdominal fat in adolescence with subsequent changes over time in a comprehensive set of circulating metabolites. This enabled us to capture early atherogenic changes occurring into young adulthood in response to adolescent adiposity and provide new evidence on when the first indications of cardiometabolic disease begin to develop in young males and females.

METHODS

Study design and study population

The Avon Longitudinal Study of Parents and Children (ALSPAC) is a population-based birth cohort study in which 14,541 pregnant women expected to deliver between 1 April and 31 of December 1991 were recruited from the former county of Avon in southwest England. Offspring (G1) alive at 1 year of age (n=13,988) have been followed since with multiple assessments, with an additional 913 children enrolled over the course of the study (18-20). Follow-up has included parent- and child-completed questionnaires, clinic attendances, and links to routine data. Research clinics were held when the G1 participants were approximately 7 years (y), 9y, 10y, 11y, 13y, 15y, and 18y. Study data among G1 cohort participants after age 22y were collected and managed using REDCap electronic data capture tools hosted at University of Bristol (21, 22). The present analyses were conducted using all eligible participants with data on total body fat mass or trunk fat at age 15y, sex, age, and at least one of the metabolomic measures at any age. This resulted in 3851 eligible participants (1810 men, 2041 women) contributing to our analyses.

Ethical approval as obtained from the ALSPAC Law and Ethics and Local Research Ethics Committee. Informed consent for the use of data collected via questionnaires and clinics was obtained from participants following the recommendations of the ALSPAC Ethics and Law Committee at the time. The study website contains details of all the data that is available through a fully searchable data dictionary and variable search tool (http://www.bristol.ac.uk/alspac/researchers/our-data/).

Assessment of total and abdominal fat mass

Dual-energy X-ray absorptiometry (DXA)-determined total fat mass and trunk fat mass were measured at mean age 15y using a GE Lunar Prodigy (Madison, WI, USA) narrow fan beam densitometer. Total body fat mass (in kg, less head) was derived from whole body DXA scans. Trunk fat mass was estimated using the automatic region of interest that included chest, abdomen, and pelvis. The scans were visually inspected and realigned where necessary.

Assessment of metabolites

Blood samples were drawn at clinic attendance of the offspring at mean ages 15y, 18y and 24y, after a minimum of a 6-hour fast. Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy from a targeted metabolomics platform (23) was performed on EDTA-plasma samples from each of these three occasions to quantify 145 traits.

The NMR spectroscopy was conducted at the Medical Research Council Integrative Epidemiology Unit (MRC IEU) at the University of Bristol, Bristol, United Kingdom, and processed by Nightingale's biomarker quantification algorithms (version 2014). This method provides quantification of lipoprotein subclass profiling with lipid concentrations within 14 lipoprotein subclasses. The 14 subclass sizes were defined as follows: extremely large VLDL with particle diameters from 75 nm upwards and a possible contribution of chylomicrons, five VLDL subclasses (average particle diameters of 64.0 nm, 53.6 nm, 44.5 nm, 36.8 nm, and 31.3 nm), intermediate-density lipoprotein (IDL, 28.6 nm), three LDL subclasses (25.5 nm, 23.0 nm, and 18.7 nm), and four HDL subclasses (14.3 nm, 12.1 nm, 10.9 nm, and 8.7 nm).

Within the lipoprotein subclasses the following components were quantified: total cholesterol, total lipids, phospholipids, free cholesterol, cholesteryl esters, and triglycerides. The mean sizes for VLDL, LDL and HDL particles were calculated by weighting the corresponding subclass diameters with their particle concentrations. Furthermore, 58 metabolites were determined, including cholesterol, triglyceride, and other lipid content in lipoprotein subclass particles, apolipoproteins, fatty acids, amino acids, and inflammatory glycoprotein acetyls.

In the present study, we excluded metabolites representing ratios between two measures. We additionally excluded diacylglycerol, fatty acid length, conjugated linoleic acid and pyruvate, which were not measured at age 24y, resulting in an outcome set of 145 metabolites.

Assessment of confounding factors

We considered the following as potential confounders of the association between fat mass at age 15y and metabolite trajectories from 15y to 24y: ethnicity (white or non-white), age at puberty onset, maternal educational level, smoking status at age 15y, alcohol intake at age 15y, medication use (hormonal contraceptives, glucocorticoids, antipsychotics/antidepressants), height at age 15y and height at age 15y squared (height^2). Age at puberty onset was estimated by the age at peak height velocity based on the SuperImposition by Translation And Rotation (SITAR) growth curve modelling of repeated height measures from age 5y to 20y (detailed previously) (24). Highest educational attainment of the participant's mother (Certificate of Secondary Education [CSE], vocational, O-level, A-level or degree) was used to indicate socioeconomic position at birth. Smoking status (ever smoked a whole cigarette yes/no) at age 15ywas recorded via questionnaire, as well as alcohol intake (ever had a whole drink yes/no) and medication use. Height was measured in light clothing without shoes to the nearest 0.1 cm using a Harpenden stadiometer.

Statistical analyses

Linear spline multilevel models were used to model trajectories from 7y to 24y, as described previously (25). For the present analysis, we included these trajectories from 15y only and used linear spline multilevel models to examine associations of total and trunk fat mass at age 15y and trajectories from 15y to 24y. Multilevel models estimate mean trajectories of the outcome while accounting for the non-independence or clustering of repeated measurements within individuals, change in scale and variance of measures over time, and differences in the number and timing of measurements between individuals (using all available data from all eligible participants under a missing-at-random assumption (26, 27). Linear splines allow knot points to be fit at different ages to derive periods in which change is approximately linear. All linear spline multilevel models included here had two linear spline periods from 15y to 18y and 18y to 24 (two levels: measurement occasion and individual). Further details for each model for the 145 metabolites included in our analysis are included in the Supplementary material. All models included robust standard errors to accommodate skewed outcome distributions.

Participants were included in the present analyses if they had data on total or trunk fat mass at age 15y, sex, age, at least one of the metabolites at 15y, 18y or 24y and data on all potential confounders. This resulted in 3851 eligible participants (1810 males, 2041 females contributing to our analyses. All analyses were performed separately for males and females

and adjusted for all potential confounders as listed previously, further information on the inclusion of confounders in models is included in the supplementary material.

We transformed total body fat mass and trunk fat using the natural logarithm due to skewed distributions and subsequently standardised these measures into SD units by generating z-scores (with a mean of zero and SD of 1). We assessed linearity of our models by using likelihood ratio tests with total and trunk fat mass treated as continuous variables and as categorical variables in quartiles in relation to all metabolites for males and females separately. We did not find evidence for non-linearity for most metabolites across time points and therefore treated both fat measures as continuous exposures. We assessed and removed outliers in the metabolites by using a cut-off of the mean and ±4 SD across the three included assessment rounds.

In addition, we centred the continuous confounding factors based on their mean values, and created dummy/binary variables for all categorical variables. We fitted a spline interaction term and sex interaction term with all confounding factors to allow confounding structures to differ y sex during the two time periods (i.e. between 15y and 18y, and between 18y and 24y).

For each sex, models directly estimate mean predicted difference in level of each metabolite at 15y (the intercept) and mean predicted difference in slopes in original units per 1-SD higher total or trunk fat mass, with sloped interpreted as change per year in each metabolite in the respective spline period. Following analyses, these estimates were then combined to provide other estimates of interest including mean predicted difference in absolute change in each metabolite level from 15y to 24y per 1-SD higher total or trunk fat mass using the sloped given by each model. The mean predicted difference in the level of each metabolite at 24y per 1-SD higher total or trunk mass was also estimated. All of the above estimates were then converted to SD units by dividing by the sex-combined standard deviation (SD) of the observed metabolite at 15y, in order to aid comparison of results between metabolites. Note that all analyses were performed in original units and converted SD units post analysis to aid comparison between results. All results in original units, including slopes for linear spline periods, are presented in the **Supplementary Material**.

The results can be interpreted as the association between 1-SD higher ln total fat mass (corresponding to 0.63 kg in men and 0.41 kg in women) or trunk fat (corresponding to 0.69 kg in men and 0.48 kg in women) with SD-unit change in a specific metabolite between 15y and 24y, i.e. over a 9 year period. The standardized mean differences between 15y and 24y of all metabolites associated with exposure to total fat mass at 15y were plotted in a circle plot to illustrate the illustrate the results.

Additional and sensitivity analyses

To reduce potential for residual confounding by hormonal factors related to puberty that we were not able to account for, we additionally examined the association between total and trunk fat mass at age 18y (log-transformed and z-scored) and changes in the metabolites between ages 18y and 24y. We calculated the absolute difference between metabolite concentrations between 18y and 24y and then standardized these differences. Because there were only two time points, we used a (non-mixed) linear regression model adjusted for

ethnicity, exact age at 18y, height at 18y, age at puberty onset and maternal educational attainment, stratified by sex.

To account for changes in total and trunk fat mass occurring after 15y, which might differentially influence changes in the concentrations of metabolites, we additionally divided participants into four groups, based on their fat mass at 15y and at 24y. The cut-offs were based on the 50th percentile of total fat mass at 15y and at 24y, calculated separately for men and women. We then established four categories: 1) individuals who stayed in the 'low fat' category between age 15y and 24y, 2) individuals who went from the 'low fat' to the 'high fat' category, 3) individuals who went from the 'high fat' to the 'low fat' category, and 4) individuals who stayed in the 'high fat' category between age 15y and 24y. We then repeated our primary analyses separately in the two categories remaining at a stable level of fat mass (category 1 and 4). Of note, the population included in this sensitivity analysis is slightly different than the population we included in our main analyses, as participants needed to have data on both total fat mass at age 15y and at 24y.

Analyses were conducted using Stata 14.0 (StataCorp, College Station, Texas, USA) and data visualisation was performed in Python and R (version 4.0.3).

RESULTS

Participant characteristics

3851 participants with measures of total or trunk fat mass at age 15y and at least one metabolomics measurement were included in analyses (**Table 1**). Of these, 53% of the participants were female, and 91% were of self-reported white ethnicity. The mean age (SD) at puberty onset was 11.7y (0.8) for females and 13.6y (0.9) for males. In females, mean body mass index at age 15y was 21.7 (3.5) kg/m^2 with a median (interquartile range) total fat of 17.2 (13.3 – 22.3) kg and median trunk fat of 7.8 (5.8 – 10.7) kg. In males, mean BMI at 15y was 20.9 (3.1) kg/m^2, with a lower median total fat (8.4 [5.8 – 13.1] kg) and trunk fat (3.7 [2.5 – 6.1] kg) than females.

Table 1. Characteristics of the first offspring generation of the Avon Longitudinal Study of Parents and Children (ALSPAC) participants included in the analyses, stratified by sex

	Women (53%)	Men (46%)
White ethnicity (%)	91	91
Height at age 15 (cm)	164.8 (6.0)	174.7 (7.6)
BMI at age 15 (kg/m^2)	21.7 (3.5)	20.9 (3.1)
Fat mass at age 15 (kg)	17.2 (13.3; 22.3)	8.4 (5.8 - 13.1)
Trunk fat at age 15 (kg)	7.8 (5.8; 10.7)	3.7 (2.5 - 6.1)
BMI at age 18 (kg/m^2)	22.8 (4.0)	22.4 (3.6)
Fat mass at age 18 (kg)	19.3 (15.0 – 25.0)	10.6 (6.8 - 16.8)
Trunk fat at age 18 (kg)	9.4 (7.1 – 12.6)	5.5 (3.4 – 8.8)
BMI at age 24 (kg/m^2)	24.8 (5.2)	24.7 (4.2)
Fat mass at age 24 (kg)	21.7 (17.2 – 28.9)	18.2 (13.8 – 24.8)
Trunk fat at age 24 (kg)	9.8 (7.2 – 14.0)	9.0 (6.4 – 12.8)
Age at puberty onset (years)	11.7 (0.8)	13.6 (0.9)
Age at puberty onset ≥15.5y (%)	5	7
Maternal education (%)		
CSE	10	9
Vocational	6	7
O Level	34	31
A Level	29	32
Degree	20	21
Ever smoked by age 15 (%)	71	58
Ever drank alcohol by age 15 (%)	87	85
Use of hormonal contraceptives (%)	14	-
Use of glucocorticoids (%)	0.3	0
Use of antipsychotics or antidepressants (%)	0.6	0.4

This table includes participants with data on sex, fat mass at age 15y and trunk fat at age 15y, and at least one metabolomic measure at any age. Abbreviations: BMI, body mass index; CSE, certificate of secondary education; O level, ordinary level; A level, advanced level. Data are presented as mean (SD or range), median (25th–75th percentile) or percentage.

Associations of total and trunk fat mass at age 15y with subsequent changes in metabolites
Patterns of associations of total and trunk fat mass at age 15y with the average rate of change in metabolites between age 15y and 24y differed substantially for males and females (see **Supplementary Table 1** and **Supplementary Table 2**).

In males, higher trunk fat at 15y was associated with an increase in almost all VLDL measures, whereas in females these associations were slightly negative or null. For example, in

males, each SD ln higher trunk fat mass at age 15y (corresponding to 0.69 kg and 0.48 kg in women), was associated with a 2.05 SD (95% CI 0.54 – 3.57) increase in cholesterol in large VLDL, and in women with a 0.05 SD (95% CI -0.67 – 0.77) change. We observed similar patterns for LDL measures.

In contrast, we found no strong evidence that higher trunk fat mass at 15y was associated with changes in total cholesterol in high-density lipoprotein (HDL) in males and females. Higher trunk fat mass at age 15y was associated with an increase in total serum triglycerides in men (2.10 SD, 95% CI 0.77 – 3.44), and but evidence of this change was weak in females. In males, higher trunk fat mass at 15y was associated with an increase in apolipoprotein B (1.90 SD, 95% CI 1.13 – 2.66), whereas in females there was no strong evidence of this change (0.29 SD (-0.54 – 1.13).

The results were similar for total fat mass (see **Figure 1** and **Supplementary table 2**). We observed comparable patterns of association for VLDL measures, as well as for LDL and HDL measures. For instance, a 1 SD ln (corresponding to 0.63 kg in males and 0.41 kg in females) higher total fat at 15y was associated with 2.06 SD (95% CI 0.54 – 3.58) change in cholesterol in large VLDL in males, and a 0.03 SD (95% CI -0.68 – 0.75) change in cholesterol in large VLDL in females. In addition, total fat at 15y was associated with an increase in total serum triglycerides in males (2.11 SD, 95% CI 0.77 – 3.45), but not in females. In males, total fat at 15y was associated with an increase in apolipoprotein B (1.88 SD, 95% CI 1.11 – 2.65), whereas in females there was weak evidence of this (0.27 SD (-0.56 – 1.10).

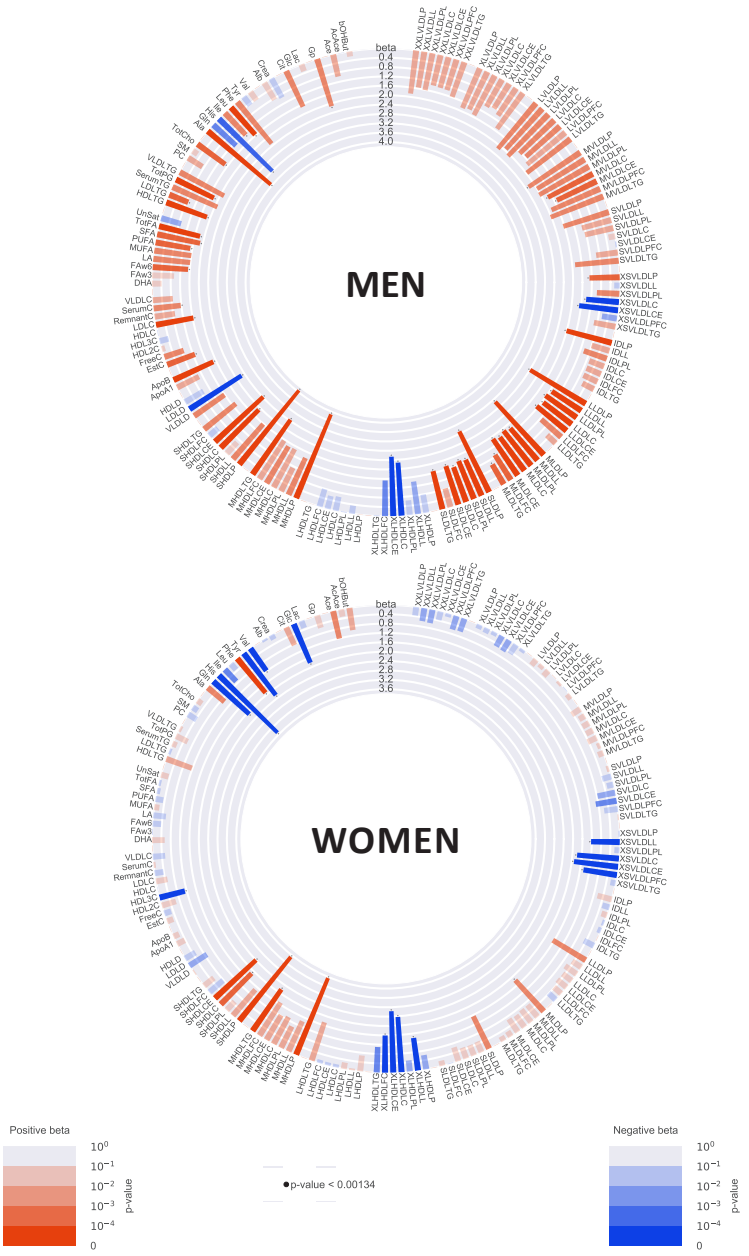

Figure 1. The circle plots show the associations between total fat mass at age 15y and the average rate of change in the individual metabolites from age 15y to age 24y in the ALSPAC. The metabolomic measures are indicated at the outer circle. A red bar indicates a positive rate of change, whereas a blue bar indicates a negative rate of change. The colour intensity and height of the bar indicate the magnitude of association. A black dot above a bar indicates association at the level of $p < 0.00134$.

Associations of total fat mass at 18y and change in metabolomic measures between 18y and 24y
The associations of total fat mass at age 18y and the changes in metabolites between age 18y and 24y were less pronounced than the results of our main analyses, which examined the change from age 15y onwards (see **Supplementary Table 3**). In males, we observed that higher fat mass at age 18y was associated with a decrease of 0.16 SD [95% CI -0.07; -0.25] in triglycerides in large LDL between age 18y and 24y. We also observed a decrease in triglycerides in large LDL in females (-0.44 SD, CI -0.25; -0.62). Furthermore, we observed strong decreases in several measures of XXL- to XL-VLDL in females related to fat mass at age 18y, in line with the findings of our main analyses.

Associations of stable amounts of total and trunk fat mass with metabolites
At age 15y, the median total fat mass for males was 8.5 kg, whereas for females it was 17.3 kg. In males, the median increased to 18.3 kg at age 24y, in females this was 22.2 kg. In total, of the 1127 males and 1683 females eligible for the sensitivity analysis, 771 males remained in the same category of fat mass (either 'low' or 'high') between age 15y and 24y, and 1252 females remained at a stable total fat mass.

Overall, we observed similar patterns in the associations of fat mass at age 15y and the rate of change of metabolites in individuals who remained in the same category of fat mass, as compared with the complete population (see **Supplementary Table 4**). We observed an association between total fat mass at age 15y and increased rate of change in total cholesterol in HDL in males (0.76 SD [95% CI 0.08 – 1.45] per log total fat mass in kg). In females, we observed 0.96 SD decrease in triglycerides in LDL (95% CI -1.68; -0.24) per SD ln fat mass at age 15y. Lastly, we observed that total fat mass at age 15y was associated with a decreased rate of change in glucose levels in males (-1.80 SD, 95% CI -2.61; -0.98).

DISCUSSION

In this population-based birth cohort of adolescents, we examined the sex-specific associations of total and trunk fat mass with early changes over time in atherogenic metabolites. Overall, our results suggest that higher total and trunk fat at adolescence were associated with increasing concentrations of apolipoprotein-B-containing lipoprotein particles, indicative of a more atherogenic progression, but that these adverse changes are only apparent in males. In addition, our findings suggest that this result is consistent for individuals after puberty and individuals who remained at a stable amount of fat mass (either low or high) during adolescence and young adulthood. This indicates that our results are not driven by changes related to puberty or increases in fat mass.

Previous studies suggested that men are at a higher risk of cardiovascular disease than women until after age 75y (27). One potential explanation is that cardiovascular disease is understudied in women, and symptoms might present differently than in men (28), leading to underdiagnosis and undertreatment of cardiovascular disease in women (29). However, we observed that atherogenic changes are already apparent at a young age. Secondly, men tend to store body fat in the abdomen, where women are more likely to store fat at the hips and thighs (17). It is well-established that abdominal adiposity, and in particular visceral

adipose tissue located around the organs, is strongly related to insulin resistance (28) and risk of type 2 diabetes mellitus (9) raising cardiovascular disease risk (29).

One mechanism explaining the association between total fat mass at adolescence and young adulthood and a more atherogenic metabolic profile is adipocyte hypertrophy. As a result of hypertrophy, adipose tissue becomes dysfunctional, ultimately leading to insulin resistance and metabolic disturbances (30-32). Spalding et al. observed that the number of adipocytes is set before adulthood, and the expansion of adipocyte number ends around the age of 16.5y in individuals with obesity and 18.5y in lean individuals (33). Taken together, this suggests that adipocytes respond to increases in fat mass by expansion during young adulthood, and thereby contribute to adipocyte hypertrophy, ultimately leading to metabolic disturbances as we observed in our study.

Childhood BMI and adult BMI were shown to be strongly genetically correlated (34). This suggests that the associations between childhood BMI and cardiometabolic diseases in adulthood may be explained by persistence of BMI from childhood into adulthood, reflected by partial genetic overlap and a common onset. In line, a recent MR analysis showed that body size in childhood was associated with an increased risk of coronary artery disease (35). However, when childhood body size was analysed in a multivariable framework with adult body size, this effect attenuated. Further MR analyses showed body size in childhood to be associated with concentrations of 42 metabolomic measures, including VLDL cholesterol and triglycerides, amino acids, glycoprotein acetyls, and HDL cholesterol (16). Although these associations were indicative of a more atherogenic cardiometabolic profile, most associations also attenuated in multivariable MR models with adult body size, with exception of the amino acids leucine, isoleucine and tyrosine. Taken together, the results of these studies indicate that the effects of childhood adiposity are mainly mediated via body size during adulthood. In addition, this suggests that there is an opportunity to reduce the cardiometabolic consequences of body size during childhood, as childhood adiposity is often carried over into adulthood (2).

In a cross-sectional analysis in ALSPAC, it was previously observed that the absolute levels of lipids in VLDL are higher in men, whereas other cardiovascular traits, such as absolute levels of cholesterol in LDL particles, Apolipoprotein B and the inflammatory glycoprotein acetyls were higher in women throughout adolescence. These results in women are not in line with our findings: however, we assessed the sex-specific longitudinal changes in the metabolomic measures related to adiposity, taking into account the correlation between the repeated measures of the metabolomic measures.

The increased risk of cardiovascular disease related to adiposity is already reflected in the metabolic profile of young adults. For example, BMI in young adulthood has been associated with higher circulating VLDLs, monounsaturated fatty acids, saturated fatty acids and branched-chain amino acid levels, as well as lower plasma large HDL concentrations (13). Findings from another prospective study in ALSPAC suggested that the atherogenic consequences of adiposity on the metabolic profile were stronger and apparent at a younger age in men than in women (36). For example, LDL cholesterol, triglycerides in VLDL and Apolipoprotein B were all found to be strongly associated with adiposity in men throughout young adulthood, in line with the results of our study.

Previous studies have linked the identified adiposity-related metabolomic measures to cardiometabolic disease events occurring in adulthood. In an observational study, NMR-based measures of circulating cholesterol and triglycerides in VLDL and LDL particles, Apolipoprotein B and glucose are strongly and consistently associated with the risk of myocardial infarction (37). MR analyses showed that both LDL cholesterol and triglycerides play a causal role in the development of coronary heart disease (38, 39), as well as Apolipoprotein B (39).

Limitations that should be considered include missing data and loss to follow-up. However, by using multilevel modelling, we used all available data from all eligible participants under a missing-at-random assumption. In addition, earlier studies in ALSPAC using the metabolomic measures compared the characteristics of included and excluded participants and these were found to be highly similar (36, 40). Secondly, the exposure trunk fat does not differentiate between the location of the body fat in the trunk, for example body fat located in the chest, or subcutaneously or viscerally in the abdomen. Third, during adolescence and into young adulthood the participants are experiencing pubertal changes, such as changes in hormonal levels. Although we adjusted our analyses for age of puberty onset, residual confounding might still be present. Results from sensitivity analyses assessing the relation of total fat mass at age 18y and the change in metabolomic measures between age 18y and 24y, thereby largely ruling out changes related to puberty, were however similar as the main results. Lastly, most of the participants of ALSPAC were of white European ethnicity. Therefore, the results of our study need to be confirmed in other ethnic groups.

Strengths of our study are the use of measures of both overall as well as abdominal adiposity from DXA scans. Additionally, we included a wide range of atherogenic metabolomic measures as outcomes, measured at multiple occasions throughout adolescence and young adulthood. To the best of our knowledge, we are the first study to examine changes in levels of lipoprotein particles indicative of a more atherogenic risk profile in relation to adiposity in a cohort of adolescents and young adults. Lastly, results from several sensitivity analyses that accounted for changes in adiposity and metabolomic measures related to puberty and gains in total fat mass during young adulthood suggest that our results are robust.

In conclusion, the present study shows that there are already atherogenic changes visible in metabolomic measures related to total and trunk fat mass at a young age. Our results further indicate that there is a difference in risk for cardiometabolic disease for males and females, which is already apparent at a young age. Therefore, adolescence is a critical period for the prevention of adiposity-related changes in atherogenic risk factors in males.

COMPETING INTERESTS

None declared.

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SUPPLEMENTARY MATERIAL

The supplementary material can be found at
https://figshare.com/s/d516d56b49b16a7b87a3

CHAPTER 4

The relation between adult weight gain, adipocyte volume and the metabolic profile at middle age

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ABSTRACT

Purpose: Weight gain during adulthood increases cardiometabolic disease risk, possibly through adipocyte hypertrophy. We aimed to study the specific metabolomic profile of adult weight gain, and to examine its association with adipocyte volume.

Methods: Nuclear magnetic resonance-based metabolomics were measured in the Netherlands Epidemiology of Obesity (NEO) study (n=6 347, discovery) and Oxford Biobank (n=6 317, replication). Adult weight gain was calculated as the absolute difference between BMI at middle age and recalled BMI at age 20. We performed linear regression analyses with both exposures BMI at age 20 years and weight gain, and separately with BMI at middle age in relation to 149 serum metabolomic measures, adjusted for age, sex and multiple testing. Additionally, subcutaneous abdominal adipocyte biopsies were collected in a subset of the Oxford Biobank (n=114) to estimate adipocyte volume.

Results: Mean (SD) weight gain was 4.5 (3.7) kg/m^2 in the NEO study and 3.6 (3.7) kg/m^2 in the Oxford Biobank. Weight gain, and not BMI at age 20 nor middle age, was associated with concentrations of 7 metabolomic measures after successful replication, which included polyunsaturated fatty acids, small to medium low-density lipoproteins and total intermediate-density lipoprotein. One SD weight gain was associated with 386 μm^3 (95% CI 143 – 629) higher median adipocyte volume. Adipocyte volume was associated with lipoprotein particles specific for adult weight gain.

Main conclusions: Adult weight gain is associated with specific metabolomic alterations of which the higher lipoprotein concentrations were likely contributed by larger adipocyte volumes, presumably linking weight gain to cardiometabolic disease.

Abbreviations: BCAA, branched chain amino acids; NEO, Netherlands Epidemiology of Obesity; nm, nanometer; OBB, Oxford Biobank; PUFA, polyunsaturated fatty acid.

INTRODUCTION

It is well established that weight gain during adulthood, as a result of an increase in body fat mass, is associated with a higher risk of type 2 diabetes, coronary artery disease, and (all-cause) mortality (1-3). Although there is a constant turnover of adipocytes throughout life, adipocyte number remains fixed during adulthood, and therefore the expansion of adipose tissue in response to weight gain in adults is due to an increase in adipocyte volume (4,5). Previous studies have shown that adipocyte expansion, also known as adipocyte hypertrophy, is associated with increased systemic insulin resistance, and thereby a worsening metabolic profile (6-8).

