UNIVERSITY OF TURKU

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ABSTRACT

Total energy intake, physical activity, body size and body composition determine total energy expenditure. An imbalance between total energy intake and total energy expenditure causes obesity. Overweight and obesity remains the most prevalent, modifiable cardiovascular risk factor for children and adolescents. In adolescence, obesity is associated with adverse non-invasive, subclinical cardiovascular phenotypes. In adulthood, these subclinical cardiovascular phenotypes and specific, obesity-related serum metabolomic profiles are predictive of future cardiovascular disease (including stroke and myocardial infarction) and cardiovascular disease mortality.

To investigate the association between determinants for total energy expenditure with the severity of obesity, with obesity-related cardiometabolic risk factors and with subclinical cardiovascular phenotypes in children and adolescents with obesity.

The present study was based on data from participants of the Childhood Overweight Biorepository of Australia (COBRA) and a derivative, longitudinal study-arm entitled COBRA-CVR (COBRA-Cardiovascular Risk). COBRA is comprised of 438 children and adolescents aged 6–18 years with overweight and obesity. COBRA-CVR includes a subset of 101 former COBRA-participants with obesity, aged up to 25 years, who were followed-up an average of 5.5 years later for the COBRA-CVR sub-study.

Among youth with obesity, low grip strength (a global measure of muscular strength) was associated with poorer cardiometabolic health indicated by higher systolic blood pressure, higher low-density lipoprotein-cholesterol, higher carotid intima-media thickness (cIMT) and higher continuous metabolic syndrome score. Compositional analysis of macronutrients revealed that the dietary proportion of proteins is negatively associated with total energy intake, irrespective of whether carbohydrates or fats were the diluents. Several adiposity measures (including body mass index, waist circumference, bioimpedance related percentage body fat) showed associations consistent in direction and strength with metabolites that are associated with cardiovascular disease risk. Particularly, adiposity-related metabolomic profiles in post-pubertal males resembled profiles from adults that are predictive for cardiovascular disease and mortality. Increasing the severity of obesity from early to late adolescence was associated with increased pulse wave velocity (PWV) in males and with reduced carotid elasticity in females. Moreover, blood pressure was positively associated with PWV and glycoprotein acetyls (GlycA, a measure of chronic inflammation) was negatively associated with carotid elasticity. Male sex was the only factor associated with increased cIMT.

Obesity in youth is a multifactorial disease of pandemic scale that is in need of methods for prevention and risk stratification to alleviate the associated negative public health impacts. This study provides evidence for approaches that are clinically translatable to youth with overweight or obesity: to reduce total energy intake by ensuring sufficient protein intake; and factors that could help identify those at higher risk for adverse cardiometabolic outcomes.

KEYWORDS: Childhood, adolescence, obesity, hand grip strength, protein leverage hypothesis, metabolomics, subclinical cardiovascular phenotypes

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TIIVISTELMÄ

Lihavuus johtuu energian saannin ja kulutuksen epätasapainosta. Se on lapsilla keskeinen sydän- ja verisuonitautien riskitekijä. Teini-ikäisillä lihavuus lisää varhaisten, oireettomien valtimomuutosten esiintyvyyttä. Aikuisilla nämä valtimomuutokset sekä lihavuuteen liittyvät metaboliset muutokset ennustavat sydän- ja verisuonitapahtumia ja niihin liittyvää kuolleisuutta.

Väitöskirjatutkimuksen tavoitteena oli selvittää ravitsemuksen vaikutuksia lapsuusiän lihavuuden vaikeusasteeseen ja tutkia kehon koostumuksen yhteyttä veren metabolomiikkamuuttujiin sekä varhaisiin valtimomuutoksiin. Lisäksi tutkimuksessa arvioitiin käden puristusvoiman yhteyttä varhaisten valtimomuutosten ilmenemiseen.

Väitöskirjatutkimus on osa australialaista Childhood Overweight Biorepository of Australia (COBRA)-tutkimusta ja sen jatko-osaa COBRA-CVR. COBRA-tutkimukseen osallistui 438 iältään 6–18-vuotiasta ylipainoista tai lihavaa lasta, joista 101 osallistui COBRA-CVR-jatkotutkimukseen.

Ravintoainesanalyysin perusteella ravinnon proteiiniosuus oli sokeri- ja rasvakulutuksesta riippumatta tutkimusryhmässä käänteisesti yhteydessä kokonaisenergiansaantiin. Metabolomiikka-analyyseissä eri kehonkoostumusmittarit korreloivat useisiin metaboliitteihin, joiden on todettu ennustavan valtimotautiriskiä. Yhteydet olivat vahvimpia teini-ikäisillä pojilla. Lihavuuden vaikeutuminen teini-iässä oli yhteydessä varhaisiin valtimomuutoksiin, pojilla lisääntyneeseen valtimoiden jäykkyyteen ja tytöillä alentuneeseen kaulavaltimon joustavuuteen. Lisäksi verenpaine korreloi suoraan valtimoiden jäykkyyteen ja inflammaatiotekijä glykoproteiini-A käänteisesti kaulavaltimon joustavuuteen. Alentunut käden puristusvoima oli yhteydessä korkeampiin systolisen verenpaineen, LDL-kolesterolin, kaulavaltimon seinämäpaksuuden ja metabolisen oireyhtymän riskipisteytyksen arvoihin.

Ravintoainesanalyysi voi antaa lisätietoa painonhallintaan. Etenkin teini-ikäisillä pojilla vaikea lihavuus on yhteydessä metabolisiin muutoksiin. Lihavuuden vaikeutumisen estäminen teini-iässä saattaisi vähentää varhaisten valtimomuutosten esiintymistä. Käden puristusvoimamittauksilla voi olla mahdollista arvioida lihavien lasten alttiutta valtimotaudin riskitekijöille.

AVAINSANAT: Lapsuus, teini-ikä, lihavuus, metabolomiikka, varhaiset valtimomuutokset, puristusvoima

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Abbreviations

AAA	Aromatic amino acids
AEE	Activity-related Energy Expenditure
AIC	Akaike information criterion
BCAA	Branched-chain amino acids
BIA	Bioimpedance analysis
BMI	Body mass index
BMR	Basal metabolic rate
CDC	Centres for Disease Control and Prevention
cIMT	Carotid intima-media thickness
COBRA	Childhood Overweight BioRepository of Australia
COBRA-CVR	Cardiovascular Risk in COBRA
CRP	C-reactive protein
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DEE	Diet-induced energy expenditure
DXA	Dual x-ray absorptiometry
FDR	False discovery rate
FFQ	Food frequency questionnaire
FFM	Fat free mass
HbA1c	Haemoglobin A1c
hs-CRP	High-sensitivity C-reactive protein
HDL-C	High-density lipoprotein cholesterol
IOTF	International Obesity Task Force
IR	Insulin resistance
i3C	International Childhood Cardiovascular Cohort
LDL-C	Low-density lipoprotein cholesterol
MetS	Metabolic syndrome
MI	Myocardial infarction
NHANES	National Health and Nutrition Examination Survey
NMR	Nuclear magnetic resonance
OR	Odds ratio
PCA	Principal component analysis
PAL	Physical activity level
PL	Protein leverage
PLH	Protein leverage hypothesis
PWV	Pulse-wave velocity
REE	Resting energy expenditure
RR	Relative risk
SBP	Systolic blood pressure
SD	Standard deviation
SEIFA	Socio-economic indexes for areas
SES	Socio-economic status
SSB	Sugar-sweetened beverage
TEE	Total energy expenditure
TEI	Total energy intake
T2DM	Type 2 diabetes mellitus
UK	United Kingdom
US	United States
VLDL	Very low-density lipoprotein
WC	Waist circumference
WtH	Waist to height

WHO	World Health Organization
%>95th BMI-centile	Percentage level above the 95th body mass index centile
%BF	Percentage body fat
%TF	Percentage truncal fat
%EC	Percentage energy from carbohydrates
%EF	Percentage energy from fats
%EP	Percentage energy from proteins
95%CI	95% confidence interval

List of Original Publications

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I-IV. Additional unpublished data is also presented.

I Laitinen TT, **Saner C**, Nuotio J, Sabin MA, Fraser BJ, Harcourt B, Juonala M, Burgner DP, Magnussen CG. Lower grip strength in youth with obesity identifies those with increased cardiometabolic risk. Obes Res Clin Pract. 2020 May-Jun;14(3):286-289. doi: 10.1016/j.orcp.2020.04.004.

II **Saner C**, Tassoni D, Harcourt BE, Kao KT, Alexander EJ, McCallum Z, Olds T, Rowlands AV, Burgner DP, Simpson SJ, Raubenheimer D, Senior AM, Juonala M, Sabin MA. Evidence for protein leverage in children and adolescents with obesity.
Obesity (Silver Spring). 2020 Apr;28(4):822-829. doi: 10.1002/oby.22755.

III **Saner C**, Harcourt BE, Pandey A, Ellul S, McCallum Z, Kao KT, Twindyakirana C, Pons A, Alexander EJ, Saffery R, Burgner DP, Juonala M, Sabin MA. Sex and puberty-related differences in metabolomic profiles associated with adiposity measures in youth with obesity. Metabolomics. 2019 May 3;15(5):75. doi: 10.1007/s11306-019-1537-y.

IV **Saner C**, Laitinen TT, Nuotio J, Arnup SJ, Harcourt BE, Bekkering S, McCallum Z, Kao KT, Alexander EJ, Janner M, Magnussen CG, Sabin MA, Juonala M, Burgner DP. Modest decrease in adiposity associates with improved arterial stiffness in children and adolescents with severe obesity. Submitted and reviewed by Pediatrics.

1 Introduction

Globally, the prevalence of obesity in youth aged 5-19 years has increased nearly eight-fold over the last four decades (Di Cesare et al.,2019), affecting close to one in five children or adolescents in the United States (US) in 2015-2016 (Skinner et al.,2018) and approximately one in twelve children aged 2-17 years in Australia in 2017-2018 (Australian Bureau of Statistics, 2018). In Europe, the corresponding prevalence of obesity amongst children aged 2-13 years was 5.7% between 2011-2016 with highest rates in Mediterranean countries and lowest rates in countries in central Europe (Garrido-Miguel et al.,2019). Obesity is caused by a genetic susceptibility for weight gain, an obesogenic environment and genome-environment interactions inducing epigenetic changes (Swinburn et al.,2002; Thaker,2017). Weight gain as a consequence of genes appear rather low based on genome-wide association studies. In contrast, factors related to the obesogenic environment people live in contribute to a greater extent. Ultimately, all these factors, hereinafter termed determinants of obesity, adversely influence the ratio of total energy intake (TEI) to total energy expenditure (TEE) (Lobstein et al.,2015). TEE is determined by physical activity, TEI, body size and body composition, and interactions between those factors (Westerterp,2017). Efficient treatment options for obesity in childhood and adulthood are limited and the likelihood for a child with obesity to have a weight in the obese category in adulthood is ~80% (Freedman et al.,2001; Ward et al.,2017). Preventive factors related to the TEI/TEE ratio are therefore paramount. Also, there is evidence to suggest that prevention of obesity in early childhood is cost-effective, particularly due to obesity related cardiovascular and metabolic disease and mortality later in the life-course (Gortmaker et al.,2015; Trasande et al.,2009). Therefore, the overall aim of this study was to investigate factors that contribute to an imbalanced TEI/TEE ratio and measures or methods that allow risk stratification for obesity-related cardiometabolic outcomes in children and adolescents.

The specific aims I and II of this study focus on factors that are novel in their application to children and adolescents with obesity and are related to TEI and TEE, respectively. The main determinants for TEE are body size, body composition, TEI and physical activity (Westerterp,2017). Combined, these determinants impact on the three main components of TEE that are resting energy expenditure (REE, ~ 55-65% of TEE), diet-induced energy expenditure (DEE, ~10%) and activity-induced energy expenditure (AEE, ~25-35%) (Westerterp,2017). REE is fairly constant at a given body composition and size, and the contribution of DEE on TEE is rather small. In contrast, AEE is the most variable component of TEE, and overall, reflects the sum of physical activity (Washburn et al.,2014). The gold standard to assess TEE is the doubly labelled water method (Schoeller et al.,1982). For physical activity, many methods are proposed, each providing information on different facets of physical activity (Sylvia et al.,2014). Recently, a number of studies have examined the utility of hand grip strength in large population studies. Hand grip strength is a global measure of muscular strength and increases with physical activity training. Studies in adolescence and senescence have shown associations between lower grip strength and higher cardiovascular disease (CVD) and all-cause mortality (Kim et al.,2017; Lopez-Jaramillo et al.,2014; Ortega et al.,2012). Hand grip strength is simple to assess and appears to have predictive value for various health outcomes in adulthood. However, data on grip strength in children and adolescents with obesity, particularly for obesity-related adverse cardiometabolic outcomes, are lacking.

Besides TEE, TEI is the most obvious determinant of human weight. TEI results from the proportional energy intake from carbohydrates, fats and proteins, each contributing roughly 50%, 30% and 20% to the total energy intake in a standard diet (National Health and Medical Research Council,2006, updated September 2017). Differences in the macronutrient composition, particularly changes in the energy derived from proteins, has been the background for the protein leverage hypothesis. Using nutritional geometry in animals and adult humans, the founders of the protein leverage mechanism suggested that the proportion of energy from proteins may have a disproportionately high impact on TEI (Simpson et al.,2005). This mechanism, while increasing TEI may also contribute to increasing weight in people living in westernised countries with an abundancy of food supply - a concept termed protein leverage

hypothesis. Whether protein leverage exists in childhood and adolescence, and whether protein leverage contributes to weight change in children with obesity has not been investigated.

Once an imbalanced TEI/TEE ratio has caused weight gain and subsequent obesity, the human body will physiologically defend the new weight status, which may lead to adverse health-related consequences (Schwartz et al.,2004). In fact, obesity in youth is associated with a plethora of adverse health-related consequences, of which adult cardiometabolic diseases are the most important for public health and healthcare systems (Olshansky et al.,2005). Therefore, specific aim III of this study focusses on a systems biology approach, i.e. metabolomics, as a method for CVD risk-stratification and risk prediction in children and adolescents with obesity. Obesity-related cardiometabolic risk factors in youth include elevated systolic blood pressure (SBP) and diastolic blood pressure (DBP), elevated total cholesterol and triglycerides (Skinner et al.,2015), insulin resistance (IR) (Friedemann et al.,2012) and elevated markers for a low-grade, chronic inflammation (Schipper et al.,2012). Similar to excessive weight, these cardiometabolic risk factors share a strong tendency to track, or persist, from childhood into adulthood (Juhola et al.,2011; Ward et al.,2017). Furthermore, in adulthood they are associated with CVD and type 2 diabetes (T2DM), and ultimately with increased mortality rates (Caprio et al.,2020; Lindberg et al.,2020a; Reilly et al.,2011). Over the past two decades there has been a rapid increase in volume, variety and velocity of medical data - summarized under the term "Big data" (Hulsen et al.,2019). Novel methods derived from Big data analysis ideally are accurate and cost-effective in improving risk-stratification and risk prediction for cardiovascular disease beyond the level from traditional cardiometabolic risk factors (Hlatky et al.,2009). Metabolomics may be such a method, providing information on large amounts of metabolites that are a consequence of the individual`s genetic background and environment. Results from metabolomic analysis reflect an individual`s phenotype more closely than analysis based on other omics-platforms such as genomics, transcriptomics or proteomics (Rattray et al.,2018). In adulthood, metabolomic studies have revealed specific profiles comprised of metabolites that are caused by increasing body mass index (BMI) levels (Holmes et al.,2014; Wurtz et al.,2014). Such metabolomic profiles have been associated with cardiometabolic risk factors (Tulipani et al.,2016; Zhao et al.,2016), type 2 diabetes (Wang et al.,2011), and future CVD (Holmes et al.,2018). However, limited analogous data are available in children and adolescents with obesity. This is unfortunate, as this is a population with high risk for the development of cardiometabolic diseases, where early detection would seem most beneficial for the long-term outcome.

Aim IV of this study will cover the relation between changes in adiposity measures and changes in traditional cardiovascular risk factors over time on changes in large artery properties. Changes in large artery properties in this study will be reflected by a comprehensive assessment of subclinical cardiovascular phenotypes in children and adolescents. Subclinical cardiovascular phenotypes illustrate intermediate outcomes of the process of arteriosclerosis before the manifestation of CVD. They can be assessed non-invasively, their method of assessment is well-tolerated and overall, these phenotypes are increasingly used in youth for research purposes. Such phenotypes comprise the carotid intima-media thickness (cIMT), pulse-wave velocity (PWV) and the carotid artery elasticity. In youth, they are associated with various, pro-atherogenic conditions such as type 1 diabetes, chronic kidney disease and various other health issues (Urbina et al.,2009a). There is cumulative evidence for an association between excessive weight and adverse subclinical cardiovascular phenotypes starting in early life. In childhood, the number and severity of traditional cardiovascular risk factors are associated with subclinical cardiovascular phenotypes in early adulthood (Juonala et al.,2010; Koivistoinen et al.,2011). In adulthood, these phenotypes are predictive for future CVD and mortality (Boesen et al.,2015; Lorenz et al.,2007; Mitchell et al.,2010). Longitudinal cohort studies have also shown that former adolescents with overweight or obesity, who regain normal body mass index (BMI) by adulthood can equalize their most cardiovascular risk factors to those who had a BMI always in the healthy range, but results for cIMT are inconsistent (Buscot et al.,2018; Juonala et al.,2011). However, studies in children and adolescents with obesity that investigate the association between longitudinal adiposity measures or key cardiovascular risk factors (such as blood pressure, blood lipids, fasting glucose levels or inflammatory markers) with subsequent subclinical cardiovascular phenotypes are sparse. Such studies could inform

the clinician as to which participants and which exposure measurements could be prioritized as intervention targets.

In conclusion, children and adolescents with obesity are a vulnerable group, especially for adverse cardiometabolic health outcomes and CVD-related mortality in adulthood. Effective treatment options in youth are limited, and the persistence of obesity and obesity related cardiometabolic risk factors into adulthood highlights the importance of primary and secondary prevention. Better understanding of factors that contribute to obesity, markers or methods that identify the most vulnerable of children or adolescents with obesity and markers or methods that allow risk prediction are needed to tackle the obesity pandemic and its adverse health effects. In a cohort of children and adolescents with obesity, this study aimed to investigate the association between determinants for an adverse TEI/TEE ratio with the severity of obesity, with obesity-related cardiometabolic risk factors and with subclinical cardiovascular phenotypes. The specific aims tested were: i) the association between grip strength, a determinant for TEE, with obesity-related cardiometabolic outcomes; ii) the protein leverage hypothesis as a contributor to increased TEI and weight gain; iii) associations between adiposity measures and CVD-specific metabolomic profiles; and iv) associations between changes in adiposity measures and changes in traditional cardiovascular risk factors over time on subclinical cardiovascular phenotypes.

2 Review of the Literature

2.1 Adiposity and adiposity-related mortality in adulthood

The World Health Organization (WHO) defines overweight or obesity as an accumulation of excess body fat to the extent that it may have adverse health-effects (WHO). In adults, overweight and obesity are determined by a body mass index (BMI) greater or equal to 25 kg/m^2 and 30 kg/m^2, respectively. In the United States (US), the most recent analysis of trends for the prevalence of obesity amongst adults showed a continuous rise, from 33.7% in 2007/2008 reaching 39.6% in 2015/2016 (Hales et al.,2018). Obesity in adulthood is associated with increased morbidity and mortality, with CVD as the major contributor (Afshin et al.,2017; McGee et al.,2005). Further details of evidence for obesity-related mortality arose from a meta-analysis in four continents including 239 prospective studies and more than 10 million participants: the results showed a J-shaped curve for the association between BMI and all-cause mortality with the vertex situated at a normal-weight BMI between 20-25kg/m^2 (Di Angelantonio et al.,2016). Detailed results showed that hazard ratios per 5-units increase in BMI for all-cause mortality were higher for younger adults (35-49 years versus 70-89 years), higher for males versus females and the major underlying causes for mortality were (ranked by priority) coronary heart disease, stroke, respiratory disease and cancer (Global et al.,2016). A corresponding meta-analysis from the United Kingdom showed a relative risk (RR) for overall mortality of 1.20 [95%CI 1.12-1.29] for men and 1.28 [95%CI 1.18-1.37] for women and specifically for death from CVD of 1.45 [95%CI 1.33-1.59] for men and 1.53 [95%CI 1.38-1.69] for women with obesity, compared to normal weight counterparts (McGee et al.,2005).

An estimated 70% of adults with obesity included in studies investigating measures of adult BMI and overall and disease-specific mortality rates were not obese in childhood or adolescence (Simmonds et al.,2016). However, based on increasing prevalence rates for obesity in childhood and the persistence of excessive weight from childhood into adulthood, the fraction of adults with obesity that were already obese in childhood or adolescence is likely to increase over the next decades. The next section will discuss the prevalence of overweight and obesity and demographic characteristics of overweight and obesity in the paediatric age range over the last 5 decades.

2.2 Prevalence of overweight and obesity in children and adolescents

In countries such as the US, Brazil, Mexico, Saudi Arabia and China, the prevalence of overweight and obesity (determined by the 85th and 95th centile of BMI reference charts, see section 2.4) in childhood was below 15% in 1972 (Lobstein et al.,2015). However, within just 3 decades, the average weight in US children has increased by 5 kg (Lobstein et al.,2015). And this trend is ongoing: the most recent evaluation of US data revealed that the prevalence of obesity in US youth aged 2-19 years has increased from 13.9% in 1999/2000 to 18.5% in 2015/2016 (Craig M. Hales,2017). Globally, 370 million children and adolescents were estimated to be affected by obesity in 2016 ("Global Nutrition Report: Shining a Light to Spur Action on Nutrition,"2018).

Detailed demographic analysis, including the effects from sex, ethnicity/race, household income and education level on obesity rates in childhood were published based on data from the US National Health and Nutrition Examination Survey (NHANES). Overall, sex did not show any differences, but variation was seen according to ethnicity/race with higher rates for Hispanic (25.8%), followed by non-Hispanic black (22%), non-Hispanic white (14.1%) and non-Hispanic Asian youth (11%) (Craig M. Hales,2017). Analysing a similar NHANES dataset, the severity of obesity across the paediatric lifespan was shown to increase with age, regardless of sex and ethnicity/race (Hall et al.,2018). Individuals with highest household income showed a lower prevalence (10.9%), compared to those with lowest income (18.9%) and those with higher level of education (college graduate) had a lower prevalence (9.6%) compared to those with lower level of education (high school graduate or less) with a prevalence of 21.6% (Ogden CL,2018).

2.3 Overview of obesity-related health effects in childhood and adulthood

Increasing weight during childhood is associated with a plethora of short-term and long-term consequences affecting mental and somatic health. Whereas adverse health consequences from obesity separately for childhood and adulthood age are well known, there is limited evidence for BMI in childhood to represent an independent predictor for adult morbidity.

Short-term, mental health outcomes for children with obesity include higher risks for depression and anxiety (>30% increase) (Lindberg et al.,2020b; Sutaria et al.,2019) and for low self-esteem and behavioural disorders (Rankin et al.,2016) when compared to normal-weight counterparts. Short-term somatic consequences compared to normal weight children include a higher risks for asthma (relative risk 1.29) (Lang et al.,2018), non-alcoholic fatty liver disease (34.2% for obese versus 7.6% for children with normal weight) (Anderson et al.,2015) and musculoskeletal problems (Krul et al.,2009). Children with obesity have a high risk for an adverse cardiometabolic profile including low-grade systemic inflammation (Singer et al.,2017), high blood pressure (Du et al.,2019; Wuhl,2019), dyslipidaemia (Skinner et al.,2015), type 2 diabetes (Galuska et al.,2018) and adverse subclinical cardiovascular phenotypes (Urbina et al.,2009a) which is discussed in detail in sections 2.6 and 2.8.

Obesity in adulthood is strongly associated with increased morbidity from many diseases such as diabetes (20% increase of the risk to develop T2DM for each 1kg/m^2 increase in BMI (Hartemink et al.,2006)), CVD (Khan et al.,2018), obesity-related hypoventilation syndrome (Mokhlesi,2010), non-alcoholic fatty liver disease (NAFLD)(Loomba et al.,2013), cancer (Nomura et al.,2010) and chronic kidney disease (Coresh et al.,2007). Obesity in adulthood is also associated with increased all-cause mortality, predominantly due to CVD (Prospective Studies et al.,2009), respiratory disease and cancer (Berrington de Gonzalez et al.,2010; Global et al.,2016).

Systematic reviews and cohort-studies have investigated the relation between childhood and adult BMI, and between childhood BMI and adult morbidity with conflicting findings. There is convincing evidence from systematic reviews (Simmonds et al.,2015) and growth trajectory simulations (Ward et al.,2017) that a child with obesity will remain in the obese weight category in adulthood. Also, there are associations between higher BMI in children aged 12 years and older with adult manifestation of type 2 diabetes mellitus (OR 1.70, 95%CI 1.30-2.22) and coronary heart disease (OR 1.30, 95%CI 1.16-1.47) from a systematic review and meta-analysis (Llewellyn et al.,2016). However, the predictive ability for BMI in childhood for type 2 diabetes and cardiovascular disease in adulthood is overall low. In studies where the association between childhood BMI and adult comorbidities were adjusted for adult BMI levels, the associations for childhood BMI as predictor regressed to the null (Lloyd et al.,2010, 2012; Park et al.,2012; Umer et al.,2017). This is largely explained by the fact that most adults (~70%) with obesity and CVD had a normal weight during their childhood 4-7 decades ago (Llewellyn et al.,2016; Simmonds et al.,2016). No other measure than BMI has shown any benefits for the determination of obesity in childhood or to predict adult obesity and morbidity. A collection of the most commonly used measures is discussed in the following section (Simmonds et al.,2015).