According to the 'lipid overflow' or 'adipose tissue expandability' hypothesis, adipose tissue becomes dysfunctional when the capacity of hypertrophic adipocytes to expand is exceeded (9,10). This in turn, leads to 'lipid overflow' and the accumulation of triglycerides in visceral adipose tissue and ectopic fat deposition in normally lean organs such as the heart, skeletal muscles, pancreas, and liver (9-11). Compared to subcutaneous adipocytes, adipocytes in the visceral depot have a high secretion rate of non-esterified fatty acids, very low-density lipoproteins and cytokines, such as IL-6 and TNF-alpha, thereby inducing a systemic low-grade inflammatory state and oxidative stress (11-14). Finally, intracellular non-esterified fatty acid accumulation in non-adipose tissues leads to impaired insulin signalling and insulin resistance (15).

We previously demonstrated in the Netherlands Epidemiology of Obesity (NEO) cohort that middle-aged individuals who gained body weight during adulthood had relatively more visceral fat and liver fat than weight-stable individuals (16). Additionally, participants with adult-onset weight gain were more insulin resistant, which was partly mediated by fat deposition in the visceral area and in the liver (17). Furthermore, body mass index (BMI) in adulthood has been associated with higher circulating very-low density lipoprotein, monounsaturated fatty acids, saturated fatty acids and branched-chain amino acid levels, as well as lower plasma large high density lipoprotein concentrations (18,19). Whilst studies have described the metabolic changes after short-term weight loss interventions (20,21), the metabolomic profile associated with long-term adult weight gain has not been defined, but can produce detailed novel insights linking body weight at different stages over the life course, adult weight gain and the development of cardiometabolic disease.

Here, we aimed to study the concentrations of metabolomic measures at middle age that were specifically associated with adult weight gain, as opposed to those associated with BMI at age 20 or BMI at middle age, in the NEO study (discovery (22)) and to replicate these findings in the Oxford Biobank (OBB (23)). Additionally, we aimed to examine the relation between adult weight gain and its specific metabolomic measures with abdominal adipocyte volume in a subpopulation of the OBB.

MATERIALS AND METHODS

Study design and study population

Netherlands Epidemiology of Obesity Study (discovery cohort)

The NEO study is a population-based cohort study of 6 671 individuals aged 45–65 years, with an oversampling of individuals with BMI ≥27 kg/m^2, living in the greater area of Leiden (in the West of the Netherlands). All inhabitants aged between 45 and 65 years from one municipality (Leiderdorp) were invited to participate irrespective of their BMI, allowing for a reference distribution of BMI. The study design and population are described in detail elsewhere (22) and in the **Supplementary Methods** (24). The Medical Ethical Committee of the LUMC approved the NEO study. All participants provided written informed consent.

Oxford Biobank (replication cohort) and abdominal adipose tissue biopsies

The OBB is a population-based cohort study of randomly selected healthy men and women living in Oxfordshire, United Kingdom. The study includes 7 185 individuals aged 29 to 56 years old. The exclusion criteria for the Oxford Biobank were history of myocardial infarction, diabetes mellitus type 1 or 2, heart failure, untreated malignancy, other ongoing systemic diseases, or ongoing pregnancy. Study recruitment criteria and population characteristics are described in detail elsewhere (23) and in the **Supplementary Methods** (24). The Oxford Biobank protocol is approved by the Oxfordshire Clinical Research Ethics Committee and all participants have provided informed consent.

In a subset of 114 participants in the OBB with data on recalled body weight at age 20 and metabolomic measures, subcutaneous abdominal adipose tissue biopsies were performed originally for other purposes than described in the present study, and were collected after the baseline assessment. All participants within the subset were recalled based on their genotype (25-27) and were matched for sex, age and BMI. The subset and the subcutaneous abdominal adipose tissue biopsies, which were used to calculate adipocyte volume and adipocyte weight (28), are described in more detail in the **Supplementary Methods** (24).

Weight change during adulthood

In the NEO study, height without shoes was measured with a vertically fixed, calibrated tape measure. Body weight was measured and percent body fat was estimated by the Tanita bio impedance balance (TBF-310, Tanita International Division, United Kingdom) without shoes and 1 kg was subtracted to correct for weight of clothing. BMI at baseline was calculated by dividing the weight in kg by the height in meters squared.

Recalled body weight at the age of 20 years was based on self-report. The general questionnaire included the question ‘How much did you weigh (approximately) when you were 20 years old?’ BMI at age 20 years was calculated by dividing body weight at age 20 in kg by the height in meters squared at middle age with the assumption that height did not majorly change during adulthood. Weight gain was calculated by subtracting recalled BMI at age 20 years from BMI at middle age. As a sensitivity analysis, we also calculated relative weight gain as (body weight at middle age (kg) – body weight at age 20 years (kg)) / body weight at age 20 years (kg) * 100.

In the OBB, height and body weight were measured at study inclusion. Information on recalled body weight at age 20 was obtained using questionnaires, similar as in the NEO study, which was used to calculate BMI at age 20 years and adult weight gain.

Metabolomic measures in the NEO study and the OBB

In both the NEO study and the OBB, a high-throughput proton nuclear magnetic resonance (NMR) metabolomics platform (29) (Nightingale Health Ltd., Helsinki, Finland) was used to quantify 149 lipid and metabolomic measures in blood plasma samples. Details of the experimentation and applications of the NMR metabolomics platform have been described previously (29), as well as coefficients of variation for the metabolomic measures (30), and can be found in the Supplementary Methods (24).

Statistical analyses

In the NEO study, persons with a BMI of 27 kg/m^2 or higher are oversampled. To correctly represent associations for the general population, adjustments for the oversampling of participants with a BMI≥27 kg/m^2 were made. This was done by weighting individuals towards the BMI distribution of participants from the Leiderdorp municipality (31), whose BMI distribution was similar to the BMI distribution of the general Dutch population (32). Consequently, the results from all analyses apply to a population-based study without oversampling of individuals with a high BMI. Baseline characteristics of the NEO study (discovery cohort) and the OBB (replication cohort) are presented as mean (SD), median (interquartile range) or proportion (%). We calculated Pearson's correlations coefficients between adult weight gain, BMI at age 20 and BMI at middle age.

For the analyses in the discovery cohort using the metabolomic measures as outcome, we used a hypothesis-free approach. To correct for multiple testing, an alpha that has been corrected for the number of independent tests/metabolomic measures was used, obtained by considering the correlation matrix between the NMR metabolomic measures (33). In the present study, 37 out of the 149 metabolic measures were independent and therefore the alpha value was corrected by dividing 0.05 by 37 (alpha = 1.34×10^{-3}). We used multivariable-adjusted linear regression models to examine the associations of adult weight gain with all NMR metabolomic measures adjusted for confounders. All concentrations were standardized to a mean of 0 and standard deviation of 1. Linear regression modelling was performed with adult weight gain and BMI at age 20 years as exposure variables and the metabolomic measures as outcome variable, adjusted for sex and age. Adult weight gain and BMI at age 20 years were included in the same model, as adult weight gain since age 20 years is dependent on initial BMI at age 20 years. A second model was additionally adjusted for the considered confounders use of glucose-lowering medication, including both oral medication and insulin, and statins and fibrates. Other covariates (e.g., food intake, physical activity) were considered as possible mediators and therefore not included. In addition, we performed linear regression analyses with the exposure BMI at middle age and the levels of metabolomic measures as the outcome, adjusted for sex and age. Finally, we repeated the linear regression analyses of adult weight gain and metabolomic measures separately for men and women, because we expected there could be differences in the concentrations of metabolomic measures between men and women based on previous literature (34). In addition, we used relative adult weight gain (percentage) as exposure.

Based on these analyses, we selected the metabolomic measures specific for adult weight gain on the basis of statistical significance. First, we examined which metabolomic measures were only statistically significantly associated with adult weight gain, and not with BMI at age 20 or BMI at middle age. Similarly, we selected metabolomic measures specific for BMI at age 20 years or BMI at middle age. The remaining metabolomic measures showed overlap in their associations with adult weight gain, BMI at age 20 years and BMI at middle age.

In the OBB, as our replication cohort, we performed the same linear regression analyses as in the NEO study, with adult weight gain and BMI at age 20 years as the exposures and NMR-based metabolomic measures as the outcome, adjusted for sex and age. In addition, we performed linear regression analyses with BMI at middle age as exposure and all NMR-based metabolomic measures as the outcome, adjusted for sex and age. For this analysis, we included all metabolomic measures that were associated with BMI at middle age, BMI at age 20 years or adult weight gain in the NEO cohort after considering multiple testing. We performed the replication analyses using a hypothesis-testing approach ($p<0.05$). From the results in OBB, we determined whether metabolomic measures were specifically associated with BMI at middle age, BMI at age 20 years or adult weight gain. Metabolomics measures of particular interest to this study where those that were only associated with adult weight gain and not with BMI at age 20 years or BMI at middle age.

In the OBB subset with data on weight gain and abdominal adipocyte volume, linear regression was used to examine the association between BMI at age 20, adult weight gain, and BMI at middle age as exposures, and the abdominal adipocyte volume as outcome. Adult weight gain and BMI at age 20 years were included in the same model. The exposures were standardized to a mean of 0 and standard deviation of 1 to allow comparisons. Similarly, we analysed the relationship between adipocyte volume (exposure) and the metabolomic measures specific for adult weight gain.

Analyses were performed using Stata 14 (StataCorp LP, College Station, Texas, US).

RESULTS

Characteristics of the study populations

We included 6347 individuals from the NEO study and 6317 individuals from the OBB in our analyses (**Table 1**). Both cohorts comprised of a similar proportion of men (43%) and women. Mean recalled body weight at age 20 years was likewise similar (NEO: 65.7 kg [SD 11.3], OBB: 66.2 kg [SD 12.8]). Participants of the OBB were younger (mean age 42 years, ranging from 29 to 56 years) than those in NEO (mean 56 years, ranging from 45 to 65 years) at inclusion. Despite the difference in mean age between the cohorts, the annual absolute adult weight gain was similar in both cohorts (0.13 [SD 0.11] kg/year in the NEO study and 0.16 [SD 0.19] kg/year in the OBB).

Adult weight gain was positively correlated with BMI at middle age (0.78 in the NEO study, and 0.67 in the OBB), but not with BMI at age 20 (correlation coefficient
-0.17 in NEO and -0.18 in the OBB). BMI at middle age was correlated with BMI at age 20 by 0.49 in the NEO study and 0.61 in the OBB.

Table 1. Characteristics of the study populations: the Netherlands Epidemiology of Obesity study (NEO, discovery cohort) and the Oxford Biobank (OBB, replication cohort)

Characteristic	NEO (N=6347)	OBB (N=6317)
Sex (% men)	43	43
At age 20 years		
Body weight (kg)	65.7 (11.3)	66.2 (12.8)
Body Mass Index (kg/m^2)	21.9 (2.7)	22.5 (3.4)
At age 20 years		
Age (years, range)	56 (45 – 65)	42 (29 – 56)
Time between age 20 and middle age (years)	36 (31 – 41)	22 (17 – 26)
BMI at age 24 (kg/m^2)	24.8 (5.2)	24.7 (4.2)
Body weight (kg)	79.1 (15.9)	76.1 (15.9)
Height (m)	1.73 (0.1)	1.71 (0.1)
Body Mass Index (kg/m^2)	26.3 (4.5)	25.8 (4.5)
Waist circumference (cm)	92.1 (13.4)	86.7 (12.8)
Relative weight gain (%)	20.9 (17.5)	15.7 (16.5)
Absolute weight gain (kg)	13.4 (11.1)	9.9 (10.7)
Absolute weight gain (kg/m^2)	4.5 (3.7)	3.6 (3.7)
Annual weight gain (kg/m^2)	0.13 (0.11)	0.16 (0.19)
Fasting glucose (mmol/L)	5.3 (5.0 – 5.7)	5.2 (4.9 – 5.5)
HOMA-IR	1.9 (1.2 – 2.9)	2.7 (2.0 – 3.6)
Triglycerides (mmol/l)	1.0 (0.7 – 1.5)	0.9 (0.7 – 1.3)
HDL-C (mmol/l)	1.6 (0.5)	1.4 (0.4)
LDL-C (mmol/l)	3.5 (1.0)	3.3 (0.9)
Total cholesterol (mmol/l)	5.7 (1.1)	5.2 (1.0)
Glucose-lowering medication (%)[a]	5.1	0
Glucose-lowering medication (%)[b]	15.4	0

[a] Use of glucose-lowering medication included oral medication and insulin. [b] Use of lipid-lowering medication included fibrates and statins. Results in the NEO study were based on analyses weighted towards the BMI distribution of the general population (N = 6347). Abbreviations: BMI, body mass index; HOMA-IR, homeostatic model assessment insulin resistance; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol. Data are presented as mean (SD or range), median (25th–75th percentile) or percentage

Metabolomic measures in the NEO study and replication in the OBB

The circle plots presented in Figure 1 show the associations between adult weight gain, BMI at age 20 years and BMI at middle age with the metabolomic measures in the NEO study. In the model (age and sex adjusted) including BMI at age 20 years and adult weight gain

simultaneously, absolute adult weight gain was associated with concentrations of 111 metabolomic measures at middle age. Furthermore, in the same model, BMI at age 20 years was associated with the concentrations of 48 metabolomic measures. BMI at middle age was associated with the levels of 105 metabolomic measures. Of the 111 metabolomic measures linked with adult weight gain during adulthood, 7 were not associated with BMI at either age 20 years or middle age and therefore considered as specific for adult weight gain, 47 were shared with metabolomic measures associated with both BMI at age 20 and BMI at middle age, and 53 were common with metabolomic measures associated with BMI at middle age (**Supplementary Table 1** (24)).

Of the 111 metabolomic measures which showed a significant association with adult weight gain in the NEO study, 107 (96%) were successfully replicated in the OBB. The Venn diagram (**Figure 2**; detailed summary statistics data of OBB can be found in **Supplementary Table 2** (24)) shows the overlap in the associations between adult weight gain, BMI at age 20 and BMI at middle age, and the 107 metabolomic measures replicated in the OBB (35).

Adult weight gain was specifically associated with the concentration of 7 metabolomic measures in the NEO study, which included omega-3 (0.02 SD per 1 kg/m^2 weight gain [$p=5.6\times10^{-5}$] and omega-6 fatty acids (0.02 SD [$p=6.5\times10^{-4}$]), sub particles related to small to medium low-density lipoproteins (e.g. phospholipids in small LDL: 0.02 SD [$p=1.4\times10^{-4}$]), and total intermediate density lipoprotein (0.01 [$p=6.5\times10^{-4}$]), and were all successfully replicated in the OBB. In addition, none of the investigated metabolomic measures were only associated with BMI at age 20 years, and only 2 metabolomic measures were associated with the measured BMI at middle age (notably free cholesterol in small HDL particles and free cholesterol in IDL particles).

To visualize consistency between the two datasets, we also plotted the regression coefficients (in standard deviation per 1 kg/m^2 gain in BMI) of the associations between adult weight gain and metabolomic measures in the NEO study and the OBB (**Figure 3**). For both populations, we observed negative associations between weight gain and HDL (green dots), whilst positive associations were detected for LDL and VLDL particles (pink dots), as well as for most amino acids (red dots), triglycerides (light pink) and fatty acids (brown dots).

Results were similar after additional adjustment for use of medication (**Supplementary Table 3** (24)). Absolute adult weight gain was associated with similar changes in concentrations of metabolomic measures in both men and women (**Supplementary Table 4** (24)). Results were also similar whether we used relative or absolute weight gain as exposures in the analyses (**Supplementary Table 5** (24)).

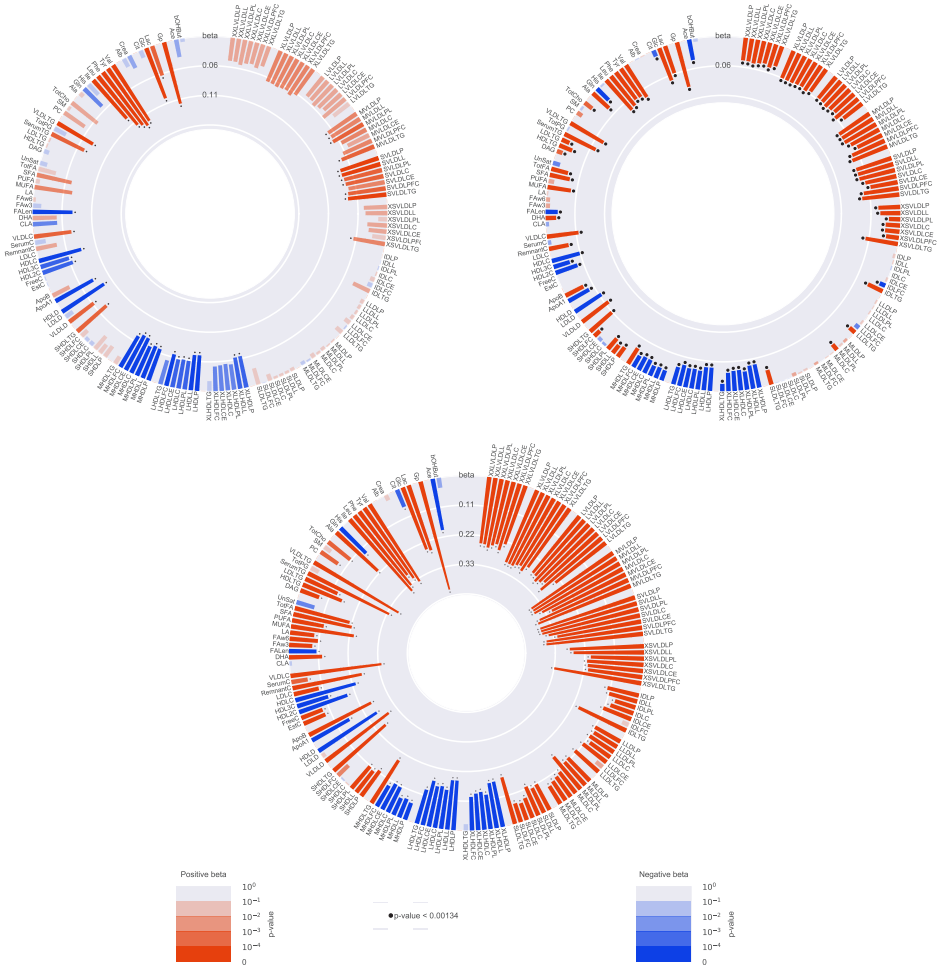

Figure 1. The circle plots show the associations between BMI at age 20 years (upper left), BMI at middle age (upper right), adult weight gain (middle) and the individual metabolomic measures in the NEO study. The metabolomic measures are indicated at the outer circle. A red bar indicates a positive association, whereas a blue bar indicates a negative association. The colour intensity and height of the bar indicate the strength of the association, a black dot above a bar indicates statistical significance ($p < 0.00134$)

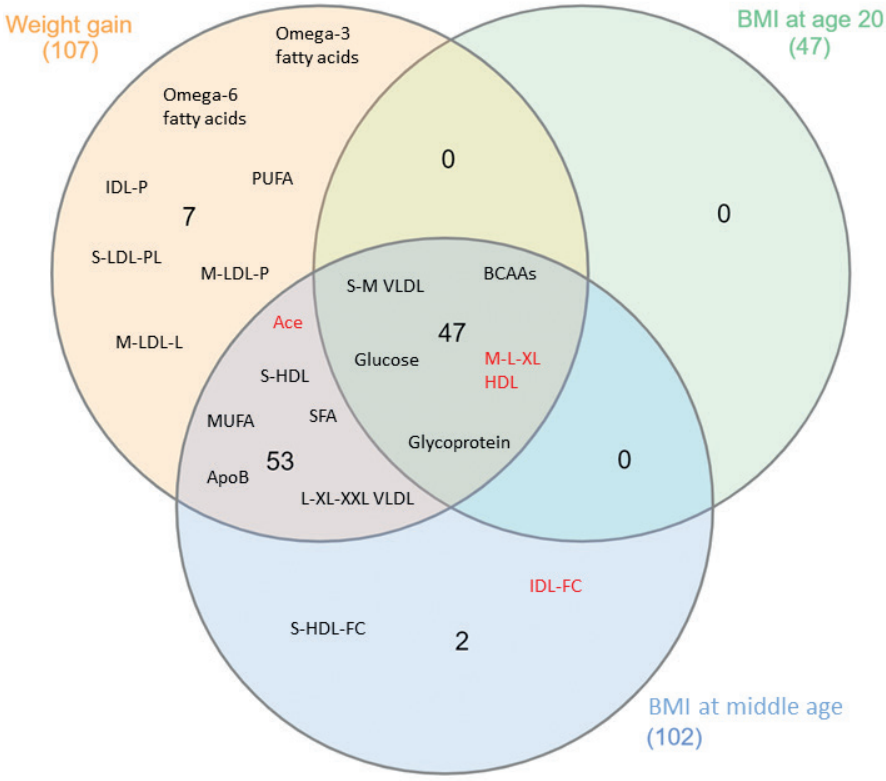

Figure 2. Venn diagram showing the overlap in the associations between adult weight gain, BMI at age 20 years and/or BMI at middle age and the metabolomic measures after successful replication in the OBB. Names in black indicate a positive association with this metabolomic measures, names in red indicate a negative association. Due to the large number of included metabolomic measures, and for illustrative purposes, we listed the classes of the associated metabolomic measures instead of exact names. Both adult weight gain and BMI at middle age were associated with 52 metabolomic measures, whereas weight gain was specifically associated with 7 metabolomic measures. 47 metabolomic measures showed overlapping associations with all three exposures

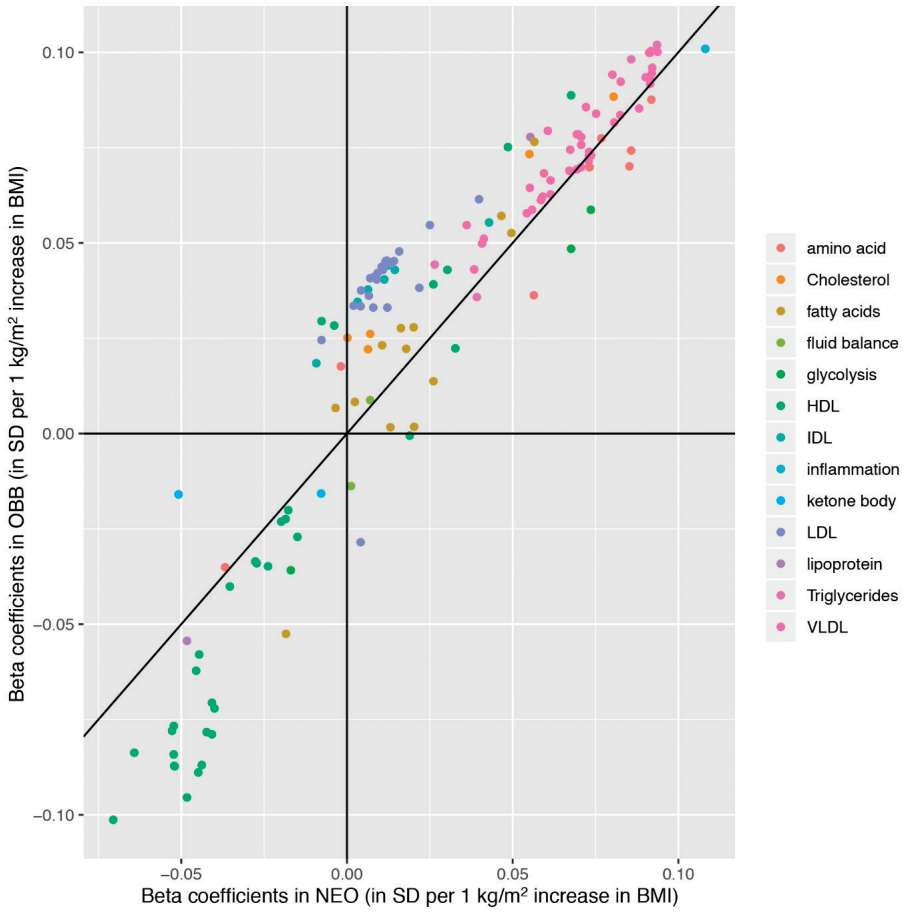

Figure 3. Correlation plot between linear regression coefficients in the NEO study and the OBB of the association between weight gain and the metabolomic measures (in SD per 1 kg/m² increase in BMI). The different colours of the dots indicate different subclasses of metabolomic measures

Cell study in subcutaneous abdominal adipocytes in a subset of the OBB

Associations between adult weight gain and abdominal adipocyte volume

Of the 114 participants with data on weight gain and abdominal AT histology, 50 (44%) were men and the mean age and BMI at tissue biopsy were 46 years (SD 6.6) and 26.2 kg/m² (3.8). Mean adult weight gain was 3.8 kg/m² (3.5) whilst median adipocyte volume was 3 782 (2 941 – 4 450) µm³.

After adjustment for sex and age at biopsy, an 1-SD increase in adult weight gain was associated with 386 µm³ (95% CI 143 – 629) larger median adipocyte volume, and a 1-SD increase in BMI at age 20 and BMI at middle age were associated with 240 µm³ (24 – 456) and 383 µm³ (160 – 605) increased adiposity volume, respectively (**Table 2**).

Table 2. Associations between BMI at age 20, weight gain during adulthood and BMI at middle age (in kg/m^2) and abdominal adipose tissue cell volume in the Oxford Biobank (n=114)

	Median AT cell volume (μm3) Beta (95% CI)
Standardized BMI at age 20 (SD)[a]	240 (24 - 456)
Standardized adult weight gain (SD)[b]	386 (143 – 629)
Standardized BMI at middle age (SD)	383 (160 – 605)

All analyses are adjusted for sex and age at adipose tissue biopsy. a: additionally adjusted for adult weight gain, b: additionally adjusted for BMI at age 20 years. Abbreviations: BMI, body mass index; AT, adipose tissue; CI, confidence interval; SD, standard deviation. SD BMI at age 20: 2.7, SD adult weight gain: 3.5, SD BMI at middle age 3.8.

Associations between abdominal adipocyte volume and adult weight gain-specific metabolomic measures

After adjustment for sex and age at biopsy, higher median cell volume was associated with higher circulating levels of phospholipids in small LDL (0.21 SD [0.05 – 0.37] per SD of adipocyte volume), total medium LDL particles (0.20 SD [0.02 – 0.37] and lipids in medium LDL (0.20 SD [0.02 – 0.37]) (see **Table 3**). In contrast, median cell volume was not associated with levels of either omega-3 or omega-6 fatty acids, or the sum thereof (i.e. total polyunsaturated fatty acids).

Table 3. Associations between adipocyte volume and 7 adult weight gain-specific metabolomic measures in the Oxford Biobank (n=114)

	Omega-3 FA (SD)	Omega-6 FA (SD)	Polyunsaturated FA (SD)	S-LDL-PL (SD)	M-LDL-P (SD)	IDL-P (SD)	M-LDL-L (SD)
	Beta (95% CI)	Beta (95% CI)	Beta (95% CI)	Beta (95% CI)	Beta (95% CI)	Beta (95% CI)	Beta (95% CI)
Median cell volume (SD)	0.02 (-0.14 – 0.18)	0.10 (-0.07 – 0.28)	0.09 (-0.08 – 0.26)	0.21 (0.05 – 0.38)	0.20 (0.02 – 0.37)	0.17 (-0.004 – 0.35)	0.20 (0.02 – 0.37)

The table shows the associations between standardised adipocyte volume as exposure, and 7 standardised adult weight gain-specific metabolomic measures as the outcomes, adjusted for sex and age. Abbreviations: SD, standard deviation; CI, confidence interval; FA, fatty acids; S-LDL-PL, phospholipids in small low-density lipoproteins; M-LDL-P, total medium LDL; IDL-P, total intermediate density lipoprotein; M-LDL-L, lipids in medium LDL

DISCUSSION

The aim of this study was to investigate the metabolomic measures specifically associated with adult weight gain, because these metabolomic measures can provide insight in the mechanisms underlying the development of cardiometabolic disease as a consequence of adult weight gain.

Using data from two large cohorts, used for discovery and replication, we showed that adult weight gain over a median period of 36 years was associated with seven specific metabolomic measures, namely omega-3 fatty acids, omega-6 fatty acids and their sum (polyunsaturated fatty acids), phospholipids within small LDL, total IDL particles, and total medium-sized LDL particles and lipids within these particles. The regression coefficients of the association between adult weight gain and the metabolomic measures in the NEO study and the OBB were similar in direction and size, which indicates robust replication of our findings in the OBB. While previous studies specifically focused on short-term changes in body weight, the analyses performed in the present study highlight specific biochemical disturbances associated with adult weight gain that could possibly link adult weight gain and the onset of cardiometabolic disease.