2.4 Adiposity measures in children and adolescents

Body mass index (BMI) is the accepted standard measure to determine overweight and obesity for children 2 years of age and older and adults (Freedman et al.,2009b; Styne et al.,2017). BMI is calculated by body weight in kg divided by height in meters squared. BMI thresholds in adulthood are static, sex-independent (i.e. 25kg/m^2 for overweight, and BMI >30kg/m^2 for obesity) (WHO,1995), and well related to morbidity and mortality (WHO,1998). In childhood and adolescence, measures such as BMI or the percentage body fat need to take into account the changes in anthropometry related to normal growth, sex, and puberty. Hence, BMI-values need to be compared to age and sex-adjusted reference-data from large populations and transformed into centiles or standard deviations (SD) or z-scores for comparability.

The two most widely used classification system for BMI are the Centers for Disease Control (CDC) growth charts (based on data from NHANES) (Ogden et al.,2014) and the International Obesity Taskforce (IOTF) cut points (Cole et al.,2000). For completeness, the World Health Organisation (WHO) is the third most common classification system (de Onis et al.,2007) (see Table 1). According to the NHANES reference, overweight and obesity is determined by a BMI exceeding the 85th or 95th centile, corresponding to ~ 1.44 or 1.96 BMI z-scores (CDC,2020; Ogden et al.,2014), whereas the IOTF curves were fitted to align with adult BMI thresholds at the age of 18 years (Cole et al.,2000).

Whereas for overweight, the IOTF and the 85th CDC centile are comparable, the IOTF cut-off for obesity is higher than the 95th CDC centile, rather corresponding to the 97th CDC centile among school-aged children. Such differences between classification systems may complicate comparisons across studies (Flegal et al.,2006).

Table 1. Cut-offs for overweight and obesity in childhood

	Overweight and obesity cut-off	**Background**	**Age coverage**	**Year of publication**
CDC	BMI ≥ 85th centile BMI ≥ 95th centile	Five national surveys in US children and adolescents from 1963 to 1994	2-18 years	2000 (Kuczmarski et al.,2002)
IOTF	BMI ≥ 85th centile BMI ≥ 95th centile	Nationally representative growth data from 6 countries including Brazil, Great Britain, Hong Kong, Netherlands, Singapore and United States	2-18 years	2000 (Cole et al.,2000)
WHO	BMI ≥ 1 SD BMI ≥ 2 SD	National Center for Health Statistics / WHO growth reference data from 1977	0-18 years	2007 (de Onis et al.,2007)

CDC: Centers for Disease Control; IOTF: International Obesity Taskforce; WHO: World Health Organization; SD: standard deviation; BMI: body mass index

Children and adolescents with severe obesity (>97th centile, i.e. > 3 BMI-SD) are at high risk for future cardiovascular and metabolic disease (Friedemann et al.,2012). In this subgroup, substantial weight increments are reflected in small increments in tenths or hundredths of percentiles, standard deviations or z-scores (Flegal et al.,2009; Woo,2009). Therefore, the American Heart Association and the Endocrine Society recommend to use the percentage above the 95th BMI centile (%>95th BMI-centile) rather than centiles, standard deviations or z-scores (Flegal et al.,2009). The %>95th BMI-centile is the ratio of the individual`s BMI divided by the relevant 95th BMI-centile for an age- and sex-matched individual multiplied by 100%: e.g. the 95th BMI-centile for a male adolescent 14 years is 26 kg/m^2. If

the adolescents` BMI is 32kg/m^2, the %>95th BMI-centile is (32/26) x 100% = 123%). The %>95th BMI-centile is a continuous variable, starting from the 95th BMI centile (i.e. the cut-off for obesity). The %>95th BMI-centile measure revealed advantages in monitoring children and adolescents with severe obesity (Cole et al.,2005; Flegal et al.,2009) due to an improved visual tracking on the growth chart for clinical purposes (Gulati et al.,2012) and a solid correlation with the number and the severity of cardiometabolic risk factors (such as abnormal cholesterol, SBP and haemoglobin A1c-values (HbA1c)) (Skinner et al.,2015).

BMI, BMI centiles, BMI z-scores and the %>95th BMI are reflecting sheer mass rather than adiposity, i.e. these measures cannot quantify body fatness and lean mass (Prentice et al.,2001). However, the level of agreement for BMI predicting body fatness improves with higher BMI-levels, reaching a moderately high sensitivity of 70-80%, with a specificity of 95% when compared with the dual X-ray absorptiometry (DXA) as the reference method (Freedman et al.,2009a). Similar, the strength of the association between BMI and CVD risk increases with increasing levels of BMI according to a systematic review and meta-analysis investigating 63 studies with nearly 50`000 children (Friedemann et al.,2012). Measures other than BMI aim to quantify and characterise body fatness, as excessive fat is the main compartment associated with obesity-related cardiometabolic disease (Barker,2005; Fortuno et al.,2003; Prentice et al.,2001).

The most accurate, yet cumbersome method to assess body fat is the deuterium dilution method. It calculates total body water and fat-free mass according to an algorithm that uses the difference in secreted markers between baseline and post ingestion. As this method is not feasible for general practise, I will subsequently review the most common clinically assessed measures that were also used as exposures for this study.

In children with overweight (i.e. between the 85th and 95th centile), waist circumference (WC) was suggested superior to predict excess body fatness and adverse risk factor levels (Freedman et al.,2009a). In adults, a waist to height-ratio (WtH), a derivative of waist circumference, has been suggested superior to WC and BMI to predict CVD, T2DM (Ashwell et al.,2012) and central obesity (Ashwell et al.,2014). Studies in children and adolescents advised a WtH-ratio cut-off of 0.5 to assess cardiovascular risks (McCarthy et al.,2006a) with other studies proposing higher or lower cut-offs (Graves et al.,2014; Nambiar et al.,2010; Ribeiro et al.,2010). However, WC and WtH-ratio require age- and sex-specific cut-offs, both are subject to larger measurement errors when compared to BMI (Ulijaszek et al.,1999), both require physical contact which may be impractical in some settings, and the amount of additional information for risk prediction when BMI is already known may be little (Freedman et al.,2007).

Bioimpedance analysis (BIA) is another commonly used method to differentiate body weight into fat mass and fat-free mass. A single frequency alternating electrical current of 50 kHz estimates the amount of total body water. As fat contains little water, impedance is a surrogate for fat-free body mass and body fat can be calculated from the total weight subtracting the fat-free body mass. Limitations to BIA include that the resistance of arms and legs (due to lower diameters) represent almost 90% of the impedance while only representing ~ 50% of the body weight. Hence, changes in muscle mass disproportionally impact on BIA results. The second limitation is based on the assumption that fat-free mass consists of 73% water. Factors such as hydration status, a full bladder or exercise will therefore affect the results. These limitations in mind, the validity of BIA results is based on reference data comparing results with dual-energy X-ray absorptiometry (DXA) or – more cumbersome - stable isotope dilution methods. Such reference data, adjusted for sex in children aged 6-18 years have been published, illustrating a comparable development of body fatness until puberty in both sexes with males proportionately decreasing and females gaining body fat during puberty (McCarthy et al.,2006b). A study comparing BIA and DXA results for body fatness in obese adolescents before and after a weight management intervention have shown acceptable mean differences (0.4 to 2.1%) of body composition measures with large limits of agreements (~15% for fat free mass). The study group concluded that bioimpedance may be a valuable tool to assess body composition at a group level but inaccurate for an individual assessment (Wan et al.,2014).

2.5 Determinants of obesity in childhood and adolescence

Obesity is a multifactorial disease involving genetic and obesogenic, environmental factors as drivers for excessive weight gain (Agostoni et al.,2011; Sabin et al.,2010). In brief, from a genetic point of view, obesity is seen as a multiallelic condition with high heritability but overall small individual allelic effects (Lee,2013). The use of genome-wide association studies have revealed more than 100 risk alleles that contribute to a predisposition for weight gain. However, their individual effect seems rather low at ~0.17 kg/m2 per additional risk allele (Speliotes et al.,2010). Also, obesity phenotypes have shown large variations even amongst groups with almost identical genotypes (McGarvey,1991) and obesity-associated genes fail to explain the rapid onset of the obesity-epidemic over the last 4 decades (Herrera et al.,2011). In contrast to those risk alleles, the presence of specific monogenic or syndromic forms of obesity are exceedingly rare. Phenotypes caused by syndromic forms of obesity may include specific features such as short stature, developmental disabilities and functional and structural disorders of sensory organs (Sabin et al.,2011). Most of the monogenic forms are involved in the regulation of satiety and hunger such as those forms affecting the leptin/leptin receptor system (Mantzoros et al.,2011).

Other than genetics, various environmental and behavioural factors have been identified to adversely impact on the TEI/TEE ratio (Swinburn et al.,1999) and to promote childhood obesity. They include factors related to antenatal and maternal conditions, socioeconomic status, lifestyle or behaviour, sleep characteristics and the food environment people live in. The effect of each of those factors may be small, but the cumulative effect of a series of such factors that make up the daily routine of entire populations is substantial. As a consequence, understanding the environmental and behavioural determinants of obesity during childhood and adolescence is key for preventive strategies (Woo Baidal et al.,2012). Many determinants discussed below are integrated in international clinical guidelines for prevention of childhood obesity (Barlow et al.,2007; Cuda et al.,2018; Styne et al.,2017).

More recently, associations between environmental chemical exposures, particularly between endocrine disrupting chemicals (EDCs) and adverse effects on weight status have gained recent attention (Braun,2017). EDC`s are chemical compounds that may alter hormonal actioning and signalling (Zoeller et al.,2012) and there are concerns that such alterations may have higher impact during the period of infancy and childhood, when hormonal homeostasis is detrimental for normal growth and development. EDC classes that have been investigated for possible associations with weight status include phthalates, bisphenol A, triclosan and perfluoroalkyl substances. The body of evidence for an association between antenatal or postnatal exposure and subsequent weight status increases, however the overall findings are inconsistent (Andersen et al.,2013; Braun et al.,2014; Buckley et al.,2016a; Buckley et al.,2016b; Buser et al.,2014; Hoepner et al.,2016; Johnson et al.,2014; Li et al.,2015; Maresca et al.,2016; Mora et al.,2017). Various mechanisms of action of these EDC have been proposed: i) perfluoroalkyl substances are associated with impaired birth weight. Decelerated intrauterine growth followed by accelerated infant growth is associated with higher adiposity and cardiometabolic risk markers later in life (Perng et al.,2016); ii) phthalates might interfere with the metabolism of thyroid hormones, glucocorticoids and androgens; iii) bisphenol A may act in oestrogen signalling and iv) triclosan can disrupt gonadal and thyroid hormone circuits (more details are reviewed in (Braun,2017). Further research in large population studies will be required including a large set of known behavioural and environmental factors to adjust for confounding to estimate the contribution of ECD on weight gain. The next sections will discuss those known factors, divided in two periods: the first covering the first 1000 days of life starting from conception, the second covering the later period of childhood and young adolescence.

2.5.1 Determinants of obesity during the first 1000 days of life from conception

The origins for the development of childhood overweight or obesity can be identified very early in life with some studies declaring the period from 0-5 years as critical for the attainment of an excessive weight status in (Cunningham et al.,2017; Cunningham et al.,2014). In addition, a recent systematic review showed that origins of obesity in childhood can be identified as early as the first 1000 days of

life, starting from day 1 of conception until the second birthday of the child (Woo Baidal et al.,2016). A summary of the aforementioned study is provided as follows divided into two periods: i) an antenatal till birth, followed by ii) a postnatal from birth till the second anniversary.

i. For the first period, the study reported evidence for gestational diabetes mellitus, higher maternal pre-pregnancy BMI, excessive gestational weight gain and maternal tobacco smoking to be associated with childhood overweight. Limited evidence was found for the mode of delivery (Caesarean section versus vaginal delivery) as a risk factor for the development of overweight or obesity. Maternal age was not, and increasing maternal parity was inversely associated with childhood overweight (Woo Baidal et al.,2016).

ii. During the second period from birth until the second birthday, factors consistently associated with later child overweight were: higher birthweight, rapid weight gain, and changes in the faecal microbiome composition (with higher concentrations of *Staphylococcus aureus* being associated with childhood overweight). In addition, based on three large US prospective studies, low socioeconomic status (SES) was consistently identified as a risk factor for later childhood obesity. Inconsistent evidence was found for behavioural factors including sleep quantity or screen time, and no studies investigated the effect of physical activity / active play time in infancy on later obesity development. For breastfeeding, the evidence for an association with childhood overweight was inconsistent, however, the authors concluded that breastfeeding may provide a protective effect. Nutritional factors such as the early introduction of solid foods before 4 months, maternal intake of coffee or tea during infancy and high protein intake were found to increase the risk for childhood obesity (Woo Baidal et al.,2016).

2.5.2 Determinants of obesity in older children and adolescents

Table 2 illustrates an overview of risk factors for the development of obesity in older children and young adolescents, including factors related to the following categories: screen time, sleep characteristics, food environment, socioeconomic status and neighbourhood factors. Included were cohort studies, populational data, systematic reviews and meta-analysis where available. Factors specifically related to energy intake and energy expenditure are discussed in the subsequent sections 2.5.3 and 2.5.4.

There is substantial multi-collinearity between the factors investigated amongst studies. E.g. screen time is associated with higher BMI but also with reduced sleep duration, which is a risk factor *per se* (Zhao et al.,2018). Similar, the consumption of sugar-sweetened beverages (SSB) is associated with increased BMI levels, however this relation is partly mediated by lower socio-economic status (SES) (Chung et al.,2018). Specific methodological problems arise for studies that investigate the effect from SSB. First, there is considerable heterogeneity in the definition of an SSB (Bucher Della Torre et al.,2016). Second, SSB are a marker of a poor-quality diet (Ambrosini et al.,2013), therefore, it is difficult to isolate the independent effect from SSB. Second, higher levels of physical activity may confound the results as physical activity is related to higher sports drink intake in some studies (Tomlin et al.,2013).

When investigating the effects from SES, a couple of specific characteristics are important. First, various definitions and metrics are commonly used in studies investigating SES. Some authors use country-specific indices, whereas others combine measures of parental income and occupation as exposure measure. Irrespective of the exact measure used, SES is believed to be one of the strongest risk factors for developing obesity in childhood, particularly in countries with "Western lifestyles" (Magnusson et al.,2014; Stamatakis et al.,2010). Also, there is evidence for an aggravation of associations between SES and rates of obesity in childhood: In many populations with higher SES, the incidence of obesity in childhood has generally slowed down or even reversed in recent years (Chung et al.,2016; Cunningham et al.,2014). In contrast, there is limited evidence for a similar development in countries with people exposed to lower SES (Cunningham et al.,2014; Morgen et al.,2014). Factors related to total energy intake, particularly the macronutrients composition and total energy expenditure are discussed in the following sections 2.5.3 and 2.5.4.

Table 2. Studies investigating environmental, socio-economic, behavioural, food-related and early-life risk factors for obesity in childhood and adolescence

	Measures	Study site	Metrics on study design	Outcome	Reference
Screen time	TV watching assessed by 2015 Youth Risk Behaviour Surveys in one US cohort of high school students	US	High school students mean age 16y / n = 24`800 / Nationally representative sample / cross-sectional	>5 hours TV watching is associated with - consumption of SSB, OR = 2.72 [95%CI 2.23 to 3.32] - risk for obesity, OR = 1.78 [95%CI 1.40 to 2.27]	(Chaput,2017)
	Associations between screen time and ponderal index (weight/height^3), skinfold thickness and bioimpedance-related fat mass (kg/m^5)	UK	The Child Heart and Health Study in England (CHASE) cohort / cross-sectional survey / aged 9–10 years / n = 5887	Compared to 1 or less hours of screen time per day, reported screen time of 3 or more hours was associated with higher - ponderal index 1.9% [95%CI 0.5% to 3.4%] - skinfold thickness 4.5% [96%CI 0.2% to 8.8%] - fat mass index 3.3% [95%CI 0.0% to 6.7%]	(Nightingale et al.,2017)

	Overall screen time and adiposity	World	Systematic review of published systematic reviews	Medium-quality reviews by - Costigan *et al*. (Costigan et al.,2013) showed in 32/33 studies a strong relationship between overall screen time and weight status including 7/8 studies with low risk for bias. - Ekris *et al*. (van Ekris et al.,2016) reported strong evidence for an association between screen time and BMI and BMI z-score - Carson *et al*. (Carson et al.,2016) reported a strong association between screen time and unfavourable body composition in 11/13 longitudinal studies, 4/4 case-control studies and 26/36 cross-sectional studies	(Stiglic et al.,2019)
Sleep	Sleep time and obesity	World	2-102 years / n (children) = 30`002 / Meta-analysis of cross-sectional studies	Pooled OR for short sleep duration (<10 hours) and obesity: - 1.89 [95%CI 1.46 to 2.43]	(Cappuccio et al.,2008)
	Association between Insufficient sleep time (9 hours for children, 8 hours for adolescents, according to a Consensus Statement of the American Academy of Sleep Medicine (Paruthi et al.,2016)) and overweight/obesity	Greece	Population data, school-based health survey in youth aged 8-17 years / n = 177`091 (40% of population)	Adjusted OR between insufficient sleep time: - 1.26 [95%CI 1.21 to 1.31] with increased screen time - 1.21 [95%CI 1.17 to 1.25] with overweight/obese	(Tambalis et al.,2018)
	Sleep duration and subsequent weight gain	US, NZL, Canada	0-18 years / n = 10`959 / systematic		(Magee et al.,2012)

			review including 7 longitudinal studies with children	Shorter sleep duration significantly predicts subsequent weight gain in 7/7 longitudinal studies	
	Increasing versus decreasing sleep time by 1.5 hours per night for 7 days	US	8-11 years / n = 37 / randomized cross-over trial	Less sleep is associated with increased energy intake (-134 kcal/d, $P < 0.05$) and increased weight (0.22 kg, $p < 0.001$)	(Hart et al.,2013)
Food environment	Effect of exposure to 30-second commercials embedded in cartoon-video	US	2-6 years / n = 46 / randomized controlled trial	Children exposed to video-commercials were significantly more likely to choose the advertised item	(Borzekowski et al.,2001)
	Effect of consuming SSB and household income	Canada	4-5 years / n = 2103 / longitudinal follow-up study	RR for overweight >3x higher for children with regular SSB consumption living in low-income households compared to non-consuming children in sufficient income households	(Dubois et al.,2007)
	Association between SSB and obesity	World	Review of systematic literature reviews / children aged 6 months to 19 years	9 reviews concluded there was a direct association between the SSB consumption and obesity in children and adolescents, 4 did not find an association. Two meta-analysis with highest quality scores showed discrepant results	(Keller et al.,2015)
	Systematic analysis of the methodology of cohort and experimental studies investigating the influence of SSB on the risk of obesity in childhood and review of conclusions in light of the methodological quality	World	Review including 29 cohort studies and 3 randomized controlled trials	More than half the studies presented with methodological issues that may affect the conclusions drawn. Studies that passed methodological assessment indicated positive associations between SSB and the development of obesity, particularly in overweight individuals	(Bucher Della Torre et al.,2016)

	Systematic review and meta-analysis on the quantitative effect of screen advertising of food on children's dietary intake	World	Children and adolescents aged 2-18 years, mean 8.8 years	Exposure to 4.4 minutes of food advertising on average would increase a child's food consumption by 60.0 kcal [95%CI 3.1 to 116.9] Playing an advergame with food cues for 5 minutes would increase consumption on average by 53.2 kcal [95%CI 31.5 to 74.9]	(Russell et al.,2019)
Various factors	Effect of early life risk factors >3 years of life and obesity rate at age 7	UK	Children until age 7 years / n = 8234 / longitudinal follow-up study	Relative risk associated with obesity at age 7 years: - parental obesity (both parents): adjusted OR 10.44 [95%CI 5.11 to 21.32]; - very early adiposity rebound: OR 15.00 [95%CI 5.32 to 42.30]; - > 8h TV time/week at 3 years: OR 1.55 [95%CI 1.13 to 2.12]; - catch-up growth: 2.60 [95%CI 1.09 to 6.16]; - birth weight, per 100g: 1.05 [95%CI 1.03 to 1.07]; - Less than 10.5 hours sleep at age 3 years: OR 1.45 [95%CI 1.10 to 1.89]	(Reilly et al.,2005)
Socioeconomic Status (SES)	Associations between income-status and ethnicity on weight status	US	Grades 1,4,7,10 / n = 111`799	For every 1%-increase in the proportion of low-income status, the prevalence of overweight/obesity increased by 1.17% Race/ethnicity was no longer associated with overweight/obesity prevalence, when adjusted for income-status	(Rogers et al.,2015)
		US			

Associations between SES: weighted measure of parental income, both parent`s educational attainment and prestige of occupation, with child`s BMI at Kindergarten		Early Childhood Longitudinal Birth Cohort (ECLS-B) / n = 14`000 Children / born 2001 in the US	Association between SES, race, birthweight, smoking status and family-dinner with overweight or obesity at Kindergarten entry: - OR for children in lowest SES quintile compared to highest SES quintile for overweight/obesity 1.7 [95%CI 1.3 to 2.2]; - OR for Black versus White children 1.57 [95%CI 1.21 to 2.05] and for Hispanic versus White 1.65 [95%CI 1.31–2.08]; - OR per 100gr increase in birthweight 1.07 [95%CI 1.06-1.08]; - OR for parental smoking 1.40 [95%CI 1.17–1.67]; - OR for family-dinner 0.96 [95%CI 0.93–0.99]	(Williams et al.,2018a)
Systematic review of associations between SES and childhood-adolescent weight status in asset-rich countries (SES = parental education, income and occupation)	Data from the 27 richest OECD countries	Systematic review between 1990-2013, youth between 0-21 years, 158 papers included	Bivariable: Associations between SEP and childhood-adolescent weight status were: - inverse for 60.4% of the studies, null for 18.7% and 20.9% of studies identified another dependant variable (e.g. age, sex or ethnicity) Multivariable: Associations between SEP and childhood-adolescent weight status were: - inverse for 51.1%, null for 20% and 27.8% identified another dependant variable	(Barriuso et al.,2015)

	Combined Index of Community Socio-Educational Advantage (ICSEA) including parents' occupation and education, school geographical location and the proportion of Indigenous students. Outcome: BMI	AUS	Longitudinal follow up study of the Australian Capital Territory Physical Activity and Nutrition Survey (ACTPANS) between 2006 to 2018	Compared to the highest ICSEA quintile, individuals with lowest ICSSEA had an adjusted prevalence ratio of 1.52 [95%CI 1.25 to 1.84] for overweight and obesity Corresponding adjusted prevalence ratios for overweight or obesity were: - 1.56 [95%CI 1.37 to 1.78] for 0-2 days per week of physical activity (60m/d) compared to 6-7 days per week - 1.44 [95%CI 1.23 to 1.68] for >/=5h TV and computer time versus 0-1h per weekday - 1.44 [95%CI 1.19 to 1.76] for Indigenous status versus non-Indigenous status	(Yang et al.,2019)
Neighbourhood factors	Physical activity environment, Nutrition environment characteristics	US	age 6-11 years / n=730 / cross-sectional	For favourable physical activity environment & nutrition environment: - Likelihood for obesity 7.7% vs 15.9%	(Saelens et al.,2012)
	SEIFA-scores (SEIFA = areas in ranked according to relative socio-economic advantage and disadvantage, based on 5-yearly Census data)	AUS	Age 2-18 years / n= 444 / cross-sectional	Neighbourhood education/occupation score negatively associated with BMI. Higher family education positively associated with lower BMI	(Juonala et al.,2019)
	Proximity to unhealthy food outlets and weight outcomes	New York City, US	3`507`542 student-year observations 2009-2013	Living > 0.025 miles away was associated with lower risk for obesity and BMI z scores (decreased ratio of obesity between 2.5-4-4%).	(Elbel et al.,2020)

Abbreviations: TV: television; SSB: sugar-sweetened beverage; BIA: bioimpedance analysis; OR: Odds ratio; RR: relative risk; 95%CI : 95% confidence interval; US: United States; UK: United Kingdom; NZL: New Zealand, AUS: Australia ; OECD: Organisation for Economic Cooperation and Development; SES: Socio-economic status; n: number of individuals; SEIFA-scores : Australian Socio-Economic Indexes for Areas -score

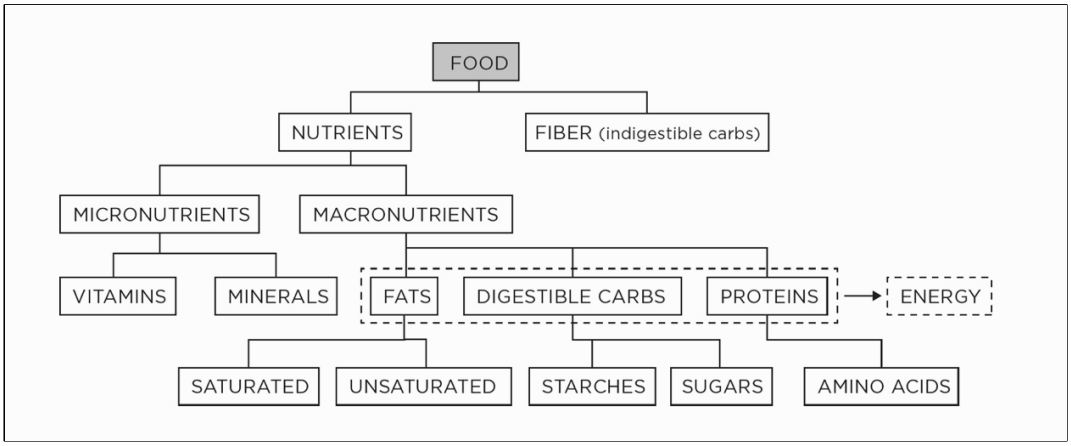
2.5.3 Total energy intake

Obesity is caused by a mismatch between TEI and TEE (Han et al.,2010; Jequier et al.,1999; Swinburn et al.,2002). The total energy surplus of energy intake for normal growth among US children and adolescents between 1988–1994 to 1999–2002 was estimated to be 110–165 kilocalories per day. For a child to become an overweight adolescent, the same figure over a 10-year period was estimated at 678-1017 kcal/d (Wang et al.,2006). In accordance, data from the 1977-1978 Nationwide Food Consumption Survey and the 1989-1991 and the 1994-1997 Continuing Surveys of Food Intake by Individuals showed that children overall increased their TEI by almost 200 calories per day between the assessment of the surveys, along with increasing rates of overweight and obesity (Nielsen et al.,2002).

So far, many studies focussed on identifying solitary food products that may be responsible for excessive energy intake and elevated BMI levels. Consequently, foods with high carbohydrate-content such as pizza or SSB were often targeted. In fact, SSB contribute up to ~40% of the added sugars in the US (McGuire,2016). They increase the TEI in youth (Malik et al.,2013) and are linked to adult cardiometabolic disease outcomes (Malik et al.,2010). However, as discussed in the previous section 2.5.2, there are methodological issues with addressing the particular effect from SSB on childhood BMI levels and many other food sources are subject to confounding by other determinants of obesity in childhood.

A different approach was to look at the amount and composition of macronutrients in daily nutrition. Macronutrients include carbohydrates, fats and proteins and their amount consumed determines the total energy intake (see Figure 1). Proteins contain less energy than the equivalent mass amount of fats, and their percentage contribution to the daily TEI is just about 20% compared with 50% from carbohydrates and 30% from fats in a standard diet (National Health and Medical Research Council,2006, updated September 2017). Nonetheless, subtle changes in the percentage energy intake from proteins may have a disproportionately high impact on TEI, a mechanism termed protein leverage (PL). Based on this mechanism, the protein leverage hypothesis (PLH) was formulated (Simpson et al.,2005), which posits that in humans, PL contributes to weight gain and obesity.

Figure 1. Food and its nutritional and energy-providing components



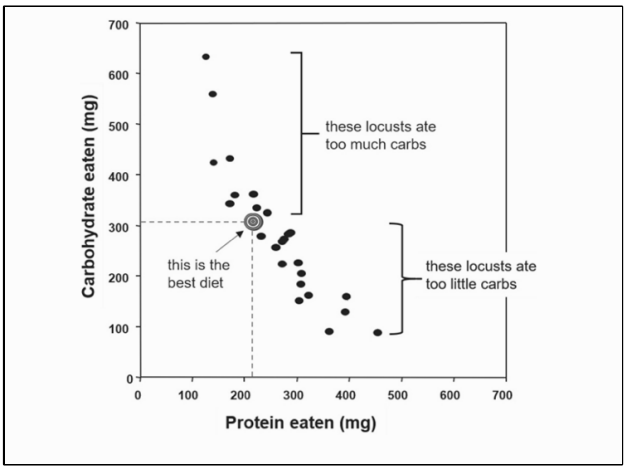
Energy is provided by the intake of the three macronutrients: carbohydrates, fats and proteins (dashed boxes). With permission from Stephen Simpson and David Raubenheimer, authors of "Eat Like Animals", March 2020.

2.5.3.1 Protein leverage hypothesis

The protein leverage hypothesis predicts that diets low in the proportion of energy from proteins stimulate a compensatory increase in food intake and hence TEI to attempt to achieve a certain absolute protein intake (Simpson et al.,2005). This hypothesis is based on studies in various insects, birds, fish and mammals, using an approach termed nutritional geometry. The foundation of this hypothesis is that first, separate appetite systems control the intake of different macronutrients to guide the animal towards an optimally balanced diet to achieve best possible outcomes for growth, survival and reproduction; and second, in situations of unbalanced food supply, an adequate intake of proteins takes precedence over carbohydrates and fats (Berthoud et al.,2012; Raubenheimer et al.,1997; Simpson et al.,2014).

Before the development of the protein leverage hypothesis, the novel concept of nutritional geometry was used in a study with locusts in 1991 (Raubenheimer D.,1993). The authors investigated the effect from foods with various proportions of carbohydrates and proteins (ranging from high protein/low carb to high carb/low protein) on the time span until the locusts successfully molted to become winged adults or died beforehand. Foods with one specific composition of proteins and carbohydrates were provided to the animals *ad libitum*. As plotted in figure 2, the relation between carbohydrates and proteins followed a specific distribution. From this plot, the authors identified the intake point that best represented the healthiest nutrition where the animals` growth and survival was balanced. The resulting plot also showed that locusts who ate too much carbohydrates lined up vertically close to a protein intake of about 150 mg, which is close to their protein-target at approximately 210 mg. However, to do so, they needed more time to become winged adults, which is associated with higher risk of being victim to predators and they became obese, a very unusual outcome in insects. In contrast, those locusts fed on a low carb diet increased their protein intake, at costs of a much lower intake of carbohydrates. Effects from a low carbohydrate/high protein diet were that these animals became lean and less likely to survive to adulthood. This experiment illustrated the existence of different appetite systems for carbohydrates and proteins with different outcomes for growth and survival based on the diet that was eaten. Also, the experiment showed that locusts had a preference for proteins over carbohydrates when the nutrition provided does not allow to reach the target intake.

Figure 2. Plot of intakes of locusts fed ad libitum on diets with various proportions of carbohydrates and proteins



The bull`s eye illustrating the target diet, the diet that supported best growth and survival. With permission from Stephen Simpson and David Raubenheimer, authors of "Eat Like Animals", March 2020.

Subsequent works identified multiple appetite-systems in many animal species that aim for a balanced diet that is specific for the animal and allows the best for survival, growth and reproduction. The first locust-similar experiment involving humans was performed in an isolated chalet in the Swiss Alps, the results of which were published in 2003 (Simpson et al.,2003). The experiment lasted one week, with 10 individuals involved. The first two days, participants were allowed to eat at their discretion from a buffet including a large variety of foods and the macronutrients composition, proportional energy intake per macronutrient and total energy intake was meticulously reported. Then, on days three and four, half the individuals where allocated to a high protein buffet and the other half of the participants to a high carbohydrate and high fat diet, still allowed to eat as much as they liked. Subsequently, they returned to the original buffet for the last two days of the experiment. Results from the analysis of the first two days showed that participants consumed food with a proportion of energy from proteins at 17% that is well fitting with the suggested 15-20% for people all over the world. In the second phase of the experiment, all participants aimed to maintain their protein intake at a similar level as during the first two days. But to do so, those allocated to the high-fat high-carbohydrate diet had to increase their total energy intake by 35% to reach their target intake of proteins. In contrast, those allocated to the high-protein diet decreased their energy intake by 38% compared to the free-choice period. The main conclusion was that similar to the experiment in locusts, the human appetite for proteins seemed to determine the total energy intake. This experiment was the background of the protein leverage hypothesis that predicts that diets low in the proportion of energy from proteins stimulate a compensatory increase in food intake and hence TEI to attempt to achieve a certain absolute protein intake.

Further data supporting protein leverage in adult humans were based on experimental studies, systematic compilations of data from numerous trials, and analyses of cohort and population data. Figure 3 illustrates how to test for protein leverage involving data from a compilation of clinical trials and experimental datasets (Gosby et al.,2014; Raubenheimer et al.,2015a; Raubenheimer et al.,2015b). A few premises to understand need to be mentioned:

1. The mechanism of protein leverage causes an increase in total energy intake when the proportion of energy from protein in a diet is diluted by either fats and/or carbohydrates.
2. The strength of the protein leverage mechanism is diluted by the content of fibre. Fibre is – similar to proteins – associated with satiety, however does not add on to the total caloric energy intake (Roberts et al.,2014).
3. The strength of protein leverage depends on the extent to which protein intake is prioritized over the regulation of other nutrients and on the actual percentage level of energy from protein sources.

Figure 3 A illustrates the total energy intake on the y-axis and the protein range from 5% to 54% on the x-axis, and figure 3 B illustrates a subset of the range of percentage protein values that is commonly found in diets of free-living human populations ranging from 10-30%. The resulting relation is typical for a fitted line following a power calculation. The power calculation describes the relation between food intake (in grams) and percent dietary protein (p) to maintain a constant, targeted intake of proteins (P, in grams). In other words, to maintain a targeted intake of proteins (P in grams) on a diet containing a given proportion of proteins (p), $P \times p^{L}$ grams of food must be eaten. The coefficient L describes the strength of protein leverage. Assuming full protein leverage, i.e. L = -1, small changes in p will cause substantial changes in total intake (e.g. if the target intake of protein is 100g, a diet containing 20% protein will cause an intake of 500 gr of food. If the dietary percent protein is reduced to 16%, 625gr of this particular diet needs to be consumed to meet the protein target). Assuming no protein leverage, i.e. L=0, changes in p will not cause changes in total energy intake

A detailed discussion is in section 4.2.9. The grey and green dashed line in Figure 3 B illustrate the extreme outcomes. For the grey dashed line represents complete protein leverage (L = -1), i.e.

individuals will maintain their protein intake at a fixed value (chosen here the mean value of the dataset at 386 kcal/d). The green dashed line indicates the outcome if total energy intake was maintained at a fixed level ($L = 0$) (chosen here is the mean value of the total energy intake from the dataset at 2086 kcal/d). However, the true results (blue and red dots) are following a power function with a strength of leverage L of -0.525 that approached but did not achieve total protein prioritization.

Figure 3. Data on protein leverage in adult humans

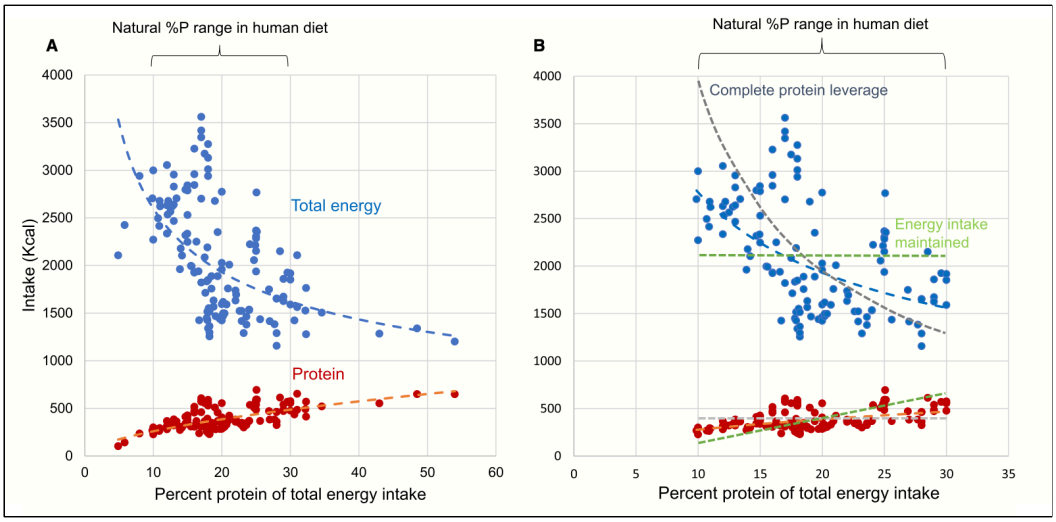


Figure 3 shows how to test for protein leverage. The plot includes data on TEI on the y-axis and the proportion energy (in %) from protein on the x-axis. The effect of protein leverage is best represented using a power calculation according to the following baseline formula: Required food intake = P x p^L. Figure 2 B illustrates two hypothetical extreme outcomes: the grey dashed line for complete protein leverage (L= -1), where the targeted protein intake would remain at a fixed value and one where total energy intake would remain stable (i.e. no protein leverage, green dashed line). Plotted data suggest a partial protein leverage with a strength of leverage L at ~ -0.5.

The protein leverage as a contributor to the US obesity pandemic was formulated by the founders of the protein leverage hypothesis in 2005 (Simpson et al.,2005). They observed data from the Food and Agriculture Organization of the United Nations database of nutrient availability. The data showed that the average diet composition between 1961 to 2000 in the US changed significantly, with a decrease of the proportion of proteins from 14% to 12.5%. The relevant study, revealing that a decline of 1.5% of energy from protein sources between 1961 and 2013 was accompanied by an increase in TEI of 950 kcal per capita was published in 2019 (Hall,2019).

Studies in infants have shown positive associations between a higher percentage of energy intake from protein sources with later development of obesity (Weber et al.,2014a). No data on protein leverage in children or adolescents are available.

2.5.4 Total energy expenditure

TEE is the total amount of energy needed to maintain homeostasis in biological systems. TEE is comprised of mainly three parts including resting energy expenditure (REE, ~55-65% of TEE), diet-induced energy expenditure (DEE, ~10%) and activity-induced energy expenditure (AEE, ~25-35%) (Westerterp,2017). The latter is the most variable portion for TEE, mostly related to the sum of physical activity (Washburn et al.,2014). The main determinants for TEE are body size, body composition, TEI and physical activity (Westerterp,2017). The gold standard to evaluate TEE in humans is the doubly labelled water (DLW) technique.

2.5.4.1 Assessment of resting energy expenditure and basal metabolic rate

REE is the amount of energy needed to maintain physiologic functions including respiration, cardiac and body temperature regulation (Rachel K. Johnson,2001). A surrogate measure for REE is the basal metabolic rate (BMR). BMR is measured under strict criteria: the individual needs to be completely rested, fasted for at least 12 hours, free from any stress and the environment should be thermoneutral (Henry,2005). Primarily, the measurement of BMR in the early 20th century was used to diagnose hypo- and hyperthyroidism. However, the interest for BMR raised in the late 20th century when BMR was used to investigate the development of human obesity and to estimate people`s energy requirement (previously, food intake surveys were used for similar questions). Based on this novel application for BMR, accurate measures using easily accessible anthropometric data were warranted (Henry,2005). After equations utilizing body surface areas as a proxy, the Food and Agriculture Organization, World Health Organization (WHO) and United Nations University (UNU) Joint Expert Consultation on Energy and Protein Requirements which met in Rome 1981, requested Schofield to produce novel formulas to predict BMR for both sexes in various age groups (Schofield,1985a). The equations by Schofield rely on the parameters age, sex and weight and were based on a sample size of 7173 BMR measurements. Other equations including different age groups and body compositions were subsequently performed including one from the WHO (WHO,1985).

The generalizability of the Schofield BMR results was found to be limited. Most of the studies reported overestimation of BMR results (Henry,2005), with a few exceptions in children that reported underestimation of BMR results (Bandini et al.,1990; Livingstone et al.,1992). Likely, this was related to ethnicity (Europeans and North Americans formed the majority), and the overrepresentation of Italians (47%), who were subsequently found to have a higher BMR per kilogram bodyweight. Due to the effect of body composition on REE (higher body weight resulting in higher REE and TEE), specific equations for children and adolescents with overweight or obesity, including various parameters such as fat-free mass, fat mass and pubertal stage were generated (Lazzer et al.,2014; McDuffie et al.,2004; Tverskaya et al.,1998). Amongst those, the two formulas provided by Lazzer in 2014, including pubertal stage (Tanner I-V) and gender and either weight and height or fat-free mass and fat mass are probably the most accurate based on comparisons with indirect calorimetry using the Bland and Altmann Method and cross-validation (Lazzer et al.,2014).

Irrespective of the method used to assess BMR, Goldberg suggested using BMR levels to identify invalid food intake reports when TEE measures are not available (Goldberg et al.,1991). This approach evaluates the reported energy intake by multiplying BMR with an estimated physical activity level (PAL, see later). The resulting measure reflects a minimum required energy intake for maintenance of basal metabolism, i.e. for homeostasis. Depending on the PAL used in the equation, this approach allows exclusion of under-reporters with high specificity.

2.5.4.2 Assessment of activity induced-energy expenditure (AEE)

There is no standard way to assess AEE, i.e. physical activity energy expenditure (Dencker et al.,2011). However, DEE is rather constant at 10% of TEE in an average mixed diet (Westerterp,2004). Utilizing BMR as a surrogate for REE and provided TEE is known, AEE can be calculated as follows:

$$AEE = 0.9 \times TEE - REE$$

AEE is determined by body movement (i.e. physical activity) and body composition, and both are related to each other as physical activity requires energy from stored sources in fat or muscle (Westerterp,2013). It requires more energy to move a large body, as a consequence, AEE results need to be adjusted for body size.

2.5.5 Physical activity, physical exercise and physical fitness

Physical activity is defined as any bodily movement produced by skeletal muscle that results in energy expenditure (Caspersen et al.,1985). Physical fitness is defined by the American College of Sports and Medicine as a set of attributes that people have or achieve that relates to the ability to perform physical activity (Wilder et al.,2006). Components of physical activity include the measurement of body composition, cardiorespiratory endurance, muscular fitness, and musculoskeletal flexibility (Wilder et al.,2006). Maximal oxygen uptake (VO2max) is the measure to define cardiorespiratory endurance, whereas muscular fitness is composed of muscular strength and muscular endurance. Musculoskeletal flexibility rather focuses on joints and associated structures such as ligaments and muscles that cross the joints (Wilder et al.,2006).

In the next sections, study-specific methods for the assessment of physical activity and physical fitness are reviewed and related to adverse cardiometabolic health-outcomes due to obesity.

2.5.5.1 Assessment of physical activity

Physical activity is vital for achieving and maintaining a healthy weight (Poorolajal et al.,2020) and an important factor to prevent or improve obesity-related health problems (Janssen et al.,2010; Tremblay et al.,2011) such as high blood pressure (Aguilar-Cordero et al.,2020; Vale et al.,2015). Recommendations from various health care departments, professional and scientific organisations require children and adolescents to perform 60 minutes of moderate-to-vigorous physical activity for six or more days a week (Services,Last reviewed May 29, 2019; WHO,2011). However, data from the 2016 National Survey of Children`s Health revealed that only 24% of children and youth meet the recommended guidelines (US Department of Health and Human Services). For the assessment of physical activity, many methods are proposed, each providing information on different facets. (Sylvia et al.,2014)

If TEE and BMR are known, the total PAL of a person can be expressed in one measure, resulting from the ratio of TEE/BMR. A PAL of 1.2 is considered for chair-bound or bedridden individuals, a PAL level of 1.55 is for those with low activity levels and a PAL of 2.4 is for professional athletes (Black et al.,1996).

However, the DLW technique to assess TEE is cumbersome and expensive, hence other methods are commonly used. They may be based on subjective (self-report questionnaires), or objective methods (e.g. direct observation, indirect calorimetry, accelerometers) (Strath et al.,2013; Sylvia et al.,2014).

2.5.5.2 Accelerometery

Accelerometers assess the quantity and quality of physical activity including the amount of sedentary time (e.g. sleep) (Rowlands,2007). They have proven practicability in children already at preschool age (Pate et al.,2006), are unobtrusive and easy to use (Rowlands,2007).

Guidelines regarding the validity of accelerometer data and cut-offs used to categorize intensity have been published (Colley et al.,2010; Colley et al.,2011). The CHASE study (Child Heart And Health Study in England) in primary school children across ethnic groups showed inverse associations between the amount of time spent physically active and adiposity measures, insulin resistance, C-reactive protein levels, low-density lipoprotein cholesterol (LDL-C) and positive associations with systolic blood

pressure and high-density lipoprotein cholesterol (HDL-C) (Owen et al.,2010). In an even larger cohort, including nearly 30`000 individuals aged 4-18, the intensity rather than the amount of physical activity was found to be the main determinant for variation of cardiometabolic risk factors (Tarp et al.,2018). A meta-analysis including 6009 children and adolescents from eight studies of the ICAD (International Children's Accelerometery Database) consistently concluded that increases in the amount of moderate intensity are associated with beneficial cardiometabolic health (Renninger et al.,2020).

2.5.5.3 Grip strength, a global measure of muscular strength

Muscular strength and muscular endurance indicate overall muscular fitness. Hand grip strength is a global measure of muscular strength and increases with physical activity training. Hand grip strength can be easily measured by participants gripping a dynamometer with maximum force and the maximum measured result, normalized for body weight after two attempts on each hand is used for further analysis according to standardised protocols (CDC,2011). Recently, a number of studies investigated effects from hand grip strength in large population studies.

The variability of handgrip strength (further termed grip strength) is influenced by behavioural and lifestyle factors and up to 56% by heritability according to a systematic review and meta-analysis (Zempo et al.,2017). In a nationally representative sample of 8469 Australian children aged 7-15 years, adiposity, fat-free mass and cardiorespiratory fitness were identified as correlates for grip strength (Fraser et al.,2020). Higher levels of grip strength in childhood are associated with lower continuous metabolic syndrome scores (a composite measure including the percentage body fat, SBP, triglycerides, HDL-C and glucose) in both boys and girls (Peterson et al.,2014). Besides inverse associations with adverse cardiovascular risk factors, a systematic review revealed benefits from higher muscular strength for bone and psychological health and cognitive ability (Smith et al.,2014).

Muscular fitness (assessed by grip strength and standing long jump tests) has shown to be inversely associated with future cardiovascular disease risk scores (combined outcome including results for skinfolds, systolic blood pressure, insulin, glucose, triglycerides, total cholesterol and HDL-C) after a 2-year follow-up (Castro-Pinero et al.,2019). With respect to the future risk for T2DM, Australian and European data have shown inverse associations between grip strength and adult levels of insulin resistance and beta cell function, both precursors to the onset of T2DM (Fraser et al.,2018; Grontved et al.,2013).

In adulthood, higher grip strength has been associated with a 20-35% lower risk for premature mortality due to any cause or cardiovascular disease, independent of blood pressure and BMI as shown in a Swedish study of >1 million male adolescents, followed-up for over 24 years (Ortega et al.,2012). Similarly, a study in more than half a million participants from the UK Biobank (including 54% women), aged 40-69 years, showed increased hazard ratios per 5 kg lower grip strength for all-cause mortality of 1.20 in women and 1.16 in men, and a cause-specific mortality from cardiovascular disease of 1.19 in women and 1.22 in men (Celis-Morales et al.,2018). Grip strength and adiposity have been shown to independently predict higher mortality risk, with grip strength being capable to partly attenuate the risk arising from excess adiposity (Kim et al.,2017).

Data on children investigating grip strength and subclinical cardiovascular phenotypes is sparse: one study in children aged 11-12 years with healthy weight demonstrated an inverse association between muscular strength and cIMT, independently of cardiorespiratory fitness and central adiposity (Melo et al.,2015). Furthermore, data in youth with obesity investigating the impact of grip strength on cardiometabolic risk factors and vascular health is lacking.

2.6 Cardiovascular risk factors in children and adolescents with obesity

Evidence for an association between increasing weight and cardiovascular risk in early life arose from pathological investigations by the Bogalusa Heart Study (Berenson et al.,1998) and the Pathobiological

Determinants of Atherosclerosis in Youth (PDAY) (McMahan et al.,2006). These investigations proved a weight-dependent increase in the presence of fatty streaks and fibrous plaques in post-mortem analysis in otherwise healthy children and young adults aged between 2-34 years.

Increasing BMI in childhood and adolescence is associated with more adverse levels of each core cardiovascular risk factors including elevated blood pressure (Freedman et al.,2012), dyslipidaemia (Margolis et al.,2014), elevated inflammatory biomarkers (Schipper et al.,2012; Skinner et al.,2010) and altered glucose metabolism (such as insulin resistance or T2DM (Abbasi et al.,2017). Also, the prevalence of cardiovascular risk factors correlate with the weight category: 37% of normal weight, 49% of overweight and 61% of US adolescents with obesity aged 12 to 19 years had at least one cardiovascular risk factor according to the NHANES survey from 1999 to 2008 (May et al.,2012). Amongst children and adolescents aged 6-19 years with severe obesity, determined by the ≥ 120% of 95th BMI-centile, the odds ratio (OR) compared to normal-weight counterparts for high blood pressure were 5.3; for high total cholesterol 2.4; for low high-density lipoprotein-cholesterol (HDL-C) 7.3; for high triglycerides 4.5; for high LDL-C 2.3; and for high fasting glucose 2.7 (Li et al.,2016).