One mechanism thought to be responsible for the adverse cardiometabolic consequences of adult weight gain is adipocyte hypertrophy (4,7,8). We observed that adult weight gain was associated with larger adipocyte size at middle age irrespective of BMI at age 20 years. In addition, BMI at age 20 was associated with enlarged adipocytes at middle age as well, irrespective of adult weight gain, albeit this association was less strong than that of adult weight gain. BMI in adulthood is the aggregate of BMI at age 20, after growth and development during childhood and puberty have ceased, and subsequent weight gain during adulthood. Spalding *et al*. observed that the number of adipocytes is set before adulthood, however expansion of adipocyte number ends around the age of 16.5 years in individuals with obesity and 18.5 years in lean individuals (4). In line with our results, this suggests that weight gain during adolescence contributes to an increased adipocyte size as well, however to lesser extent than weight gain during adulthood. As a result of hypertrophy, adipose tissue becomes dysfunctional, ultimately leading to insulin resistance and metabolic disturbances (6-8). Accordingly, we observed an association between adipocyte volume and three out of the seven metabolomic measures that were specifically associated with adult weight gain, particularly phospholipids in small LDL, total medium LDL particles and lipids in medium LDL. However, it is important to note that these metabolomic measures are highly correlated, as well as three other adult weight gain-specific metabolomic measures, omega-3 and omega-6 fatty acids and their sum (polyunsaturated fatty acids).

Overall, our findings are consistent with the results of previous longitudinal studies investigating the effects of body weight gain on the plasma metabolome (18,36,37). These studies identified increases in VLDL and LDL particles, mono- and polyunsaturated fatty acids, saturated fatty acids, branched-chain amino acids and products of glycolysis to be most strongly associated with weight gain during follow-up ranging from 6 to 9 years. In contrast, weight loss in older adults was associated with decreased circulating glycerol levels and increased HDL diameter (20). Additionally, a recent randomized clinical trial found a reduction in plas-

ma BCAAs as a results of weight loss and showed that this was associated with decreased hepatic and intra-abdominal fat after the two-year intervention (21).

Earlier studies have linked the metabolomic measures we found to be specifically associated with adult weight gain to cardiometabolic disease. Low-density lipoprotein particles are a causal risk factor for cardiovascular disease identified by randomized controlled trials and Mendelian randomization studies (38).

In observational studies, NMR-based measures of circulating LDL particles are strongly and consistently associated with the risk of cardiovascular disease in observational studies, also specifically small and medium LDL particles (39). On the other hand, in randomised controlled trials, the replacement of dietary saturated fatty acids or carbohydrates by polyunsaturated fatty acids resulted in lower levels of LDL cholesterol (40,41). In the present study, we focused on circulating polyunsaturated fatty acids instead of the dietary intake. The levels of polyunsaturated fatty acids in tissues and blood might not be an accurate biomarker of polyunsaturated fatty acid intake, as they are affected by metabolic processes (41,42).

In a large pooled analysis of individual data from prospective cohort studies (n=45, 637), higher concentrations of three circulating omega-3 fatty acids, alpha-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid, were associated with a lower risk of fatal coronary heart disease (43). In contrast, both intake of dietary omega-3 fatty acids and circulating levels of omega-3 fatty acids were not associated with risk of type 2 diabetes in a meta-analysis of longitudinal studies (44).

Omega-6 fatty acids mainly include linoleic acid. Higher circulating levels of linoleic acids were associated with a lower risk of cardiovascular disease across multiple prospective observational studies including a total of 68,659 individuals (45). In the same dataset, higher levels of linoleic acid were associated with a lower risk of type 2 diabetes (46), which itself is an important risk factor for cardiovascular disease (47).

Strengths of our study included two large population-based studies, and robust replication of our study results in a younger cohort. A limitation that needs to be considered is the use of recalled body weight at age 20 years in both studies, which we used to calculate absolute adult weight gain. However, previous studies have shown that recalled body weight is strongly related with measured weight at the same age (48). By adjusting for age, we took into account the period between age 20 years and middle age, which differs between participants. Additionally, most of the participants of the NEO study and Oxford Biobank were of Caucasian ethnicity. Therefore, the results of our study need to be confirmed in other ethnic groups. Another limitation is the lack of a measure of adipocyte hyperplasia, or the number of adipocytes, as both adipocyte hyperplasia and hypertrophy influence the amount of fat mass (4). However, previous research showed that after the age of 20 years adipocytes mainly respond by hypertrophy during weight gain (4). Our study has an observational cross-sectional design: however, our results are in line with longitudinal studies on adult weight gain and the metabolite profile (18,36,37). Another limitation is the use of the p-value as an arbitrary cut-off to determine which metabolomic measures we considered being associated with our exposures in our discovery and replication cohort. Hereby, we might

have missed metabolomic measures also associated with adult weight gain. Additionally, it is likely that metabolomic measures are shared between adult weight gain and BMI at middle age. And last, the present study used a rather limited metabolomics platform mostly containing lipoprotein (sub)particles. Investigating the metabolomic pathways altered by adult weight gain would require alternative platforms.

Our results indicate that adult weight gain was specifically and robustly associated with a higher concentration of seven metabolomic measures, including some specific lipoproteins and polyunsaturated fatty acids. Although this should be further investigated in more detail in future studies, our observations may help explain how adult weight gain increases the risk of cardiometabolic disease.

DUALITY OF INTEREST

Dr. Dennis Mook-Kanamori is a part-time research consultant at Metabolon, Inc. All other authors declare to have no conflict of interest.

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SUPPLEMENTARY MATERIAL

The supplementary material can be found at
https://figshare.com/s/02e0b7bd24b4ef7f95f1

CHAPTER 5

Adult weight change in relation to visceral fat and liver fat at middle age: The Netherlands Epidemiology of Obesity study

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ABSTRACT

Objective: We aimed to investigate the associations between weight change during adulthood and the amount of abdominal subcutaneous fat, visceral fat and liver fat at middle age.

Methods: The Netherlands Epidemiology of Obesity (NEO) study is a population-based cohort of 6 671 middle-aged men and women. We calculated the percentage of weight change during adulthood based on body weight at middle age and recalled body weight at age 20. Abdominal subcutaneous and visceral adipose tissue were assessed by magnetic resonance imaging (MRI), in addition to hepatic triglyceride content by ^{1}H-MR spectroscopy in a random subgroup (maximum of n=2 580). With multivariable linear regression analysis, we examined the associations between categories of adult weight change, body mass index (BMI) at age 20 and measures of abdominal adiposity at middle age, adjusted for age, sex, ethnicity, lifestyle factors, menopausal status, parity, use of medication and total body fat at middle age.

Results: In 2 399 participants (54% women), individuals who gained more than 50% of body weight during adulthood had 1.96 (95% CI: 1.64; 2.33) times more visceral adipose tissue at middle age and 2.39 (95% CI: 1.70, 3.36) times more hepatic triglyceride content than weight maintainers (weight change between -5% and 5%). Associations with abdominal subcutaneous adipose tissue were weaker: participants who gained more than 50% of their body weight had 1.54 (95% CI: 1.38, 1.72) times more abdominal subcutaneous adipose tissue compared with weight maintainers.

Conclusions: In this population-based study, adult weight gain was associated with relatively more visceral adipose tissue and hepatic triglyceride content at middle age than abdominal subcutaneous adipose tissue. Overall, our study suggests that weight maintenance during adulthood plays an important role in limiting excess visceral adipose tissue and hepatic triglyceride content at middle age.

INTRODUCTION

Obesity is a well-established risk factor for development of cardiometabolic diseases such as type 2 diabetes and coronary heart disease (1). Adiposity in early adulthood and weight gain during adulthood have both been associated with a considerable increased risk of major chronic diseases in middle-aged individuals, including type 2 diabetes, cardiovascular disease and obesity-related cancers (2-6). Alternatively, individuals who maintained a body mass index of 18.5 to 25.0 kg/m^2 during adulthood had the lowest risk of these chronic diseases, and all-cause mortality (2, 7).

During adult weight gain, excess adipose tissue is stored in different areas of the body, depending on various factors including genetic variation, sex, age and lifestyle (8-12). Abdominal adiposity is characterized by an increased storage of excess fat in the abdominal subcutaneous and visceral adipose tissue depots. Visceral adipose tissue has a high secretion rate of cytokines such as TNF-α and IL-6, promoting local inflammation and oxidative stress (13). In addition, as a result of the hyperlipolytic state of visceral adipose tissue, non-esterified fatty acids are released into the circulation, subsequently leading to metabolic abnormalities in the liver and increased hepatic glucose production (14-17). These mechanisms contribute to an overflow of lipids that cannot be stored in the subcutaneous adipose tissue, eventually resulting in accumulation of fat in and around the organs, including the visceral area, liver, skeletal muscles, heart and pancreas (18). Previous studies have shown that both visceral adipose tissue and liver fat accumulation are better predictors of the metabolic syndrome than abdominal subcutaneous adipose tissue (19, 20). Additionally, excess visceral adipose tissue and liver fat are important risk factors for type 2 diabetes and cardiovascular disease (19, 21-26).

To date, the importance of adult weight gain in the accumulation of visceral fat and liver fat has not been well described. Only few studies investigated the depots in which body fat is preferentially stored during adult weight gain. A four-year follow-up study in normal-weight premenopausal women (N=65) showed that a gain in body weight was associated with excess visceral adipose tissue after four years (27). In contrast, it was observed that an increase in body fat induced by short-term weight gain during 100 days of overfeeding was not accompanied by accumulation of visceral adipose tissue in a cohort study in men (N=24) (28). Additionally, self-reported adult weight gain was associated with fat deposition in the liver in a large Asian study population (N=21 496) (29).

Because both adult weight gain and excess visceral adipose tissue are strongly associated with insulin resistance and type 2 diabetes (1-6, 21, 22, 30, 31), we hypothesized that individuals with the largest weight gain during adulthood have more visceral adipose tissue and a higher hepatic triglyceride content at middle age. Therefore, the aim of this study was to investigate the associations of adult weight change with visceral adipose tissue and hepatic triglyceride content, irrespective of total body fat at middle age, in a population-based study.

METHODS

Study design and population

The Netherlands Epidemiology of Obesity (NEO) study is a population-based, prospective cohort study in 6 671 individuals aged 45–65 years, with an oversampling of individuals with a BMI ≥27 kg/m^2. The study design and population are described in detail elsewhere (32).

Men and women living in the greater area of Leiden (Western Netherlands) were invited by letters sent by GPs and municipalities and by local advertisements. They were invited to respond if they were aged between 45 and 65 years and had a self-reported BMI of 27 kg/m^2 or higher. In addition, all inhabitants aged between 45 and 65 years from one municipality (Leiderdorp) were invited to participate irrespective of their BMI, allowing for a reference distribution of BMI.

Participants were invited to a baseline visit at the NEO study centre Leiden University Medical Center after an overnight fast. Prior to this study visit, participants completed a general questionnaire at home to report demographic, lifestyle and clinical information. The participants were asked to bring all medication they were using in the month preceding the study visit, which was recorded by research nurses. At the study centre, participants completed a screening form, asking about anything that might create a health risk or interfere with magnetic resonance imaging (most notably metallic devices, claustrophobia or a body circumference of more than 1.70 meter). Of the participants who were eligible for magnetic resonance imaging (MRI), approximately 35% were randomly selected to undergo MRI.

For the present analysis, we included participants who underwent MRI of the abdomen (n= 2 580), in addition to ^{1}H-magnetic resonance spectroscopy of hepatic triglyceride content. We excluded participants with images of insufficient quality to estimate abdominal subcutaneous or visceral adipose tissue (n=11), with missing recalled weight at age 20 (n=79) or with a BMI at age 20 below 14.0 kg/m^2 (n=2). Additionally, we excluded participants with missing data on total body fat (n=4), ethnicity (n=3), educational level (n=25), smoking (n=2) and physical activity (n=55), resulting in 2 399 participants who were included in the present analysis. Hepatic triglyceride content was available in 1 948 of these, due to technical failures and an insufficient quality of the measurements to estimate liver fat content. The Medical Ethical Committee of the Leiden University Medical Center approved the design of the study. All participants gave their written informed consent.

Data collection

Weight change during adulthood

Recalled body weight at age 20 was based on self-report. The general questionnaire included the question 'How much did you weigh (approximately) when you were 20 years old?' BMI at age 20 was calculated by dividing body weight at age 20 in kilograms by the measured height in meters squared at middle age, with the assumption that height did not majorly change during adulthood. Relative weight change was calculated by subtracting body weight at age 20 from measured body weight at middle age, divided by body weight at age 20, multiplied by 100%.

Abdominal adiposity and liver fat at middle age
Height without shoes was measured with a vertically fixed, calibrated tape measure with precision of 0.1 cm. Body weight and percent body fat were measured by the Tanita bio impedance balance (TBF-310, Tanita International Division, UK) without shoes and 1 kg was subtracted to correct for the weight of clothing. BMI at baseline was calculated by dividing the weight in kilograms by the height in meters squared. Waist circumference was measured between the border of the lower costal margin and the iliac crest with the precision of 0.1 cm.

Abdominal subcutaneous and visceral fat depots were directly assessed by MRI (1.5 Tesla MR imaging, Philips Medical Systems, Best, Netherlands) using a turbo spin echo imaging protocol (300/20; flip angle, 90°; section thickness, 10 mm; section gap, 2mm). At the level of the fifth lumbar vertebra, three transverse 10 mm slices were obtained during one breath-hold. By using in-house-developed software (MASS; Leiden University Medical Center, Leiden, the Netherlands), abdominal subcutaneous and visceral fat areas were quantified by converting the number of pixels to centimetres squared for all three slices, allowing a semi-automated detection of the subcutaneous and visceral adipose tissue area. The mean of abdominal subcutaneous and visceral adipose tissue areas was used in the analysis. Hepatic triglyceride content was quantified using ^{1}H-magnetic resonance spectroscopy of the liver. An 8 ml voxel was positioned in the right lobe of the liver, avoiding gross vascular structures and adipose tissue depots. Sixty-four averages were collected with water suppression (repetition time = 2900 msec; echo time = 23 msec [2900/23]). Without changing any parameters, spectra without water suppression, with a repetition time of 10 seconds and with four averages were obtained as an internal reference. Spectra were not corrected for frequency drift and were analysed while blinded to all study parameters. Spectra were initially included when automatic fitting was successful. When line shapes were distorted by eddy currents or as a result of poor shimming, spectral data were rejected. Hepatic triglyceride content relative to water was calculated as (signal amplitude of triglyceride) / (signal amplitude of water) * 100. Fatty liver was defined as a hepatic triglyceride content of ≥5.56% (33).

Covariates
On the baseline questionnaire, participants reported ethnicity by self-identification in eight categories which we grouped into white (reference) and other. Level of education was grouped into high versus low education (reference) according to the Dutch education system. Tobacco smoking was reported in three categories: current smoker, former smoker and never smoker. Alcohol consumption was reported in the Food Frequency Questionnaire (FFQ) and expressed as grams of alcohol consumed per day. Participants reported the frequency and duration of their physical activity during leisure time on the Short Questionnaire to Assess Health-enhancing activity (SQUASH), which we expressed in MET-hours per week. Use of antidepressants, antipsychotics, thyroid medication, corticosteroids and/or hormonal treatments in the month preceding the study visit was recorded by research nurses. In women, we grouped the use of contraceptives and hormone replacement therapy into current, past and never (reference) users of oestrogens. Menopausal status was classified as premenopausal, perimenopausal (menopausal during last year) or postmenopausal, according to information on oophorectomy, hysterectomy and self-reported state of menopause in the questionnaire.

Statistical analyses
In the NEO study, individuals with a BMI of 27 kg/m^2 or higher are oversampled. To correctly represent associations for the general population (34), adjustments for this oversampling were made. Adjustment was done by weighting all participants towards the BMI distribution of participants from the Leiderdorp municipality (35), whose BMI distribution was similar to the BMI distribution of the general Dutch population (36). All results were based on weighted analyses. Consequently, the results apply to a population-based study without oversampling of individuals with a BMI≥27 kg/m^2. As a result of the weighting procedure, the numbers of participants per group are presented as proportions.

Baseline characteristics of the study population were expressed as mean (SD), median (25th, 75th percentiles) or as percentage, stratified by categories of weight change. We categorized weight change during adulthood on the basis of the distribution of weight change in the reference population of Leiderdorp: weight change of more than -5%, between -5% and 5% (weight maintenance: reference category), 5% to 25%, 25% to 50% and ≥50%. The majority of participants fell into the weight gain categories. However, according to the distribution of weight change in the Leiderdorp population, who had a BMI distribution similar to the Dutch general population, this is a typical representation of weight change in the Dutch general population (**Figure S1**).

We performed linear regression analyses to examine the associations of adult weight change with waist circumference, abdominal subcutaneous and visceral adipose tissue, and hepatic triglyceride content at middle age, compared with the reference category of weight maintenance during adulthood. Crude models were adjusted for sex and age (model 1). In model 2, we additionally adjusted for BMI at age 20, because the percentage of weight change since age 20 depends on the initial BMI at age 20. Model 3 was additionally adjusted for ethnicity, education, smoking, alcohol consumption, physical activity, menopausal status, use of medication known to affect body weight (antidepressants, antipsychotics, thyroid medication, corticosteroids and hormonal treatments) and parity. Finally, in model 4, we additionally adjusted for total body fat at middle age to investigate to what extent weight change during adulthood was specifically associated with measures of abdominal adiposity instead of merely overall adiposity. Because of the skewed distribution of hepatic triglyceride content and to facilitate interpretation, values of waist circumference, abdominal subcutaneous and visceral adipose tissue and hepatic triglyceride content were all transformed using the natural logarithm. Regression coefficients and corresponding 95% confidence intervals (CI) were back transformed and expressed as ratios, which can be interpreted as relative changes in measure of abdominal adiposity, compared with that measure in the reference category of weight maintenance during adulthood. For example: a ratio of 2 for visceral adipose tissue in individuals who gained 5% to 25% of body weight during adulthood indicates that these individuals have twofold more visceral adipose tissue at middle age than individuals who maintained their body weight during adulthood. Because men and women have different patterns of body fat distribution (8-12), we repeated the analyses stratified by sex.

For the next analyses, we stratified the study population based on BMI at age 20, according to WHO criteria: <18.5 kg/m^2 (underweight), 18.5-25.0 kg/m^2 (normal range, reference), 25.0-30.0 kg/m^2 (overweight) and ≥30.0 kg/m^2 (obese) (37). We used linear regression mo-

dels to examine the associations between BMI at age 20 strata and waist circumference, abdominal subcutaneous and visceral adipose tissue and hepatic triglyceride content at middle age, compared with the reference category. In model 1, we adjusted for sex and age. In model 2, we additionally adjusted for ethnicity, education, smoking, alcohol consumption, physical activity, menopausal status, use of medication known to affect body weight (antidepressants, antipsychotics, thyroid medication, corticosteroids and hormonal treatments) and parity. Finally, in model 3, we adjusted for total body fat at middle age.

Subsequently, we performed joint analysis of the associations between weight change during adulthood and measures of abdominal adiposity within the BMI at age 20 strata, using participants with BMI at age 20 strata between 18.5 and 25.0 kg/m^2 and with weight change ≥-5% to <5% as a reference. The analysis was adjusted for sex, age, ethnicity, education, smoking, alcohol consumption, physical activity, menopausal status, use of medication known to affect body weight (antidepressants, antipsychotics, thyroid medication, corticosteroids and hormonal treatments), parity and total body fat at middle age.

We repeated all analyses after excluding participants who reported weight loss, while creating a new reference category of 0-5% relative weight change. We performed a sensitivity analysis to correct for potential measurement error of adult weight gain, in which the regression analysis was corrected for the correlation between recalled past weight and measured past weight reported in a previous study (r=0.87) (42). Additionally, we repeated all analyses after exclusion of participants who reported alcohol consumption of more than 4 units per day.

Analyses were performed with STATA Statistical Software version 12.1 (Statacorp, College Station, Texas, USA). Figures were constructed with GraphPad Prism version 7.02 (GraphPad Software Inc, La Jolla California, USA)

RESULTS

Characteristics of the study population

A total of 2 399 participants (54% women) were analysed in the present study, of whom 1948 had measurements of hepatic triglyceride content. Mean (SD) age of the study population was 56(6) years, mean BMI was 25.9(4.0) kg/m^2, and mean percentage of adult weight gain was 19.5(16.5) %. Characteristics of the study population stratified by the five weight change categories are presented in **Table 1**.

More women than men gained more than 25% of body weight during adulthood than participants who gained less than 25% of body weight or remained at a stable body weight. In addition, participants who gained more than 25% of body weight more often had a low education. Waist circumference, abdominal subcutaneous and visceral adipose tissue and hepatic triglyceride content were higher in participants who gained more than 25% of body weight than in the categories of less than 25% weight gain.

Table 1. Characteristics of participants of the Netherlands Epidemiology of Obesity (NEO) study with measurements of abdominal subcutaneous and visceral adipose tissue by magnetic resonance imaging, stratified by adult weight change (N=2 399)

	Weight change categories				
	<-5%	**≥-5% to <5%**	**≥5 to <25%**	**≥25% to <50%**	**≥50%**
Proportion of population (%)	4.5	11.0	54.1	25.4	5.0
Sex (% men)	17	18	51	48	36
Age (years)	54 (4)	57 (4)	55 (5)	56 (7)	56 (8)
Ethnicity (% white)	100	95	96	96	91
Education (% high)	48	50	51	38	34
Weight at age 20					
Recalled weight at age 20 (kg)	71.3 (8.6)	67.3 (7.6)	66.5 (9.3)	64.5 (14.1)	58.8 (14.7)
BMI at age 20 (kg/m^2)	24.6 (2.2)	22.8 (1.9)	21.8 (1.9)	21.3 (3.4)	20.0 (4.0)
Change in weight (%)	-7.6 (-32.2; -5.2)	1.7 (-5.0; 4.8)	14.8 (5; 24.9)	32.6 (25; 49.8)	58.8 (50; 130.4)
Smoking (% current)	18	18	14	14	11
Alcohol consumption (g/day)	7 (1-21)	10 (2-15)	11 (3-22)	8 (2-22)	7 (1-21)
Physical activity (MET-h/week)	27 (11-58)	38 (25-56)	30 (16-53)	27 (15-45)	24 (11-41)
Use of medication[1] (% yes)	13	8	17	10	19
In women:					
Postmenopausal (% yes)	41	80	51	65	69
Use of sex hormones (% current)	11	2	11	8	7
Number of liveborn children	2 (0-2)	2 (1-2)	2 (2-3)	2 (2-3)	2 (1-3)
Body weight (kg)	64.0 (6.9)	67.9 (7.9)	76.4 (11.1)	86.5 (18.6)	95.0 (23.6)
BMI (kg/m^2)	22.1 (1.6)	23.0 (2.0)	25.0 (2.4)	28.5 (4.7)	32.3 (6.7)
Waist circumference (cm, M/W)	90 (6)/76 (6)	88 (6)/77 (6)	96 (7)/ 83 (7)	104 (12)/94 (14)	111(15)/104(17)
Total body fat (%, M/W)	18 (3)/30 (4)	20 (3)/31 (4)	24(4) / 35 (4)	28(7) / 41 (6)	32(9) / 45 (9)

	Weight change categories				
	<-5%	**≥-5% to <5%**	**≥5 to <25%**	**≥25% to <50%**	**≥50%**
Abdominal subcutaneous adipose tissue (cm^2, M/W)	125 (125-159) /165 (117-223)	140 (99-182)/177 (130-226)	187(154- 222) /230 (184-281)	241(199-298)/309 (258-376)	300(254-273)/ 403 (331-485)
Visceral adipose tissue (cm^2, M/W)	50 (44-66)/21 (14-37)	50 (32-79)/36 (24-53)	97 (75-131)/51 (36-72)	135 (103-178)/89 (63-114)	170 (137-132)/125 (94-159)
Hepatic triglyceride content[2] (%, M/W)	2.2 (0.9-2.6)/0.9(0.7-1.6)	1.8 (1.0-3.8)/1.2 (0.8-1.8)	3.5 (1.9-7.1)/1.6 (1.1-3.6)	6.0 (3.5-14.1)/3.4 (1.6-8.4)	11.1 (3.8-20.3)/8.1 (3.7-19.0)
Fatty liver[2] (%, HTGC>5.56%)	6	8	25	44	66

[1]Use of medication includes thyroid hormone, antithyroid preparations, antipsychotic and antidepressant use and systemic corticosteroids. [2]n=1 948. Abbreviations: BMI, body mass index; MET, metabolic equivalent of task; M, men; W, women. Data are presented as mean (SD), median (25^{th}-75^{th} percentile/range) or percentage. Results were based on analyses weighted towards the BMI distribution of the general population (N=2 399)

Adult weight gain and measures of abdominal adiposity at middle age

A gain in body weight was associated with more abdominal adiposity at middle age (**Table 2**). After adjustment for potential confounding factors including total body fat, participants who gained more than 50% of their body weight showed 1.96 (95% CI: 1.64; 2.33) times more visceral adipose tissue and 2.39 (95% CI: 1.70, 3.36) times more hepatic triglyceride content than weight maintainers. Participants who gained more than 50% of their body weight had 1.54 (95% CI: 1.38, 1.72) times more abdominal subcutaneous adipose tissue and their waist circumference was 1.18 (95% CI: 1.15, 1.22) times higher compared with the reference category. Results were similar for men and women (men: **Table S1**, women: **Table S2**). After excluding all participants who reported a loss of body weight, the results remained similar (**Table S3**). **Table S4** shows the association between adult weight change and measures of insulin resistance, uncorrected and corrected for the measurement error in recalled body weight. Results of both analyses are similar. Furthermore, results on hepatic triglyceride content remained similar when we excluded 216 participants with alcohol consumption of more than four units per day (results not shown).

Table 2. Ratios with 95% confidence intervals in measures of abdominal adiposity by categories of weight change during adulthood, compared with weight maintenance (N=2 399)

	Weight change categories								
	<-5%		≥-5% to <5%	≥5% to <25%		≥25% to <50%		≥50%	
	Ratio	95% CI	Reference	Ratio	95% CI	Ratio	95% CI	Ratio	95% CI
Waist circumference	0.99	0.95; 1.03	1	1.08	1.06; 1.10	1.20	1.18; 1.23	1.31	1.28; 1.35
Model 2	0.93	0.90; 0.96	1	1.12	1.10; 1.13	1.26	1.24; 1.28	1.42	1.40; 1.45
Model 3	0.93	0.90; 0.96	1	1.11	1.10; 1.13	1.25	1.23; 1.28	1.41	1.39; 1.44
Model 4	0.98	0.94; 1.01	1	1.05	1.04; 1.07	1.12	1.09; 1.14	1.18	1.15; 1.22
Abdominal subcutaneous adipose tissue	0.98	0.84; 1.13	1	1.34	1.23; 1.47	1.83	1.67; 2.00	2.32	2.11; 2.56
Model 2	0.82	0.73; 0.92	1	1.48	1.37; 1.60	2.10	1.95; 2.27	2.94	2.71; 3.19
Model 3	0.80	0.72; 0.90	1	1.45	1.35; 1.57	2.07	1.93; 2.22	2.88	2.66; 3.11
Model 4	0.96	0.87; 1.06	1	1.20	1.11; 1.30	1.37	1.25; 1.49	1.54	1.38; 1.72
Visceral adipose tissue	0.83	0.64; 1.06	1	1.73	1.50; 1.99	2.64	2.30; 3.03	3.60	3.11; 4.16
Model 2	0.70	0.55; 0.88	1	1.90	1.67; 2.16	3.01	2.65; 3.42	4.53	3.97; 5.18
Model 3	0.67	0.54; 0.83	1	1.86	1.65; 2.11	2.93	2.59; 3.32	4.36	3.83; 4.97
Model 4	0.85	0.68; 1.05	1	1.46	1.29; 1.65	1.72	1.50; 1.98	1.96	1.64; 2.33
Hepatic triglyceride content[1]	1.00	0.73; 1.38	1	1.70	1.42; 2.04	2.92	2.42; 3.54	5.41	4.31; 6.78
Model 2	0.81	0.59; 1.12	1	1.88	1.58; 2.25	3.33	2.77; 3.99	6.96	5.59; 8.68
Model 3	0.84	0.61; 1.15	1	1.85	1.55; 2.22	3.24	2.70; 3.89	6.69	5.38; 8.32
Model 4	1.09	0.78; 1.53	1	1.34	1.11; 1.63	1.62	1.27; 2.07	2.39	1.70; 3.36

[1]n=1 948. Results were based on analyses weighted towards the BMI distribution of the general population (N=2 399), and were derived from beta coefficients with 95% confidence intervals from linear regression analyses and expressed as ratios of outcome measures compared with weight maintenance during adulthood. Abbreviations: CI, confidence interval. Adjusted for sex and age; 2: additionally adjusted for BMI at age 20; 3: Adjusted for 2 + ethnicity, education, smoking, alcohol consumption, physical activity, menopause status, use of antidepressants, antipsychotics, thyroid, corticosteroids or hormonal use and parity; 4: Adjusted for 3 + total body fat

BMI at age 20 and measures of abdominal adiposity at middle age

We observed a higher BMI and total body fat, larger waist circumference, more abdominal subcutaneous and visceral adipose tissue and a higher hepatic triglyceride content at middle age in participants who were overweight or obese at age 20 (**Table S5**).