Up to 80% of children with obesity will remain obese in adulthood (Juonala et al.,2011; Ward et al.,2017). As a consequence, paediatric obesity is increasingly associated with obesity-related co-morbidities in adulthood including CVD , T2DM and non-alcoholic fatty liver disease (Daniels et al.,2005; Morrison et al.,2007).

2.6.1 Blood pressure

The overall prevalence of hypertension amongst US children and adolescents is ~3-5% and up to 25% amongst children with obesity (Flynn et al.,2017; Hansen et al.,2007). Studies in adults have shown a near linear association between BMI with SBP and DBP (Hall,2003). However, not every individual with obesity has a blood pressure in the hypertensive range. Higher proportions of body fat in visceral and retroperitoneal parts have been shown to increase the risk for higher BP values (Tchernof et al.,2013). Increasing fat mass causes a higher total blood flow to supply the extra adipose tissue causing an overall higher resting tissue blood flow. This in turn is accompanied by a limited blood flow reserve that is normally needed during exercise, partly explained by endothelial dysfunction (Raitakari et al.,2004). This functional vascular dilatation and higher blood flow increases cardiac output and increases peripheral resistance, contributing to the development of hypertension and left ventricular hypertrophy (Hall,2000). Impaired renal-pressure natriuresis causing increased renal sodium absorption is another major mechanism to initiate higher blood pressure levels. As a result of impaired pressure natriuresis, higher BP measures are needed to maintain normal natriuresis in individuals with excessive weight (Hall,1997). Factors related to obesity-induced renal-pressure natriuresis impairment include: i) physical compression of the kidneys due to retroperitoneal fat tissue (Hall et al.,2014); ii) an activated renin-angiotensin-aldosterone system and activated mineralocorticoid receptors independent of aldosterone (Hall et al.,2012); iii) a dysfunctional, activated sympathetic nervous system (Hall et al.,2015); and iv) an impaired proatrial natriuretic peptide control. Levels of natriuretic peptides are inappropriately low in obese hypertensive individuals compared to lean normotensive individuals despite higher salt intake (Asferg et al.,2013) and their release in response to a volume load was shown to be impaired (Savoia et al.,2009).

The assessment of blood pressure among children necessitates appropriate methods to avoid false measures including i) repeated measures within one visit (Alpert et al.,2006), ii) assessment on multiple, different visits (McNiece et al.,2007), iii) using appropriate cuff sizes (Pickering et al.,2005), and iv) using the auscultatory method (Urbina et al.,2015). Auscultatory BP values, gained by aneroid and mercury devices are comparable (Duncombe et al.,2017). In contrast, oscillatory devices, despite increasingly used, have some limitations compared to auscultatory methods. First, they systematically overestimate SBP and DBP compared to auscultatory devices (Chio et al.,2011). Second, target organ damage such as elevated left ventricular mass or higher PWV are better predicted by auscultatory devices (Urbina et al.,2015). Third, oscillatory devices show a large within-visit variation with

inaccurately high readings particularly at the first measurement (Negroni-Balasquide et al.,2016). Fourth, rather than directly measuring SBP or DBP, the devices us a proprietary algorithm to calculate the SBP and DBP from the mean arterial pressure (Alpert,2010). However, if the device is approved for children and adolescents and if the aforementioned limitations are accounted for, the oscillatory method can be used as an alternative for BP assessment in children and adolescents (Duncombe et al.,2017).

Ambulatory blood pressure monitors record blood pressure values every 20-30 minutes through daytime and every 45-60 minute at night. They are recommended in adulthood before starting treatment for hypertension (Siu et al.,2015). For children, the use of ambulatory blood pressure measurements is recommended for the diagnosis of hypertension, to detect for non-dipping of blood pressure measures overnight, to avoid white coat hypertension or masked hypertension, and should be strongly considered in children with obesity and other conditions (Flynn et al.,2017). Non-dipping refers to the inability of the blood pressure regulating system to reduce the overall mean blood pressure values by 10% over night. White coat hypertension is an entity describing isolated office hypertension with normal values outside the office setting. In a cost-effectiveness analysis of 24-hour ambulatory blood pressure measurements, a proportion of 46% of children suspected of hypertension were diagnosed with white coat hypertension (Swartz et al.,2008). Masked hypertension is referred to the identification of hypertension in children based on ambulatory blood pressure measurements with normal blood pressure readings in the office setting. Masked hypertension is well described at a rate of 5-25%, particularly for children with obesity (Lurbe et al.,2005; Maggio et al.,2008)

It is recommended for children with obesity to test for blood pressure at every visit encounter (Lo et al.,2014) and every diagnosis of hypertension warrants exclusion of secondary causes for hypertension (Lurbe et al.,2016). Once hypertension is diagnosed, an echocardiography is recommended to assess cardiac structure and function. High blood pressure in childhood correlates with higher blood pressure in adolescence and with higher risk of hypertension in adulthood (Chen et al.,2008; Juhola et al.,2011). With obesity and dyslipidaemia, hypertension was confirmed a key modifiable risk factor in childhood for elevated cIMT in adulthood (relative risk 1.9 for cIMT >95th centile) (Koskinen et al.,2018). Rising blood pressure levels from as low as 115 mmHg systolic and 75 mmHg diastolic are linearly associated with increased mortality among adults (Lewington et al.,2002).

For children, age-, sex- and height-related, normative blood pressure reference charts based on auscultatory technique from ~50`000 children and adolescents have been updated in 2017 (Flynn et al.,2017). The updated definitions of blood pressure categories and stages are illustrated in table 3. Two important changes were implemented in the 2017 reference charts compared to the previous ones from 2004 (National High Blood Pressure Education Program Working Group on High Blood Pressure in et al.,2004): first, only children and adolescents with normal weight (BMI <85th centile) were included; and second, for adolescents aged 13 years and older, adult guidelines for classifying elevated stage 1 and stage 2 hypertension were used (Whelton et al.,2018). The rationale behind these changes were i) to avoid introducing bias due to the strong association between overweight/obesity and hypertension, and ii) the predictive value of adult cut-offs is strongly related to CVD-outcomes whereas the paediatric cut-offs are based on their statistical distribution (Daniels,2016).

Table 3. Blood pressure categories in children

Children age 1-13 years	Children >13 years
Normal BP: <90th centile	Normal BP: <120/<80mmHg
Elevated BP: ≥90th centile to <95th centile or, 120/80 mmHg to <95th centile (whichever is lower)	Elevated BP: 120 to 129 / <80mmHg
Stage 1 HTN: ≥95th centile to <95th centile +12 mmHg, or 130/80 to 139/89 mmHg (whichever is lower)	Stage 1 HTN: 130/80 to 139/89mmHg
Stage 2 HTN: ≥95th centile +12 mmHg, or ≥140/90 mmHg (whichever is lower)	Stage 2 HTN: ≥140/90mmHg

Abbreviations: BP: blood pressure; HTN: hypertension.

Excluding overweight and obese children caused the 2017 cut-off values for blood pressure categories to be lower than the equivalent from 2004. One recent study compared the validity of the two hypertension definitions, investigating i) the difference in prevalence of hypertension in childhood and ii) the predictive value of being diagnosed with hypertension by either the 2004 or 2017 reference charts towards an adult diagnosis of hypertension, metabolic syndrome or left ventricular hypertrophy (Du et al.,2019). The prevalence of hypertension was 7% and 11%, respectively, as defined in the 2004 and 2017 guidelines and the associations with adult outcomes were overall similar. However, the proportion of individuals who developed adult left ventricular hypertrophy increased from 12% to 19% using the newer guidelines from 2017 (Du et al.,2019).

2.6.2 Dyslipidaemia

Normative levels for lipids – cholesterol and triglycerides – in children and adolescents change with age and pubertal development (Expert Panel on Integrated Guidelines for Cardiovascular Health Risk Reduction in Children Adolescents,2011). Lipid concentrations increase significantly over the first year of life and then increase more slowly until ages 9-11 years, when they more closely reflect adult levels. During puberty the total cholesterol and LDL-C levels decrease as much as 10% to 20% or more, most likely due to elevated sex hormone levels before returning to baseline around the second decade of life (Elkins et al.,2019). Accepted levels for children and adolescents <19 years of age are summarized in table 4.

Table 4. Acceptable, borderline and high plasma lipid measures for children and adolescents

Category	Acceptable, mg/dl (mmol/l)	Borderline mg/dl, (mmol/l)	High mg/dl, (mmol/l)
Total cholesterol	<170 (<4.3)	170–199 (4.3-5.1)	≥200 (≥5.1)
LDL-C	<110 (<2.8)	110–129 (2.8-3.3)	≥130 (≥3.4)
Non–HDL cholesterol	<120 (<3.1)	120–144 (3.1-3.7)	≥145 (≥3.7)
Triglycerides			
0–9 years	<75 (<0.8)	75–99 (0.8-1-1)	≥100 (≥1.1)
10–19 years	<90 (<1.0)	90–129 (1.0-1.5)	≥130 (≥1.4)
HDL-C	>45 (>1.2)	40–45 (1.0-1.2)	<40 (<1.0)

Adapted from Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents, National Heart, Lung, and Blood Institute (2011). LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol.

Dyslipidaemia disorders are categorized into primary and secondary forms. Primary forms include hereditary forms such as familial hypercholesterolaemia and hereditary hypertriglyceridaemia whereas secondary forms are caused by non-hereditary conditions such as obesity, hypothyroidism, uncontrolled diabetes, hepatic and renal dysfunction or the use of medications such as corticosteroids, retinoids or oral contraceptive drugs (Elkins et al.,2019).

In children and adolescents with obesity, the prevalence of dyslipidaemia correlates with the severity of obesity. In a cross-sectional study with children and adolescents aged 3-19 years who were included in the National Health and Nutrition Examination Survey from 1999 to 2012, individuals were categorized according to the %>95th BMI centile into class I (95th BMI centile - 120% of the 95th BMI-centile), class II (≥120 - <140%) and class III (≥140%) (Flegal et al.,2009) and the distribution of cardiometabolic risk markers was evaluated (Skinner et al.,2015). Among 12-19 years old, levels of total cholesterol, non-HDL cholesterol and triglycerides increased with weight classes and HDL-C decreased. However, LDL-C did not show a similar association with weight categories above the 95th BMI-centile (Skinner et al.,2015) in this age group. A recent study aimed to evaluate the additive effect on discriminating individuals aged 12-18 years with a high (study-specific) cIMT >90th centile, when adding dyslipidaemia into a model including BMI, blood pressure, age and sex. The AUC of the model including lipid measures was slightly higher at 0.701 versus 0.688 for the model without lipid measures (Koskinen et al.,2018).

The 2011 Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents, National Heart, Lung, and Blood Institute guidelines recommend a universal screening for dyslipidaemia at the age of 9-11 and after puberty at the age of 17-21 years (Expert Panel on Integrated Guidelines for Cardiovascular et al.,2011). Selective screening is based on underlying risk factors, co-morbidities or positive family history (Elkins et al.,2019). This approach allows an early detection of primary and secondary lipid abnormalities (including familial hypercholesterolaemia, the prevalence of the heterozygous state was estimated at around 1 in 200 to 500 individuals (Singh et al.,2015)), and the identification of those who may respond favourable to lifestyle modification.

2.6.3 Inflammation

Obesity induces and maintains a chronic low-grade inflammation that triggers the development of many non-communicable diseases, including metabolic syndrome, type 2 diabetes mellitus, non-alcoholic fatty liver disease, CVD and various cancers (Christ et al.,2019). Adipose tissue`s core function is to store energy that can be released in times of starvation. However, if available in excess, it is also involved in inflammatory processes (Hajer et al.,2008). Subsequently, obesity-related mechanisms that trigger a chronic inflammation and common inflammatory markers that are associated with obesity are discussed. The focus will be on inflammatory markers that are linked with obesity and arteriosclerosis and a novel measure for chronic low-grade inflammation is introduced.

Several obesity-related mechanisms contribute to this inflammatory response: The intake of a Westernized diet (i.e. energy-dense, fatty, salty, sugary diets and ultra-processed foods that are low in fibres but rich in additives for longer storage and palatability) is driving excessive weight gain (Mozaffarian,2016) and correlates with elevated serum inflammatory markers such as C-reactive protein (CRP) and interleukin-6 (Lecube et al.,2019; Lopez-Garcia et al.,2004). Diet-induced alterations in the gut microbiome composition and gut-leakage are discussed as mediators between this association (Wan et al.,2019). Finally, an abundance of fatty acid substrates, exceeding the storage capacity of adipocytes has shown to cause adipocyte stress (Crewe et al.,2017) that induces pro-inflammatory changes in number and function of adipose tissue-resident macrophages (McLaughlin et al.,2017).

In children, inflammatory markers such as CRP (Kapiotis et al.,2006; Patel et al.,2006; Weiss et al.,2004), interleukin-6 (Kapiotis et al.,2006) and oxidised LDL Cholesterol, a marker of oxidative stress (Norris et al.,2011) have been shown to correlate in a linear manner with increasing weight

categories from normal weight (<85th BMI centile) to overweight (between 85th and 95th BMI centile), obesity (>95th BMI centile) and extreme obesity (≥1.2 times the 95th BMI-centile). Conversely, anti-inflammatory markers such as interleukin-10 (Mattos et al.,2016) and adiponectin (Orlando et al.,2019; Stefan et al.,2002) are reduced in children with increasing weight status. There is evidence to suggest that the obesity-related inflammatory condition is crucial for rapidly progressing arteriosclerosis in obese individuals (Rocha et al.,2009), as arteriosclerosis *per se* is an inflammatory process (Libby et al.,2002). Studies in children and adolescents, investigating cross-sectional associations between inflammatory markers and non-invasive subclinical atherosclerosis outcomes (such as flow-mediated dilatation, cIMT, PWV) have shown that inflammatory markers remain significantly associated with subclinical atherosclerosis outcomes after adjustment for BMI (Di Bonito et al.,2016; Giannini et al.,2009; Jarvisalo et al.,2002; Lopez-Garcia et al.,2004).

High-sensitivity CRP (hs-CRP) is the most widely used inflammatory marker in the context of obesity and CVD. In the Framingham Study, the relative risk (RR) for future stroke for the highest quartile of hs-CRP compared with the lowest was 2.0 for men and 2.7 for women (Rost et al.,2001) with similar results from corresponding studies restricted to men (Ridker et al.,1997) and women (Ridker et al.,2000). However, another study did not find an association between hs-CRP and hard cardiovascular endpoints when adjusted for other cardiovascular risk factors (Folsom et al.,2001). Similar, according to the Cardiovascular Risk in Young Finns Study, hs-CRP did not show a benefit to predict future cIMT measures in adulthood (Juonala et al.,2006b).

2.6.3.1 Glycoprotein acetyls, a marker for risk prediction for adverse health

Glycoprotein acetyls (GlycA), initially identified as N-acetylated carbohydrate side-chains of acute-phase plasma glycoproteins, were first described in 1987 as a broad nuclear magnetic resonance (NMR) spectroscopy signal (Bell et al.,1987). The NMR technique utilizes the magnetic behaviour of hydrogen nuclei. Similar to a compass needle, hydrogen nuclei behave like little magnets and align (energetically stable) or oppose (unstable) to an externally applied magnetic field. By applying a combination of specific radio frequency waves (usually at 60-100 MHz) and an external magnetic field, hydrogen nuclei may flip from the stable alignment to the less stable alignment. This particular situation – the flipping of the hydrogen nuclei – is known as the resonance condition and can be detected. Hydrogen makes up >60% of the atoms of a human body. Most of them are bound in molecules with a specific structure. For a given radio frequency, hydrogen atoms bound to molecules will require a slightly different magnetic field applied to it to achieve the resonance condition. The necessary strength of the magnetic field will depend on the environment of the hydrogen atom, i.e. on the structure of the molecule that incorporates the hydrogen atom. Resulting spectra from NMR spectroscopy will therefore show peaks of signals from hydrogen atoms in different molecules. The y-axis of such spectra reflects the number of hydrogen atoms in a similar environment/molecule, whereas the x-axis gives the chemical shift that is a standardised value (in parts per million (ppm)) of the required strength of the magnetic field to achieve the resonance condition.

In 1987, broad peaks at a range of the chemical shift between 2.04 - 2.08ppm were identified and assigned to N-acetyl protons of N-acetylated carbohydrate side-chains that were associated with acute-phase plasma glycoproteins (Bell et al.,1987). And in 2015, this particular NMR signal received its new name – glycoprotein acetyls (GlycA) (Otvos et al.,2015). Human plasma contains many glycoproteins with N-acetylated sidechains including α_1-antitrypsin, transferrin, haptoglobin, α_1-acid glycoprotein (acute-phase glycoproteins), and immunoglobulins and fibrinogen. Most of them are produced and secreted by the liver, mediated by interleukin-6 (Gabay et al.,1999), and the concentration of GlycA will rise or fall according to acute and chronic inflammation (Gornik et al.,2008). Recent studies performing high-throughput NMR metabolomics profiling of large prospective cohorts have introduced glycoprotein acetyls (GlycA) as a strong predictive biomarker for long-term risk of morbidity and mortality from diverse diseases (Connelly et al.,2017). Diseases, for which GlycA was predictive include CVD (Akinkuolie et al.,2014; Duprez et al.,2016), certain cancers (Fischer et al.,2014), T2DM (Akinkuolie et al.,2015; Connelly et al.,2016), non-alcoholic fatty liver (Kaikkonen et al.,2017) and

severe infections (Ritchie et al.,2015), many of which share a chronic inflammatory component (Connelly et al.,2017). Also, GlycA have been associated with and is predictive for all-cause mortality in adults (Akinkuolie et al.,2014; Fischer et al.,2014; Otvos et al.,2015), and is associated with obesity in young adults (Wurtz et al.,2014).

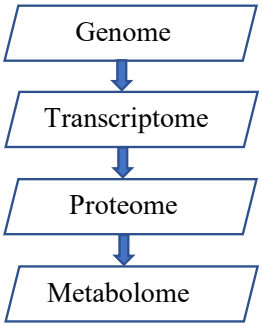
GlycA offers particular advantages to solitary inflammatory markers such as hs-CRP: first, hs-CRP and various cytokines are of much lower concentrations and therefore, they contribute negligibly to the measured GlycA signal. Therefore, GlycA may reflect different aspects of inflammatory conditions that provide an additional value in risk prediction (Otvos et al.,2015). Second, hs-CRP needs at least two serial measurements due to within-individual variability before making clinical decisions (DeGoma et al.,2012). A recent study investigating risk prediction capability of GlycA and hs-CRP for incident hospitalization and mortality in two large Finnish cohorts showed that GlycA had additional predictive value beyond that of hs-CRP (Kettunen et al.,2018).

Data on GlycA in childhood and adolescence is sparse: one study in 27 obese, prediabetic adolescent Latinos illustrated improved GlycA values after a lifestyle intervention (Olson et al.,2019); another study attributed a mediating effect of GlycA in children with overweight or obesity aged 11 years on microvascular venular calibre (Liu et al.,2020); and a recent study investigated the association between GlycA and hs-CRP and measures for inflammatory immune measures revealing that GlycA may be a better measure of inflammation in children and has potential value as a measure of chronic inflammation from very early childhood (Collier et al.,2019).

2.7 Metabolomics, BMI and cardiovascular risk

Metabolomics is a rather novel tool to study disease pathogenesis and to identify novel biomarkers for future disease risk (Monteiro et al.,2013). Metabolomic studies refer to the identification and quantification of small molecule metabolic products in biological specimens (e.g. urine, blood, organs) at a specific point in time. The resulting metabolome is considered a top-down extract that covers genetic, transcriptional and translational information all of which are influenced by environmental factors (Zhang et al.,2013) (see figure 4).

Figure 4. Omics hierarchy



Omics hierarchy, illustrating the direction of the flow of information from the genome towards the metabolome and intersected omics-platforms, where the information is processed.

Two general approaches are used in metabolomic studies, targeted and nontargeted. Targeted metabolomics investigate a selected group of metabolites, often chemically or metabolically related for precise quantification and interpretation for specific research questions. In contrast, non-targeted approaches aim to discover and quantify as many metabolites as possible (McGarrah et al.,2018).

Metabolomic studies in adults have revealed distinct metabolic patterns related to increasing BMI, to cardiometabolic risk factors (Ho et al.,2016; Holmes et al.,2014; Tulipani et al.,2016; Wang et al.,2011; Welsh et al.,2018; Zhao et al.,2016) and to CVD events (Holmes et al.,2018). Using Mendelian randomisation, Wurtz et al. have shown in young adults (aged 26 years) that increasing BMI is an antecedent for such metabolic patterns (Wurtz et al.,2014). In healthy and obese adults, NMR metabolomic analyses have also shown sex-dependent variation, in particular for levels of branched chain amino acids that are related to future T2DM development (Vignoli et al.,2018; Xie et al.,2014).

Data in children and adolescents are sparse, particularly in those with obesity. Particularly in this age group, sex and pubertal development need to be considered as covariates, given their impact on body composition (Loomba-Albrecht et al.,2009) and cardiometabolic risk factors (Reinehr et al.,2015). These caveats in mind, metabolomics may be a promising tool with higher predictive capacity for adult cardiometabolic disease compared to BMI (Umer et al.,2017).

2.8 Continuous metabolic syndrome score

Cardiometabolic risk factors tend to cluster, particularly in individuals with obesity. Therefore, a common concept in adulthood is to summarize this co-occurrence of risk factors, including elevated blood pressure, abdominal obesity, elevated triglycerides and glucose, and low level HDL-C, under the umbrella-term metabolic syndrome (National Cholesterol Education Program Expert Panel on Detection Evaluation and Treatment of High Blood Cholesterol in Adults,2002). The pathophysiology is largely explained by a selective insulin resistance with continuous hepatic lipogenesis and continuing hepatic gluconeogenesis (Rask-Madsen et al.,2012). Along with insulin resistance, a concomitant chronic inflammation and ectopic fat distribution are the main components of metabolic syndrome (Magge et al.,2017). However, the substitutes for the definition of metabolic syndrome vary between organisations. The most widely used definition is the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III), founded 2001 and updated in 2005, including any 3 or more of the following conditions (Grundy et al.,2005):

- Fasting glucose >5.6mmol/l or treatment for elevated glucose
- HDL-Cholesterol <1.0mmol/l
- Triglycerides >1.7mmol/l
- Obesity (waist circumference >102 in man or 88cm in woman)
- Hypertension: BP >130/85mmHg or on treatment for medication)

The International Diabetes Federation (IDF) updated in 2006 warrants three of the aforementioned conditions with a more stringent waist circumference of 94 cm for man and 80 cm for woman, a higher threshold for HDL-Cholesterol in woman of 1.3mmol/l and including individuals diagnosed with T2DM. As a consequence of the more stringent criteria used by the IDF, the percentage of individuals diagnosed with metabolic syndrome using the NHANES 1999 to 2002 dataset was slightly higher for the IDF criteria at 39% compared to the ATP III criteria with 34.5%. However, the two definitions overlapped for 93% of the subjects evaluated for the presence or absence of metabolic syndrome (Adams et al.,2005).

In children and adolescents, the utility of the metabolic syndrome beyond the significance of its contributors remains questionable (Goodman et al.,2007; Steinberger et al.,2009). Uncertainties are around the specific factors and their thresholds to use, as for many factors the risk likely is a continuum. Also, there are ethnic differences in the rates of obesity and cardiometabolic risk factors (Ogden et al.,2014). Last, the diagnosis of metabolic syndrome in childhood is very unstable with up to 50% of children diagnosed no longer qualify for the diagnosis when reassessed within 3 weeks (Gustafson et al.,2009)) or within 9 years (Stanley et al.,2014), irrespective of weight change over the time observed. An approach to solve the problems around thresholds and ethnic differences was to develop continuous metabolic syndrome scores based on BMI z-score, HDL-cholesterol, triglycerides, fasting glucose, and systolic blood pressure (Gurka et al.,2012). Until such continuous metabolic syndrome risk scores have

a clinical applicability, the current recommendations are to identify and treat single risk factors (Magge et al.,2017).

2.9 Subclinical cardiovascular phenotypes

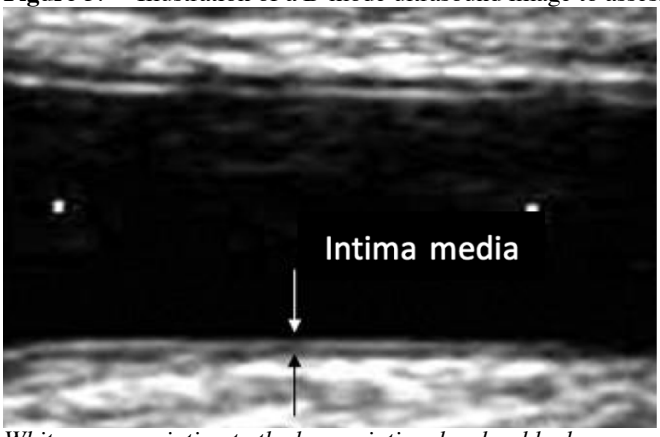
Subclinical cardiovascular phenotypes reflect the severity of atherosclerosis prior to clinical manifestation of CVD, and therefore are used as intermediate outcomes to assess CVD risk in youth. Subclinical cardiovascular phenotypes include the measurements of cIMT, carotid-femoral PWV and arterial elasticity. These measures can be assessed non-invasively, e.g. via ultrasound, are reproducible, well-tolerated and safe for children and adolescents (Urbina et al.,2009b).

CIMT and PWV are the most widely used measures. In childhood, the severity and the number of co-existing cardiovascular risk factors are associated with elevated cIMT (Juonala et al.,2010) and PWV (Koivistoinen et al.,2011) in adults. CIMT and PWV assessed in adulthood are associated with and predictive for CVD and mortality (Lorenz et al.,2007; Mattace-Raso et al.,2006; Mitchell et al.,2010). Specifically, a systematic review showed that for every 0.1mm cIMT, the risk for future myocardial infarction (MI) increases by 10% to 15%, and for future stroke by 13% to 18% (Lorenz et al.,2007). Adding cIMT to the Framingham Risk Score revealed some small, but significant improvement for the 10-year risk prediction of first-time stroke and MI, particularly in those with intermediate risk (Den Ruijter et al.,2012). Arterial elasticity describes vascular diameter changes during the systole-diastole cycle (Boesen et al.,2015). In adulthood, arterial elasticity is associated with the severity of atherosclerosis, carotid plaque presence, incident stroke (Boesen et al.,2015) and all-cause mortality (van Sloten et al.,2014).

2.9.1 Carotid intima-media thickness

The method to assess intima-media thickness was first described by Pignoli et al. in 1986, illustrating a close correlation of *in vitro* aortic intima-media thickness when assessed with ultrasound and light microscopy (Pignoli et al.,1986). Commonly the right, far wall cIMT is assessed with B mode ultrasound imaging, illustrating the arterial wall layers as shown in figure 5. Using semi-automated edge-detecting software allows accurate quantification of the IMT which has further improved reproducibility (Peters et al.,2012). The use of standardized protocols by trained sonographer has revealed reliable IMT measures, independent of whether the proximal part, the bulb or the internal carotid arteries were scanned (Riches et al.,2010). For cIMT assessment in children, a simultaneous ECG gating is recommended due to changes in IMT during the cardiac cycle (Rueb et al.,2017).

Figure 5. Illustration of a B-mode ultrasound image to assess arterial intima-media thickness



White arrow pointing to the lumen-intima border, black arrow pointing to the adventitia-media border. Distance between arrows is the intima-media thickness.

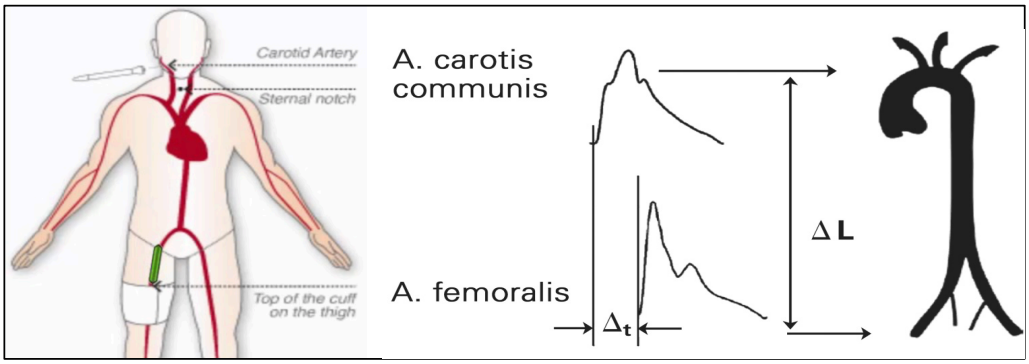
Cardiovascular risk factors in childhood predict young adult`s cIMT, however the strength of this relation depends on age as shown in a large dataset including 4 prospective cohorts with 4380 participants (the Cardiovascular Risk in Young Finns Study, Childhood Determinants of Adult Health study, Bogalusa Heart Study and the Muscatine Study). The number of childhood cardiovascular risk factors (total cholesterol, triglycerides, blood pressure and BMI) in their highest quintile was predictive for the highest decile in cIMT in young adulthood (20-45 years) for children aged 9, 12, 15 and 18 years: OR were: for 9 years old 1.37 [95%CI 1.16 to 1.61], p-value=0.0003; for 12 years old 1.48 [95%CI 1.28 to 1.72], p-value<0.0001; for 15 years old 1.56 [95%CI 1.36 to 1.78], p-value<0.0001; and for 18 years old 1.57 [95%CI 1.31 to 1.87], p-value<0.0001 (Juonala et al.,2010).

With respect to BMI, studies from the international Childhood Cardiovascular Cohort (i3c) consortium, systematic reviews and other longitudinal studies have shown that increasing BMI levels during childhood are predictive for cIMT in adulthood, and remain predictive after adjustment for other cardiovascular risk factors (Ajala et al.,2017; Dwyer et al.,2013; Juonala et al.,2006a; Oikonen et al.,2013; Oren et al.,2003; Petkeviciene et al.,2015; Raitakari et al.,2003). A recent study from the i3c consortium involving individuals aged 12-18 years old with 23.4 years of follow-up data, a BMI in the obese range was the risk factor most strongly associated with cIMT values >90th centile (relative risk 3.7 [95%CI 2.0-7.0], followed by male sex (relative risk 2.7 [95%CI 2.0-2.6], hypertension (relative risk 1.9 [95%CI 1.3-2.9] and high LDL-C (relative risk 1.6 [95%CI 1.1-2.1]) (Koskinen et al.,2018).

2.9.2 Pulse-wave velocity

PWV is the most widely used measure of arterial stiffness and best correlated with cardiovascular disease mortality when assessed between the carotid and femoral artery (Mitchell et al.,2010). PWV for the purpose of this study was calculated according to the method shown in figure 6. Faster PWV means higher arterial stiffness along the investigated arterial vasculature, and hence more progressed atherosclerosis and cardiovascular disease risk. In children and adolescents, PWV is highly reproducible and a well-tolerated measure of arterial stiffness, when assessed with applanation tonometry (Lowenthal et al.,2014). Other methods to assess PWV include the Arteriograph®, a method where oscillations serve as indirect measure for pulsatile pressure changes in the artery (Baulmann et al.,2008). Probably the best non-invasive method to study the artery propagation wave is the MRI method as no geometric assumptions need to be made (Joly et al.,2009). A more complete review including current available methods is provided by Pereira et al. (Pereira et al.,2015). Besides different devices, PWV may also be assessed in different arterial fields, i.e. brachial PWV versus carotid-femoral PWV, revealing different results that are dependent amongst others on arterial wall composition.

Figure 6. Assessment of carotid-femoral pulse-wave velocity



Left: A doppler ultrasound device detects the pulse wave at the carotid bulb, a thigh cuff detects the pulse wave at the femoral artery. Right: Pulse-wave velocity is calculated out of ratio between the Δlength (distance between carotid bulb and proximal cuff margin at the thigh) and the Δ_t (time interval between the detection of the pulse wave at the two sites).

PWV in healthy children varies with age, sex and blood pressure and various reference data, the largest a multicentre study involving 1008 children aged 6-20 years, exist (Reusz et al.,2010). According to a meta-regression analysis involving 9 studies on the relation between age and PWV in childhood, every year of ageing increases PWV by 0.12m/s per year (Stoner et al.,2020).

Studies investigating the impact of body composition measures on PWV revealed opposing findings. One meta-analysis included 14 studies and revealed a 0.45m/s increase in PWV in children with obesity compared to children with normal weight (Hudson et al.,2015). Limiting factors in this study were that different methods to assess PWV were compared (e.g. carotid-femoral versus brachial PWV), and when studies with high risk of bias were excluded, the association was no longer significant. A more recent meta-analysis including studies with overall high quality and >100 individuals found mixed associations between body composition measures and PWV (Stoner et al.,2020): of 10 cross-sectional studies investigating the association between body composition (such as BMI, DXA, and percentage body fat) and PWV, four reported a positive association, two a negative and another four found no association (Stoner et al.,2020).

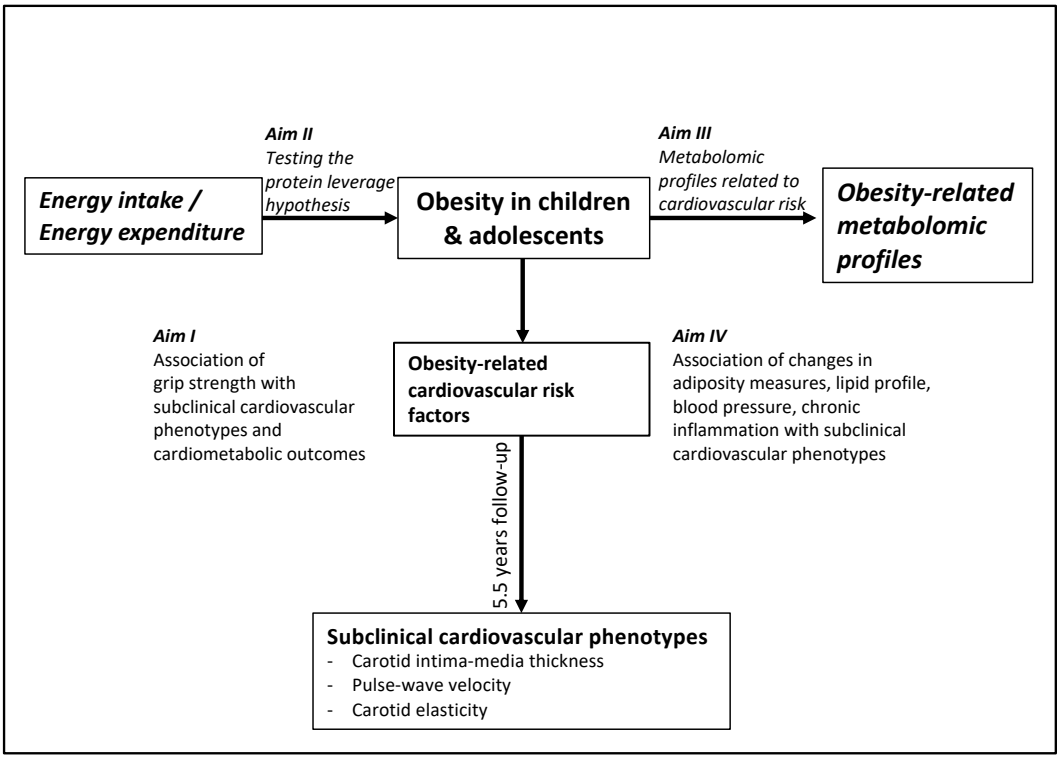
2.9.3 Carotid elasticity

Elastic properties of arteries can be assessed using various metrics (e.g. absolute distension, distensibility, Young`s Elastic Modulus), reflecting structural (i.e. the amount of elastic fibres) and functional characteristics (i.e. changes in artery diameter according to arterial blood pressure) (Boesen et al.,2015). Decreased arterial elasticity has been associated with diabetes (Prenner et al.,2015), hypertension (Mitchell,2014) and obesity (Tokita et al.,2009), and it correlates with the degree of atherosclerosis and risk for incident stroke (Boesen et al.,2015).

A commonly used measure for carotid elasticity in children (Liu et al.,2019) and adults (Juonala et al.,2005) is assessed with ultrasound calculating the systolic-diastolic diameter change, normalized to the diastolic diameter and pulse pressure. In the Special Turku Coronary Risk Factor Intervention Project for Children study, the number of ideal cardiovascular health metrics according to the American Heart Association (Lloyd-Jones et al.,2010) was directly related to carotid elasticity in adolescents (Pahkala et al.,2013). Also, cardiovascular risk factors defined as values at or above the age- and sex-specific 80th percentile for LDL-C, blood pressure, skinfold thickness, at or below the 20th percentile for HDL-C and smoking were directly related with arterial elasticity in adulthood after adjustment for risk factors identified in adulthood (Juonala et al.,2005).

3 Aims of the Study

Figure 7. Study aims



Overview to illustrate the study aims

Studies for this thesis aimed to investigate the association between determinants for an adverse TEI/TEE ratio with the severity of obesity, with obesity-related cardiometabolic risk factors and with subclinical cardiovascular phenotypes. As depicted in figure 7, the specific aims were:

I. to investigate the association of grip strength, a global measure of muscle strength, on cardiovascular risk factors and subclinical cardiovascular phenotypes among obese youth;

II. to investigate the relation between the dietary macronutrient composition with TEI and BMI in children and adolescents with obesity;

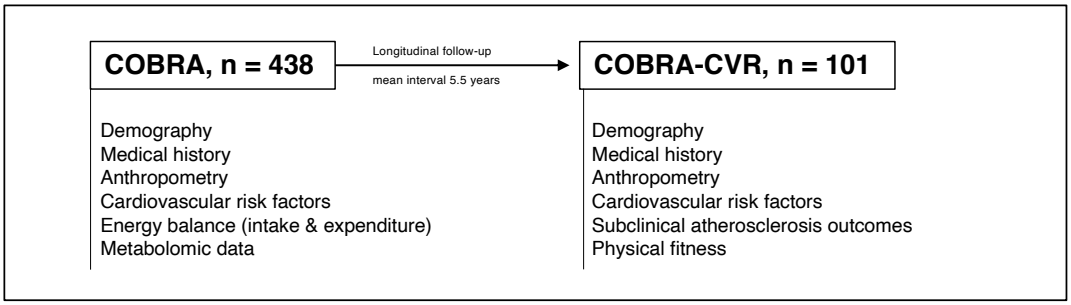
III. to investigate the relation between clinically assessed adiposity measures and a metabolomic platform with metabolites assessed for cardiometabolic risk in sera of children and adolescents with obesity stratified for sex and pubertal development;

IV. to investigate the association of change in adiposity measures and cardiovascular risk factors from early (10.2 years) to late adolescence (15.7 years) on non-invasive subclinical cardiovascular phenotype assessment in children and adolescents with obesity.

4 Subjects and Methods

4.1 Description of the study cohort

Figure 8. Illustration of the COBRA and COBRA-CVR follow-up cohort



4.1.1 COBRA – Childhood Overweight Biorepository of Australia

The Childhood Overweight Biorepository of Australia (COBRA) is a single centre cohort study, located at the Royal Children`s Hospital (RCH), a tertiary care paediatric hospital in Australia (Sabin et al.,2010). Research projects including COBRA participants are conducted in collaboration with the onsite research institute, the Murdoch Children`s Research Institute (MCRI), providing the epidemiologic and statistical research support and infrastructure.

Since 2009, COBRA participants are recruited from the RCH Weight Management Service, a tertiary care outpatient clinic to provide support to families and healthcare professionals to assist with weight management for infants, children and adolescents. Reviewed by the local RCH ethical board, a broad ethical consent was obtained from COBRA participants, or in the case of minors from their legal representatives, to collect detailed demographic and anthropometric data, medical and family history and questionnaire data. The consent also included the collection and storage of blood and urine samples at the local MCRI biobanking facility for future, ethically approved, research projects.

The COBRA biorepository was set up as a library of biological and health-related information from a large number of individuals with overweight or obesity (overweight and obesity determined the 85th and 95th percentile cut-offs of US-derived data produced by the Centers for Disease Control and Prevention (http://www.cdc.gov)). This library aimed to provide answers to research questions related but not limited to:

I. identify the factors contributing to overweight and obesity in children and adolescents;
II. identify weight-related co-morbidities at presentation; and
III. identify factors that contribute to future, weight-related co-morbidities.

4.1.2 COBRA-CVR – Cardiovascular Risk in COBRA

COBRA-Cardiovascular Risk (COBRA-CVR) is a longitudinal study-arm of COBRA (see figure 8). To be eligible to participate in COBRA-CVR, the criteria were:

- former COBRA participant;
- consent to be re-contacted and permission to use collected data and stored biological samples;
- aged 6-18 years;

- ability to understand and provide consent (or to provide a legally acceptable representative who can understand and provide consent);
- not meeting any of the exclusion criteria.

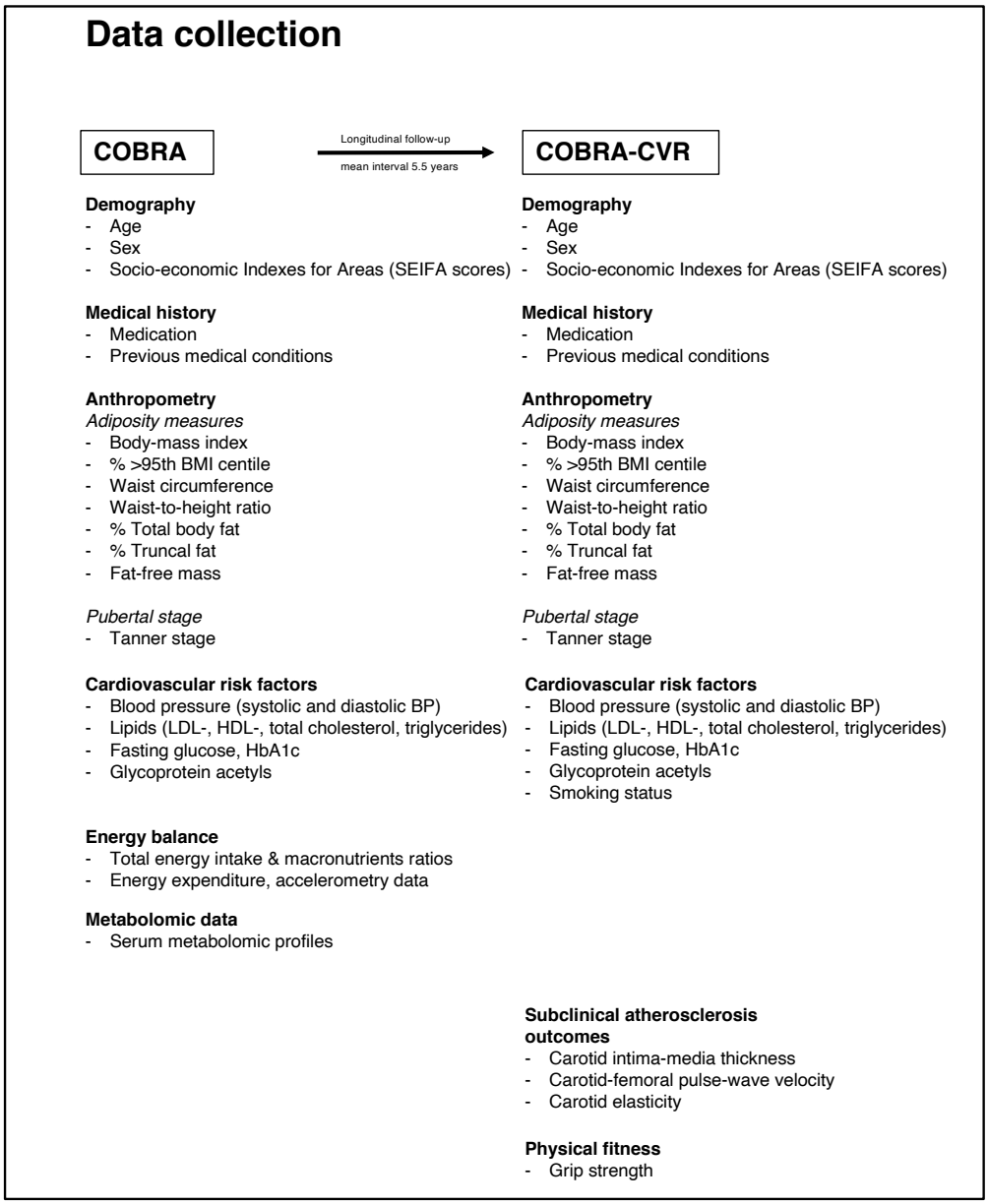
Exclusion criteria for the COBRA-CVR study were:

- children, adolescent or representative with intellectual impairment to give informed consent;
- families whose level of English precluded adequate understanding to give informed consent;
- primary or secondary immunodeficiency;
- adolescents with conditions requiring current or recent (within the previous three months) immunosuppressive medication, including oral or IV steroids (inhaled or topical steroids were not exclusion criteria);
- history of acute infection requiring hospitalisation within the last four weeks;
- children with central venous access or indwelling prosthetic devices.

4.2 Collected data for COBRA and COBRA-CVR

Data used for this thesis were obtained from participants at the time-point of enrolment for COBRA and at the time-point of participating for COBRA-CVR (see figure 9).

Figure 9. Overview and comparison of collected data for COBRA and COBRA-CVR



4.2.1 Acquisition of demographic, medical history and anthropometric data

Demographic information was collected at the study visits for COBRA and COBRA-CVR, including date of birth, age, sex and contact details to obtain postcodes for the SEIFA-scores. Socioeconomic status was measured using SEIFA-scores of Relative Socioeconomic Advantage and Disadvantage according to the participant's postal code at COBRA-CVR (Liu et al.,2017). The SEIFA score is a standardized score compiled from census data summarizing information about economic and social conditions of people and households according to their living area. The score is scaled at a numeric mean of 1000 and a standard deviation of 100. A higher score represents an area of more advantage and less disadvantage.

Data from medical history were collected at the study visits according to the inclusion/exclusion criteria. This information was sourced from the participant, their parent/guardian, and their hospital medical records.

Anthropometric measures included height (cm) and weight (kilograms). Height was measured without shoes to the nearest 0.5 cm, using a fixed Harpenden stadiometer (Holtain Ltd., Crymych, Dyfed, UK). Weight was assessed in light clothes to the nearest 0.1 kilogram. Adiposity measures included BMI, BMI z-score, %>95th BMI-centile, % total body fat (%BF), % truncal fat (%TF), fat-free mass (FFM), WC and WtH-ratio.

BMI was calculated according to the formula weight in kilograms divided by height in meters squared and then converted into age- and sex-specific BMI z-scores using the US CDC growth reference charts (Kuczmarski et al.,2000). %BF and %TF were assessed by a four-point bioimpedance device, Tanita BC-418 segmental body composition analyzer (Tanita Corporation, Tokyo, Japan), which was previously validated for the use in children > 6 years (McCarthy et al.,2006b). The FFM was calculated according to the formula FFM = weight (kilograms) × (100 − %BF) and then used to calculate BMR according to the Schofield equation (Schofield,1985b).

Pubertal development was assessed with an orchidometer and graded according to Tanner stages by a specialist paediatric endocrinologist or a consultant general paediatrician. Tanner 1 was considered pre-pubertal, Tanner 2 to 3 was peri-pubertal, and Tanner 4 to 5 was post-pubertal (Tanner,1986).

4.2.2 Acquisition of cardiovascular risk data

Blood pressure measurements at COBRA enrolment were performed with a manual sphygmomanometer (Welch Allyn, Macquarie Park, Australia) by auscultation of the right brachial artery when the participant was sitting, quiet and calm. At COBRA-CVR, the right brachial SBP, DBP, mean arterial pressure, and pulse pressure and heart rate were measured using a SphygmoCor XCEL system (AtCor Medical Pty Ltd., Naperville, USA) after a 5-minute rest in supine position. The mean of three measurements was used. The cuff-size was fitted according to published clinical practise guidelines (Flynn et al.,2017) and the updated 2017 clinical guidelines were used to define normal or elevated blood pressure and stage I and II hypertension (Flynn et al.,2017).

Lipid profiles at the COBRA and COBRA-CVR time-points included total cholesterol, HDL-C, LDL-C and triglycerides. They were derived from a NMR spectroscopy platform (Nightingale Health, Helsinki, Finland) (Saner et al.,2019). Results were obtained from serum blood samples that were collected after a minimum six hour fast. LDL-C, HDL-C and total cholesterol levels derived from metabolomics profiling are lower than a standard lipid profile. 139 pairs of NMR and standard laboratory LDL-C levels were compared, available from analysis performed in COBRA participants using the Bland Altmann method (Altman et al.,2017). The mean difference and limits of agreement between the Nightingale LDL-C and standard laboratory LDL-C was -1.1mmol/l [95%CI -1.9 to -0.3mmol/l]. A regression model was used to calculate LDL-C levels that were comparable to standard laboratory LDL-C levels.

Fasting glucose and HbA1c were analysed on fresh blood using standard methods (fluoride oxalate plasma on VITROS 5600, Ortho Clinical Diagnostics, Australia; and EDTA plasma on BIO-RAD D10, Gladesville, Australia, respectively). Type 2 diabetes mellitus or prediabetes was diagnosed according to standard criteria of the American Diabetes Association (American Diabetes,2019).

Glycoprotein acetyls (GlycA) is a measure for chronic inflammation and predictive for cardiovascular disease and mortality (Akinkuolie et al.,2014; Lawler et al.,2016) and was derived from the NMR spectroscopy platform.

4.2.3 Acquisition of energy balance data

Energy intake

TEI and relevant proportions of energy intake from macronutrients (carbohydrates %EC; fats %EF and proteins %EP) were collected using the Australian Child and Adolescent Eating Survey Food-frequency questionnaire (ACAES-FFQ) (Watson et al.,2009) between the years 2010 and 2018. As part of the Australian Eating Survey suite of food questionnaires, these questionnaires have undergone comprehensive evaluation for validity and reproducibility, and they can be self-administered or completed by parents for young children. Reproducibility and comparative validity for this survey have previously been established (Watson et al.,2009). The ACAES-FFQ is a 120 item semiquantitative FFQ with 15 supplementary questions regarding age, use of vitamin supplements, food behaviours, and sedentary behaviours. While completing the survey, a list of the most commonly eaten foods by Australians are given and the participant indicated the frequency of consumption of these foods. The ACAES-FFQ was sent to the University of Newcastle for scanning, and nutrient analysis was assessed using FoodWorks software (version 8.0.3553; Xyris Software, Xyris, Australia) to elicit TEI, %EP, %EC and %EF. Absolute measures of energy from dietary carbohydrate, fat, and protein were calculated by multiplying grams of intake by 16.7 kJ/g for carbohydrate and protein and 37.7 kJ/g for fat (National Health and Medical Research Council,2006, updated September 2017).