Compared with the reference group (18.5-25.0 kg/m^2) and adjusted for potential confounding factors, a higher BMI at age 20 was associated with a relatively higher waist circumference, abdominal subcutaneous and visceral adipose tissue and hepatic triglyceride content (**Table 3**). However, after additional adjustment for total body fat at middle age, we observed that a higher BMI at age 20 was associated with a relatively lower visceral adipose tissue and lower hepatic triglyceride content at middle age compared with the reference category. Obesity (BMI ≥30 kg/m^2) at age 20 was associated with 0.78 (95% CI: 0.67, 0.92) times less visceral adipose tissue and 0.60 (95% CI: 0.39, 0.93) times less hepatic triglyceride content at middle age. BMI >30.0 kg/m^2 at age 20 remained associated with a relatively higher waist circumference (1.05, 95% CI: 1.03, 1.07) and abdominal subcutaneous adipose tissue (1.11, 95% CI: 1.03, 1.20) at middle age. The results on hepatic triglyceride content remained similar after additional exclusion of 216 participants who consumed more than four units of alcohol per day (results not shown).

Table 3. Ratios with 95% confidence intervals in measures of abdominal adiposity by categories of BMI at age 20, compared with BMI 18.5 – 25.0 kg/m^2 at age 20 (N=2 399)

	BMI at age 20 categories (kg/m^2)						
	<18.5		**18.5 – 24.9**	**25.0 – 29.9**		**≥30.0**	
Proportion	8.1%		82.3%	8.7%		0.9%	
	Ratio	**95% CI**	**Reference**	**Ratio**	**95% CI**	**Ratio**	**95% CI**
Waist circumference	0.96	0.94; 0.98	1	1.11	1.09; 1.14	1.23	1.16; 1.30
Model 2	0.96	0.94; 0.98	1	1.11	1.08; 1.13	1.20	1.14; 1.27
Model 3	0.99	0.97; 1.00	1	1.02	1.01; 1.04	1.05	1.03; 1.07
Abdominal subcutaneous adipose tissue	0.92	0.85; 0.99	1	1.39	1.30; 1.49	1.81	1.50; 2.19
Model 2	0.91	0.84; 0.98	1	1.37	1.29; 1.47	1.71	1.42; 2.06
Model 3	1.00	0.95; 1.05	1	1.07	1.03; 1.10	1.11	1.03; 1.20
Visceral adipose tissue	1.02	0.91; 1.15	1	1.29	1.16; 1.43	1.49	1.09; 2.05
Model 2	1.00	0.90; 1.11	1	1.26	1.14; 1.40	1.40	1.04; 1.88
Model 3	1.14	1.03; 1.25	1	0.90	0.85; 0.96	0.78	0.67; 0.92
Hepatic triglyceride content[1]	0.98	0.73; 1.31	1	1.35	1.12; 1.63	1.26	0.66; 2.39
Model 2	0.95	0.72; 1.25	1	1.33	1.11; 1.59	1.19	0.65; 2.18
Model 3	1.10	0.86; 1.42	1	0.90	0.75; 1.07	0.60	0.39; 0.93

[1]n=1 948. Results were based on analyses weighted towards the BMI distribution of the general population (N=2 399), and were derived from beta coefficients with 95% confidence intervals from linear regression analyses and expressed as ratios of outcome measures compared with BMI 18.5 – 25.0 kg/m^2 at age 20. Abbreviations: BMI, body mass index; CI, confidence interval. Adjusted for sex and age; 2: additionally adjusted for ethnicity, education, smoking, alcohol consumption, physical activity, menopause status, use of medication (antidepressants, antipsychotics, thyroid, corticosteroids, hormonal use) and parity; 3: Adjusted for 2 + total body fat.

Relative contributions of weight change and BMI at age 20

Within each category of BMI at age 20, we observed that a higher gain in body weight was associated with a relatively higher waist circumference, abdominal subcutaneous and visceral adipose tissue and hepatic triglyceride content, compared with the reference category (≥-5% - <5% weight change and BMI 18.5-25.0 kg/m^2 at age 20, **Figure 1**). The strongest associations were observed for visceral adipose tissue, for individuals with BMI at age 20 of 25-30 kg/m^2 who gained more than 50% of weight (2.17, 95% CI: 1.51, 3.13) and individuals with BMI at age 20 of more than 30 kg/m^2 who gained 25-50% of weight (1.98, 95% CI: 1.41, 2.80). In addition, strong associations were observed for hepatic triglyceride content for individuals with a BMI at age 20 of 25-30 kg/m^2 who gained more than 50% of weight (3.11, 95% CI: 1.57, 6.18).

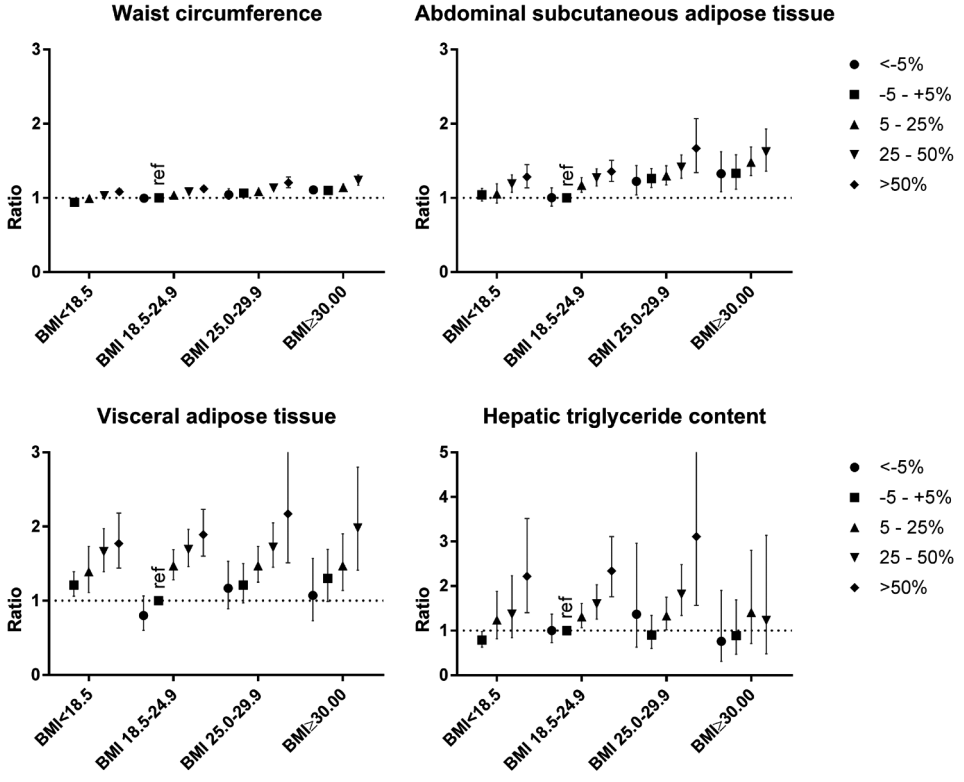

Figure 1. Weight gain is associated with a relatively higher amount of abdominal adiposity within each BMI category at age 20. Results were based on analyses weighted towards the BMI distribution of the general population (N=2 399, HTGC; n=1 848), and were derived from beta coefficients with 95% confidence intervals from linear regression analyses and expressed as ratios of outcome measures compared with weight maintenance during adulthood. Linear regression models were adjusted for sex, age, ethnicity, smoking, alcohol consumption, physical activity, menopause status, use of medication (antidepressants, antipsychotics, thyroid, corticosteroids, hormonal use), parity and total body fat.

DISCUSSION

The aim of our study was to investigate the association between adult weight change and measures of abdominal adiposity (waist circumference, abdominal subcutaneous adipose tissue and visceral adipose tissue) and hepatic triglyceride content at middle age, irrespective of total body fat. In this study, we consistently observed that weight gain was associated with a relatively higher amount of abdominal adiposity within each BMI category at age 20, compared with BMI 18.5-25.0 kg/m^2 at age 20 and weight maintenance during adulthood as a reference. Adult weight gain was more strongly associated with a relatively higher amount of visceral adipose tissue and hepatic triglyceride content than of waist circumference and abdominal subcutaneous adipose tissue. This result underlines the importance of measuring visceral adipose tissue and hepatic triglyceride content, since measuring waist circumference alone seems to underestimate the consequences on abdominal adiposity after adult weight gain. Additionally, our results suggest that adult weight gain is associated with relatively more storage of excess fat in the visceral area and the liver, compared with storage in the subcutaneous adipose tissue depot. In contrast, after adjustment for total body fat at middle age, we observed that overweight and obesity at age 20 were associated with relatively less visceral fat and liver fat at middle age compared with normal weight at age 20. Notwithstanding that on an absolute level all measures of abdominal adiposity at middle age were highest in those with overweight or obesity at age 20, these results may suggest that individuals who were already overweight or obese at age 20 have less visceral adipose tissue and hepatic triglyceride content relatively to their amount of total body fat at middle age compared with individuals who had a normal weight at age 20. In our study, we included a small group that lost more than 5% of body weight since age 20. However, because this group is heterogeneous, with weight loss ranging from 5% to more than 30% and it was not reported whether their weight loss was intentional or unintentional (e.g. due to wasting as a result of underlying disease), we are not able to determine the associations of intentional weight loss with measures of abdominal adiposity.

Two short-term overfeeding studies, one in normal weight, male twin pairs aged 21±2 years and the other in normal weight men and women aged 30±6 years, demonstrated that weight gain was associated with increases in both abdominal subcutaneous and visceral adipose tissue (28, 38). A prospective cohort study in 65 lean premenopausal women, aged 22 to 47 years, observed that weight gain was associated with an increased accumulation of visceral adipose tissue relative to total body fat (27), in line with the results of our study. In an Asian cohort of 21 496 participants (29), it was observed that the prevalence of non-alcoholic fatty liver disease increased proportionally with a larger weight gain since age 20. The association of weight gain with the prevalence of non-alcoholic fatty liver disease was even stronger in individuals with a normal weight, in accordance with our study where individuals who were overweight or obese at age 20 had relatively less hepatic triglyceride content at middle age.

The biological mechanism underlying these observations may be the limited capacity of subcutaneous adipose tissue to expand and store lipids. It was previously shown that the number of adipocytes remained constant in both lean and overweight individuals after age 20 (39). This indicates that during adult weight gain, the size of adipocytes is increasing in order to store excess lipids, but not adipocyte number. However, when adipocytes in the

subcutaneous adipose tissue increase in size, their ability to store lipids decreases. As a result, excess lipids will 'spill over' and will be stored in the visceral compartment or deposited at ectopic sites, such as the liver, heart, muscles and pancreas (25). Here, the excess visceral fat and ectopic fat may exert their detrimental effects by secreting cytokines and fatty acids and thereby inducing an inflammatory state (13). However, individuals who were overweight at age 20, may have higher adipocyte numbers and therefore enhanced capacity to store excess lipids subcutaneously, in agreement with our findings.

Additionally, it has been shown that metabolically abnormal, obese postmenopausal women (obesity according to their BMI and with an impaired insulin sensitivity) had twice as much visceral adipose tissue than metabolically normal, obese postmenopausal women (obesity according to their BMI, but with high insulin sensitivity) (40). Strikingly, they observed an association between early onset of obesity during adolescence and a more favourable metabolic profile, e.g. higher insulin sensitivity. The positive association between duration of obesity and variation in insulin sensitivity was reported earlier in a case-control study in 42 non-diabetic obese subjects (41).

Strengths of our study are the large population size and the availability of measures of abdominal subcutaneous and visceral adipose tissue by MRI and of hepatic triglyceride content by ^{1}H-MRS, providing more accurate measures of abdominal adiposity than waist circumference, and information on a wide range of potential confounding factors. This enabled us to investigate the specific associations of weight gain and BMI at age 20 with waist circumference, abdominal subcutaneous and visceral adipose tissue and hepatic triglyceride content after adjusting for total body fat at middle age.

This study also has some limitations that need to be considered. First, we calculated BMI at age 20 using recalled weight at age 20. Therefore, weight at age 20 might be misclassified. However, previous studies have shown that recalled weight is strongly correlated with measured weight (42, 43) and our sensitivity analyses correcting for the measurement error in recalled body weight gave similar results. Second, one of the contraindications of undergoing MRI was a waist circumference over 1.70 meter. Therefore, we might have missed a small number of severely obese individuals in our analyses. Third, because study participants were selected on having a self-reported BMI of 27 kg/m^2 or higher, weight gain patterns might be different in this overweight population. However, the distribution of weight gain in the study population was similar to that of the reference population of Leiderdorp. Finally, because our study population included predominantly white men and women, the results of our study need to be confirmed in other ethnic groups.

In conclusion, our study indicated that adult weight gain is associated with more abdominal adiposity, in particular with more visceral adipose tissue and hepatic triglyceride content, within all BMI categories at age 20. This implies that weight maintenance during adulthood plays an important role in limiting excess visceral adipose tissue and liver fat accumulation and its detrimental effects on cardiometabolic health. Future prospective studies need to investigate to what extent excess visceral adipose tissue and hepatic triglyceride content mediate the associations between adult weight gain and risk of cardiometabolic diseases.

CONFLICT OF INTEREST STATEMENT

All authors declare to have no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary information is available at International Journal of Obesity's website: https://www.nature.com/articles/s41366-018-0163-5#Sec17

CHAPTER 6

The association between adult weight gain and insulin resistance at middle age: mediation by visceral fat and liver fat

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ABSTRACT

We aimed to investigate the role of the amount of visceral fat and liver fat in the association between adult weight change and insulin resistance at middle age. In the Netherlands Epidemiology of Obesity study, adult weight change was calculated with recalled body weight at age 20 years and measured body weight at middle age. Measures of insulin resistance were calculated using both fasting and postprandial glucose and insulin concentrations. Visceral fat was assessed by magnetic resonance (MR) imaging and liver fat by proton-MR spectroscopy (N = 1758). We examined the association between adult weight change and insulin resistance with linear regression, adjusted for confounding factors. To investigate mediation, we additionally adjusted for total body fat, visceral fat, and liver fat. In participants who gained ≥50% of body weight during adulthood, homeostatic model assessment for insulin resistance (HOMA-IR) was 3.22 (95% CI 2.76; 3.77) times higher than in weight maintainers. In a joint model, total body fat mediated this association for 8.1% (95% CI −9.2; 25.4), visceral fat for 32.0% (18.6; 45.4%) and liver fat for 22.5% (15.0; 30.1). The association between adult weight gain and insulin resistance at middle age is largely mediated by both visceral fat and liver fat.

INTRODUCTION

Adult weight gain and obesity are well-established causal risk factors for the development of type 2 diabetes mellitus, cardiovascular disease and obesity-related cancers (1–3). In line with these findings, adult weight gain was strongly associated with increased insulin resistance in multiple studies (4–9).

It is well-established that abdominal adiposity, and in particular visceral adipose tissue, is strongly related to insulin resistance and risk of type 2 diabetes mellitus, also after adjustment for total body fat (10–15). In a previous analysis, we observed that larger gain in body weight during adulthood was associated with more visceral fat and liver fat at middle age, compared with weight maintenance (16). This finding is in agreement with the 'lipid overflow' hypothesis, which postulates that lipids are stored in the visceral area and in and around organs (ectopic fat) when the subcutaneous adipose tissue has reached its limited storage capacity (17,18). In addition to visceral adipose tissue, excess liver fat has been associated with insulin resistance (19), as well as with a higher risk of type 2 diabetes mellitus, cardiovascular disease and mortality (20).

As both excess visceral fat and liver fat are strongly associated with increased insulin resistance and type 2 diabetes, we hypothesized that the association between adult weight change and insulin resistance is largely mediated by both excess visceral fat and liver fat. Therefore, the aim of this study was to investigate to what extent the association of adult weight change with insulin resistance is mediated by the amounts of visceral fat and liver fat at middle age.

METHODS

Study Design and Study Population

The Netherlands Epidemiology of Obesity (NEO) study is a population-based cohort study of 6671 individuals aged 45–65 years, with an oversampling of individuals with body mass index (BMI) ≥ 27 kg/m^2, living in the greater area of Leiden (in the West of the Netherlands). All inhabitants aged between 45 and 65 years from one municipality (Leiderdorp) were invited to participate irrespective of their BMI, allowing for a reference distribution of BMI. The study design and population are described in detail elsewhere (21).

Participants were invited to a baseline visit at the NEO study center of the Leiden University Medical Center (LUMC) after an overnight fast. Prior to the study visit, participants completed a general questionnaire at home to report demographic, lifestyle and clinical information. At the study center, participants completed a screening form, asking about anything that might create a health risk or interfere with magnetic resonance imaging, e.g., presence of metallic devices, claustrophobia and a body circumference > 1.70 m. Of the eligible participants, 2580 participants were randomly selected to undergo magnetic resonance imaging (MRI) (21). This subset of participants from the NEO cohort has similar characteristics as the participants from the NEO cohort not participating in this subset, apart from a slightly higher BMI and more individuals with a history of cardiovascular disease in the subset that did not

participate (15). The Medical Ethical Committee of the LUMC approved the NEO study. All participants provided written informed consent.

For the present study, we performed cross-sectional analyses with baseline measurements of the NEO study. Hepatic triglyceride content was available in 2086 of the participants who underwent MRI, due to technical failures or an insufficient quality of the measurements to estimate liver fat content. We excluded participants with images of insufficient quality to estimate visceral adipose tissue (n = 11), with missing data on recalled body weight at age 20 years (n = 60), and with a BMI at age 20 below 14.0 kg/m^2 (n = 1). Additionally, we excluded participants who used glucose-lowering medication (n = 129), were non-fasting at baseline (n = 1), or had missing data on postprandial glucose at 30 min (n = 38), at 150 min (n = 30) or fasting insulin (n = 3), total body fat at baseline (n = 3), ethnicity (n = 2), educational level (n = 15) and physical activity (n = 35), resulting in 1758 participants (913 men, BMI range 20.1–39.6 kg/m^2 and 845 women, BMI range 18.2–45.3 kg/m^2) who were included in the analyses. The majority of our study population was of Caucasian ethnicity (96.3%). Other ethnicities that were represented in our study sample are African (0.5%), Turkish (0.3%), South-East Asian (0.5%), Hindu (0.1%), and ethnicities other than aforementioned (2.3%).

Data Collection

Weight Change during Adulthood

At the baseline study visit, height without shoes was measured with a vertically fixed, calibrated tape measure. Body weight was measured and percent body fat was estimated by the Tanita bio impedance balance (TBF-310, Tanita International Division, UK) without shoes and 1 kg was subtracted to correct for weight of clothing. BMI at baseline was calculated by dividing the weight in kg by the height in meters squared.

Recalled weight at the age of 20 years was based on self-report. The general questionnaire included the question 'How much did you weigh (approximately) when you were 20 years old?'. BMI at age 20 years was calculated by dividing body weight at age 20 in kg by the height in meters squared at middle age with the assumption that height did not majorly change during adulthood. Relative weight change was calculated by subtracting weight at age 20 from measured baseline weight, divided by weight at age 20, and multiplied by 100% (16).

Visceral Fat and Liver Fat at Middle Age

Visceral adipose tissue was directly assessed by MRI (1.5 Tesla MR imaging, Philips Medical Systems, Best, Netherlands) using a turbo spin echo imaging protocol with the following imaging parameters: 300/20; flip angle, 90°; section thickness, 10 mm, section gap, 2 mm. At the level of the fifth lumbar vertebra, three transverse slices were obtained during one breath-hold (21). Imaging parameters were: TR = 300 ms; TE = 20ms; flip angle = 90°; slice thickness = 10 mm, slice gap = 2 mm. Visceral fat areas were quantified by converting the number of pixels to centimeters squared for all three slices and totaling the areas of the three sections, using in-house-developed software (MASS; Leiden University Medical Center, Leiden, the Netherlands).

Hepatic triglyceride content was quantified using ^{1}H-magnetic resonance spectroscopy of the liver (21). An 8 mL voxel was positioned in the right lobe of the liver, avoiding gross vascular structures and adipose tissue depots. Sixty-four averages were collected with water

suppression (repetition time = 2900 ms; echo time = 23 ms (2900/23). Data points (1024) were collected by using a 1000-Hz spectral line. Without changing any parameters, spectra without water suppression, with a repetition time of 10 s and with four averages were obtained as internal reference. Hepatic triglyceride content relative to water was calculated as (signal amplitude of triglyceride)/(signal amplitude of water) × 100. Spectra were not corrected for frequency drift. Spectral data were analyzed while blinded to all study parameters, including age, sex, LV function and dimensions, BMI, waist circumference, visceral adipose tissue, and total body fat. Spectra were initially included when automatic fitting was successful. When line shapes were distorted by eddy currents or as a result of poor shimming, spectral data were rejected.

Measures of Insulin Resistance at Middle Age

Fasting blood samples were drawn from the antecubal vein after 5 min rest of the participant, after an overnight fast of at least 10 h. Within 5 min after drawing a fasting blood sample, all participants consumed a liquid mixed meal (400 mL) that contained 2.5 megajoule (MJ), of which 16 percent of energy (En%) was derived from protein, 50 En% from carbohydrates and 34 En% from fat. Subsequently, blood samples were drawn after 30 and 150 min. Plasma glucose concentrations were determined by enzymatic and colorimetric methods (Roche Modular Analytics P800, Roche Diagnostics, Mannheim, Germany; CV < 5%) and serum insulin concentrations were determined by an immunometric method (Siemens Immulite 2500, Siemens Healthcare Diagnostics, Breda, The Netherlands; CV < 5%) at the Department of Clinical Chemistry and Laboratory Medicine of the LUMC [21]. From fasting glucose and insulin concentrations, we calculated the Homeostasis Model Assessment for Insulin Resistance (HOMA-IR), a marker of hepatic insulin resistance (22). HOMA-IR was calculated as fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5 (22, 23). Matsuda Insulin Sensitivity Index (ISI) was calculated as 10,000/square root (fasting glucose (mg/dL) × fasting insulin (μU/mL)) × ($meanglucose_{0\text{-}150}$ × $meaninsulin_{0\text{-}150}$) (24–26).

Covariates

Ethnicity was self-identified in the questionnaire and regrouped into Caucasian and other. Highest level of education was reported in ten categories according to the Dutch education system and regrouped in two categories: Low education (no education, primary school or lower vocational education) and high education (higher vocational education, university and postgraduate education). Smoking status was self-reported. Alcohol consumption was reported on the food frequency questionnaire and expressed as grams/day (27). Participants reported the frequency and duration of their usual physical activity during leisure time in the Short questionnaire to assess health-enhancing physical activity (SQUASH), which was expressed in hours per week of metabolic equivalents (28,29). Family history of diabetes mellitus and myocardial infarction were reported as having any parent or sibling with diabetes mellitus or not (reference group).

Statistical Analyses

In the NEO study, individuals with BMI ≥ 27 kg/m^2 are oversampled. To correctly represent associations for the general population (30), adjustments for the oversampling were made. This was done by weighting all participants towards the BMI distribution of participants from the Leiderdorp municipality (31), whose BMI distribution was similar to the BMI dis-

tribution of the general Dutch population (32). All results were based on weighted analyses and are therefore generalizable to a population-based study without oversampling of individuals with BMI ≥ 27 kg/m^2 (21). As a consequence of the weighting procedure, numbers of participants per category are presented as percentages.

Characteristics of the study population at middle age were expressed as mean (SD), median (25th, 27th percentiles or range) or as percentage, stratified by categories of weight change. We explored the distribution of weight change in the reference population of Leiderdorp in an earlier study, and observed that the majority of the population gained weight between age 20 years and middle age in this era (16). Therefore, we decided to stratify categories of adult weight change as: weight loss of more than 5%, weight change between −5% and 5% (weight maintenance: reference category), weight gain of 5% to 25%, 25% to 50%, and ≥50%.

We performed linear regression analyses to examine the associations of adult weight change with fasting and postprandial glucose and insulin concentrations and with measures of insulin resistance at middle age, compared with the reference category of weight maintenance during adulthood. Potential confounding factors were defined a priori based on biological knowledge from previous studies. Crude models were adjusted for sex and age (model 1). In model 2, we additionally adjusted for BMI at age 20, because the percentage of weight change since age 20 depends on initial BMI at age 20. In model 3, we additionally adjusted for ethnicity, education, smoking, alcohol consumption, physical activity, and family history of diabetes. Because of a skewed distribution, values of fasting and postprandial glucose and insulin, HOMA-IR and Matsuda ISI were all transformed to the natural logarithm. Regression coefficients and corresponding 95% confidence intervals (95% CI) were back transformed and expressed as ratios, which can be interpreted as the relative changes in insulin resistance in a certain weight change category, compared with insulin resistance in the reference category of weight maintenance. For example: a ratio of 2 for HOMA-IR in individuals who gained 5% to 25% of body weight during adulthood indicates that these individuals have twofold higher HOMA-IR values at middle age than individuals who maintained their body weight during adulthood.

Subsequently, we examined mediation in the association between adult weight change and insulin resistance at middle age by total body fat, visceral fat, and liver fat at middle age according to the method proposed by Baron and Kenny (33). This method is based on comparing the regression coefficient of the association between an exposure and outcome, and the regression coefficient of the association between the exposure and outcome adjusted for the mediating variable. First, we checked whether the exposure–outcome, exposure–mediator, and mediator–outcome associations were present in our study. Associations between adult weight change and total body fat, visceral fat, and liver fat were previously described for the NEO population (16), as well as the associations between total body fat, visceral fat, and liver fat and insulin resistance (HOMA-IR) (15). The association between adult weight change and insulin resistance was examined in the present study. Secondly, we checked the assumption of no interaction between the exposure and mediator by examining the interaction between adult weight change and total body fat, visceral adipose tissue

and hepatic triglyceride content in the association with insulin resistance. We attempted to avoid mediator-outcome confounding in the mediation analyses by adjusting for measured potential confounding factors.

Additional to Baron and Kenny's mediation method, we used structural equation modelling (SEM) to evaluate the effect of several mediators on the relation between adult weight change and insulin resistance adjusted for possible confounding factors, expressed as a percentage of mediation (34). By multiplying the regression coefficients of the exposure-mediator and the mediator-outcome model, we were able to calculate the separate and combined indirect effects of total body fat, visceral fat and liver fat on the association between adult weight change and insulin resistance, with their corresponding 95% CIs. We divided the indirect effects by the total effect, which is the sum of the direct and indirect effects, to calculate the percentage of mediation by the different mediators and corresponding 95% CI.

We also conducted several sensitivity analyses. We repeated all analyses with absolute adult weight change between age 20 years and middle age in kilograms, instead of the relative weight change as a percentage. We repeated the analyses with relative weight change as a continuous variable. Additionally, we assessed whether there was interaction by sex in the association between adult weight change and insulin resistance by including a product term of sex and adult weight change, and repeated all analyses for men and women separately.

RESULTS

Characteristics of the Study Population

A total of 1758 individuals (54% women) were analyzed in the present study. Mean (SD) age of the study population was 55 (6) years, mean BMI at age 20 years was 21.8 (2.6) kg/m^2, mean BMI at middle age was 25.8 (3.9) kg/m^2, and mean percentage of adult weight change was a gain in weight of 19.1% (16.0%).

Characteristics of the study population stratified by five weight change categories are presented in **Table 1**. The proportion of women was higher in participants who gained more than 25% of body weight between age 20 and middle age than in participants who gained less than 25% during adulthood. Additionally, participants who gained more than 25% of body weight were less likely to be highly educated, and waist circumference, total body fat, visceral fat and liver fat at middle age were higher than in participants who gained less than 25% of body weight, in both men and women. Median fasting plasma glucose, fasting serum insulin and HOMA-IR were higher in individuals who gained more than 25% of body weight, compared with individuals who gained less than 25% of body weight, whereas Matsuda ISI was lower.

Table 1. Characteristics of participants of the Netherlands Epidemiology of Obesity (NEO) study, aged 45 to 65 years, with measurements of visceral adipose tissue and hepatic triglyceride content by magnetic resonance imaging and spectroscopy, stratified by adult weight change (N = 1758).