Energy expenditure

A subgroup of COBRA participants had physical activity measured by Actical accelerometers (Philips Respironics, Philips, USA) worn on the left hip on an elasticized belt continuously for 7 days, including water-based activities and sleep. Accelerometer results were considered valid for participants with data on three or more weekdays and one or more weekend days, with a wearing time of at least 600 minutes and a maximum non-wear time of 360 minutes in each 24-hour period, as per published guidelines regarding acceptability (Colley et al.,2010). Actical accelerometery physical activity intensity was categorized according to published intensity cut-off points (Colley et al.,2011).

4.2.4 Acquisition of grip strength data

Grip strength was measured in kilograms using a digital dynamometer (T.K.K. 5401; Takei Scientific Instruments Co., Ltd, Japan), which has the highest criterion validity and reliability in adolescent populations (Espana-Romero et al.,2010). As grip span has been shown to affect grip strength performance, hand span of participants was measured to determine the optimal grip span for the dynamometer based on age- and sex-specific equations for children and adolescents (Ruiz et al.,2006). Grip strength was then measured twice on both hands from participants according to protocols from the 2011-12 NHANES (CDC,2011). Results were adjusted for body weight due to reported weight-related influence (Aasa et al.,2003; Milliken et al.,2008) and subsequently categorized into low, moderate and high grip strength according to previously published normalized values (see table 5) (Peterson et al.,2016).

Table 5. Sex-specific normalized grip strength categories for children and adolescents

Normalized grip strength category (kg/kg)	**Boys**	**Girls**
Low	≤0.33	≤0.28
Moderate	>0.33 to ≤0.45	>0.28 to ≤0.36
High	>0.45	>0.36

Normalized grip strength categories as the ratio of raw grip strength (kg) is divided by body weight (kg)

4.2.5 Acquisition of metabolomic data

Metabolomic analysis from stored serum samples was performed on the Nightingale® NMR spectroscopy platform as previously described (Kettunen et al.,2012; Soininen et al.,2009). A total of 73 metabolites, capturing the majority of variation within the dataset, were used in analyses, including lipid subclass concentration, composition, size and ratios and concentrations of apolipoproteins, cholesterols, fatty acids, glycerides, phospholipids, amino acids, glycolysis related products, albumin, creatinine and glycoprotein acetyls.

4.2.6 Acquisition of subclinical cardiovascular phenotypes

Carotid intima-media thickness
The right common carotid artery was assessed in the supine participant with their head turned 45° to the left, according to on-site standardized protocols (Liu et al.,2019). Simultaneous ECG gating was performed to allow the assessment of cIMT at end-diastole (R-wave of ECG) due to changes in cIMT during the cardiac cycle in children (Rueb et al.,2017). Ultrasound images were obtained using a 10-MHz linear array probe (Vivid I; GE Healthcare, Parramatta, NSW, Australia). Real-time B-mode ultrasound cine loops over five heartbeats were captured in triplicate. The qualitatively best loop was further processed using Carotid Analyzer (Medical Imaging Applications, Coralville, Iowa, USA), a commercially available semiautomatic edge-detection software program. Carotid IMT was measured ~10 mm proximal to the carotid bulb, over a distance of 5 to 10 mm. The far wall cIMT, averaged from the mean of five frames at end-diastole was used in the analysis.

Carotid-femoral pulse wave velocity
The pulse-wave velocity was determined using the SphygmoCor XCEL (AtCor Medical, Australia) (Butlin et al.,2013) in the supine participant after a 5-minute rest. The PWV was calculated as the ratio of the distance of the carotid-femoral arterial segment and the time interval needed for the pulse wave to travel this distance. The distance was measured with a non-flexible tape measure using the following anatomical landmarks: from the carotid pulse to the suprasternal notch to the right femoral artery (estimated midway the crease between thigh and torso when the knee was bent) to the proximal end of the thigh cuff. The time interval was measured from simultaneously recorded waveforms by applanation tonometry at the carotid pulse and the cuff at the upper right thigh. The mean of the three measures was used for analysis (Liu et al.,2019).

Carotid elasticity
Carotid elasticity (%/mmHg) was calculated as previously described in adults (Juonala et al.,2005) and children (Liu et al.,2019) according to the formula:

$$\frac{\left(\frac{LDmax - LDmin}{LDmin}\right)}{\Delta P} X\ 100\%$$

The maximum (LDmax) and minimum (LDmin) lumen diameter was calculated with the Carotid Analyser software (Medical Imaging Applications, Coralville, IA, USA). Pulse pressure (ΔP) was calculated as the difference between systolic and diastolic blood pressure.

4.2.7 Reproducibility of cIMT results

Subclinical cardiovascular phenotypes outcome data were assessed by a PhD applicant after a training period of three months by expert examiners on-site. To test for reliability, a random sample of 30 de-identified loops with five heartbeats of B-mode images (~25 frames per heartbeat) to assess cIMT were re-analysed by a second reader. The results were compared using the Bland Altman method (Bland et al.,1986a).

4.3 Statistical analyses

All analyses were performed using the software R® 3.4.3 (R Foundation, Vienna, Austria). Figures were drawn using the R-package *ggplot2* or Microsoft PowerPoint (Microsoft Corporation, WA, USA). A significance level was set at $p < 0.05$.

All data were checked for plausibility prior to analysis. Values incompatible with life were removed from the dataset. Unlikely but plausible values were retained.

Variables included in analysis were checked for their symmetry of distribution according to visual inspection of histograms and by using the skew function of the e1071-R-package (David Meyer, 2018).

4.3.1 Sample size estimation

There are no equivalent data available for grip strength (aim I), macronutrients analysis (aim II) or metabolomic profiles (aim III) in children and adolescents with obesity. The impact of evidence provided by the design for aim IV was considered highest. Therefore, a sample size estimation for aim IV was performed to detect an effect size meeting the criteria of $\alpha = 0.05$ and $\beta = 0.2$ (allowing for 80% power) for PWV. Published data on PWV between obese versus normal weight adolescents showed mean and SD differences of 3.7 m/s ± 0.34 versus 4.02 m/s ± 0.44 (Nunez et al.,2010). Unadjusted correlation coefficients from a large population-based study for BMI and PWV are reported at 0.21 (Baier et al.,2018). Using the R-package *pwr*, the minimum necessary samples size was calculated assuming a correlation coefficient $r = 0.3$, indicating a medium effect size, resulting in a minimum number of 84 individuals. To assure an adequate sample size the ethics application included a minimum of 84 individuals with a maximum of 120 individuals

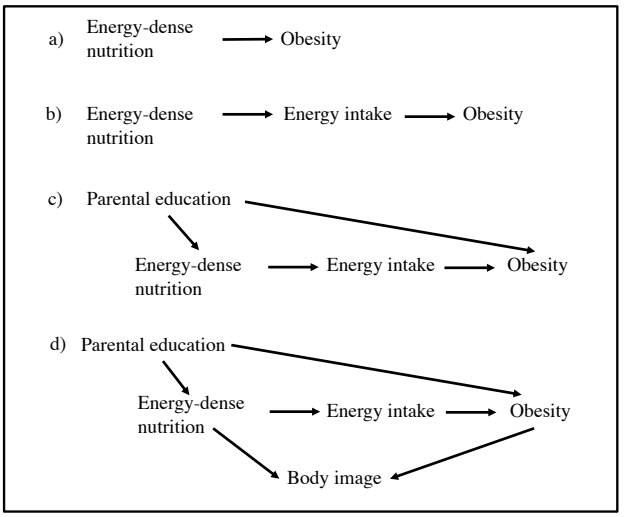
4.3.2 Descriptive statistics

Participant characteristics, study exposures, outcomes and covariates were described in mean and standard deviation (SD) for continuous and number (%) for categorical variables including the range from min to max.

4.3.3 Inferential statistics

Directed acyclic graphs (DAG) were used to define the nature of the variables from an analysis perspective. A DAG is an illustration of a research hypothesis that includes the variables of interest ordered according to their causal relations to each other. This method facilitates the definition of exposures, confounders, mediators, colliders and outcomes for the inferential analysis (Greenland et al.,1999). See figure 10 for an illustration adapted from (Williams et al.,2018b).

Figure 10. Illustration of the definition of variables according to their relation to exposures and outcomes



a) Energy-dense nutrition (exposure) causes obesity (outcome). b) Energy-dense nutrition acts on obesity through the mediator of increased energy intake. c) Parental education causes increased intake of energy-dense nutrition and obesity and is therefore a confounder. d) Body image is a collider in the path from the consumption of energy-dense nutrition to obesity.

Adjustments for mediators (i.e. variables that are on the causal pathway between exposures and outcomes) was occasionally performed to assess direct effects of the exposure variable on the outcome and to evaluate mediation of an additional variable (Baron et al.,1986). Colliders - descendant of both the exposure and outcome – were not included to avoid the finding of associations between variables where there are none (collider bias).

Regression analyses were the main method used for assessing the direction and strength of associations between variables in most studies (see aim-specific statistical analysis). Adjustment was performed for true confounders, such as age and sex, to describe the total effect between exposure and outcomes.

4.3.4 Aim-specific statistical analysis

Aim I: to investigate the association of grip strength, a global measure of muscle strength, on cardiovascular risk factors and subclinical cardiovascular phenotypes among obese youth

Data preparation
Participants were divided into categories of normalized grip strength according to previously defined thresholds described in the methods (table 5).

Confounding by age, sex, and pubertal stage was tested before considering adjustment for these variables. Similar, within the relationship of grip strength and the subclinical cardiovascular phenotypes, BMI z-score may act as a mediator (Fraser et al.,2018) or confounder (Fraser et al.,2016), hence results for BMI z-score adjusted analysis were provided.

Main analysis
The associations between normalized grip strength categories (independent variable) and cardiometabolic, preclinical markers (dependent variables) were estimated using linear regression.

Aim II: to investigate the relationship between macronutrient composition and total energy intake and BMI (specifically the protein leverage hypothesis) in children and adolescents with obesity.

Modelling the relation between macronutrients and total energy intake using power functions

The relation between the proportion of energy from protein sources and the total energy intake follows a power function. To determine the strength of the leverage from macronutrients on total energy, power functions were fitted. The default formula for a power law solution adapted for PL is characterized by the following equation:

$$Required\ food\ intake\ (in\ grams) = P\ x\ p^{L}$$

where P is the targeted intake of protein in grams, p is the proportion of protein from a given diet, and L is the strength of leverage. P and p are derived from the analysis, and the exponent L reveals the strength of leverage. Assuming full protein leverage that is, L = −1, small changes in the proportion of energy from protein (p) will cause substantial changes in total energy intake. Assuming no protein leverage that is, L = 0, changes in p will not cause any changes in total energy intake (Raubenheimer et al.,2019).

Compositional data analysis to explain total energy intake from macronutrient composition

Multiple regression with compositional predictors (e.g., percentages of energy from relevant macronutrients) warrant a different approach to simple linear regression as covariance among the percentages of different components may cause inferential errors. For example, an increase in %EP must necessarily lead to a decrease in %EF and/or %EC.

Mixture models, also known as Scheffés` polynomials, are an analytical framework that is based on multiple regression and robust to the analysis of outcomes with compositional predictors (Lawson et al.,2016). To test the sensitivity of our results to the choice of analysis, the total energy intake and BMI z-score data was analysed as a function of dietary macronutrient content (%EC, %EF, and %EP) in a mixture model framework, which allows to test for effects of all three macronutrients simultaneously.

Five different models were fitted with increasing complexity for each outcome. Model 1 was a null model, which assumes no effect of dietary composition on the outcomes. Models 2 through 5 were mixture models, corresponding to equations 1 through 4 in Lawson and Willden (Lawson et al.,2016) to test for linear additive through increasingly complex nonlinear interactive effects of macronutrients on total energy intake and BMI z-score. Noteworthy that model 2, which tests for linear effects, is identical to the partition substation model commonly used in nutritional epidemiology (Song et al.,2018; Willett et al.,1997). To select among models, an information theoretic approach based on Akaike information criterion (AIC) was used (Akaike,1992). Of the five models fitted, the model with the smallest AIC was selected. In the event that two models were within two AIC points of one another, the simplest model was selected. Where the null model is favoured, no effect of macronutrient composition on the outcome is inferred, with subsequent models suggesting more complex effects of diet composition on the outcome of interest.

Mixture models were implemented using the "MixModel" function in the R package mixexp (The R Foundation for Statistical Computing, Vienna, Austria). For interpretation, the predictions from the AIC-favoured model were plotted as surfaces on a right-angle mixture triangle (Raubenheimer,2011).

Aim III: to investigate the relation between clinically assessed adiposity measures and a metabolomic platform with cardiometabolic interests in sera of children and adolescents with obesity.

Data preparation
Adiposity measures (BMI, total %BF, %TF, WC and WtH-ratio but not BMI z-scores) and metabolites were scaled to SD-units to facilitate the comparison of estimates across metabolite measures.

Multivariate analysis of metabolomic data
Principal component analysis (PCA), a common approach in multivariate data analysis, was used to investigate the dataset`s variability and to uncover clusters within the dataset (Worley et al.,2013). The value of PCA is to reduce the dataset`s variability into principal components that explain most (PC1), second most (PC2), and so forth, of the variability. For illustration, the principal components one and two were plotted including their relation to age and sex. The percentage contribution to the variability of the dataset is given in a table for the most important five principal components.

PCA was used to gain an informative understanding of the dataset structure. PCA aims to identify class differences from a multivariate dataset. A class may refer to any relevant characteristic such as sex, use of a specific medication or different genetic traits. The precondition to detect such bias within the dataset is that the within-class variation must be sufficiently less than the between-class variation.

Main analysis and adjustments for multiple comparisons
Multiple linear regression was applied for a 1 standard deviation change in each adiposity measure (predictor) and the metabolite measure (outcome), adjusted for age and sex as confounders. In addition, linear regression modelling was applied to sex and puberty-specific datasets to illustrate trends for metabolites in these subgroups. A Benjamini-Hochberg (Hochberg et al.,1990) false discovery rate of 0.1 was used to adjust for multiple testing.

Aim IV: to investigate the association of adiposity measures and cardiovascular risk factors over time on a comprehensive, non-invasive set of subclinical cardiovascular phenotypes in children and adolescents with obesity.

Selection of covariates: sex, age, socioeconomic status, pubertal status and smoking status

The analysis accounted for factors that were potential confounders of the association between adiposity measures or cardiovascular risk factors and subclinical atherosclerosis. Selection was based on evidence

in the literature and included socioeconomic status, age, sex, and pubertal status. The confounding for subclinical atherosclerosis outcomes by age, sex and socioeconomic status is well described (Liu et al.,2017). In contrast, for pubertal status the evidence is less clear. Pubertal status may be on the causal pathway between adiposity measures and outcomes and hence act as a mediator. Results including pubertal status as a covariate were therefore provided in a supplementary table. Similar, smoking status (within the last 30 days) (Liu et al.,2017) was assessed at the COBRA-CVR visit but not included in the analysis because it may act as a mediator and the sample size was too small for robust analysis.

Models including BMI z-score or %BMI>95th centile were not adjusted for age and sex as they are based on BMI centiles that already standardized for age and sex.

Estimation of effect measures
Multiple linear regression was used to assess the association between adiposity measures and cardiovascular risk factors (at visit 2) on subclinical atherosclerosis outcomes (at visit 2). Models were adjusted for available confounders including: the relevant adiposity measure or cardiovascular risk factor at COBRA visit, age (COBRA and COBRA-CVR visits), sex and SEIFA scores.

Because the exposure and outcome measures are assessed at the same time (at the COBRA-CVR visit) it is necessary to establish the direction of the association. This was achieved by conditioning the effect estimate on the exposure at the COBRA visit (a preceding time-point). Also, from a mechanistic point of view, adiposity and cardiovascular risk factors are both preceding, causative factors for atherosclerosis that change slowly. As described above, the interest was in the total effect of the exposures, therefore the models were not adjusted for likely mediators (systolic blood pressure or LDL-C), as suggested by Westreich and Greenland (Westreich et al.,2013). The estimates of the associations of each adiposity measure or cardiovascular risk factor at the COBRA-CVR visit with subclinical cardiovascular phenotypes was visualised by tables and forest plots.

To address the effect of increasing versus decreasing the %>95th BMI-centile over time on subclinical atherosclerosis outcomes, the %BMI >95th centile was dichotomized depending on whether participants increased or decreased their percentage level of BMI>95th during the visit-interval. The resulting binary variable was used as the exposure, with the subclinical atherosclerosis measures at the COBRA-CVR visit as outcomes, adjusted for age at both visits and SEIFA scores. The analysis was stratified for sex.

5 Results

Four aims were studied in different subpopulations of the COBRA cohort. To allow comparison of relevant characteristics, the results of the aim-specific subpopulations are illustrated in combination with the results from the original cohort the subpopulation was derived from. Aim IV was based on longitudinal data from COBRA-CVR participants with data collected at COBRA enrolment and at COBRA-CVR participation; Participant characteristics are shown for both time-points. Subclinical cardiovascular phenotypes were analysed for aim I and aim IV and results shown in section 5.1.6.

5.1 Participant characteristics

5.1.1 Participant characteristics for COBRA-CVR and aim I

From the original COBRA biorepository (n= 438), a total of 101 participated in the COBRA-CVR follow-up. From COBRA, a total of 30 individuals were excluded due to a BMI < 95th CDC centile or age below 6 years, resulting in 408 individuals suitable for recruitment. Of those, 115 declined participation for COBRA-CVR, 96 had lapsed contact details, 74 were excluded after three failed attempts and 22 had relocated inter-state. Finally, a total of 101 participants were recruited and had data collected for COBRA-CVR. Of those, a total of 43 were eligible for addressing aim I. Participants with no grip strength data and a BMI <95th BMI centile were excluded (see table 6).

Table 6. Characteristics for participants of COBRA-CVR and eligible participants for aim I

	COBRA-CVR			Grip strength		
Characteristic	**n**	**Mean (SD)**	**Range**	**n**	**Mean (SD)**	**Range**
Sex, males (%)	101	52 (51.5)		43	21 (49)	
Age (y)	101	15.7 (3.7)	6.1-24.3	43	14.8 (3)	8.7-19.9
Weight (kg)	101	101.7 (30.5)	40.8-187.7	43	107.9 (27.2)	57.3-179.4
Height (m)	101	1.67 (0.13)	1.26-1.95	43	1.67 (0.12)	1.38-1.95
BMI (kg/m^2)	101	35.7 (7.9)	18.2-60.9	43	38.1 (6.9)	27.0-60.9
BMI z-score	101	2.27 (0.61)	-0.53-3.39	43	2.53 (0.29)	2.03-3.12
%>95th BMI-centile	101	131.5 (26.4)	75.6-202	43	141.1 (21.1)	113.5-200.4
n <100%		9			0	
n ≥100% - <120%		26			6	
n ≥120% - <140%		33			18	
n ≥140%		33			19	
Waist circumference (cm)	79	105.0 (16.4)	66-153	41	108.9 (13.2)	82-139
Waist-to-height ratio	79	0.62 (0.08)	0.43-0.83	41	0.62 (0.07)	0.54-0.83
Truncal fat %	99	36.4 (9.5)	15-58.8	42	39.6 (7.9)	22.6-58.8
Total body fat %	99	40.6 (9.9)	18.6-67.8	42	44.4 (7.2)	30.4-60.8
Tanner stage (%)				43		
n pre-pubertal		10 (10)			5 (12)	
n peri-pubertal		16 (16)			6 (14)	
n post-pubertal		75 (74)			32 (74)	

Results are given in mean and standard deviation (SD), absolute number of participants (n) and ratios (%). The range provides maximum and minimum levels of the relevant characteristics. Standard international units are used such as years (y), kilograms (kg) and meters (m)

5.1.2 Cardiovascular risk factors for COBRA-CVR and eligible participants for aim I

Table 7 illustrates cardiovascular risk factors in the subgroup of COBRA-CVR participants who were eligible to participate for aim I. Also illustrated are the cardiovascular risk factors for the COBRA-CVR cohort from where participants were recruited for comparison.

Table 7. Cardiovascular risk factors for COBRA-CVR and participants eligible for aim I

	COBRA-CVR			**Grip strength**		
Cardiovascular risk factor	**n**	**Mean (SD)**	**Range**	**n**	**Mean (SD)**	**Range**
Systolic BP (mmHg)	100	126 (13)	96-162	43	127.7 (13.1)	104-156
Diastolic BP (mmHg)	100	69 (8)	50-89	43	69.4 (8)	52-81
Normal BP	28			18		
Elevated BP	33			4		
Stage I Hypertension	19			16		
Stage II Hypertension	20			5		
LDL-C (mmol/l)	98	1.76 (0.58)	0.9-4.6	43	1.4 (0.52)	0.7-3.7
Triglycerides (mmol/l)	98	1.15 (0.40)	0.6-2.5	43	1.3 (0.44)	0.7-2.5
GlycA (mmol/l)	98	1.11 (0.11)	0.88-1.40	43	1.16 (0.09)	0.93-1.34
HbA1c (%)	94	5.2 (0.4)	4.1-6.5	43	5.3 (0.4)	4.1-6.3
Fasting glucose (mmol/l)	94	4.6 (0.5)	3.7-7.1	43	4.7 (0.5)	3.7-6.7
Diabetes	8			0		
Pre-Diabetes	8			6		
Smoking status						
Never smoked	92			43		
Current smoker	9			1		

Cardiovascular risk factors for participants eligible for aim I and for all COBRA-CVR. Numbers are given in mean and standard deviation (SD), absolute number of participants (n) and ratios (%). The range provides maximum and minimum levels of the relevant characteristics. Standard international units are used such as millimetres of mercury (mmHg), millimole per litre (mmol/l). BP: blood pressure; LDL-C: Low-density lipoprotein cholesterol; GlycA: glycoprotein acetyls; HbA1c: Haemoglobin A1c.

5.1.3 Participant characteristics – for COBRA and aims II and III

Table 8 displays demographic and anthropometric characteristics for the COBRA cohort and the relevant characteristics for the eligible participants for aims II and III. For aim II, 203 individuals had food frequency questionnaires completed. Of those, a total of 137 fit the age criteria (i.e. >6 years), were classified as obese, had plausible energy intake according to a Goldberg cut-off of >1.2 and were considered for further analysis. For aim III, 269 individuals had blood samples collected and stored for metabolomic analysis. Of those, 50 individuals were excluded due to age (<6 years) and/or a BMI z-score below 2.0. An additional five individuals were excluded due to analytical errors. Finally, 214 participants with serum derived metabolomic data were included in the analysis.

Table 8. Participant characteristics for COBRA and eligible participants for aims II and III

	Visit 1, COBRA		**Protein leverage hypothesis**		**Metabolomics**	
Characteristic	**n**	**Mean (SD)**	**n**	**Mean (SD)**	**n**	**Mean (SD)**
Sex, males (%)	438	206 (47)	137	68 (50)	214	101 (47)
Age (y)	438	11.1 (3.6)	137	11.3 (2.7)	214	11.9 (3.1)
Weight (kg)	438	78.4 (31.7)	137	77.6 (26.3)	214	85.9 (30.2)
Height (m)	438	1.51 (0.19)	137	1.52 (0.15)	214	1.55 (0.16)
BMI (kg/m^2)	438	32.6 (7.36)	137	32.5 (6.0)	214	34.5 (7.1)
BMI z-score	438	2.53 (0.49)	137	2.47 (0.27)	214	2.49 (0.24)
Waist circumference (m)	355	1.01 (0.20)			169	1.06 (0.19)
Waist-to-height ratio	355	0.67 (0.09)			169	0.69 (0.08)
Truncal fat %	313	36.8 (9.1)			175	38.2 (8.7)
Total body fat %	329	42.8 (8.4)			182	44.3 (7.8)
Tanner stage (%)	371		137		214	
n pre-pubertal		165 (45)		66 (48)		83 (39)
n peri-pubertal		93 (25)		40 (29)		58 (27)
n post-pubertal		113 (30)		21(23)		75 (74)

Demographic and anthropometric data for COBRA participants and eligible participants for aims II and III. Numbers are given in mean and standard deviation (SD), absolute number of participants (n) and ratios (%). The range provides maximum and minimum levels of the relevant characteristics. Standard international units are used such as years (y), kilograms (kg) and meters (m). BMI: body mass index.

5.1.4 Participant characteristics for the COBRA-CVR longitudinal subsample

From the original COBRA biorepository (n= 438), a total of 101 participated for COBRA-CVR. Table 9 shows characteristics of the 101 COBRA-CVR participants at baseline and at follow-up, mean interval 5.5 years.