	Weight change categories				
	Loss of >5%	**Weight Maintenance -5% to <5%**	**Gain of ≥5% to <25%**	**Gain of ≥25% to <50%**	**Gain of ≥50%**
Proportion of population (%)	4.5	11.2	55.0	24.7	4.6
Sex (% men)	22	39	50	46	38
Body weight at age 20					
Recalled weight at age 20 (kg)	73.7 (8.0)	67.2 (7.6)	66.2 (9.3)	64.5 (14.1)	59.0 (14.5)
BMI at age 20 (kg/m^2)	25.1 (2.1)	22.6 (1.9)	21.7 (1.9)	21.3 (3.3)	19.9 (4.1)
Change in weight (%, range)	−7.6 (−32.2; −5.8)	1.9 (−4.9; 4.8)	14.9 (5.0; 24.9)	32.3(25.0; 49.8)	57.2 (50.0;102.8)
Characteristics at middle age					
Age (years)	53 (3)	57 (4)	55 (5)	55 (7)	56 (8)
Ethnicity (% Caucasian)	100	96	96	98	91
Education (% high)	45	53	51	37	31
Smoking (% current)	19	21	13	13	11
Physical activity (MET-h/week)	27 (11-58)	38 (25-56)	30 (16-53)	27 (15-45)	24 (11-41)
Alcohol (g/day)	4 (1–21)	10 (4–16)	11 (3–23)	8 (2–21)	8 (1–21)
Physical activity (MET-hours/week)	27 (19–58)	42 (28–56)	31 (17–53)	26 (14–44)	20 (10–42)
Body weight (kg)	66.0 (6.2)	67.9 (7.8)	76.0 (11.2)	86.3 (18.7)	94.6 (23.3)
BMI (kg/m^2)	22.4 (1.4)	22.8 (1.9)	25.0 (2.4)	28.5 (4.6)	31.9 (6.6)
Waist circumference (cm, M/W)	90(5)/76(5)	87(6)/77(7)	96(7)/82(7)	105(12)/93(13)	110(14)/103(16)
Total body fat (%, M/W)	18(3)/32(3)	20(3)/31(4)	24(3)/35(4)	28(7)/41(6)	31(8)/44(10)
Visceral adipose tissue (cm^2, M/W)	50(44–66)/21(14–37)	50(19–79)/ 36(24–47)	98(76–133)/49(35–69)	135(104–173)/88(59–113)	158(131–210)/118(94–156)
Hepatic triglyceride content (%, M/W)	2.2(0.9–2.6)/0.9(0.7–1.6)	1.8(1.0–3.6)/1.2(0.7–1.7)	3.5(2.0–7.0)/1.6(1.1–3.6)	6.0(3.5–14.0)/3.4(1.6–8.4)	11.8(3.8–20.8)/7.7(3.7–18.8)

	Weight change categories				
	Loss of >5%	**Weight Maintenance --5% to <5%**	**Gain of ≥5% to <25%**	**Gain of ≥25% to <50%**	**Gain of ≥50%**
In women [a]:					
Postmenopausal (% yes)	37	78	51	66	69
Current use of sex hormones [b] (%)	4	3	11	7	3
Insulin resistance at middle age					
Family history of diabetes (% yes)	31	22	26	24	31
Family history of myocardial infarction (% yes)	26	34	39	47	48
Fasted plasma glucose (mmol/L)	4.8 (4.5–5.1)	5.1 (4.8–5.3)	5.2 (4.9–5.6)	5.5 (5.2–5.9)	5.6 (5.3–6.1)
Fasted serum insulin (mU/L)	5.5 (4.1–6.5)	5.4 (3.6–7.1)	7.3 (5.2–9.9)	10.5 (7.6–14.7)	13.0 (8.6–21.6)
HOMA-IR	1.1 (0.8–1.5)	1.2 (0.8–1.6)	1.7 (1.2–2.4)	2.6 (1.8–3.7)	3.2 (2.1–5.5)
Matsuda ISI	2.4 (2.1–2.7)	2.1 (1.8–2.5)	1.8 (1.5–2.1)	1.4 (1.0–1.8)	1.1 (0.6–1.6)

[a] 54% of study population. [b] Use of sex hormones included oral contraceptive and hormonal replacement therapy. Results were based on analyses weighted towards the BMI distribution of the general population (N = 1758). Abbreviations: BMI, body mass index; MET, metabolic equivalent of task; M, men; W, women; HOMA-IR, homeostatic model assessment insulin resistance; Matsuda ISI, Matsuda insulin sensitivity index. Data are presented as mean (SD), median (25^{th}–75^{th} percentile/range) or percentage.

Adult Weight Change and Insulin Resistance at Middle Age

As shown in **Figure 1**, each higher category of change in body weight during adulthood was associated with higher fasting and postprandial glucose and insulin concentrations at middle age, after adjustment for sex, age, BMI at age 20, ethnicity, education, smoking, alcohol consumption, physical activity, and family history of diabetes.

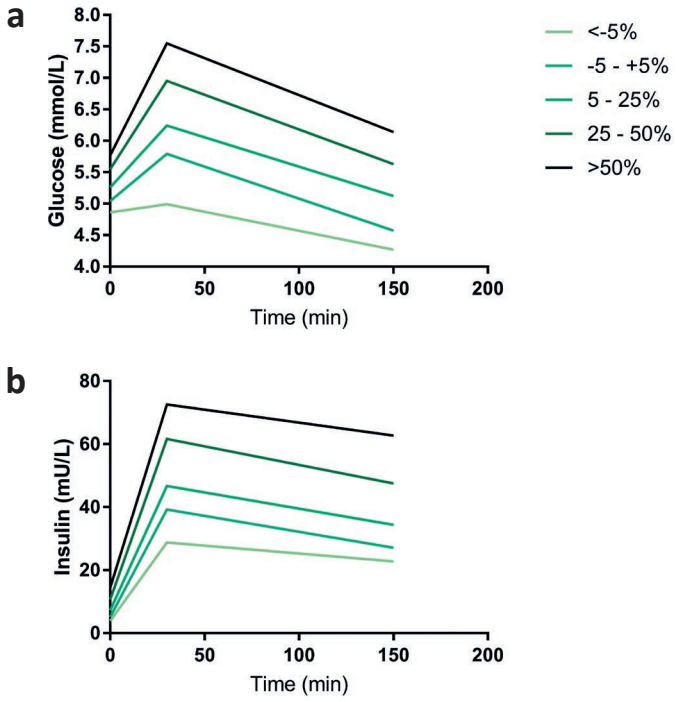

Figure 1. Estimated means of (a) glucose (mmol/L) and (b) insulin (mU/L) blood concentrations fasting, and at t = 30 and t = 150 min after a mixed meal challenge, stratified by adult weight change (N = 1758) and adjusted for sex, age, BMI at age 20, ethnicity, education, smoking, alcohol consumption, physical activity, and family history of diabetes.

After adjustment for potential confounding factors (model 2, **Table 2**), HOMA-IR was 1.37 (95% CI 1.20; 1.56) times higher in participants who gained 5–25% of body weight, 2.04 (1.79; 2.34) times higher in participants who gained 25–50% of body weight, and 2.65 (2.24; 3.14) times higher in those who gained ≥50% of body weight during adulthood than in weight maintainers. The ratio of relative change in the Matsuda ISI was 0.76 (95% CI 0.68; 0.84) in participants who gained 5–25% of body weight during adulthood, 0.51 (0.46; 0.58) in participants who gained 25–50% of body weight compared with weight maintainers, and 0.40 (0.34; 0.47) in participants who gained ≥50% of body weight in model 2. After additional adjustment for BMI at age 20 (model 3), associations between adult weight change and both HOMA-IR and Matsuda ISI became slightly stronger (Table 2). Adjustment for sex, age and BMI at age 20 had the largest impact on the associations between adult weight gain and insulin resistance.

Table 2. Relative change with 95% confidence intervals in measures of insulin resistance and insulin sensitivity for categories of weight change during adulthood, compared with weight maintenance (N = 1758).

	Model 1		Model 2		Model 3	
	Ratio	**95% CI**	**Ratio**	**95% CI**	**Ratio**	**95% CI**
HOMA-IR						
< −5.0%	0.85	0.56; 1.30	0.85	0.56; 1.30	0.73	0.47; 1.12
−5% to 5% (ref)	1		1		1	
5–25%	1.38	1.21; 1.57	1.37	1.20; 1.56	1.47	1.30; 1.67
25–50%	2.14	1.87; 2.44	2.04	1.79; 2.34	2.28	2.01; 2.59
>50%	2.78	2.34; 3.30	2.65	2.24; 3.14	3.22	2.76; 3.77
Matsuda ISI						
< −5.0%	1.22	0.92; 1.63	1.23	0.92; 1.63	1.40	1.05; 1.86
−5% to 5% (ref)	1		1		1	
5–25%	0.75	0.67; 0.84	0.76	0.68; 0.84	0.71	0.64; 0.79
25–50%	0.49	0.44; 0.55	0.51	0.46; 0.58	0.47	0.42; 0.52
>50%	0.38	0.32; 0.44	0.40	0.34; 0.47	0.34	0.30; 0.39

Results were based on analyses weighted towards the BMI distribution of the general population and were derived from beta coefficients with 95% confidence intervals from linear regression analyses and expressed as ratios of outcome measures compared with weight maintenance during adulthood. Abbreviations: CI, confidence interval; HOMA-IR, homeostatic model assessment insulin resistance; Matsuda ISI, Matsuda insulin sensitivity index; ref, reference group. Model 1: Adjusted for sex and age; 2: additionally adjusted for ethnicity, education, smoking, alcohol consumption, physical activity and family history of diabetes; 3: additionally adjusted for BMI at age 20.

When using absolute adult weight change (in kg) instead of relative weight change, results were similar (**Table S1**). Additionally, we repeated the analyses with adult weight change as continuous variable (per 10% of weight change, **Table S2**), showing similar results.

Mediation Analyses

We did not observe interaction between the mediators (total body fat, visceral adipose tissue and hepatic triglyceride content) and adult weight change in the association with HOMA-IR or Matsuda ISI (**Table S3**).

As presented in **Figure 2** and **Table S4**, after adjustment for total body fat at middle age, the association between adult weight change and HOMA-IR attenuated across all weight change categories. For example, individuals who gained ≥50% of body weight during adulthood had 1.87 (1.48; 2.36) times higher mean HOMA-IR values. After additional adjustment for visceral fat, the association attenuated further to 1.49 (1.17; 1.89), while after adjustment

for liver fat to 1.59 (1.27; 2.00). When we adjusted the association between adult weight change and HOMA-IR for total body fat, visceral fat and liver fat in one model, the association was 1.38 (1.09; 1.74) for individuals who gained ≥50% of body weight. For Matsuda ISI, the pattern of associations was in opposite direction, but similar to HOMA-IR (**Figure 2** and **Table S4**).

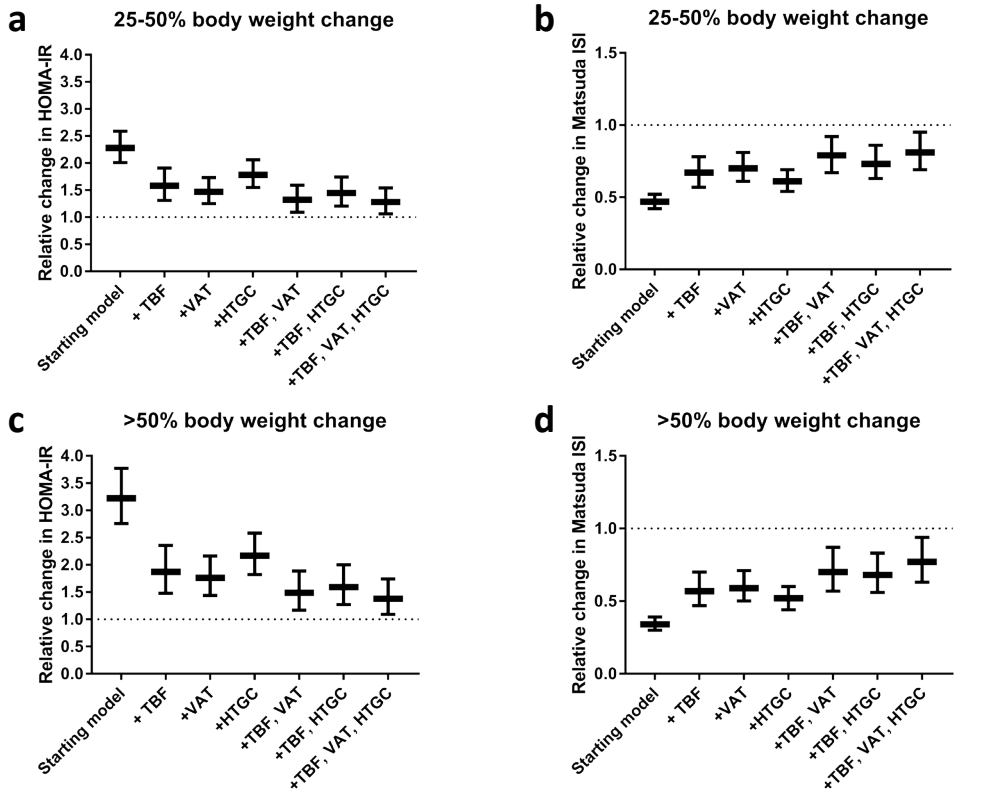

Figure 2. Relative changes (95% CI) of measures of HOMA-IR and Matsuda Index for participants who gained 25–50% of body weight (a,b) or >50% of body weight (c,d) after addition of mediators, compared to weight maintainers. TBF, total body fat; VAT, visceral adipose tissue; HTGC, hepatic triglyceride content. Starting model (model 3) was adjusted for sex, age, BMI at age 20, ethnicity, education, smoking, alcohol consumption, physical activity and family history of diabetes.

Results for sex-stratified mediation analyses are presented in **Table S5** (men) and **Table S6** (women). We observed similar patterns of mediation by visceral fat and liver fat in men and women. Additionally, we did not observe interaction by sex in the association between adult weight change and HOMA-IR or Matsuda ISI (p-value for interaction 0.53 and 0.67, respectively).

In addition, we also performed SEM analyses to estimate the indirect effects of adult weight change through total body fat, visceral fat and liver fat as a percentage of its total effect (**Table 3**). Separately, total body fat, visceral adipose tissue and hepatic triglyceride content all had an indirect effect in the association between adult weight change and HOMA-IR (**Table 3**). However, when the joint mediating effect of total body fat and visceral fat or liver fat was considered, the indirect effect via total body fat disappeared in women, but remained in men. When all three mediators were included in the model, the percentage of mediation of the total association between adult weight change and HOMA-IR was 32.0% (95% CI 18.6; 45.4) for visceral fat and 22.5% (15.0; 30.1) for liver fat. Similar percentages of mediation were observed for Matsuda ISI (results not shown).

Table 3. Analysis of indirect effects of the mediators total body fat, visceral adipose tissue and hepatic triglyceride content in the association between adult weight gain and HOMA-IR.

		All (N = 1758)		Men (N = 913)		Women (N = 845)	
		% of Total Effect	95% CI	% of Total Effect	95% CI	% of Total Effect	95% CI
Total effect		100		100		100	
Indirect effect through:							
TBF alone		34.2	16.6; 51.9	42.2	20.6; 63.9	27.3	−0.4; 55.0
VAT alone		44.1	31.3; 56.9	31.9	19.3; 44.6	51.2	29.6; 72.8
HTGC alone		28.3	20.9; 35.8	25.8	14.9; 36.8	29.1	19.1; 39.1
	TBF	13.0	−4.4; 30.3	29.8	9.0; 50.6	−1.7	−0.29.9; 26.5
	VAT	41.6	28.7; 54.4	28.5	16.1; 41.0	51.7	29.4; 73.9
TBF + HTGC	TBF	22.5	4.5; 40.5	28.6	7.6; 49.7	16.6	−12.3; 45.5
	HTGC	27.0	19.4; 34.6	24.2	13.4; 35.0	28.1	17.4; 38.8
TBF + VAT + HTGC	TBF	8.1	−9.2; 25.4	20.2	−0.4; 40.9	−3.2	−30.8; 24.4
	VAT	32.0	18.6; 45.4	22.5	10.2; 34.7	39.5	15.5; 63.4
	HTGC	22.5	15.0; 30.1	21.8	11.6; 32.0	21.9	10.6; 33.3

Results were based on analyses weighted towards the BMI distribution of the general population and were derived from multiplied path coefficients with 95% confidence intervals from structural equation modelling (path analysis) and expressed as indirect effects in the association between adult weight change (per 10% weight change) and insulin resistance. Indirect effects were divided by total effects to calculate the percentage mediated. Abbreviations: CI, confidence interval; HOMA-IR, homeostatic model assessment insulin resistance; TBF, total body fat; VAT, visceral adipose tissue; HTGC, hepatic triglyceride content. Indirect effects were adjusted for sex, age, BMI at age 20, ethnicity, education, smoking, alcohol consumption, physical activity and family history of diabetes.

DISCUSSION

The aim of our study was to investigate the association between adult weight change and insulin resistance at middle age, and to what extent this association is mediated by visceral fat and liver fat. In this population-based study of 1758 men and women, we observed that a gain in body weight during adulthood as small as 5% was already associated with more insulin resistance compared with weight maintenance during adulthood. Stronger associations with insulin resistance were observed for more excessive weight gain during adulthood. When considering the combined mediation effect, by adjusting for total body fat, visceral fat and liver fat in one model, we observed that the association between adult weight change and insulin resistance was 8.1% mediated by total body fat, 32.0% by visceral fat and 22.5% by liver fat. After adjustment for total body fat, visceral fat or liver fat separately, we observed the largest attenuation of the association between adult weight change and insulin resistance after adjustment for total body fat or visceral fat, compared with adjustment for liver fat. However, this result can be explained by the strong correlation between total body fat, visceral fat and liver fat, and thereby their overlapping mediation roles.

After adjustment for total body fat, visceral fat and liver fat at middle age, insulin resistance at middle age of individuals who had gained more than 50% of body weight was still 1.38-fold higher than the insulin resistance of weight maintainers. This difference can be explained by either residual confounding or measurement error in the assessment of total body fat, because total body fat was estimated by bioelectrical impedance analysis (BIA). The estimation of total body fat percentage by BIA showed good absolute agreement (intraclass correlation coefficient 0.90, 95% CI 0.89; 0.91) with total body fat percentage by dual-energy X-ray absorptiometry (DXA) which was available in a small subset of the NEO study (N = 915). Alternatively, the remainder of the association between weight change and insulin resistance might be mediated by ectopic fat deposition in organs other than the liver, such as the heart, pancreas, kidneys, and skeletal muscles (18). Because this information is not available in the NEO study, we were unable to investigate this hypothesis.

A meta-analysis based on 15 observational studies showed that the risk of type 2 diabetes mellitus was increasing in line with an increasing gain in body weight during adulthood, suggesting a dose-response association (35), in agreement with the results of our study. Also in line with our results, in a cross-sectional study of 153 middle-aged women HOMA-IR was higher in women who had gained more than 30 kg of body weight since the age of 20 than in women who had gained less than 10 kg (9). A study in Japanese adults (N = 399) also showed that weight gain since age 20 years was associated with higher HOMA-IR (36). The authors noted that this association attenuated after adjustment for BMI at middle age, suggesting that the association between weight gain and HOMA-IR was largely explained by the participants' BMI at middle age. In our study, we adjusted for total body fat, visceral fat and liver fat and showed that the association between adult weight change and insulin resistance is mostly mediated by visceral fat and liver fat.

The biological mechanism underlying these observations could be a reflection of the eventual limited capacity of subcutaneous adipose tissue to store lipids during weight gain (14,18). When the capacity threshold of adipose tissue is reached, lipids will be stored in the visceral

area and subsequently will be deposited in ectopic sites such as the liver, heart, muscles and pancreas (18,37). Here, the visceral fat cells will exert their detrimental effects by secreting cytokines and non-esterified fatty acids (NEFAs) or very low-density lipoproteins (14,38–41). Elevation in release of pro-inflammatory cytokines such as IL-6 and TNF-α may induce a low-grade inflammatory state and oxidative stress, eventually leading to insulin resistance. Intracellular NEFAs inhibit insulin signaling, which will also lead to insulin resistance (42).

Strengths of our study include the large study population, data on many potential confounding factors, and the availability of directly assessed visceral adipose tissue by MRI and hepatic triglyceride content by ^{1}H-MRS, providing more accurate measures of abdominal adiposity than waist circumference. This enabled us to assess the mediating effects of visceral fat and liver fat after taking mediation via total body fat into account.

A limitation that needs to be considered is assessment of insulin resistance by HOMA-IR and Matsuda ISI. The golden standard measurement of insulin resistance is the hyperinsulinemic euglycemic clamp (43). However, this is not feasible in a large study population. Instead, we calculated HOMA-IR and Matsuda ISI based on both fasting and postprandial glucose and insulin concentrations, which are valid surrogate measures for insulin resistance in large population-based studies (44). The fact that the results of these two different proxies of insulin resistance were similar, suggests that our findings are robust. Second, we calculated BMI at age 20 using recalled weight at age 20. Therefore, body weight at age 20 and weight change during adulthood might have been misclassified. However, previous studies have shown that recalled weight is highly correlated with measured weight at the same age (45), and in a previous study we showed that the association between adult weight change and measures of (abdominal) adiposity did not markedly change after correction for measurement error in recalled weight at age 20 years (16). Third, the majority of our study population was Caucasian, therefore the results of our study need to be confirmed in other ethnic groups.

In conclusion, our results indicate that the association between adult weight gain and insulin resistance at middle age is largely mediated by visceral fat and liver fat at middle age, which is increasingly important given the growing prevalence of abdominal obesity and non-alcoholic fatty liver disease (46,47). Our results suggest that weight maintenance during adulthood plays an important role in preventing accumulation of excess visceral fat and liver fat and thereby insulin resistance and eventually, type 2 diabetes at middle age and older age. Future prospective studies need to investigate the precise mechanisms by which visceral fat and liver fat lead to insulin resistance, and ways to reduce or prevent visceral fat and liver fat accumulation.

CONFLICTS OF INTEREST

All authors declare that there is no conflict of interest associated with this manuscript.

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SUPPLEMENTARY MATERIAL

The supplementary material can be found at
http://www.mdpi.com/2077-0383/8/10/1559/s1

CHAPTER 7

Preventive factors and underlying pathways of incident cardiometabolic disease in obesity

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In preparation

ABSTRACT

Introduction: Not all individuals with obesity develop chronic cardiometabolic disease. We aimed to identify risk factors that may help to prevent cardiometabolic disease in people with obesity.

Methods: The Netherlands Epidemiology of Obesity study is a prospective cohort study including 6671 middle-aged men and women. Incident diagnoses of diabetes and cardiovascular disease were collected through medical records from general practitioners during ten years of follow-up. With Cox proportional hazards models, we first calculated hazard ratios (HR) with 95% confidence intervals (CI) for incident cardiometabolic disease (including type 2 diabetes mellitus, myocardial infarction and cerebrovascular events) in individuals with body mass index (BMI) ≥27 kg/m^2, related to measures of body fat distribution, metabolic syndrome, and lifestyle factors, adjusted for appropriate confounding factors. Second, we calculated the synergy index (SI) to investigate interaction between risk factors and obesity on an additive scale.

Results: After exclusion of participants with a history of cardiometabolic disease at baseline (n=860), who were not in a fasting state (n=21) or who were lost to follow-up (n=120), 5670 participants (54% women, 42% obesity) were analysed. During a total of 37,034 person-years follow-up, 383 participants were diagnosed with incident cardiometabolic disease. In participants with BMI ≥27 kg/m^2, all studied risk factors related to body fat distribution and metabolic syndrome were associated with increased risk of incident cardiometabolic disease. High fasting plasma glucose was particularly harmful in those with obesity (HR 7.62 [5.63 – 10.31]) as was smoking (being a smoker with obesity was associated with a 3-fold increased risk of cardiometabolic disease compared with non-smokers without obesity (HR 2.89 [2.12 – 3.95])

Discussion: We observed that abdominal adiposity, smoking and hyperglycaemia were particularly harmful to cardiometabolic disease risk in individuals with obesity. A healthy lifestyle, preventing or reducing abdominal obesity, and treatment of metabolic risk factors may help to prevent cardiometabolic disease in people with obesity.

INTRODUCTION

Obesity, as defined by a body mass index (BMI) of 30 kg/m^2 or higher, is a well-established causal risk factor for type 2 diabetes and cardiovascular diseases (1). However, importantly, many individuals with obesity remain free of cardiometabolic disease (2). Body fat distribution, metabolic consequences of obesity, and lifestyle behaviour may differ considerably between individuals with obesity and may in part explain why some people with obesity develop cardiometabolic disease and others do not.

It is well-established that abdominal obesity is driving the increased cardiometabolic risk associated with obesity (1, 3-5). Within the abdomen, fat is stored subcutaneously or in the visceral area. According to the so-called 'lipid overflow' hypothesis, when the capacity of hypertrophic adipocytes to expand is exceeded, lipids 'overflow' and accumulate in the visceral area and in normally lean organs as the heart or the liver (ectopic fat) (3, 6, 7). Excess visceral adipose tissue is associated with a systemic low-grade inflammatory state (3, 8-11), insulin resistance (11), dyslipidaemia, hepatic steatosis, and thereby an increased risk of cardiometabolic disease (12).

Next to body fat distribution and metabolic risk factors, lifestyle factors including smoking, physical activity, alcohol intake, and diet quality have been associated with incidence of chronic diseases and disease-free life expectancy (13). Insights in factors that increase the risk of cardiometabolic disease in individuals with obesity may yield particular targets for interventions, which show the largest reduction in risk in this high-risk population to develop cardiometabolic disease.

The present study aimed to investigate the association between a wide range of risk factors related to body fat distribution, metabolic syndrome, and lifestyle with incident cardiometabolic disease in people with obesity. Thereby, we aimed to identify risk factors that may help to prevent cardiometabolic disease in individuals with obesity.

METHODS

Study design and study population

The NEO study is a population-based, prospective cohort study of individuals aged 45–65 years, with an oversampling of individuals with overweight or obesity. Men and women aged between 45 and 65 years with a self-reported BMI of 27 kg/m^2 or higher, living in the greater area of Leiden (in the West of the Netherlands) were eligible to participate in the NEO study. In addition, all inhabitants aged between 45 and 65 years from one municipality (Leiderdorp) were invited, irrespective of their BMI.

Recruitment of participants started in September 2008 and completed at the end of September 2012. In total, 6671 participants have been included, of whom 5217 with a BMI of 27 kg/m^2 or higher. The study design and population are described in detail elsewhere (14).

Participants were invited to visit the NEO study center of the LUMC for a baseline study visit after an overnight fast and were asked to bring all medication they were using in the month preceding the study visit. Prior to the study visit, participants completed a general questionnaire at home to report demographic, lifestyle and clinical information.

At the study center, participants underwent an extensive physical examination, including anthropometry and blood sampling. In addition, participants completed a screening form, asking about anything that might create a health risk or interfere with magnetic resonance imaging, e.g., presence of metallic devices, claustrophobia and a body circumference > 1.70 m. Of the eligible participants, 2580 participants were randomly selected to undergo magnetic resonance imaging (MRI).

During ten years of follow-up, new diagnoses of cardiometabolic diseases have been collected by extraction of medical data from the electronic health records of general practitioners. Time of follow-up was defined as the number of days between the baseline of the study and the date of diagnosis, or censoring due to death, loss to follow-up, or the end of the follow-up (extraction date at the GP in 2018), whichever came first.

For the present study, participants with a medical history of myocardial infarction (n=114), cerebrovascular accident (n=128) or type 2 diabetes at baseline (n=618) were excluded from the analyses. In addition, individuals who did not come to the study center in a fasting state (n=21) were excluded, as well as 120 participants who were lost to follow-up.

The Medical Ethical Committee of the LUMC approved the NEO study and all participants provided written informed consent.

Risk factors for cardiometabolic disease in a population with obesity

Risk factors related to body fat distribution

Body height without shoes was measured with a vertically fixed, calibrated tape measure. Body weight was measured and percent body fat was estimated by the Tanita bio impedance balance (TBF-310, Tanita International Division, United Kingdom) without shoes and 1 kg was subtracted to correct for weight of clothing. BMI at baseline was calculated by dividing the weight in kg by the height in meters squared. Weight gain during adulthood was calculated by subtracting recalled BMI at age 20 years from BMI at middle age, as measured during the baseline visit of our study. We calculated relative weight gain as (body weight at middle age (kg) – body weight at age 20 years (kg)) / body weight at age 20 years (kg) * 100. Waist circumference was measured between the border of the lower costal margin and the iliac crest with the precision of 0.1 cm.

Visceral adipose tissue was directly assessed by MRI (1.5 Tesla MR imaging, Philips Medical Systems, Best, Netherlands) using a turbo spin echo imaging protocol (300/20; flip angle, 90º; section thickness, 10 mm, section gap, 2 mm). At the level of the fifth lumbar vertebra, three transverse slices were obtained during one breath-hold. Visceral fat areas were quantified by converting the number of pixels to centimetres squared for all three slices and totalling the areas of the three sections, using in-house-developed software (MASS; Leiden

University Medical Center, Leiden, the Netherlands). The mean of visceral adipose tissue content was used in the analyses.

Hepatic triglyceride content was quantified using ^{1}H-magnetic resonance spectroscopy of the liver. An 8 mL voxel was positioned in the right lobe of the liver, avoiding gross vascular structures and adipose tissue depots. Sixty-four averages were collected with water suppression (repetition time = 2900 ms; echo time = 23 ms [2900/23]). Without changing any parameters, spectra without water suppression, with a repetition time of 10 s and with four averages were obtained as internal reference. Hepatic triglyceride content relative to water was calculated as (signal amplitude of triglyceride)/(signal amplitude of water) × 100. Fatty liver was defined as a hepatic triglyceride content of ≥5.56% (15).

Metabolic risk factors
Systolic and diastolic blood pressure were obtained by an OMRON™ digital sphygmomanometer at the left arm. Fasting blood samples were drawn from the antecubal vein after 5 min rest of the participant, after an overnight fast of at least 10 h. Fasting plasma glucose and serum cholesterol and triglyceride concentrations were determined using standard clinical chemistry methods (Roche Modular P800 Analyzer, Roche Diagnostics, Mannheim, Germany) in the central clinical chemistry laboratory of the LUMC (14). Serum LDL cholesterol was calculated using the Friedewald formula (16).

Concentrations of CRP were determined using a high sensitivity CRP assay (TINA-Quant CRP HS system, Roche, Germany and Modular P800, Roche, Germany).

Lifestyle risk factors
Habitual dietary intake of all participants was estimated using a semiquantitative self-administered 125-item Food Frequency Questionnaire (FFQ) (17, 18). In this questionnaire, participants reported their frequency of intake of foods during the past month (times per day, week, month, never). This was combined with the assessment of serving size (spoons of potatoes, pieces of fruit, etc). Dietary intake of nutrients and total energy was estimated using the Dutch Food Composition Table (NEVO-2011). Based on the FFQ, we calculated the adapted version of the Dutch Healthy Diet(DHD)-index for each participant, which is a continuous score ranging from 0 and 130 and represents the adherence to the Dutch Guidelines for Healthy Diet of 2015 as described by the Health Council of the Netherlands and originally consists of fifteen components (19). A higher score means a better adherence to the 2015 Dutch Guidelines for a Healthy Diet. Alcohol consumption was reported in the FFQ and expressed as grams of alcohol consumed per day.

Tobacco smoking habits were reported and divided into three categories: current smoker, former smoker, and never smoker. Participants reported the frequency and duration of their physical activity during leisure time on the Short Questionnaire to Assess Health-enhancing activity (SQUASH), which we expressed in MET-hours per week

Incident cardiometabolic disease
New diagnoses of cardiometabolic disease, including type 2 diabetes mellitus, myocardial infarction and cerebrovascular accident, were extracted from electronic health records of the participants. Extraction was based on 1) the International Classification of Primary

Care (ICPC), coding used by GPs to indicate health problems, 2) screening of predefined key words in the descriptions in the GP database, and 3) prescription of specific medication, registered according to the Anatomical Therapeutic Chemical (ATC) codes or by screening medication names (only for type 2 diabetes and cerebrovascular accident). The index date was defined as the first date of an ICPC-coded diagnosis, strong indication for the diagnosis based on key words or prescription of relevant medication.

Diagnosis of type 2 diabetes mellitus was indicated by T90 (diabetes mellitus), T90.02 (diabetes mellitus type 2) or the presence of any keywords (e.g., synonyms of (type 2) diabetes mellitus). In addition, the medication list of participants was checked for the use of insulin, metformin and sulfonylurea derivative; participants using these medications were considered to have diabetes mellitus type 2.
Diagnosis of myocardial infarction was indicated by ICPC K75 (recent myocardial infarction) or K76.02 (myocardial infarction > 4 weeks ago) or presence of any keywords. Keywords included synonyms of myocardial infarction, presence of chest pain, or mention of cardiovascular surgery procedures such as coronary artery bypass grafting (CABG) and angioplasty. In case only a key word was found and an ICPC was lacking in the search, the electronic health record was manually checked to confirm the diagnosis.

Diagnosis of cerebrovascular accident was indicated by K90 (cerebrovascular accident), or one of its subtypes K90.01 (subarachnoid haemorrhage), K90.02 (intracerebellar haemorrhage) or K90.03 (cerebral infarction), or presence of keywords. Keywords included synonyms of cerebrovascular accident or haemorrhage. The medication list of participants was checked for the use of specific anticoagulants.

In case of uncertainty, the general practitioner of the participants was contacted to confirm the (date of) diagnosis.

Statistical analyses
The characteristics of the study population were expressed as percentages, mean and standard deviation or median and interquartile range, stratified by BMI <27 kg/m^2 and BMI ≥27 kg/m^2 based on the inclusion criteria of the NEO study. We first examined risk factors in association with cardiometabolic disease in individuals with BMI≥27 kg/m^2, and subsequently examined the interactions between the risk factors and obesity (BMI>30 kg/m^2) in relation to risk of cardiometabolic disease.

We calculated the incident rates with 95% confidence interval of type 2 diabetes, myocardial infarction, cerebrovascular accident and the composite outcome cardiometabolic disease. Second, we tested for the proportional hazard assumption by making log-log plots. Cox proportional hazards models were used to calculate hazard ratios (HRs) with 95% confidence intervals to examine the association between the risk factors and the composite outcome in the population with BMI 27 kg/m^2 or higher. Results were presented as the hazard ratios with the accompanying 95% confidence intervals, with the reference category coded as the category with the lowest risk.

Because some individuals might experience more than one event, we first analysed the oc-

currence of first ever cardiometabolic disease event. In addition, we analysed the separate outcomes using cause-specific hazards (20), where cases were censored at the development of the first event. For example, some participants might be diagnosed with type 2 diabetes, and later during follow-up suffer from a myocardial infarction. In this example, the participants were counted as cases in the cause-specific hazard of type 2 diabetes (first event), but were censored in the cause-specific hazard of myocardial infarction.

Cox proportional hazards models were used to examine the presence of interaction between the relation between obesity and risk factors in relation to the risk of cardiometabolic disease on an additive scale. Continuous exposures were standardised using the population mean and standard deviation. Results were presented as the hazard ratios with the accompanying 95% confidence intervals, with the reference category coded as the category with the lowest. By this means, the reference category was the exposure to both preventive factors. The cut-offs (either clinical cut-off or median-based) of the risk factors can be found in **Supplementary Table 1**.

Based on these analyses, we calculated the synergy index (SI), which can be interpreted as the excess risk from exposure to both exposures jointly, relative to the risk from exposure without presence of interaction (21, 22). Synergy index can then be calculated as follows:
$S= [HR^{++}] - 1]/[HR^{+-} - 1)+(HR^{-+} - 1)]$.

As a sensitivity analysis, we excluded all participants with missing data on lifestyle factors or metabolic risk factors, and repeated the interaction analyses for all exposures, adjusted for sex and age. In all analyses, we adjusted the first model for sex and age, and the second model was additionally adjusted for BMI. In our multivariable-adjusted model, we additionally adjusted the analyses with exposures related to body fat distribution for lifestyle factors included in our analyses (Dutch Healthy Diet Index, physical inactivity, alcohol consumption and smoking). The analyses with exposures related to metabolic syndrome were additionally adjusted for lifestyle factors and measures of body fat distribution (BMI, adult weight gain, total body fat, waist circumference, visceral fat and liver fat) (see **Figure 1**).

Analyses were performed with STATA Statistical Software version 14.1 (Statacorp, College Station, TX, USA). Interactions and corresponding Synergy indices and 95% confidence intervals were calculated using the icp tool in STATA.

Figure 1. Hypothesis path diagram depicting the associations between lifestyle factors, body fat distribution, metabolic syndrome and cardiometabolic disease. Analyses with one of the measures of body fat distribution as exposure and cardiometabolic disease as outcome were adjusted for all lifestyle factors. Analyses with one of the metabolic risk factors as exposure and cardiometabolic disease as outcome were adjusted for all lifestyle factors and body fat distribution measures. All analyses will be adjusted for sex and age.

RESULTS

Characteristics of the study population

The characteristics of the study population at risk stratified by BMI<27 kg/m^2 and BMI≥27 kg/m^2 are presented in **Table 1**. In the present study, 1360 participants had a BMI lower than 27 kg/m^2 (59% women) and 4310 had a BMI of 27 kg/m^2 or higher (53% women). In both groups, the mean (SD) age was 56 (6) year.

The mean (SD) body mass index in the group with BMI<27 kg/m^2 was 24.3 (17.2 – 26.9) kg/m^2, and 31.7 (27.0 – 61.2) kg/m^2 in the group with BMI≥27 kg/m^2. Overall, participants with BMI<27 kg/m^2 adhered more to the dietary guidelines, were more physically active, but consumed more alcohol. Metabolic factors were more favourable in individuals with BMI<27 kg/m^2 than in individuals with BMI≥27 kg/m^2, for example fasting glucose was lower (5.3 [0.8] mmol/l and 5.8 [1.2] mmol/l), as was the mean systolic blood pressure (128.8 [17.0] mmHg and 133.6 [16.9] mmHg).

Table 1. Characteristics of the Netherlands Epidemiology of Obesity study, stratified by body mass index (BMI)<27 kg/m^2 and BMI≥27 kg/m^2 (n=5670)

Characteristic	**BMI<27.0 kg/m^2**	**BMI≥27.0 kg/m^2**
	n=1360	n=4310
Sex (% men)	41	47
Age (years)	55.6 (6.1)	55.5 (6.0)
Ethnicity (% white)	95	95
Education (% high)	50	35
Body fat distribution		
Body Mass Index (kg/m^2)	24.3 (2.2)	31.5 (3.9)
Relative weight gain since age 20 years (%)	15.5 (12.9)	34.6 (19.0)
Body fat (%)	M: 22.3 (3.6) / W: 34.4 (5.1)	M: 30.2 (5.1) / W: 44.3 (3.9)
Waist circumference (cm)	M: 93.2 (6.7) / W: 82.2 (8.5)	M: 108.5 (9.2) / 102.0 (10.5)
Visceral adipose tissue (cm^2)[a]	M: 95.1 (44.5) / W: 54.5 (29.5)	M: 151.1 (57.6) / W: 109.9 (48.0)
Hepatic triglyceride content (%)[b]	M: 2.8 (1.8 – 5.3) / W: 1.5 (1.0 – 3.2)	M: 7.4 (3.7 – 15.0) / W: 4.4 (1.3 – 10.5)
NAFLD (>5.56% HTGC)	M: 8 W: 5	M: 22 W: 12
Metabolic factors		
Mean systolic blood pressure (mmHg)	128.5 (17.0)	133.2 (16.7)
Mean diastolic blood pressure (mmHg)	82.0 (10.2)	85.8 (10.1)
Hypertension (% yes)	30	43
Waist circumference (cm)	M: 8 W: 5	M: 8 W: 5
Blood pressure-lowering medication (%)	17	29
Triglycerides (mmol/L)	0.9 (0.7 – 1.3)	1.4 (1.0 – 1.9)
HDL-C (mmol/L)	1.6 (0.5)	1.4 (0.4)
LDL-C (mmol/L)	4.5 (1.2)	5.1 (1.3)
Lipid-lowering medication (%)[c]	6	11
Fasting glucose (mmol/L)	5.3 (0.7)	5.6 (0.7)
hsCRP (mg/L)	0.9 (0.5 – 1.8)	1.9 (1.1 – 3.8)
Triglycerides (mmol/L)	0.9 (0.7 – 1.3)	1.4 (1.0 – 1.9)
Lifestyle factors		
Dutch Healthy Diet Index (score)	71.7 (15.0)	68.4 (14.5)
Physical activity (MET/week)[d]	30.5 (16.8 – 50.8)	26.3 (12.5 – 46.0)
Alcohol consumption (grams per day)	10.0 (3.2 – 21.0)	9.1 (1.7 – 22.2)
Smoking (% current)	16	16

[a] n=2,258. [b] n=1,854 [c] Use of lipid-lowering medication included fibrates and statins. [d] Physical activity during leisure time. Abbreviations: BMI, body mass index; HOMA-IR, homeostatic model assessment insulin resistance; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol. Data are presented as mean (SD or range), median (25th–75th percentile) or percentage.

Associations of risk factors with incident cardiometabolic disease in a population with BMI≥27 kg/m²

During a median of 6.7 years of follow-up, 383 participants experienced a first event of cardiometabolic disease. The population at risk was n=5670 with 37034 person-years, resulting in an incidence rate of 1.034 per 100 person years. Of this population, the incidence rate was 0.48 per 100 person-years in individuals with BMI<27 kg/m², and 1.19 in individuals with BMI≥27 kg/m² per 100 person years (**Table 2**).

Table 2. Population at risk, their time at risk, the number of events and incidence of cardiometabolic disease (type 2 diabetes mellitus, myocardial infarction and cerebrovascular accidents) in ten years of follow-up in the NEO study.

Event	Population at risk (n)	Follow-up time (person-years)	Number of events	Incidence rate (per 100 py)
Cardiometabolic disease	5670	37034.2	383	1.03
BMI<27 kg/m²	1360	8268.6	40	0.48
BMI≥27 kg/m²	4310	28765.6	343	1.19

Abbreviations: BMI, body mass index.

Table 3 shows the association between the standardised risk factors and incident cardiometabolic disease in the population with BMI≥27 kg/m². Overall, we observed that all risk factors related to body fat distribution were associated with the risk of incident cardiometabolic disease, also after taking into account BMI at baseline. For example, one standard deviation (57.2 cm²) in visceral adipose tissue was associated with 1.45 (95% CI 1.21 – 1.75) fold increased risk of cardiometabolic disease. In addition, metabolic factors were associated with the risk of incident cardiometabolic disease. For example, one standard deviation (0.9 mmol/L) in fasting glucose was associated with 2.24 (95% CI 2.09 – 2.39) fold increased risk of cardiometabolic disease. Lifestyle was not or weakly associated with the risk of cardiometabolic disease (HR 0.90 [95% CI 0.81 – 1.00]) per standard deviation (14.5 points) in Dutch Healthy Diet index.

After adjustment for lifestyle factors and measures of body fat distribution, results were similar. The results for the cause-specific analyses were similar (data not shown).

Table 3. The association between the risk factors (standardised using the population standard deviation) and incident cardiometabolic disease in an population with BMI ≥27.0 kg/m^2, n=4310

Exposure	Standard deviation (SD)	Hazard ratio (all events)[1]	Hazard ratio (all events, adjusted for sex, age, BMI at baseline)	Hazard ratio (all events, multivariable-adjusted analysis)[2]
Body fat distribution				
Body mass index (kg/m^2)	3.9	1.63 (1.49 – 1.79)	-	1.60 (1.46 – 1.76)
Adult weight gain (%)	19.0	1.40 (1.28 – 1.52)	1.11 (1.00 – 1.24)	1.15 (0.93 – 1.43)
Total body fat (%)	8.3	2.16 (1.83 – 2.55)	1.41 (1.09 – 1.82)	1.32 (1.01 – 1.73)
Waist circumference (cm)	10.5	1.76 (1.57 – 1.96)	1.37 (1.11 – 1.70)	1.28 (1.03 – 1.59)
Change in weight (%, range)	−7.6 (−32.2; −5.8)	1.9 (−4.9; 4.8)	14.9 (5.0; 24.9)	32.3(25.0; 49.8)
Visceral adipose tissue (cm^2)	57.2	1.73 (1.48 – 2.02)	1.45 (1.21 – 1.75)	1.42 (1.18 – 1.72)
Hepatic triglyceride content (%)	9.9	1.63 (1.43 – 1.86)	1.54 (1.34 – 1.77)	1.48 (1.30 – 1.70)
Metabolic risk factors				
Systolic blood pressure (mmHg)	16.7	1.28 (1.15 – 1.41)	1.23 (1.11 - 1.37)	1.26 (1.02 – 1.56)
Diastolic blood pressure (mmHg)	10.1	1.19 (1.08 – 1.32)	1.15 (1.04 – 1.28)	1.12 (0.91 – 1.39)
Triglycerides (mmol/L)	1.0	1.20 (1.14 – 1.26)	1.19 (1.13 – 1.25)	1.13 (0.98 – 1.30)
HDL-c (mmol/L)	0.4	0.58 (0.50 – 0.67)	0.63 (0.54 – 0.73)	0.68 (0.50 – 0.92)
LDL-c (mmol/L)	0.9	1.27 (1.15 – 1.40)	1.28 (1.16 – 1.41)	1.20 (1.00 – 1.44)
Glucose (mmol/L)	0.7	2.40 (2.26 – 2.56)	2.24 (2.09 – 2.39)	1.78 (1.58 – 2.01)
hsCRP (mg/L)	3.4	1.41 (1.31 – 1.51)	1.32 (1.22 – 1.43)	1.36 (1.18 – 1.57)
Lifestyle factors				
Smoking (yes)	-	1.38 (1.09 – 1.76)	1.40 (1.10 – 1.78)	-
Dutch Healthy Diet Index (score)	14.5	0.89 (0.80 – 0.99)	0.90 (0.81 – 1.00)	-
Physical inactivity (MET/h)	33.1	0.87 (0.78 – 0.98)	0.90 (0.80 – 1.00)	-
Alcohol consumption (grams per day)	17.8	1.03 (0.93 – 1.14)	1.06 (0.97 – 1.17)	-

[1]All analyses were adjusted for sex and age; [2]The analyses with exposures related to body fat distribution were adjusted for lifestyle factors (Dutch Healthy Diet Index, physical inactivity, alcohol consumption and smoking); the analyses with exposures related to metabolic syndrome were adjusted for lifestyle factors and body fat distribution (BMI, adult weight gain, body fat percentage, waist circumference, visceral fat and liver fat

Interaction between obesity and risk factors in relation to incident cardiometabolic disease
Table 4A to **4C** show the interaction analyses and corresponding Synergy Indices for the risk factors related to body fat distribution, metabolic syndrome and lifestyle, and obesity defined as BMI≥30 kg/m^2. Of this study population, 42% had a BMI of 30 kg/m^2 or higher.

Compared with non-smokers with a BMI below 30 kg/m^2, the combination of obesity and smoking was associated with an increased risk of cardiometabolic disease (HR; 95% CI 2.93; 2.16 – 3.98) with corresponding Synergy Index of 2.29 (1.04 – 5.04). Individuals with obesity and with a fasting plasma glucose concentration of 7.0 mmol/L or higher had an 8.32 (6.25 – 11.35) fold increased risk of cardiometabolic disease compared with individuals without obesity and with normal glucose concentrations. For obesity and glucose concentrations we observed additive interaction as well, based on the Synergy Index of 2.28 (1.63 – 3.18). Finally, individuals with obesity and excess visceral fat (based on sex-specific median levels) had an increased risk of cardiometabolic disease. In addition, excess visceral adipose tissue showed additive interaction with obesity on the risk of cardiometabolic disease compared with individuals without obesity and without excess visceral adipose tissue (HR: 4.16 [2.75 – 6.30] Synergy Index 2.66 [0.94 – 7.58]).

After adjustment for lifestyle factors and measures of body fat distribution, results were similar, as well as for the complete case analyses (**Supplementary Table 2A-2C**).

Table 4A. Interaction analysis between BMI lower or higher than 30 kg/m^2 and protective factors related to body fat distribution (reference category = BMI<30 g/m^2 and lowest risk category of protective factor), n=5697.

Exposure	Hazard ratio (all events)	Hazard ratio (all events, adjusted for lifestyle factors)[1]
Adult weight gain (%, ref = no excess weight gain)	1	1
Synergy Index	1.51 (0.85 – 2.69)	1.46 (0.83 – 2.57)
No excess weight gain and BMI≥30.0 kg/m^2	2.08 (1.45 – 2.98)	2.11 (1.47 – 3.05)
Excess weight gain and BMI<30.0 kg/m^2	1.34 (0.94 – 1.90)	1.38 (0.96 – 1.96)
Excess weight gain and BMI≥30.0 kg/m^2	3.14 (2.42 – 4.07)	3.18 (2.43 – 4.15)
Body fat percentage (ref = low body fat)	1	1
Synergy Index	1.03 (0.19 – 5.45)	0.76 (0.15 – 3.76)
Low body fat and BMI≥30 kg/m^2	2.96 (0.71 – 12.41)	3.84 (0.91 – 16.12)
High body fat and BMI<30 kg/m^2	1.54 (1.03 – 2.30)	1.51 (1.01 – 2.32)
High body fat and BMI≥30 kg/m^2	3.57 (2.45 – 5.21)	3.57 (2.41 – 5.27)
Waist circumference (ref = high waist circumference)	1	1
Synergy Index	1.07 (0.71 – 1.61)	1.09 (0.71 – 1.67)
Low waist circumference and BMI≥30.0 kg/m^2	2.58 (1.94 – 3.43)	2.55 (1.91 – 3.41)
High waist circumference and BMI<30.0 kg/m^2	1.83 (1.16 – 2.86)	1.69 (1.07 – 2.66)
High waist circumference and BMI≥30.0 kg/m^2	3.56 (2.59 – 4.89)	3.44 (2.49 – 4.74)
Visceral adipose tissue (cm^2, ref = low VAT)	1	1
Synergy Index	3.06 (0.79 – 11.89)	3.15 (0.74 – 13.32)
Low VAT and BMI≥30.0 kg/m^2	1.45 (0.72 – 2.91)	1.45 (0.72 – 2.92)
High VAT and BMI<30.0 kg/m^2	1.45 (0.85 – 2.48)	1.40 (0.81 – 2.43)
High VAT and BMI≥30.0 kg/m^2	3.76 (2.46 – 5.76)	3.68 (2.38 – 5.69)
Non-alcoholic fatty liver disease (%, ref = no NAFLD)	1	1
Synergy Index	1.77 (1.00 – 3.13)	1.75 (1.00 – 3.08)
No NAFLD and BMI≥30.0 kg/m^2	2.39 (1.89 – 3.01)	2.39 (1.89 – 3.03)
NAFLD and BMI<30.0 kg/m^2	1.04 (0.63 – 1.71)	1.11 (0.67 – 1.83)
NAFLD and BMI ≥30.0 kg/m^2	3.53 (2.60 – 4.79)	3.63 (2.67 – 4.95)

All analyses were adjusted for sex and age, where applicable. [1]Additionally adjusted for lifestyle factors (Dutch Healthy Diet Index, physical inactivity, alcohol consumption and smoking)

Table 4B. Interaction analysis between BMI lower or higher than 30.0 kg/m^2 and metabolic factors (reference category = BMI<30.0 kg/m^2 and lowest risk category of protective factor), n=5697

Exposure	Hazard ratio (all events)	Hazard ratio (all events, adjusted for lifestyle factors)[1]
Blood pressure (mmHg, ref = low blood pressure)	1	1
Synergy Index	0.98 (0.63 – 1.51)	0.57 (0.21 – 1.58)
Low blood pressure and BMI≥30.0 kg/m^2	3.21 (2.19 – 4.70)	2.82 (1.08 – 7.35)
High blood pressure and BMI<27 kg/m^2	2.52 (1.60 – 3.97)	2.84 (1.08 – 7.46)
High blood pressure and BMI≥30.0 kg/m^2	4.64 (3.17 – 6.79)	3.10 (1.15 – 8.39)
Triglycerides (mmol/L, ref = low triglycerides)	1	1
Synergy Index	1.72 (0.75 – 3.97)	0.58 (0.11 – 3.15)
Low triglycerides and BMI≥30.0 kg/m^2	2.74 (2.04 – 3.67)	2.27 (1.00 – 5.15)
High triglycerides and BMI<30.0 kg/m^2	1.57 (0.68 – 3.61)	3.50 (0.99 – 12.39)
High triglycerides and BMI≥30.0 kg/m^2	4.96 (2.96 – 8.31)	3.18 (0.98 – 10.29)
HDL-c (mmol/L, ref = high HDL-c)	1	1
Synergy Index	1.63 (1.07 – 2.50)	1.80 (0.38 – 8.43)
High HDL-c and BMI≥30.0 kg/m^2	2.34 (1.81 – 3.02)	1.67 (0.91 – 3.05)
Low HDL-c and BMI<30.0 kg/m^2	1.95 (1.32 – 2.86)	1.13 (0.48 – 2.63)
Low HDL-c and BMI≥30.0 kg/m^2	4.74 (3.59 – 6.25)	2.43 (1.25 – 4.72)
LDL-c (mmol/L, ref = low LDL-c)	1	1
Synergy Index	1.20 (0.82 – 1.76)	1.67 (1.14 – 5.04)
Low LDL-c and BMI≥30.0 kg/m^2	2.71 (1.94 – 3.78)	1.58 (0.70 – 3.55)
High LDL-c and BMI<30.0 kg/m^2	1.51 (1.08 – 2.13)	1.25 (0.61 – 2.57)
High LDL-c and BMI≥30.0 kg/m^2	3.68 (2.71 – 5.00)	2.39 (1.14 – 5.04)
Glucose (mmol/L, ref = low glucose)	1	1
Synergy Index	2.39 (1.66 – 3.46)	1.88 (0.94 – 3.74)
Low glucose and BMI≥30.0 kg/m^2	1.66 (1.13 – 2.42)	1.76 (0.75 – 4.12)
High glucose and BMI<30.0 kg/m^2	3.11 (2.20 – 4.40)	3.58 (1.72 – 7.46)
High glucose and BMI≥30.0 kg/m^2	7.62 (5.63 – 10.31)	7.27 (3.33 – 15.88)
hsCRP (mg/L, ref = low hsCRP	1	1
Synergy Index	1.60 (1.04 – 2.46)	1.20 (0.47 – 3.05)
Low hsCRP and BMI≥30.0 kg/m^2	2.17 (1.58 – 2.97)	1.83 (0.90 – 3.70)
High hsCRP and BMI<30.0 kg/m^2	1.77 (1.26 – 2.49)	1.73 (0.87 – 3.44)
High hsCRP and BMI≥30.0 kg/m^2	4.10 (3.13 – 5.38)	2.86 (1.44 – 5.68)

All analyses were adjusted for sex and age, where applicable. [1]Additionally adjusted for lifestyle factors and body fat distribution (BMI, adult weight gain, body fat percentage, waist circumference, visceral fat and liver fat).

Table 4C. Interaction analysis between BMI lower or higher than 30.0 kg/m^2 and lifestyle factors (reference category = BMI<30.0 kg/m^2 and lowest risk category of protective factor), n=5697

Exposure	Hazard ratio (all events)
Dutch Healthy Diet Index (score, ref = high DHDI)	1
Synergy Index	1.36 (0.80 – 2.30)
High DHDI and BMI≥30.0 kg/m^2	2.32 (1.71 – 3.14)
Low DHDI and BMI<30.0 kg/m^2	0.96 (0.69 – 1.35)
Low DHDI and BMI≥30.0 kg/m^2	2.74 (2.06 – 3.64)
Physical activity (MET/h, ref = physically active)	1
Synergy Index	1.48 (0.91 – 2.41)
Physically active and BMI≥30.0 kg/m^2	2.37 (1.74 – 3.22)
Physically inactive and BMI<30.0 kg/m^2	1.09 (0.77 – 1.53)
Physically inactive and BMI≥30.0 kg/m^2	3.16 (2.36 – 4.22)
Alcohol consumption (ref= low alcohol consumption)	1
Synergy Index	1.08 (0.61 – 1.92)
Low alcohol consumption and BMI ≥30.0 kg/m^2	2.33 (1.75 – 3.11)
High alcohol consumption and BMI<30.0 kg/m^2	0.76 (0.54 – 1.06)
High alcohol consumption and BMI≥30.0 kg/m^2	2.17 (1.61 – 2.93)
Smoking (ref = smoking no)	1
Synergy Index	2.52 (1.00 – 6.34)
Not smoking and BMI≥30.0 kg/m^2	1.79 (1.22 – 2.61)
Smoking and BMI<30.0 kg/m^2	0.96 (0.68 – 1.36)
Smoking and BMI≥30.0 kg/m^2	2.89 (2.12 – 3.95)

All analyses were adjusted for sex and age, where applicable

DISCUSSION

The aim of the present study was to identify risk factor that may help to prevent cardiometabolic disease in people with obesity. To that extent, we investigated a wide range of established risk factors related to body fat distribution, metabolic factors, and lifestyle factors in relation to incident cardiometabolic diseases in middle-aged people with obesity during a maximum of 10 years of follow-up. In a population with BMI≥27 kg/m^2, a favourable body fat distribution and healthy metabolic profile were strongly associated with a decreased risk of cardiometabolic disease, whereas healthy lifestyle factors were not or only weakly associated with an increased risk of developing cardiometabolic disease. When considering the joint effect of the risk factors and obesity, defined as BMI≥30 kg/m^2, we observed that fasting glucose and smoking showed additive interaction with obesity on the risk of cardiometabolic disease, as well excess visceral fat. These results suggest that that preserving low glucose levels and being a non-smoker, as well as maintaining low visceral adipose tissue will largely reduce the risk of incident cardiometabolic disease in individuals in obesity.