Table 9. Baseline and follow-up characteristics of COBRA-CVR participants

	Baseline (COBRA)			Follow-up (COBRA CVR)		
Characteristic	**n**	**Mean (SD)**	**Range**	**n**	**Mean (SD)**	**Range**
Sex, males (%)	101	52 (51.5)		101	52 (51.5)	
Age (y)	101	10.2 (3.5)	3.0 -16.9	101	15.7 (3.7)	6.1-24.3
Weight (kg)	101	71.8 (29.3)	19.1-157.7	101	101.7 (30.5)	40.8-187.7
Height (m)	101	1.49 (0.20)	1.00-1.90	101	1.67 (0.13)	1.26-1.95
BMI (kg/m^2)	101	30.9 (6.2)	18.4-51.8	101	35.7 (7.9)	18.2-60.9
BMI z-score	101	2.52 (0.36)	1.70-3.90	101	2.27 (0.61)	-0.53-3.39
%>95th BMI-centile	101	135.3 (19.2)	100.5-204.4	101	131.5 (26.4)	75.6-202
n <100%		0			9	
n ≥100% - <120%		24			26	
n ≥120% - <140%		43			33	
n ≥140%		34			33	
Change in %>95th BMI centile				101	-3.8 (19.9)	-60.5 - 46.6
Waist circumference (cm)	85	96.1 (17.8)	54.0-138.0	79	105.0 (16.4)	66-153
Waist-to-height ratio	85	0.65 (0.07)	0.50-0.80	79	0.62 (0.08)	0.43-0.83
Truncal fat %	72	35.05 (7.77)	16.4-60.3	99	36.4 (9.5)	15-58.8
Total body fat %	75	41.43 (6.92)	24.7-58.0	99	40.6 (9.9)	18.6-67.8
Tanner stage (%)	96					
n pre-pubertal		50 (52)			10 (10)	
n peri-pubertal		22 (23)			16 (16)	
n post-pubertal		24 (25)			75 (74)	
SEIFA Score				101	1004.21 (64.76)	802-1134

Demographic and anthropometric data for COBRA-CVR participants at the COBRA and COBRA-CVR visits and for the individuals eligible for aim I. The mean interval between visits was 5.5 (2.1) years. Numbers are given in mean and standard deviation (SD), absolute number of participants (n) and ratios (%). The range provides maximum and minimum levels of the relevant characteristics. Standard international units are used such as years (y), kilograms (kg) and meters (m)

5.1.5 Cardiovascular risk factors for the COBRA-CVR longitudinal subsample

Cardiovascular risk factors included blood pressure, LDL-C, triglycerides, GlycA, HbA1c, fasting glucose and smoking status. Available data on these characteristics including the relevant values at the initial COBRA enrolment are shown in table 10.

The prevalence of normal blood pressure decreased from 62% (59 out of 95) to 28% (28 out of 100) at the follow-up visit. In contrast, the percentage of participants with elevated blood pressure and stage II hypertension increased during the 5.5 years interval (from 7% to 33% and from 9% to 20%, respectively). Lipid profiles assessed by NMR metabolomic profiles revealed lower LDL-C and lower triglyceride levels at the follow-up visit. GlycA remained the same at both visits. At the initial COBRA visit, two participants (2%) were diagnosed with diabetes, whereas 8 (8%) were diagnosed with diabetes at follow-up.

Table 10. Baseline and follow-up cardiovascular risk factors of COBRA-CVR participants

	Baseline (COBRA)			**Follow-up (COBRA CVR)**		
Cardiovascular risk factor	**n**	**Mean (SD)**	**Range**	**n**	**Mean (SD)**	**Range**
Systolic BP (mmHg)	95	109 (17)	80-160	100	126 (13)	96-162
Diastolic BP (mmHg)	95	66 (11)	45-95	100	69 (8)	50-89
Normal BP	59			28		
Elevated BP	7			33		
Stage I Hypertension	20			19		
Stage II Hypertension	9			20		
LDL-C (mmol/l)	67	2.54 (0.55)	1.0-4.0	98	1.76 (0.58)	0.9-4.6
Triglycerides (mmol/l)	67	1.37 (0.68)	0.4-4.6	98	1.15 (0.40)	0.6-2.5
GlycA (mmol/l)	67	1.11 (0.13)	0.9-1.6	98	1.11 (0.11)	0.88-1.40
HbA1c (%)				94	5.2 (0.4)	4.1-6.5
Fasting glucose (mmol/l)	86	4.6 (0.5)	3.5-6.1	94	4.6 (0.5)	3.7-7.1
Diabetes		2 (2)			8 (9)	
Pre-Diabetes		12 (14)			8 (9)	
Smoking status						
Never smoked				92		
Current smoker				9		

Cardiovascular risk factors in participants at the initial COBRA and follow-up COBRA-CVR visit. Numbers are given in mean and standard deviation (SD), absolute number of participants (n) and ratios (%). The range provides maximum and minimum levels of the relevant characteristics. Standard international units are used such as millimetres of mercury (mmHg), millimole per litre (mmol/l). BP: blood pressure; LDL-C: Low density lipoprotein cholesterol; GlycA: glycoprotein acetyls; HbA1c: Haemoglobin A1c.

5.1.6 Subclinical cardiovascular phenotypes for COBRA-CVR participants

Data on subclinical cardiovascular phenotypes (cIMT, PWV, elasticity), collected in COBRA-CVR participants are shown in table 11.

Table 11. Subclinical cardiovascular phenotypes for COBRA-CVR participants

Subclinical Atherosclerosis Outcomes	**n**	**Mean (SD)**	**Range**
Carotid IMT (μm)	100	457 (62)	344-608
Carotid-femoral PWV (m/s)	98	5.32 (0.87)	3.57-7.9
Elasticity (%/10mmHg)	100	4.24 (1.25)	1.84-8. 40

Subclinical cardiovascular phenotypes assessed at COBRA-CVR visit. n = number of participants with available data. Data are given in mean, SD (standard deviation), and range from minimum to maximum. IMT: Intima-media thickness; PWV: pulse-wave velocity. Units used were micrometer (μm), meter/second (m/s) and percentage change per 10 millimetres mercury (%/10mmHg).

5.2 Aim I: association between grip strength and cardiometabolic risk

Aim I was to investigate the association of grip strength, a global measure of muscle strength, on cardiovascular risk factors and subclinical cardiovascular phenotypes among obese youth;

Participant characteristics and data on cardiovascular risk factors for aim I are displayed in tables 6 and 7 with relevant data from the original COBRA-CVR cohort for comparison. Table 12 illustrates data on grip strength in all participants with normalized grip strength categories (low, moderate and high). The mean maximum grip strength was 32.2 kg. Only 4 participants (9.3 %) had a high normalized grip strength and therefore, the categories of moderate and high normalized grip strength were collapsed into one category (moderate/high) for further analysis.

Table 12. Grip strength in COBRA-CVR participants

Variable	n	Mean (SD)	Range (min-max)	%
Grip strength, kg	43	32.2 (8.3)	16.2-53.5	
Normalized grip strength	43	0.30 (0.06)	0.20-0.42	
Normalized grip strength categories				
Low	21			48.8
Moderate	18			41.9
High	4			9.3

Grip strength assessed at COBRA-CVR visit. n = number of participants with available data. Data are given in mean, SD (standard deviation), and range from minimum to maximum.

In univariable analysis, age, sex, and pubertal stage were not associated with normalized grip strength (p-values 0.40, 0.29, and 0.62, respectively). Therefore, these covariates were not included in the regression models.

Mean levels of cardiometabolic risk factors according to normalized grip strength categories (high, moderate and low) are shown in table 13. In general, participants with low normalized grip strength had poorer cardiometabolic risk measures than those with moderate/high normalized grip strength. The mean difference between normalized grip strength groups (moderate/high vs. low) was +0.05 mm for cIMT, +13 mmHg for systolic blood pressure, +0.26 mmol/l for LDL-C, and +0.36 for metabolic syndrome (MetS) score.

After adjustment for BMI z-score, the difference between groups remained largely unchanged for cIMT (+0.06 mm, +2 %, p-value = 0.003, n = 43), whereas the difference was reduced for systolic blood pressure (+9 mmHg, -30 %, p-value = 0.015, n = 43) and LDL-C (+0.23 mmol/l, -10 %, p-value = 0.10, n = 41). BMI z-score is a factor in the MetS score, and therefore did not require additional adjustment. Because systolic blood pressure and LDL-C might be intermediates between grip strength and cIMT, a model was fit including these covariates. The difference in cIMT between normalized grip strength groups remained (+0.06 mm, p-value = 0.003, n = 41).

Table 13. Adjusted means of cardiometabolic risk variables according to normalized grip strength category

		Normalized grip strength category			
Cardiometabolic risk variable	n*	**Moderate/High** Mean ± SE	**Low** Mean ± SE	Mean difference [95%CI]	p
Total cholesterol, mmol/l	41	2.91 ± 0.10	3.19 ± 0.12	0.27 (-0.04, 0.59)	0.08
LDL-C, mmol/l	41	1.21 ± 0.08	1.46 ± 0.10	0.26 (0.00, 0.51)	0.048
Triglycerides, mmol/l	41	1.14 ± 0.07	1.24 ± 0.08	0.11 (-0.12, 0.33)	0.34
HbA1c (%)	41	5.30 ± 0.09	5.36 ± 0.15	0.06 (-0.19, 0.31)	0.63
Fasting glucose (mmol/l)	41	4.69 ± 0.11	4.53 ± 0.10	-0.15 (-0.44, 0.14)	0.29
Systolic BP (mmHg)	43	120 ± 2	133 ± 3	13 (6, 20)	0.001
Diastolic BP (mmHg)	43	68 ± 2	72 ± 2	3 (-2, 8)	0.20
Continuous metabolic syndrome score	41	1.16 ± 0.09	1.52 ± 0.09	0.36 (0.11, 0.62)	0.007
Glycoprotein acetyls (mmol/l)	41	1.13 ± 0.10	1.17 ± 0.08	0.04 (-0.10, 0.01)	0.12
Carotid IMT (μm)	43	0.46 ± 0.01	0.52 ± 0.01	0.05 (0.02, 0.09)	0.002
Pulse wave velocity (m/s)	43	5.21 ± 0.17	5.42 ± 0.20	0.21 (-0.32, 0.74)	0.43
Carotid elasticity (%/mmHg)	40	0.34 ± 0.02	0.34 ± 0.02	-0.01 (-0.07, 0.05)	0.81

**Number of participants in the normalized grip strength categories were 21 (low) and 22 (moderate/high) when n=43. Participants in the normalized grip strength categories were 21 (low) and 20 (moderate/high) when n=41. Participants in the normalized grip strength categories were 19 (low) and 21 (moderate/high) when n=40. Participants in the normalized grip strength categories were 19 (low) and 19 (moderate/high) when n=38. The reduced sample sizes for some analyses are due to participants missing data on the cardiometabolic risk variable. Abbreviations: CI, confidence interval; LDL-C: low-density lipoprotein cholesterol; HbA1c: glycated hemoglobin A_{1c}; N: number of participants; SE: standard error.*

5.3 Aim II: testing the protein leverage hypothesis

Aim II was to test the relation between the macronutrient composition with TEI and BMI in children and adolescents with obesity. Particularly, the protein leverage hypothesis was tested, indicating that a lower proportion of energy derived from proteins drives excessive total energy intake and may contribute to excessive weight gain.

The main participant characteristics for aim II are displayed in table 8 with relevant data from the original COBRA cohort for comparison.

5.3.1 Total energy intake

Table 14 shows data on total energy intake and the proportional energy intake of macronutrients in eligible participants, derived from the Australian Child and Adolescent Eating Survey - Food Frequency Questionnaire (ACAES-FFQ).

The overall energy intake was at 10`330 kJ with the proportions of energy from proteins calculated at 18.4%, from carbohydrates at 50.6% and fats at 31.6%.

Table 14. Total energy intake and macronutrient composition

Variable	Mean (SD)	Range (min–max)
Total energy intake (TEI in kJ)	10`330 (2728)	5467-17`886
Total daily protein intake (g)	110 (30)	56-213
Energy intake from proteins (kJ)	1831 (497)	933-3565
% TEI protein	18.4 (3.1)	11.0-30.0
Total daily carbohydrate intake (g)	307 (95)	154-611
Energy intake from carbohydrates (kJ)	5119 (1293)	2563-10`202
% TEI carbohydrate	50.6 (6.1)	32.0-68.0
Total daily fat intake (g)	84 (27)	37-195
Energy intake from fats (kJ)	3173 (1013)	1408-7354
% TEI fat	31.6 (4.9)	21.0-51.0

Total energy intake as calculated from Australian Child and Adolescent Eating Survey - Food Frequency Questionnaire (ACAES-FFQ), given as total energy intake (TEI) and macronutrients (protein, carbohydrate and fat) in gram, kilojoules and percentages from TEI.

5.3.2 Energy expenditure, assessed by accelerometry

A subgroup of participants for aim II had accelerometry data collected. Results are illustrated in table 15. The Australian guidelines recommend 60 minutes of moderate to vigorous activity per day for children and adolescents aged 5-17 years. Of the 57 individuals with valid accelerometry data, 48 (84%) did not meet these recommendations and for the purpose of our study were considered to be physically inactive individuals (Australian Commonwealth Department of Health,2019)

Table 15. Accelerometry-data on energy expenditure

Variable	n (%)	Mean (SD)	Range
Accelerometry	57		
Ave. counts/d		249`734 (97`904)	96`841 – 493`728
MVPA (min/d)		43.4 (24.1)	3.5 - 110.8
<60min MVPA/d	47 (84%)		

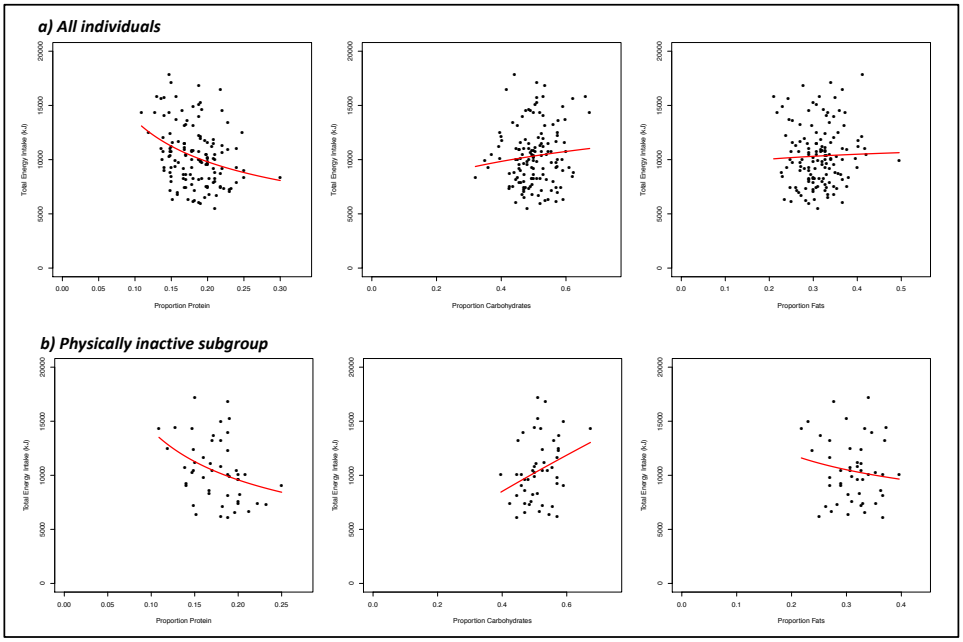
Accelerometry; Ave. counts/d: number of signals reflecting deceleration and acceleration forces counted by the accelerometer, averaged per day. MVPA: moderate to vigorous physical activity given in minutes, derived from Ave. counts/d according to published intensity cut-off points (Colley et al.,2011).

5.3.3 Bivariate modelling of each macronutrient and total energy intake

Figure 11a illustrates associations between proportion of energy derived from each macronutrient (x-axis) and the TEI (y-axis) in all individuals and in the physically inactive subgroup. In all individuals (Figure 11a), the distribution of percentage intake from macronutrients and TEI significantly followed a power function for %EP, but not for %EC or %EF. For protein, the strength of leverage for protein was -0.48 ($p < 0.001$), whereas the exponent (L) for carbohydrates and fats were 0.22 ($p=0.23$) and 0.06 ($p=0.67$), respectively (see figure 11a and table 16).

Figure 11b illustrates the associations between percentages of energy intake from each macronutrient (x-axis) and the total energy intake (y-axis) in the physically inactive subgroup. The %EP and %EC followed a power function with respect to TEI. Relevant exponents (L) for protein were -0.57 ($p < 0.05$), for carbohydrates 0.82 ($p < 0.05$) and for fats 0.82 (p 0.30) (see figure 11b and table 16).

Figure 11. Bivariate modelling between macronutrient proportions and total energy intake



Power functions between relevant percentages of energy from macronutrients and total energy intake. Scatterplots revealing associations between proportions of each macronutrient and total energy intake that followed the law of a power function (see Methods). (A) All individuals (n = 137). Small changes in proportion of energy only from protein sources resulted in substantial changes in total energy intake. (B) Physically inactive subgroup (n = 48). Proportions of proteins and carbohydrates followed a power function: for proteins, the association was negative and for carbohydrates positive (see table 16).

Table 16. Results from power functions fitted between macronutrients and TEI

All individuals, n=137		
Macronutrient	*Strength of leverage (L)*	*p value*
Protein	*-0.48*	*<0.001*
Carbohydrate	*0.22*	*0.23*
Fat	*0.06*	*0.67*

Physically inactive subgroup, n=47		
Macronutrient	*Strength of leverage (L)*	*p value*
Protein	-0.57	<0.05
Carbohydrate	*0.82*	*<0.05*
Fat	*-0.31*	*0.30*

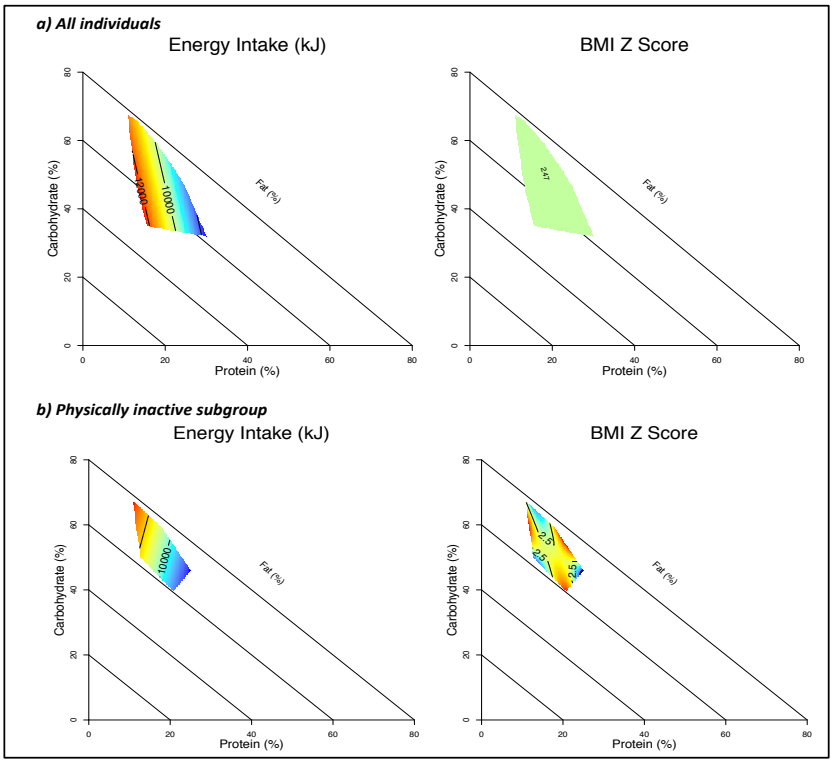
Model coefficients for fitted power functions for the whole cohort and for the physically inactive subgroup. L indicating strength of leverage for each macronutrient (-1 signifies complete leverage, 0 means no leverage). In the whole cohort, leverage on total energy intake was only significant from the proportion of protein. In the physically inactive group, leverage on total energy intake was significant from proportions of protein and carbohydrate

5.3.4 Compositional data analysis between macronutrients with TEI and BMI

To assess whether there was a relationship between the macronutrient composition of diet, TEI and BMI z-score, mixture model analysis was performed. For TEI, model 2 had the most favourable AIC (see table 17 for AIC results and table 18 for the AIC-chosen model coefficients), which suggests a linear effect of dietary macronutrients on TEI. TEI was highest in those diets with the lowest proportion energy from proteins (see 12a for visual interpretation). In this population of youths with obesity, dietary macronutrient composition was not a significant driver of BMI z-score, as the null model was favoured (see table 17 for AIC results).

Compositional data analysis in the physically inactive group revealed model 2 favoured by AIC, suggesting linear effects of dietary macronutrients on TEI (see figure 12b for visual interpretation, table 19 for AIC results and table 20 for the AIC-chosen model coefficients). For BMI z-score in physically inactive youths, compositional model 4 was favoured, suggesting complex, non-linear effects of macronutrients composition on BMI z-score: physically inactive individuals with a diet high in %EP with moderate %EC had lower BMI z-scores, whereas individuals with a diet moderate in %EP and low %EC had higher BMI z-scores (see table 19 for AIC and table 21 for the AIC-chosen model coefficients).

Figure 12. Rectangular mixed triangle to illustrate compositional data analysis from macronutrients composition on total energy intake and BMI z-scores



Right-angle mixture triangles (RMTs) illustrating (a) energy intake (kJ) and (b) BMI z-score as a function of percentage total energy from protein (x-axis), carbohydrates (y-axis) and fats (implicit-axis) in series a) for all individuals (n=137) and series b) for physically inactive subgroup (n=48). Values shown are as predicted by AIC-favoured mixture models. Areas of red and blue space correspond to high and low outcomes, respectively.

Table 17. AIC scores for five mixture-models in all individuals

Model	Energy intake (kJ)	BMI z-score
1	2559.47	37.83
2	2549.87	36.84
3	2553.16	37.11
4	2554.39	43.52
5	2554.87	38.4

AIC scores to test for effects of percentage energy from protein, carbohydrates and fats on energy intake and BMI z-scores. Models with minimal AIC scores are favoured. In the event that two models where within two AIC points of one another, the simplest model was selected. Model 1 refers to the Null model, indicating no effect from macronutrient ratios.

Table 18. Coefficients for effects of macronutrients on TEI as estimated by model 2 for the physically inactive subgroup

Model	Coefficient	Estimate	LCL	UCL
2	P	-16796	-40568	6976
	C	18594	11253	25935
	F	12343	-2316	27002

AIC favoured model 2. Coefficient`s estimates for model 2 and their lower and upper (LCL and UCL) 95% confidence limits are given

Table 19. AIC scores for five mixture-models in the physically inactive subgroup

Model	Energy intake (kJ)	BMI z-score
1	904.52	12.37
2	901.28	16.12
3	904.09	15.8
4	911	8.76
5	905.74	15.95

AIC scores to test for effects of percentage energy from protein, carbohydrates and fats on energy intake and BMI z-scores in the physically inactive subgroup. Models with minimal AIC scores are favoured. In the event that two models where within two AIC points of one another, the simplest model was selected.

Table 20. Coefficients for effects of macronutrients on TEI as estimated by model 2 for the physically inactive subgroup

Model	Coefficient	Estimate	LCL	UCL
2	P	-16796	-40568	6976
	C	18594	11253	25935
	F	12343	-2316	27002

AIC favoured model 2. Coefficient`s estimates for model 2 and their lower and upper (LCL and UCL) 95% confidence limits are given.

Table 21. Coefficients for effects of macronutrients on BMI *z*-score as estimated by model 4 for the physically inactive subgroup

Model	Coefficient	Estimate	LCL	UCL
4	P	-898.00	-1891.10	95.10
	C	0.02	-20.72	20.75
	F	-367.65	-743.02	7.72
	cubic(P, C)	1312.43	404.88	2219.99
	cubic(P, F)	-1006.34	-2759.78	747.11
	cubic(C, F)	-211.55	-667.19	244.10
	P:C	2046.05	329.75	3762.34
	P:F	2169.65	282.17	4057.13
	C:F	690.17	6.74	1373.59
	P:C:F	-3539.23	-5948.22	-1130.25

AIC favoured model 4. Coefficient`s estimates for model 4 and their lower and upper (LCL and UCL) 95% confidence limits are given.

5.4 Aim III: adiposity measures and CVD-related metabolomic profiles

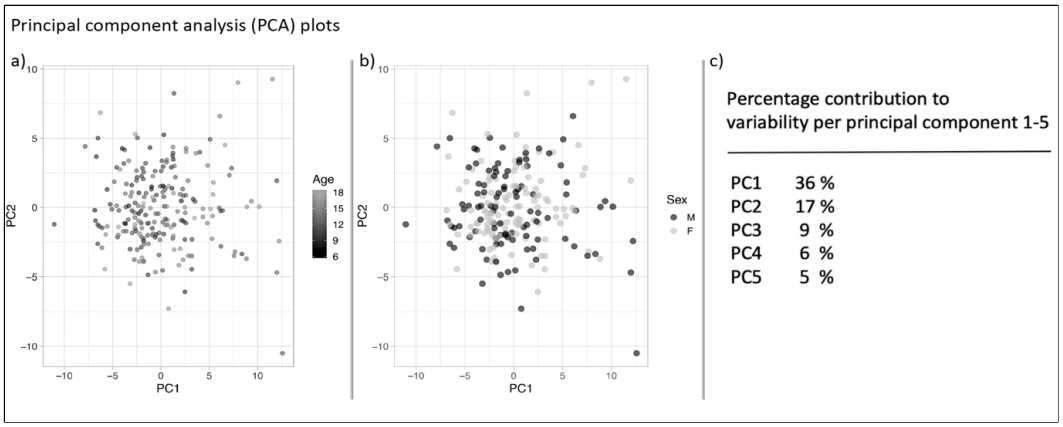
Aim III was to investigate the relation between clinically assessed adiposity measures and a metabolomic platform with cardiometabolic interests in sera of children and adolescents with obesity. Results were further stratified for sex and pubertal development (Tanner stages for pre-, peri-, and post-pubertal stages).