The results of our study demonstrate that within the population with obesity having a favourable body fat distribution, including low body fat, low waist circumference, and low levels of visceral adipose tissue and liver fat, is associated with a decreased risk of cardiometabolic disease. In a previous study using data from the NEO study, we demonstrated that weight gain during adulthood is associated with more visceral adipose tissue at middle age, compared with weight maintenance (23). This suggests that weight maintenance during adulthood plays an important role in preventing accumulation of excess visceral fat and eventually, cardiometabolic disease at middle age and older age. This finding can be explained by the association of excess visceral fat with an array of metabolic abnormalities (3, 8, 9), including systemic low-grade inflammation, insulin resistance, and dyslipidaemia, which might eventually result in an increased risk of cardiometabolic disease (3). In conclusion, our results suggest that both a favourable body fat distribution and metabolic profile contribute to a decreased risk of incident cardiometabolic disease in a population with obesity. These goals could be reached by lifestyle changes, e.g., healthy eating habits and physical exercise, that go hand in hand with weight loss (24). However, lifestyle change has been proven difficult, especially in the modern obesogenic environment (25). This underscores the need for health-promoting population-level interventions and governmental policies promoting a healthy lifestyle (25-27).

From previous literature, it is known that not everyone with obesity will develop cardiometabolic disease, and that metabolic factors, such as blood pressure, cholesterol, triglycerides, glucose concentrations and inflammatory markers likely contribute to this observation (28). This is in line with our results, as these indicate that within the population with obesity having a beneficial metabolic profile, such as low blood pressure, low triglyceride levels, low glucose levels, low high-sensitivity CRP levels and high HDL-cholesterol levels is associated with a decreased risk of cardiometabolic disease. Preserving low glucose levels will even be associated with an additive decrease in the risk of incident cardiometabolic disease in individuals in obesity, compared with individuals with both risk factors present. This might be driven by the large number of type 2 diabetes mellitus cases in the outcome of cardiometabolic disease. However, in addition to type 2 diabetes itself, glucose levels are known

to be robustly and causally associated with the risk of cardiovascular disease, as shown by Mendelian randomization analyses (29, 30). Because the number of events for myocardial infarction and cerebrovascular accident were small, we were not able to investigate the associations between risk factors and the separate outcomes of cardiometabolic disease.

In our study population of individuals with obesity, we did not observe robust associations of lifestyle factors such as dietary intake, physical activity and alcohol consumption with the risk of cardiometabolic disease. This was also the case when the analyses were not adjusted for BMI. Dietary intake, physical activity, and alcohol consumption were all based on self-report. The results of our interaction analyses indicate that being a non-smoker is associated with an additive decrease in the risk of incident cardiometabolic disease in individuals with obesity. This result might be due to an increased propensity of storing body fat centrally in smokers with obesity. It has been shown previously that smoking is associated with a decrease in overall body weight, however not with a decrease in waist circumference (31). In addition, smoking causes higher central adiposity as measured by waist circumference (32), a well-known risk factor of cardiometabolic disease. In addition to an effect on body fat distribution, smoking also has a vascular effect: in Mendelian randomization studies, smoking was associated with risk of type 2 diabetes, ischemic stroke, myocardial infarction, and heart failure (33-36).

Strengths of our study include data on many risk factors and confounding factors, and the availability of a range of directly assessed and accurate measures of body fat distribution such as visceral adipose tissue and hepatic triglyceride content. In addition, follow-up data of ten years on the development of cardiometabolic disease was available. A limitation that needs to be considered is that diagnoses of cardiovascular disease were collected by extraction of medical data from the electronic health records of general practitioners. Although we used a wide range of international codes and keywords to define diagnoses, and we checked uncertain diagnoses with the general practitioner of the study participants to ensure data quality, we might have missed or misclassified some diagnoses or dates of diagnosis. However, in general, extraction of diagnoses of type 2 diabetes mellitus and cardiovascular disease from electronic health records of general practitioners is a valid method to define cases, and is more reliable than self-report (37-39). Due to the small numbers, we were not able to perform analyses stratified by sex. Lastly, the majority of our study population was Caucasian, therefore the results of our study need to be confirmed in other ethnic groups.

In conclusion, our results suggest that both a favourable body fat distribution and metabolic profile contribute to a decreased risk of incident cardiometabolic disease in a population with obesity. Preserving low glucose levels and being a non-smoker, as well as maintaining low visceral fat, showed the largest reduction in cardiometabolic risk in this high-risk population to develop obesity-related disease. Future prospective studies need to investigate how to integrate interventions to improve body fat distribution, metabolic factors and lifestyle in the population in obesity to reduce the risk of future cardiometabolic disease.

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CONFLICT OF INTEREST

All authors declare that there is no conflict of interest associated with this manuscript.

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Supplementary Table 1. Coding of independent variables in binaries for interaction analyses.

Primary exposure	Definition	Coding
Body fat distribution		
Obesity	<30.0 kg/m^2	0 (ref)
	≥30.0 kg/m^2	1
Relative weight change	Below median in NEO study (<27.4%)	0 (ref)
	Above median in NEO study ≥27.4%	1
Total body fat	Men ≤25%, women ≤35%	0 (ref)
	Men >25%, women >35%	1
Normal waist circumference	Men <102 cm, women <88 cm	0 (ref)
	Men ≥102 cm, women ≥88 cm	1
Visceral adipose tissue	Below median in NEO study (men<132 cm^2 and women <86 cm^2)	0 (ref)
	Above median in NEO study (men≥132 cm^2 and women≥86 cm^2)	1
Non-alcoholic fatty liver disease	<5.56% liver fat content	0 (ref)
	≥5.56% liver fat content	1
Metabolic syndrome		
Blood pressure	Systolic <130 mmHg or diastolic <85 mmHg and no use of anti-hypertensive agents	0 (ref)
	Systolic ≥130 mmHg or diastolic ≥85 mmHg or use of anti-hypertensive agents	1
Triglyceride levels	<1.7 mmol/L and no use of lipid-lowering agents	0 (ref)
	≥1.7 mmol/L or use of lipid-lowering agents	1
HDL-cholesterol	≥1.0 mmol/L in men and ≥1.3 mmol/L in women and no use of medication for reduced-HDL	0 (ref)
	<1.0 mmol/L in men and <1.3 mmol/L in women or use of medication for reduced-HDL	1
LDL-cholesterol	Below median in NEO study (<4.9 mmol/L)	0 (ref)
	Above median in NEO study (≥4.9 mmol/L)	1
Normal fasting glucose	≤5.6 mmol/L	0 (ref)
	>5.6 mmol/L	1
High sensitivity (hs)-CRP	≥2.0 mg/L	1
Lifestyle factors		
Dutch Healthy Diet Index (score)	Above median in NEO study (≥69)	0 (ref)
	Below median in NEO study (<69)	1
Alcohol consumption	Below median in NEO study (men<16.6 g/day, women<5.2 g/day)	0 (ref)
	Above median in NEO study (men≥16.6 g/day, women≥5.2 g/day)	1
Smoking	Never smoking	0 (ref)
	Current smoking or former smoking	1

Abbreviations: NEO, Netherlands Epidemiology of Obesity; HDL, high-density lipoprotein; LDL, low-density lipoprotein; hs-CRP, high sensitivity C-reactive protein; MET, metabolic equivalent of task.

Supplementary Table 2A. Complete case interaction analysis between BMI lower or higher than 30.0 kg/m^2 and body fat distribution (reference category = BMI<30.0 kg/m^2 and lowest risk category of risk factor), n=1737, 111 events.

Exposure	Hazard ratio (all events)
Adult weight gain (%, ref = no excess weight gain)	1
Synergy Index	1.72 (0.66 – 4.50)
No excess weight gain and BMI≥30.0 kg/m^2	2.88 (1.53 – 5.43)
Excess weight gain and BMI<30.0 kg/m^2	0.94 (0.44 – 2.03)
Excess weight gain and BMI≥30.0 kg/m^2	4.13 (2.57 – 6.65)
Body fat percentage (ref = low body fat)	1
Synergy Index	0.44 (0.06 – 3.11)
Low body fat and BMI≥30 kg/m^2	13.3 (1.59 – 111.27)
High body fat and BMI<30 kg/m^2	2.18 (0.90 – 5.30)
High body fat and BMI≥30 kg/m^2	6.89 (2.99 – 15.87)
Waist circumference (ref = high waist circumference)	1
Synergy Index	1.28 (0.61 – 2.68)
Low waist circumference and BMI≥30.0 kg/m^2	3.63 (2.18 – 6.02)
High waist circumference and BMI<30.0 kg/m^2	1.44 (0.54 – 3.85)
High waist circumference and BMI≥30.0 kg/m^2	4.94 (2.64 – 9.23)
Visceral adipose tissue (cm^2, ref = low VAT)	1
Synergy Index	3.32 (0.92 – 11.99)
Low VAT and BMI≥30.0 kg/m^2	1.91 (0.86 – 4.26)
High VAT and BMI<30.0 kg/m^2	1.40 (0.70 – 2.78)
High VAT and BMI≥30.0 kg/m^2	5.34 (3.17 – 8.98)
Non-alcoholic fatty liver disease (%, ref = no NAFLD)	1
Synergy Index	2.46 (1.10 – 5.51)
No NAFLD and BMI≥30.0 kg/m^2	2.43 (1.20 – 4.94)
NAFLD and BMI<30.0 kg/m^2	1.83 (0.92 – 3.63)
NAFLD and BMI≥30.0 kg/m^2	6.55 (3.78 – 11.36)

All analyses were adjusted for sex and age.

Supplementary Table 2B. Complete case interaction analysis between BMI lower or higher than 30.0 kg/m^2 and metabolic risk factors (reference category = BMI<30.0 kg/m^2 and lowest risk category of risk factor), n=1737, 111 events.

Exposure	Hazard ratio (all events)
Blood pressure (mmHg, ref = low blood pressure)	1
Synergy Index	0.83 (0.40 – 1.72)
Low blood pressure and BMI≥30.0 kg/m^2	5.89 (2.72 – 12.78)
High blood pressure and BMI<27 kg/m^2	3.22 (1.23 – 8.42)
High blood pressure and BMI≥30.0 kg/m^2	6.93 (3.08 – 15.59)
Triglycerides (mmol/l, ref = low triglycerides)	1
Synergy Index	1.10 (0.33 – 3.68)
Low triglycerides and BMI≥30.0 kg/m^2	4.46 (2.29 – 7.91)
High triglycerides and BMI<30.0 kg/m^2	4.30 (1.24 – 14.98)
High triglycerides and BMI≥30.0 kg/m^2	8.24 (3.17 – 21.41)
HDL-c (mmol/l, ref = high HDL-c)	1
Synergy Index	1.77 (0.89 – 3.53)
High HDL-c and BMI≥30.0 kg/m^2	3.40 (2.10 – 5.51)
Low HDL-c and BMI<30.0 kg/m^2	1.55 (0.67 – 3.57)
Low HDL-c and BMI≥30.0 kg/m^2	6.23 (3.69 – 10.51)
LDL-c (mmol/l, ref = low LDL-c)	1
Synergy Index	1.52 (0.81 – 2.85)
Low LDL-c and BMI≥30.0 kg/m^2	3.71 (1.85 – 7.42)
High LDL-c and BMI<30.0 kg/m^2	1.62 (0.80 – 3.29)
High LDL-c and BMI≥30.0 kg/m^2	6.05 (3.24 – 11.31)
Glucose (mmol/l, ref = low glucose)	1
Synergy Index	2.68 (1.54 – 4.68)
Low glucose and BMI≥30.0 kg/m^2	2.77 (1.28 – 5.97)
High glucose and BMI<30.0 kg/m^2	4.21 (2.04 – 8.73)
High glucose and BMI≥30.0 kg/m^2	14.39 (7.56 – 27.37)
hsCRP (mg/l, ref = low hsCRP	1
Synergy Index	1.66 (0.89 – 3.08)
Low hsCRP and BMI≥30.0 kg/m^2	3.41 (1.87 – 6.22)
High hsCRP and BMI<30.0 kg/m^2	2.02 (1.02 – 4.010
High hsCRP and BMI≥30.0 kg/m^2	6.68 (3.88 – 11.50)

All analyses were adjusted for sex and age.

Supplementary Table 2C. Complete case interaction analysis between BMI lower or higher than 30.0 kg/m^2 and lifestyle factors (reference category = BMI<30.0 kg/m^2 and lowest risk category of risk factor), n=1737, 11 events.

Exposure	Hazard ratio (all events)
Dutch Healthy Diet Index (score, ref = high DHDI)	1
Synergy Index	1.19 (0.59 – 2.39)
High DHDI and BMI≥30.0 kg/m^2	3.53 (1.98 – 6.31)
Low DHDI and BMI<30.0 kg/m^2	0.96 (0.48 – 1.91)
Low DHDI and BMI≥30.0 kg/m^2	3.97 (2.29 – 6.85)
Physical activity (MET/h, ref = physically active)	1
Synergy Index	1.67 (0.80 – 3.50)
Physically active and BMI≥30.0 kg/m^2	3.14 (1.75 – 5.62)
Physically inactive and BMI <30.0 kg/m^2	1.02 (0.51 – 2.030
Physically inactive and BMI ≥30.0 kg/m^2	4.61 (2.67 – 7.97)
Alcohol consumption (ref= low alcohol consumption)	1
Synergy Index	1.28 (0.61 – 2.68)
Low alcohol consumption and BMI ≥30.0 kg/m^2	3.23 (1.79 – 5.82)
High alcohol consumption and BMI <30.0 kg/m^2	0.90 (0.46 – 1.79)
High alcohol consumption and BMI ≥30.0 kg/m^2	4.13 (2.31 – 7.38)
Smoking (ref = smoking no)	1
Synergy Index	1.29 (0.65 – 2.59)
Not smoking and BMI≥30.0 kg/m^2	3.74 (1.79 – 7.82)
Smoking and BMI <30.0 kg/m^2	1.32 (0.64 – 2.72)
Smoking and BMI ≥30.0 kg/m^2	4.96 (2.60 – 9.46)

All analyses were adjusted for sex and age.

CHAPTER 8

General discussion and summary of the main results

GENERAL DISCUSSION AND SUMMARY OF THE MAIN RESULTS

The main objective of this thesis was to study the cardiometabolic consequences of obesity and weight gain during the life course. Here, we will discuss and interpret the findings of the chapters described in this thesis. Furthermore, we will discuss the implications and future perspectives in the field of obesity, body weight gain and cardiometabolic disease.

Summary of main findings

In Mendelian randomization studies, genetic variants associated with an exposure of interest, assigned randomly at conception, are used as an instrumental variable to approximate the association between a life-long exposure to a certain risk factor and an outcome (43). Thereby, Mendelian randomization can be used to approximate a causal association between exposure and outcome using observational data in the absence of residual confounding and reverse causation. The field of Mendelian randomization is rapidly evolving, and novel methodological approaches are published every month in the scientific literature. For example, novel approaches to group genetic variants may provide insights in distinct processes underlying heterogenous, complex traits such as obesity (56, 57). In **Chapter 2**, we designed and applied another novel methodological approach to group BMI-associated genetic variants based on their expression in different tissues in the body. We hypothesized that a high BMI could result in a different cardiometabolic disease risk profile, depending on the underlying processes in different tissues that may have caused the high BMI. We identified 17 tissue-grouped gene sets, where BMI-associated genes were differentially expressed, mostly in several brain areas. These tissue-grouped BMI-associated genetic variants were used as exposures in two-sample Mendelian randomization analyses on cardiometabolic disease and anthropometry measures. We observed that tissue-grouped BMI-associated genetic variants were similarly associated with increased risks of type 2 diabetes and coronary artery disease. This suggests that the grouping of genetic variants based on tissue expression profiles does not yield a different risk profile for type 2 diabetes and coronary artery disease risk. These results were supported by findings from additional analyses, in which we randomly selected 100 or 200 genetic instruments from the 633 BMI-associated genetic variants. After we repeatedly performed Mendelian randomization analyses on T2DM and CAD with randomly sampled BMI-associated gene sets, the distribution of the effect estimates was similar to the results of the tissue-grouped MR analyses. We therefore concluded that our novel approach, based on tissue eQTL expression levels, does not suggest that the cause-specific increase in BMI (depending on the tissue expression level) gives an altered risk of cardiometabolic diseases. However, other novel approaches have been developed by others since we conducted this study, which may provide new and necessary insights to further grasp the heterogeneity of the obesity phenotype in future studies (56, 57). Furthermore, we cannot exclude the possibility that our novel analysis approach could be of value for addressing scientific questions other than the obesity-cardiometabolic disease relationship. A more refined Mendelian randomization analysis, in which more biological data is incorporated, might be an additional step towards a more personalized medicine approach and result in suitable and effective targets for interventions tailored to a specific cause underlying the high BMI. Evidence supporting this hypothesis has been provided recently by others examining insulin-like growth factor 1 (IGF-1) and type 2 diabetes mellitus: depending on certain data-driven clusters, the effect of IGF-1 on type 2 diabetes mellitus

was differential, and genes mapping to the instruments in the different clusters were part of different biological pathways (58).

Obesity in childhood and adolescence tends to carry over into adulthood (11), and there is a window of opportunity to reduce the cardiometabolic consequences of body size that unfold later in life already during childhood. However, it is unknown how body fat distribution during adolescence is associated with subsequent early changes in circulating metabolites. In **Chapter 3**, we investigated the consequences of overall and abdominal obesity at adolescence on changes in metabolomic measures during young adulthood in the Avon Longitudinal Study of Parents and Children (ALSPAC). We observed that abdominal adiposity in adolescence was associated with early changes in metabolomic measures indicative of a pro-atherogenic profile, including higher concentrations of very-low density lipoprotein, higher Apolipoprotein B and lower high density lipoprotein levels, in young adulthood mainly in men. This finding is in line with another prospective study in ALSPAC, in which the authors observed that the atherogenic consequences of adiposity on several metabolomic measures, including LDL cholesterol, triglycerides in VLDL and Apolipoprotein B, were stronger and apparent at a younger age in men than in women (59). These metabolomic measures were linked to adult onset cardiometabolic disease in previous studies. Circulating cholesterol and triglycerides in VLDL and LDL particles, Apolipoprotein B and glucose are strongly and consistently associated with the risk of myocardial infarction (40), whereas MR analyses showed that both LDL cholesterol and triglycerides play a causal role in the development of coronary heart disease (41, 42) as well as Apolipoprotein B (42).

Our results indicated that there is a difference in risk of cardiometabolic disease for men and women, which is already apparent at a young age. Based on these findings, we concluded that adolescence is a critical period for the prevention of adiposity-related changes in atherogenic risk factors in men. It is not completely clear why abdominal adiposity was specifically associated with atherogenic changes in young men and not in young women. One explanation might be that men are more likely to store body fat at the abdomen, whereas women are more likely to store fat at the hips and thighs (19). Abdominal adiposity, in particular visceral fat, is strongly related to metabolic disturbances (23, 30, 60), reflected by increased levels of atherogenic metabolomic measures as observed in our study.

In **Chapter 4, 5 and 6**, we studied several cardiometabolic consequences of body weight gain during adulthood. Body weight gain during adulthood is associated with an increased risk of cardiometabolic disease, possibly through adipocyte hypertrophy. Specific metabolomic alterations have the potential to indicate the onset of cardiometabolic disease as a consequence of adult weight gain. As we described in **Chapter 3**, metabolic alterations present the first indications of cardiometabolic disease in young men and also provide insights into underlying processes. However, the metabolomic profile associated with long-term adult weight gain has not been clearly defined. In **Chapter 4**, we investigated which metabolomic measures were specifically associated with weight gain during adulthood in the NEO study. We observed that adult weight gain, and not BMI at age 20 years nor BMI at middle age, was specifically associated with concentrations of 7 metabolomic measures, which we successfully replicated in Oxford Biobank. These metabolomic measures included omega-3, omega-6, total polyunsaturated fatty acids, small to medium low-density lipoproteins and

total intermediate-density lipoproteins. In addition, adult weight gain was associated with adipocyte size at middle age, and the adult weight gain-specific lipoprotein particles were associated with adipocyte size. Earlier studies have shown the metabolomic measures we found to be specifically associated with adult weight gain and cardiometabolic disease (39-41, 61, 62). Therefore, the results of our study highlight specific biochemical disturbances that could possibly indicate the onset of cardiometabolic disease related to adult weight gain.

We did observe similarities between the metabolomic measures identified to be associated with abdominal adiposity in adolescence (**Chapter 3**) and with weight gain during adulthood (**Chapter 4**). All are metabolomic measures indicative of atherogenic progression. In both studies, abdominal adiposity in adolescence and adult weight gain were strongly associated with the levels of low-density lipoproteins at either young adulthood or middle age, of which it is known to increase the risk of cardiovascular disease (40-42) and is a main target for pharmacological treatment to reduce atherogenic risk. In contrast with the results in **Chapter 3**, we did not observe differences in the association between adult weight gain and metabolomic measures between men and women at middle age in **Chapter 4**. This could be due to differences in study design or population: ALSPAC is a birth cohort study of young adults born in the 1990s and has a longitudinal design with repeated measures of metabolomics over time, whereas in the NEO study we performed a cross-sectional analysis of baseline measurements of middle-aged men and women. Other factors may have played a role as well: as the study in **Chapter 3** was performed in adolescents and young adults, changes in metabolomic measures might also be related to pubertal changes, such as (sex) hormone changes. In addition, adipocytes might respond differently to changes in body weight during young adulthood compared with adulthood or middle age (38). Future perspectives in the field of metabolomics related to body weight gain include prospective analyses of repeated measures of metabolomics in cohorts of middle-aged individuals. By this means, body weight trajectories can be established, which can then be related to outcomes of interest.

Differences in body fat distribution are an important contributor to differences in risk of cardiometabolic disease in individuals with obesity (23). During adult weight gain, excess adipose tissue is stored in different areas of the body, however only few studies investigated the depots in which body fat is preferentially stored during adult weight gain. In **Chapter 5**, we investigated the association between adult weight change and several measures of abdominal adiposity (waist circumference, abdominal subcutaneous adipose tissue, and visceral adipose tissue) and liver fat at middle age, taking into account overall body fat. In this study, we consistently observed that weight gain during adulthood was associated with a relatively higher amount of visceral fat and liver fat at middle age within all BMI categories at age 20 years. This implies that weight maintenance during adulthood plays an important role in limiting excess visceral fat and liver fat and their detrimental effects on cardiometabolic health. It is well-established that excess visceral fat was associated with increased insulin resistance and diabetes risk (28, 30, 31, 63). Therefore, we hypothesized that the association between adult weight gain and insulin resistance is mediated by the amount of visceral fat and liver fat at middle age. In **Chapter 6**, we confirmed that a small gain in body weight during adulthood was already associated with more insulin resistance compared with weight maintenance during adulthood. Stronger associations with insulin resistance were observed for more excessive weight gain during adulthood. In addition, when

we considered the mediating roles of visceral fat, we observed that the association between adult weight gain and insulin resistance at middle age was mediated by visceral fat for 32.0% (95% confidence interval 18.6-45.4) and by liver fat for 22% (95% confidence interval 15.0-30.1%). Our results highlight the importance of weight maintenance during adulthood to prevent the accumulation of excess visceral fat and liver fat and thereby insulin resistance and eventually, type 2 diabetes at middle age and older age.

Importantly, not everyone with obesity will develop cardiometabolic disease, and obesity-related consequences differ between individuals with a similar BMI (18). Apparently, there are additional risk factors needed for obesity to result in disease. In the last chapter of this thesis, **Chapter 7**, we aimed to answer the question which factors are needed for obesity to result in cardiometabolic disease. To this extent, we examined a range of risk factors, including body fat distribution, metabolic factors and lifestyle factors, in relation to incident cardiometabolic disease in ten years of follow-up in participants with a BMI of 27 kg/m^2 or higher in the NEO study. We observed that especially the amount of visceral fat, as well as metabolic factors, including fasting glucose levels and triglycerides, increase the risk of developing cardiometabolic disease in a population with obesity. Notably, lifestyle factors, such as diet quality and physical activity, were not associated with an increased risk of cardiometabolic disease in a population with obesity. When considering the joint effect of the risk factors and obesity, defined as BMI≥30 kg/m^2, we observed that fasting glucose and smoking showed additive interaction with obesity on the risk of cardiometabolic disease, as well visceral adipose tissue. This suggests that that preserving low glucose levels and non-smoking, as well as maintaining low visceral adipose tissue will result in an additive decrease in the risk of incident cardiometabolic disease in individuals with obesity, compared with individuals with both risk factors present. It must be noted that our results do not support the existence of a 'metabolically healthy obesity' phenotype, defined as obesity with absence of metabolic risk factors (18). As shown in a study with up to twenty years of follow-up, healthy obesity appears to be a transient state, with individuals with healthy obesity often naturally progressing to unhealthy obesity (64). In other words, although the risk of cardiometabolic disease was lowest in those with obesity without additional risk factors, it most likely remains increased compared with people without obesity.

Strengths and limitations

Strengths of the studies described in this thesis include the large population-based studies and the availability of precise and accurate measures of body fat distribution, including DXA-derived total fat mass and trunk fat in **Chapter 3**, and abdominal subcutaneous and visceral adipose tissue by MRI and hepatic triglyceride content by ^{1}H-MRS in Chapter **5, 6, and 7**. Additionally, all studies had information on a wide range of potential confounding factors. This enabled us to investigate the specific associations between adult weight gain and several outcomes of interest, taking into account confounding factors.

For a correct interpretation of our results, several limitations need to be considered. For most studies included in this thesis, we made use of data from the Netherlands Epidemiology of Obesity (NEO) study. In the NEO study, adult weight gain between age 20 years and middle age was based on recalled weight at age 20 years, which was reported by questionnaire. Therefore, body weight at age 20 years might be misclassified when participants do

not correctly remember their body weight at age 20 years. Nevertheless, previous studies have shown that recalled body weight is strongly related with measured weight with a similar time interval between measurement and recall as in the NEO study (65). In addition, as we only had information on body weight at two time points (age 20 years and middle age), we had no information on individual body weight trajectories over time (66, 67). For example, an individual gained a certain amount of weight between age 20 years and middle age: this could have happened gradually, or the person could have had a stable body weight for a long period of time, however rapidly gained weight in a short time period around middle age. Weight fluctuations, the so-called 'yo-yo effect', are also common and may result in different disease risks (68).

To investigate potential distinct effects of individual body weight trajectories, we are in need of detailed longitudinal studies in which body weight is frequently assessed, preferably by measuring the body weight at a study centre. By this means, body weight trajectories can be established, which can then be related to outcomes of interest. A limitation of this approach is that it is time-consuming and expensive. For example, the UK-based birth cohort ALSPAC started in the 1990s and the participants had measurements taken regularly (53, 69). However, the participants are now in their late twenties, which means that it will take approximately an additional twenty to thirty years before they will develop outcomes such as type 2 diabetes and cardiovascular disease.

In the NEO study, participants were selected on having a self-reported BMI of 27 kg/m^2 or higher and therefore, weight gain patterns might be different in this overweight population. We compared the distribution of weight gain in the NEO study population to the reference population of Leiderdorp, and observed that the distribution of weight gain was similar. In **Chapter 3** and **4**, we studied the metabolomic profile, derived from a metabolomics platform mostly containing lipoprotein (sub)particles. This platform is rather limited: to investigate other altered metabolomic pathways would require alternative platforms to have a complete overview. Lastly, all studies described in this thesis were performed in study populations predominantly including European men and women, and the results of our study need to be confirmed in other ethnic groups.

The studies about weight gain described in this thesis are all observational studies, and therefore we cannot exclude residual confounding or reverse causation and infer causality. Confounding of results may occur by a common cause of the exposure and the outcome variable, which is not in the causal pathway. In all studies we aimed to control for known and measured confounding factors as good as possible. To investigate the association between adult weight gain and visceral and liver fat at middle age, we adjusted the analyses for an array of confounding factors including ethnicity, educational level, smoking, alcohol consumption, physical activity, use of antidepressants, thyroid medication, corticosteroids or hormonal treatments, and menopausal status. However, residual confounding by unknown, unmeasured or inaccurately measured confounding factors may always remain.

Reverse causation may occur in studies of weight change when an underlying disease leads to weight loss as a result of muscle wasting and changes in muscle composition (70). This process is called sarcopenia, and can also happen in individuals with obesity (obese sar-

copenia). Sarcopenia with would result in an underestimation of the risk associated with obesity and weight gain, even in those with obesity. Nevertheless, our results were similar when we excluded participants with potential unintentional weight loss during adulthood. Therefore, we do not expect a large influence of potential reverse causation on our results. Additionally, reverse causation may occur when we studied the association between adult weight gain and insulin resistance at middle age: the development of insulin resistance may precede a gain in body weight (71, 72). However, weight gain was calculated using recalled weight at age 20 years and measured body weight at middle age, and insulin resistance was assessed at middle age, which indicates temporality.

Previously, studies have been performed in which participants were overfed in order to study the effects of body weight gain. A famous example is a study in identical twins by Bouchard et al., in which differences in response to overfeeding were investigated, as well as the involvement of genotypes in the different responses (73). Randomized controlled trials involving overfeeding also have been performed, however, such studies often assessed differences in dietary composition instead of merely weight gain versus weight maintenance (74). It is highly questionable whether such overfeeding studies are ethical, especially when participants need to be overfed for a long period of time (e.g., for a period comprising years or even decades) of time. As an alternative to randomized controlled trials on body weight gain or obesity, Mendelian randomization analyses can be performed. Mendelian randomization analyses make use of genetic variants associated with the exposure of interest (e.g., BMI) as an instrumental variable, assigned randomly at conception. These instrumental variables can then be related to the outcomes of interest (e.g., type 2 diabetes mellitus, cardiovascular disease) thereby mimicking randomized controlled trials.

Conclusions, implications and future perspectives

The results described in this thesis fit within the framework of the 'lipid overflow' hypothesis (21-23). Shortly, this hypothesis describes that when individuals gain body fat, this will be stored in subcutaneous adipose tissue, until the storage capacity is reached. Lipids will then overflow into the visceral compartment or stored ectopically, e.g., in normally lean tissues and organs, such as the liver. Here, the lipids will disrupt organ function and exert their harmful effects, eventually leading to increased risks of cardiometabolic disease. In the epidemiological studies described in this thesis, we observed an association between adult weight gain and adipocyte size at middle age. Adult weight gain was strongly associated with the amount of visceral fat and liver fat at middle age, which indicates that in individuals who gain excess body weight, the storage capacity in subcutaneous adipose tissue is more likely to be reached. We also observed a strong association between adult weight gain and insulin resistance, which was mediated by the amount of visceral fat. These results support the 'lipid overflow' hypothesis and highlight the cardiometabolic consequences of adult weight gain and lipid overflow (21-23). Additionally, adult weight gain was specifically related to atherogenic metabolic alterations specifically associated with adult weight gain and indicative of cardiometabolic disease. Lastly, overall and abdominal adiposity at adolescence was associated with an atherogenic metabolic profile. Adipocyte hypertrophy may be one of the explanations, as adipocytes respond to increases in fat mass by expansion during young adulthood, and thereby contribute to adipocyte hypertrophy, ultimately leading to metabolic disturbances (75-77).

As an alternative to randomized controlled trials on body weight gain or obesity, we suggest Mendelian randomization analyses, which make use of genetic variants associated with the exposure of interest as an instrumental variable, assigned randomly at conception. To identify genetic variants to be used as instrumental variables, Genome-Wide Association Studies (GWAS) need to be performed. A GWAS on weight gain during adulthood is currently ongoing, of which the results can be used to perform MR analyses to complement the results of our observational studies. It is also possible that genetic factors play a less significant role in adult weight gain, and that environmental factors may be more important (78). Studies on the interaction between genetic variants predisposing for adult weight gain and environmental factors, such as dietary composition, are needed to provide more information on the interplay between genetics and the environment in the development of obesity and cardiometabolic disease.

Characterization of genetic variants that are associated with adult weight gain during adulthood and the genes corresponding to them, can provide insights into the complex processes underlying adult weight gain. Identified genetic variants can then be grouped for novel MR approaches as we described in Chapter 2 of this thesis. Recently, MR techniques similar to our approach but from a different perspective, have been developed (56, 57). For example, in a recent study, principial component analysis was used to identify four groups of genetically driven variation in body shape and size: overall body size, adiposity, predisposition to abdominal fat deposition, and lean mass (56). These approaches can be used to unravel the heterogeneity in the genetics underlying complex diseases such as obesity. More detailed investigations of the differential causes of complex traits such as obesity and adult weight gain will allow a comprehensive overview of potential targets for interventions. In addition, genetic variants potentially provide personalized information on the risk of cardiometabolic disease beyond the traditional risk factors.

Not only the causes, but also the consequences of obesity are complex. Importantly, not everyone with obesity will develop cardiometabolic disease, and obesity-related consequences differ between individuals with a similar BMI. Body weight history plays an important role in the risk of cardiometabolic disease later in life: for cardiovascular disease, both BMI in adolescence and adulthood are associated with the risk of cardiovascular disease, whereas for type diabetes BMI and weight gain shortly before diagnosis seem to be more conclusive (34). We consistently observed that weight gain during adulthood was associated with an adverse body fat distribution, adverse metabolic profile and insulin resistance, eventually resulting in an increased risk of cardiometabolic disease at middle age. Precise mechanisms underlying these associations include the response of adipocytes to weight gain: although there is a constant turnover of adipocytes throughout life, adipocyte number remains fixed during adulthood, and therefore the expansion of adipose tissue in response to weight gain in adults is due to an increase in adipocyte volume (38, 79). Previous studies have shown that this adipocyte hypertrophy is associated with increased systemic insulin resistance, and thereby a worsening metabolic profile (75-77), as a results of adipose tissue becoming dysfunctional when the capacity of hypertrophic adipocytes to expand is exceeded (21, 22). This in turn, leads to “lipid overflow” and the accumulation of triglycerides in visceral adipose and ectopic fat deposition in normally lean organs such as the heart and the liver (21, 22, 24). Compared to subcutaneous adipocytes, adipocytes in the visceral

depot have a high secretion rate of non-esterified fatty acids, very low-density lipoproteins (VLDL), and cytokines, such as interleukin-6 and tumour necrosis factor-α, thereby inducing a systemic low-grade inflammatory state and oxidative stress (23-26). Finally, intracellular non-esterified fatty acid accumulation in non-adipose tissue leads to impaired insulin signalling and insulin resistance (27). Eventually, this will lead to an increased cardiometabolic risk, including both a high risk of developing type 2 diabetes and cardiovascular disease (22).

The results of this thesis emphasize the importance of maintaining a stable body weight during young adulthood throughout middle age. Body weight reduction has been proven difficult, as well as keeping weight off permanently, however, weight maintenance will already be beneficial for health as supported by or results. This is also supported by a study on type 2 diabetes (80), in which the authors estimated that about 1 in 5 cases of type 2 diabetes could be prevented if body weight in middle age was maintained at the population level. Since 2019, individuals with overweight or obesity in the Netherlands are eligible for a combined lifestyle intervention, integrating dietary habits, physical activity and behavioural changes, in order to promote and maintain a healthy weight (81). This multifactorial approach, including a combination of lifestyle changes such as maintaining a healthy diet, increasing physical activity and smoking cessation, was also promoted by the recent guidelines on the short- and long-term prevention of cardiovascular disease by the European Society of Cardiology (82). In addition, the guidelines underline the importance of increasing the knowledge on effective strategies to reduce body weight and maintain a healthy body weight. However, lifestyle changes have been proven difficult, especially in the modern obesogenic environment, in which high energy intake and sedentary behaviour are encouraged (83). This underscores the need for health-promoting population-level interventions and governmental policies promoting a healthy lifestyle (83-85). In the Netherlands, the National Prevention Agreement has been signed in 2018, with the goal to reduce smoking, obesity and alcohol consumption in the Dutch population (86). Measures against obesity in the National Prevention Agreement include providing combined lifestyle interventions, selling healthy foods at gyms, canteens and hospitals, and helping people make healthy food choices by healthy food logos on packaging (86). Altogether, the ambition of the National Prevention Agreement is that these measures should result in a decrease in overweight and obesity in Dutch adults from 50% to 38% by 2040 (86).

In conclusion, in this thesis we investigated several underlying mechanisms linking adult weight gain to cardiometabolic disease later in life. The results described in this thesis suggest that the cardiometabolic consequences of weight gain during both adolescence and adulthood are mediated by the amount of visceral and ectopic fat, and are reflected by a more atherogenic metabolomic profile and increased cardiometabolic risk. Once obese, preserving low glucose levels, non-smoking, and preventing abdominal obesity are important to prevent the onset of cardiometabolic disease. Overall, the results of this thesis emphasize the importance of maintaining a stable body weight during young adulthood throughout middle age. With increased attention being given to promoting a healthy lifestyle, there is potential for cardiometabolic disease prevention in promoting a healthy body weight during the life course.

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APPENDICES

Dutch summary
Acknowledgements
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DUTCH SUMMARY

Overgewicht en obesitas worden door de World Health Organisation gedefinieerd als overtollig lichaamsvet dat een risico voor de gezondheid vormt. In 1990 had nog één op de drie Nederlandse volwassenen overgewicht of obesitas, maar in 2019 was dit al gestegen tot de helft van de Nederlandse volwassenen. Deze stijgende trend is wereldwijd zichtbaar. Overgewicht en obesitas zijn eveneens een probleem bij kinderen en adolescenten: in deze groep steeg de prevalentie van obesitas van 4% in 1975 naar 18% in 2016. Kinderen en adolescenten met overgewicht of obesitas blijven vaak een te hoog gewicht houden als volwassenen.

De “body mass index” (BMI) wordt veel gebruikt om overgewicht en obesitas aan te duiden, maar BMI maakt geen onderscheid tussen vet- en spiermassa. Daarnaast geeft de BMI niet aan waar het vet is opgeslagen: in de buik of in de benen en heupen. Het meeste lichaamsvet (80-90%) wordt onderhuids opgeslagen. Buikvet kan zowel subcutaan (onder de huid) als visceraal (tussen de organen) worden opgeslagen. Volgens de ‘lipid overflow’ hypothese leidt gewichtstoename tot het groeien van vetcellen in het subcutane vetweefsel. Als de maximale opslagcapaciteit van de subcutane vetcellen is bereikt, gaat het lichaam over tot de opslag van overtollig vet in het viscerale vetweefsel of wordt het als vetdruppeltjes opgeslagen in organen die normaal geen vet opslaan zoals het hart en de lever (ectopische vetopslag). Vetcellen in het viscerale vet hebben een snellere afgifte van vrije vetzuren en ontstekingsstoffen dan subcutane vetcellen. Bij overtollig visceraal vet kan hierdoor een laaggradige ontsteking door het hele lichaam ontstaan en uiteindelijk insulineresistentie wat kan leiden tot type 2 diabetes mellitus. Eerder onderzoek heeft aangetoond dat het aantal vetcellen na jongvolwassenheid gelijk blijft, wat suggereert dat gewichtstoename in volwassenen eerder samengaat met het groter worden van vetcellen (hypertrofie) dan met het vermeerderen van vetcellen (hyperplasie), en daarmee de opslag van overtollig vet in het visceraal vetweefsel of ectopisch. Het is nog niet duidelijk waar het overtollige vet voornamelijk wordt opgeslagen wanneer mensen vooral in gewicht toenemen tijdens hun volwassen leven.

Eerdere studies hebben de gevolgen van gewichtstoename tijdens het (jong)volwassen leven onderzocht, maar veel is ook nog niet bekend. Ook zijn de moleculaire en metabole processen die de link vormen tussen gewichtstoename en cardiometabole aandoeningen nog niet in kaart gebracht. Het doel van mijn onderzoek was de cardiometabole gevolgen van obesitas en gewichtstoename tijdens de levensloop na te gaan.

Mendeliaanse randomisatiestudies vormen een alternatief voor gerandomiseerde klinische studies, aangezien deze niet altijd mogelijk, extreem duur, of niet ethisch zijn. In Mendeliaanse randomisatiestudies worden genetische varianten die samengaan met een bepaalde blootstelling gebruikt als instrumentele variabele. Omdat de genetische varianten tijdens conceptie willekeurig toebedeeld worden aan het nageslacht, wordt een gerandomiseerde klinische studie nagebootst en worden de resultaten van Mendeliaanse randomisatiestudies niet beïnvloed door verstorende factoren zoals in observationeel onderzoek, als aan een aantal aannames wordt voldaan.

Genetische varianten gerelateerd aan een verhoogd BMI kunnen gebruikt worden als instrumentele variabele in Mendeliaanse randomisatiestudies naar het verband tussen BMI en verschillende ziekte-uitkomsten, zoals type 2 diabetes en hart- en vaatziekten. Individuele genetische varianten kunnen een specifiek causaal effect hebben op een ziekte-uitkomst door een verhoogd BMI. Een deel van de heterogeniteit in de causale effecten van genetische varianten op ziekte-uitkomsten kan wellicht worden verklaard doordat genen die samengaan met een verhoogd BMI tot expressie komen in verschillende celtypen en weefsels, en daardoor andere bio-moleculaire functies hebben. Diepgaand onderzoek naar de verschillende oorzaken van complexe aandoeningen zoals obesitas kunnen leiden tot een completer beeld van de mogelijke aanknopingspunten voor (gepersonaliseerde) interventies.

In **Hoofdstuk 2** onderzochten we het verband tussen BMI en cardiometabole aandoeningen met Mendeliaanse randomisatie. Het doel van deze studie was te onderzoeken of onderliggende genetische oorzaken van een hoog BMI, zoals genexpressie in hersengebieden die een rol spelen in de verzadiging, samengaan met het risico op type 2 diabetes en hart- en vaatziekten. We onderscheidden 17 groepen van genen die samengaan met BMI en tot veranderde expressie komen in bepaalde weefsels, zoals in verschillende hersengebieden. We gebruikten de 17 groepen van genen als blootstelling in Mendeliaanse randomisatieanalyses met als uitkomst cardiometabole aandoeningen. De 17 verschillende groepen gebaseerd op expressieprofielen in weefsels, gingen allen samen met een min of meer gelijk risico op type 2 diabetes en hart- en vaatziekten. Dit betekent dat het groeperen van genetische varianten op basis van weefselexpressie niet leidt tot een verschillend risicoprofiel voor cardiometabole ziekten, en dus lijkt het onderliggend mechanisme waardoor iemand in gewicht aankomt geen rol te spelen in het risico op cardiometabole ziekten.

Metabole markers zijn kleine moleculen, tussen- of eindproducten van de stofwisseling. Voorbeelden van metabole markers zijn lipoproteïnen, aminozuren en vetzuren. Metabole markers vormen een belangrijke schakel tussen risicofactoren, zoals obesitas en leefstijlfactoren, en cardiometabole aandoeningen. Eerdere studies naar het metabool profiel hebben verschillende metabole markers aangewezen die een rol spelen in de ontwikkeling van cardiometabole aandoeningen als gevolg van een hoog BMI. BMI gaat sterk samen met hoge concentraties van *very low-density* lipoproteïnen (VLDL) en *low-density* lipoproteïnen (LDL). Ook hangt BMI sterk samen met hoge concentraties van verzadigde vetzuren en vertakte-keten-aminozuren. VLDL, LDL, verzadigde vetzuren en vertakte aminozuren zijn in eerder onderzoek in verband gebracht met een verhoogd risico op cardiometabole aandoeningen. Hoewel deze metabole markers mogelijk de link vormen tussen gewichtstoename en het ontstaan van cardiometabole aandoeningen, is het metabool profiel dat samengaat met gewichtstoename tijdens verschillende levensfases nog niet vastgesteld. Daarnaast zouden longitudinale studies kunnen helpen bij het identificeren van vroege veranderingen in het metabool profiel die zouden kunnen duiden op de ontwikkeling van cardiometabole afwijkingen in jongvolwassenen.

In **Hoofdstuk 3** onderzochten we de gevolgen van het totale vetpercentage en vetopslag rond de buik op veranderingen in metabolieten in jongvolwassenen in de Avon Longitudinal Study of Parents and Children (ALSPAC). We toonden aan dat vetopslag rond de buik samengaat met vroege veranderingen in het metabool profiel die een indicatie geven van

hart- en vaatproblemen, zoals hogere concentraties van VLDL en Apolipoproteine B en lagere concentraties van *high density* lipoproteïnen (HDL). Dit was voornamelijk zichtbaar bij jonge mannen. Onze resultaten laten zien dat adolescentie een belangrijke periode is voor het voorkómen van nadelige veranderingen in het metabool profiel gerelateerd aan het lichaamsgewicht en vetverdeling, zeker voor mannen.

In **Hoofdstuk 4**, **5** en **6** onderzochten we verschillende cardiometabole gevolgen van gewichtstoename tijdens de volwassen levensfase in de Nederlandse Epidemiologie van Obesitas (NEO) studie. Het is bekend dat gewichtstoename samengaat met een verhoogd risico op cardiometabole aandoeningen, mogelijk door het groeien van vetcellen. Het cardiometabole profiel dat samengaat met gewichtstoename in volwassenen is echter nog onbekend. In **Hoofdstuk 4** toonden we aan dat gewichtstoename specifiek samenging met de concentraties van zeven metabole markers in de NEO studie en Oxford Biobank. Deze metabole markers zijn omega-3, omega-6, meervoudige onverzadigde vetzuren, klein tot medium LDL en totaal *intermediate-density* lipoproteïne (IDL). Daarnaast was gewichtstoename gerelateerd aan de grootte van de vetcellen uit het buikvet op middelbare leeftijd, en gingen de lipoproteïnen die samengaan met gewichtstoename ook samen met de grootte van de vetcellen. Uit eerdere studies is bekend dat de metabole markers die gerelateerd zijn aan gewichtstoename ook het risico op cardiometabole aandoeningen verhogen, waardoor deze vroege markers mogelijk het begin kunnen aangeven van cardiometabole verstoringen als gevolg van gewichtstoename.

In **Hoofdstuk 5** hebben we de relatie tussen gewichtstoename vanaf 20 jaar en verschillende maten van buikvet (middelomtrek, subcutaan vet en visceraal vet) en levervet op middelbare leeftijd onderzocht. We toonden aan dat gewichtstoename samengaat met relatief meer visceraal vet en levervet op middelbare leeftijd binnen alle BMI categorieën. Uit eerder onderzoek weten we dat overmatig visceraal vet samengaat met verhoogde insulineresistentie en een hoger risico op type 2 diabetes. In **Hoofdstuk 6** onderzochten we of de hoeveelheid visceraal vet en levervet de relatie tussen gewichtstoename en insulineresistentie medieert. In dit hoofdstuk zagen we dat zelfs een kleine toename in gewicht samengaat met verhoogde insulineresistentie op middelbare leeftijd. Daarnaast vonden we aan dat de relatie tussen gewichtstoename en insulineresistentie op middelbare leeftijd voor 32% gemedieerd wordt door visceraal vet en 22% door levervet. De resultaten uit **Hoofdstuk 5** en **6** tonen aan dat het belangrijk is om een stabiel gewicht te houden tijdens de volwassen leeftijd om een toename van visceraal vet en levervet te voorkomen, en daarmee het risico op insulineresistentie en type 2 diabetes op latere leeftijd te verkleinen.

Obesitas is een sterke risicofactor voor type 2 diabetes, hart- en vaatziekten en kanker, maar niet iedereen met overgewicht of obesitas wordt ziek en de gevolgen van obesitas verschillen tussen mensen met eenzelfde BMI. In **Hoofdstuk 7** onderzochten we welke factoren bijdragen aan het voorkomen van cardiometabole aandoeningen in mensen met obesitas. Zowel de hoeveelheid visceraal vet als metabole factoren, zoals glucose- en triglyceridegehaltes in het bloed, hangen allen samen met een verhoogd risico op cardiometabole ziekten in een populatie met een BMI van 27 kg/m^2 of hoger. Daarnaast zagen we een interactie tussen verschillende risicofactoren en obesitas voor het risico op cardiometabole ziekten, waarbij het glucosegehalte in het bloed, roken en visceraal vet in combinatie met obesitas een extra verhoogd risico op cardiometabole ziekten lieten zien.

Tot slot vormt **Hoofdstuk 8** een discussie van de resultaten van de verschillende studies beschreven in dit proefschrift. In dit hoofdstuk beschrijven we ook de klinische implicaties en perspectieven voor vervolgonderzoek. Kort samengevat wijzen de resultaten beschreven in dit proefschrift erop dat de cardiometabole gevolgen van gewichtstoename tijdens (jong)volwassenheid worden gemedieerd door de hoeveelheid visceraal vet en levervet, en dat deze gevolgen weerspiegeld worden door een nadelig cardiometabool profiel en een verhoogd risico op cardiometabole ziekten. Zodra iemand overgewicht of obesitas heeft, zijn het behouden van een laag glucosegehalte, niet roken en het voorkomen van buikvet belangrijk om het risico op cardiometabole aandoeningen niet verder te verhogen. De resultaten uit dit proefschrift onderstrepen het belang van het behoud van een stabiel gewicht van jonge leeftijd tot middelbare leeftijd. Als aanvulling op alle aandacht die uitgaat naar een gezonde leefstijl, zoals bijvoorbeeld beschreven in het Nationaal Preventieakkoord, tonen de resultaten uit dit proefschrift aan dat er potentie zit in het voorkómen van cardiometabole aandoeningen door het aanmoedigen van een stabiel en gezond lichaamsgewicht gedurende de gehele levensloop.

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CURRICULUM VITAE

Inge Verkouter is geboren op 28 december 1993 in Vlaardingen. Ze behaalde haar gymnasiumdiploma in 2012 aan het Groen van Prinsterercollege in Vlaardingen. Nadat ze haar diploma behaalde, heeft zij de bachelor Biomedische Wetenschappen in Leiden gedaan. Vervolgens heeft zij in 2017 haar master Biomedical Sciences aan de Universiteit van Leiden afgerond. Haar tweede masterstage was bij de afdelingen Klinische Epidemiologie en Ouderengeneeskunde van het Leids Universitair Medisch Centrum. Haar stageonderzoek in de Nederlandse Epidemiologie van Obesitas studie kon zij vervolgen als PhD-kandidaat van januari 2018 tot april 2021, met behulp van een MSc-PhD beurs van het Leids Universitair Medisch Centrum. Tijdens haar promotietraject heeft Inge verschillende cursussen gevolgd voor de registratie als Epidemioloog B. Ook heeft zij (inter)nationale congressen bezocht en daar haar onderzoeken gepresenteerd. Begin 2020 is zij een aantal weken naar Bristol geweest om onderzoek te doen aan de Universiteit van Bristol na het ontvangen van een Building Bridges Grant vanuit het Energise!-consortium.

Momenteel werkt Inge als Medical Data Scientist bij ORTEC in Zoetermeer.

PORTFOLIO

Oral and poster presentations	Year	Credits
The association between adult weight change and insulin resistance: mediation by visceral fat and liver fat, Annual Dutch Diabetes Research Meeting (NVDO-ADDRM), Oosterbeek, the Netherlands	2017	0.25
The contribution of tissue-specific BMI-associated gene sets to cardiometabolic disease risk: a Mendelian Randomization study, Annual Dutch Diabetes Research Meeting (NVDO-ADDRM), Oosterbeek, the Netherlands	2018	0.25
The contribution of tissue-specific BMI-associated gene sets to cardiometabolic disease risk: a Mendelian Randomization study, European Atherosclerosis Society (EAS), Maastricht, the Netherlands	2019	0.50
The contribution of tissue-specific BMI-associated gene sets to cardiometabolic disease risk: a Mendelian Randomization study, Mendelian Randomization conference, Bristol, United Kingdom	2019	0.50
Adult weight change-specific metabolites in the general population, Annual Dutch Diabetes Research Meeting (NVDO-ADDRM), Wageningen, NL	2019	0.25
Adult weight change-specific metabolites in the general population, European and International Congress on Obesity (ECO), online	2020	0.50
Changes in metabolomic measures attributable to body composition during puberty and young adulthood, Annual Dutch Diabetes Research Meeting (NVDO-ADDRM), online	2020	0.25
Body fat distribution at adolescence and early changes in atherogenic metabolomic measures during young adulthood, WEON, online	2021	0.50

Consortia	Year	Credits
CVON Energise! Consortium, Dutch Heart Foundation	2018 - 2021	3.0

Congresses and symposia	Year	Credits
Netherlands Association for the Study of Obesity (NASO) Spring Meeting, Utrecht, the Netherlands	2018 - 2020	0.75
Annual Dutch Diabetes Research Meeting (ADDRM), Oosterbeek and Wageningen, the Netherlands	2017 - 2020	1.00
European Atherosclerosis Society, Maastricht, the Netherlands	2019	0.25
Mendelian Randomization Conference, Bristol, United Kingdom	2019	0.25
European Congress on Obesity (ECO), online	2020	0.25
WEON congress, online	2021	0.25

Courses, seminars and masterclasses	Year	Credits
Introduction to Clinical Epidemiology (Rothman), Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, the Netherlands	2017	3.0
Clinical Epidemiology Schiermonnikoog, Boerhaave Instituut, Leiden, the Netherlands	2018	2.0
Survival Analyses, Boerhaave Instituut, Leiden, the Netherlands	2018	1.5
Basic Methods and Reasoning in Biostatistics, Boerhaave Instituut, Leiden, the Netherlands	2019	1.5
Regression Analyses, Boerhaave Instituut, Leiden, the Netherlands	2019	1.5
Mendelian Randomization – Introductory Course, MRC Integrative Epidemiology Unit, University of Bristol, United Kingdom	2019	0.4
Mediation Analysis workshop, MRC Integrative Epidemiology Unit, University of Bristol, United Kingdom	2019	0.2
Clinical Epidemiology (Grobbee), Department of Clinical Epidemiology, Leiden University Medical Center, the Netherlands	2019	3.0
Causal Inference (Hernan), Department of Clinical Epidemiology, Leiden University Medical Center, the Netherlands	2020	3.0
Multiomics Data Integration Using R, Medical Genetics Centre (MGC), Leiden, the Netherlands	2020	1.75
Statistical Aspects of Clinical Trials, Boerhaave Instituut, Leiden, the Netherlands	2020	1.0
Basic Course Legislation and Organisation for Clinical Researchers (BROK/GCP), Graduate School, Leiden University Medical Center, the Netherlands	2021	1.0
Systematic Reviews and Meta-analyses	2021	1.0
Weekly Research Lunch, Department of Clinical Epidemiology, Leiden University Medical Center, the Netherlands	2018 – 2021	3.0
Weekly Research Lunch, Department of Endocrinology – Section Gerontology and Geriatrics, Leiden University Medical Center, the Netherlands	2018 – 2021	3.0
Weekly Capita Selecta, Department of Clinical Epidemiology, Leiden University Medical Center, the Netherlands	2018 – 2021	3.0
Bi-weekly Journal Club from the NEO study, Department of Clinical Epidemiology, Leiden University Medical Center, the Netherlands	2018 – 2021	3.0

Student monitoring and teaching	Year	Credits
Master thesis Babette de Roos	2018	1.5
Various epidemiological classes for bachelor and master students Biomedical Sciences, Medicine, and Vitality and Ageing	2018 – 2020	5.0

Grants	Year	Credits
MSc/PhD grant, Leiden University Medical Center, the Netherlands	2018	1.5
Building Bridges travel grant, CVON Energise! Consortium (Netherlands Cardiovascular Research Initiative, Dutch Heart Foundation [CVON2014-02 ENERGISE])	2019	5.0

Other	Year	Credits
Reviewing scientific epidemiological publications	2018 – 2021	1.5
Chair of the NEO Journal Club	2019 – 2021	5.0

LIST OF PUBLICATIONS

1. van der Tuin SJL, Li Z, Berbee JFP, **Verkouter I**, Ringnalda LE, Neele AE, et al. Lipopolysaccharide Lowers Cholesteryl Ester Transfer Protein by Activating F4/80(+)Clec4f(+)Vsig4(+)Ly6C(-) Kupffer Cell Subsets. J Am Heart Assoc. 2018;7(6).

2. **Verkouter I**, Noordam R, de Roos A, Lamb HJ, Rosendaal FR, van Heemst D, et al. Adult weight change in relation to visceral fat and liver fat at middle age: The Netherlands epidemiology of obesity study. Int J Obes (Lond). 2019;43(4):790-9.

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