The main participant characteristics for aim III are displayed in table 8 with relevant data from the original COBRA cohort for comparison.

5.4.1 Multivariate analysis of metabolomic data, principal component analysis

Principal component analysis (PCA) was performed to detect distinguishable clusters of individuals according to confounders (age and sex) or the variety of metabolite results. PCA did not reveal separable clusters by age or sex within the dataset (see figure 13). Principal components 1 to 5 collectively assessed 73% coverage of the data variability.

Figure 13. Principal components plot

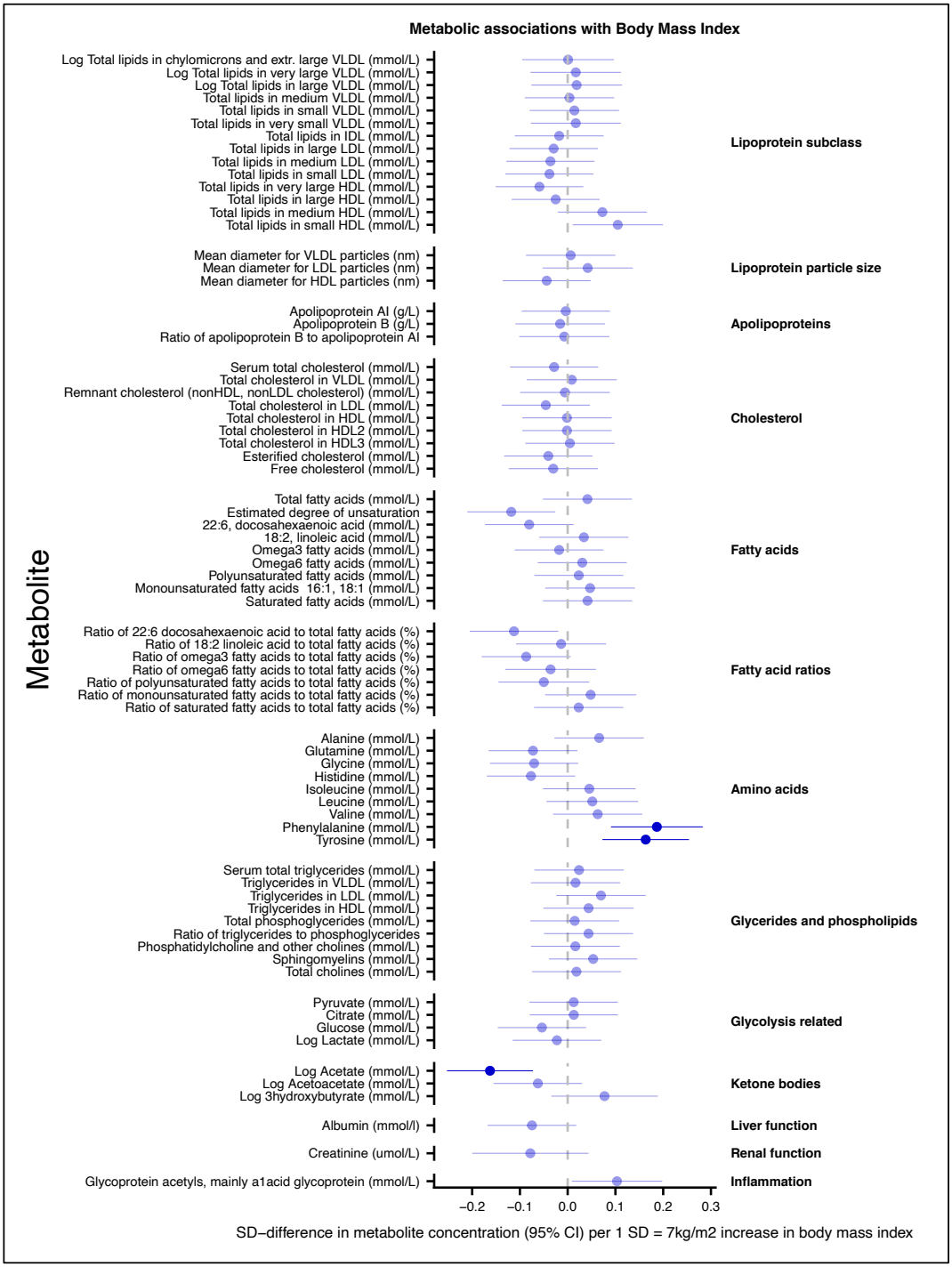


PCA plot for principal component 1 (PC1) and principal component 2 (PC2), graded for age (a) and sex (b). Section c lists percentage contribution of the top 5 principal components.

5.4.2 Associations between adiposity measures and metabolites

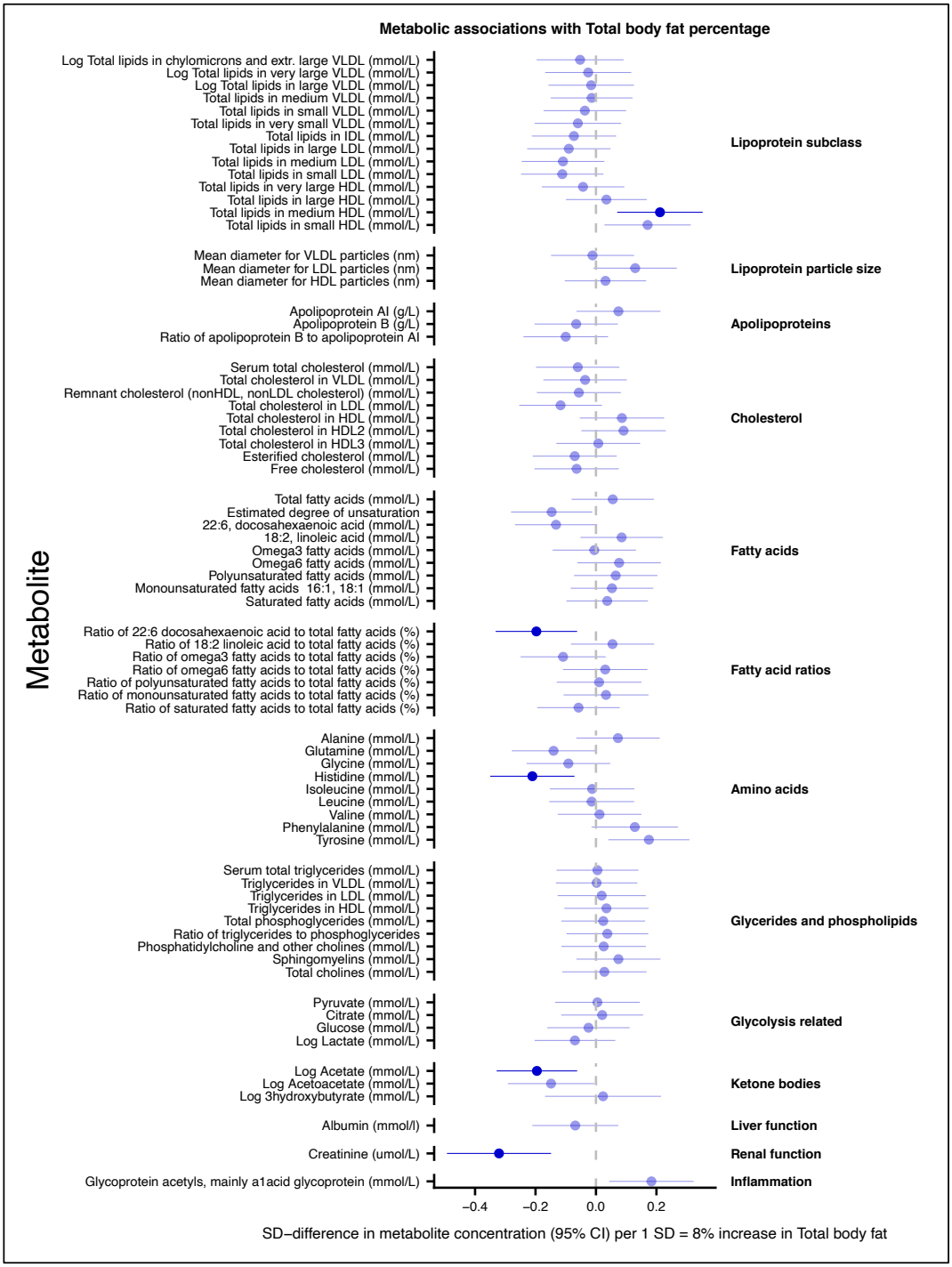
Raw BMI was positively associated with phenylalanine and tyrosine, and negatively associated with acetate, adjusted for false discovery rate (see figure 14). Associations were similar between pairs of BMI and BMI z-score, of %TF and %BF, and of WC and WtH-ratio (see forest plots in figures 14, 15 and 16 for one of each pair).

Figure 14. Forest plot with associations between raw BMI and metabolites



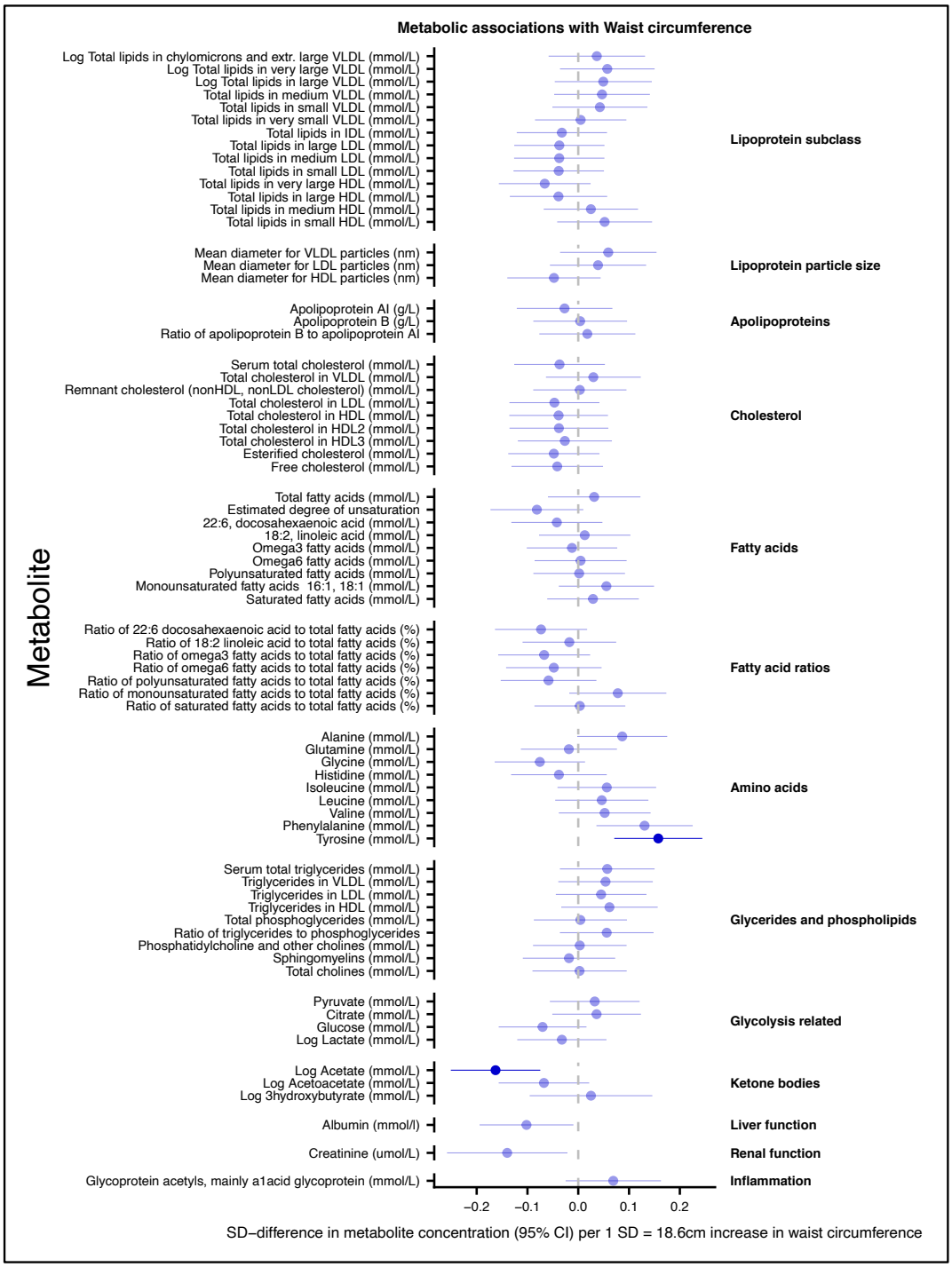
Changes in mean and 95% confidence interval per 1-SD increase in BMI. Estimates and 95% CI in bold illustrate significance after adjustment for false discovery rate (FDR, according to Benjamini-Hochberg).

Figure 15. Forest plot with associations between %BF and metabolites



Changes in mean and 95%CI per 1-SD increase in total body fat %. Estimates and 95%CI in bold illustrate significance after adjustment for false discovery rate (FDR, according to Benjamini-Hochberg).

Figure 16. Forest plot with associations between waist circumference and metabolites



Changes in mean and 95%CI per 1-SD increase in waist circumference. Estimates and 95%CI in bold illustrate significance after adjustment for false discovery rate (FDR, according to Benjamini-Hochberg).

5.4.3 All associations between various adiposity measures and metabolites

Across different adiposity measures associations were broadly similar in terms of the direction, with some variability in the strength of the associations with individual metabolites (see table 22).

Table 22. Associations between various adiposity measures and metabolites

		BMI	BMI z -score	BF%	TF%	WC	WtH
Metabolites & direction of association							
Total lipids in very large HDL (mmol/l)	neg	ns.	p < 0.05	ns.	ns.	ns.	ns.
Total lipids in medium HDL (mmol/l)	pos	ns.	ns.	**p < 0.01**	p < 0.01	ns.	ns.
Total lipids in small HDL (mmol/l)	pos	p < 0.05	ns.	p < 0.05	p < 0.05	ns.	ns.
Estimated degree of unsaturation	neg	p < 0.05	p < 0.01	p < 0.05	ns.	ns.	ns.
22:6, docosahexaenoic acid(mmol/l)	neg	ns.	ns.	ns.	p < 0.05	ns.	ns.
Ratio of docosahexaenoic acid to total fatty acids (%)	neg	p < 0.05	P < 0.05	**p < 0.01**	p < 0.01	ns.	ns.
Ratio of omega3 fatty acids to total fatty acids (%)	neg	ns.	p < 0.05	ns.	ns.	ns.	ns.
Glutamine (mmol/l)	neg	ns.	ns.	p < 0.05	p < 0.05	ns.	ns.
Histidine (mmol/l)	neg	ns.	p < 0.05	**p < 0.01**	p < 0.01	ns.	p < 0.05
Phenylalanine (mmol/l)	pos	**p < 0.001**	**p < 0.01**	ns.	p < 0.05	p < 0.01	ns.
Tyrosine (mmol/l)	pos	**p < 0.001**	p < 0.05	p < 0.05	ns.	**p < 0.001**	p < 0.05
Log acetate (mmol/l)	neg	**p < 0.001**	**p < 0.001**	**p < 0.01**	p < 0.05	**p < 0.001**	p < 0.05
Log acetoacetate (mmol/l)	neg	ns.	ns.	p < 0.05	ns.	ns.	ns.
Albumin (mmol/l)	neg	ns.	ns.	ns.	ns.	p < 0.05	p < 0.05
Creatinine (μmol/l)	neg	ns.	ns.	**p < 0.001**	**p < 0.01**	p < 0.05	p < 0.05
Glycoprotein acetyls (mmol/l)	pos	p < 0.05	ns.	p < 0.05	p < 0.05	ns.	ns.

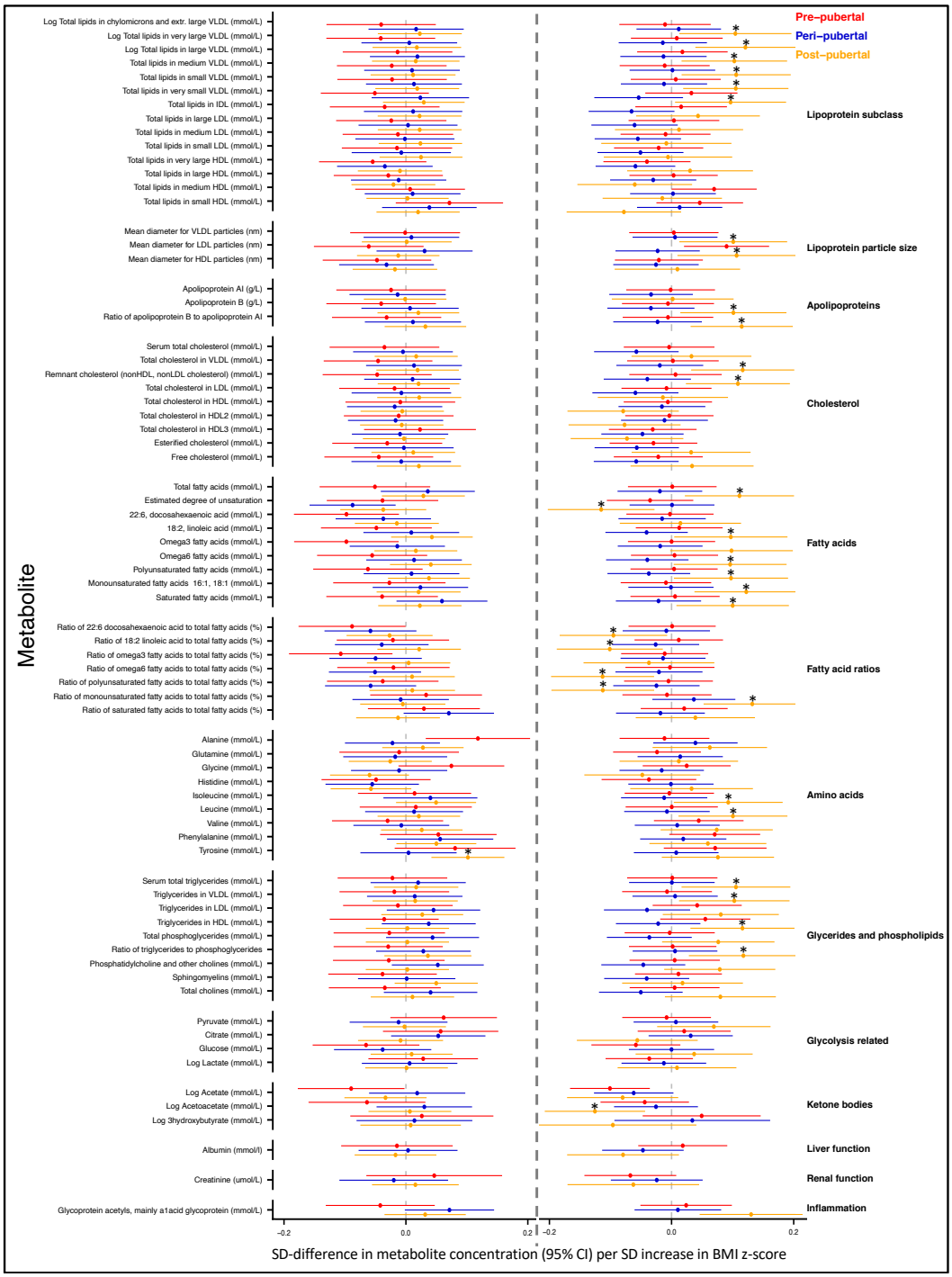
Results from multiple regression modelling with each adiposity measure as explanatory variable and the metabolite as outcome, adjusted for age and sex. In bold are the ones adjusted for false discovery rate (FDR). Abbreviations: BMI: body mass index; BF%: total body fat percentage; TF%: truncal fat percentage; WC: waist circumference; WtH: waist to height ratio; HDL: High density lipoprotein; ns: non-significant; pos: positive association; neg: negative association

5.4.4 Sex- and puberty-related associations between BMI z-score and metabolites

Figure 17 illustrates the association of BMI z-score with metabolites stratified by sex and pubertal development. In post-pubertal females, the only metabolite associated with BMI z-score after multiple comparison was tyrosine (positively associated).

In contrast, several associations were found between BMI z-score and metabolites in post-pubertal males, including: the concentration of total lipids, cholesterols and triglycerides in VLDL lipoproteins, the ratio of apolipoprotein B/A1 and the mean diameter for VLDL and LDL particles. Associations were also seen with concentrations of total fatty acids, linoleic acid, omega-6 fatty acids, and polyunsaturated, monounsaturated and saturated fatty acids (all positively associated) and the estimated degree of unsaturation (negatively associated). Further associations were found with triglycerides in HDL, total phosphoglycerides, isoleucine and leucine, glycoprotein acetyls (all positively associated) and log acetoacetate (negatively associated).

Figure 17. Associations between BMI z-score and metabolites, stratified for sex and pubertal stage



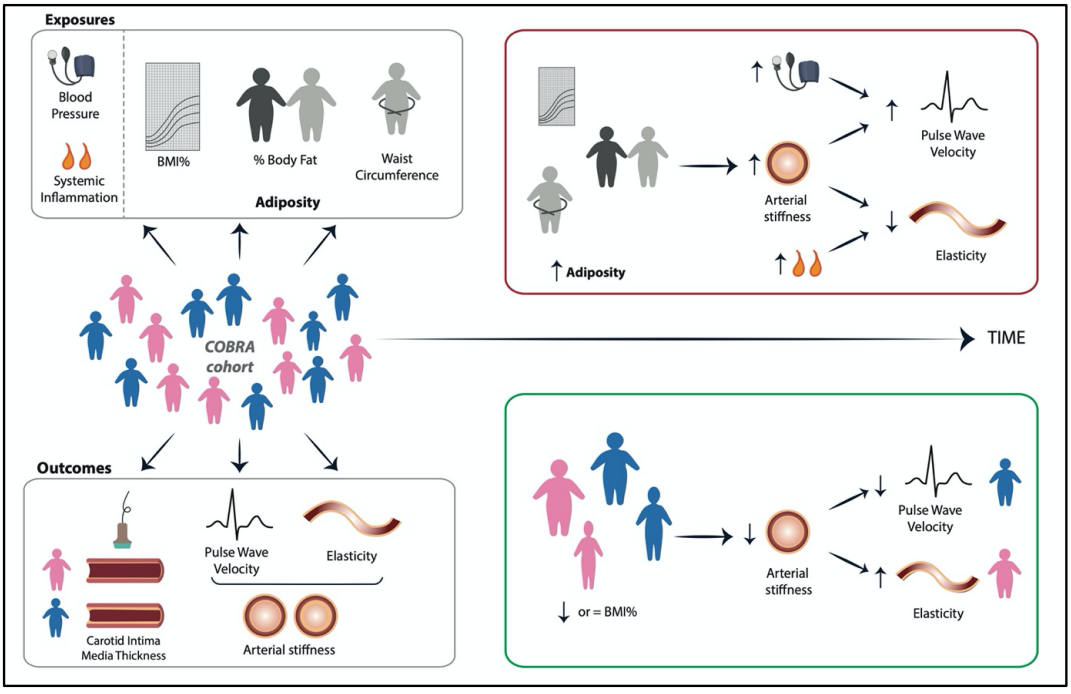
*Changes in mean and 95%CI per 1-SD increase in BMI z-score categorized by sex and pubertal stage. Pre-pubertal (red) = Tanner stage. 1. Peri-pubertal (blue) = Tanner stage 2-3. Post-pubertal (yellow) = Tanner stage 4-5). Females in the left panel, males in the right panel. In post-pubertal subgroup (yellow), * indicates association after multiple regression modelling, including adjustment for false discovery rate (FDR, according to Benjamini-Hochberg).*

5.5 Aim IV: associations of changes in adiposity measures and cardiovascular risk factors on subclinical cardiovascular phenotypes

Aim IV was to investigate the association of changes in adiposity measures or cardiovascular risk factors over time on a comprehensive, non-invasive subclinical cardiovascular phenotype assessment in children and adolescents with obesity. Participant characteristics in the longitudinal study are displayed in tables 9 & 10. Results for the subclinical cardiovascular phenotypes are shown in table 11.

Associations between age or sex and the subclinical cardiovascular phenotypes are reported with a scatterplot for illustration (figure 19). Table 23 reports the association of changes in various adiposity measures on subclinical cardiovascular phenotypes, and table 24 illustrates the association from a reference model including baseline age, sex and SEIFA scores, then each of the cardiovascular risk factors is included one at a time in the model. A graphical abstract, summarizing the main findings from this study is illustrated in figure 18.

Figure 18. Graphical abstract for aim IV



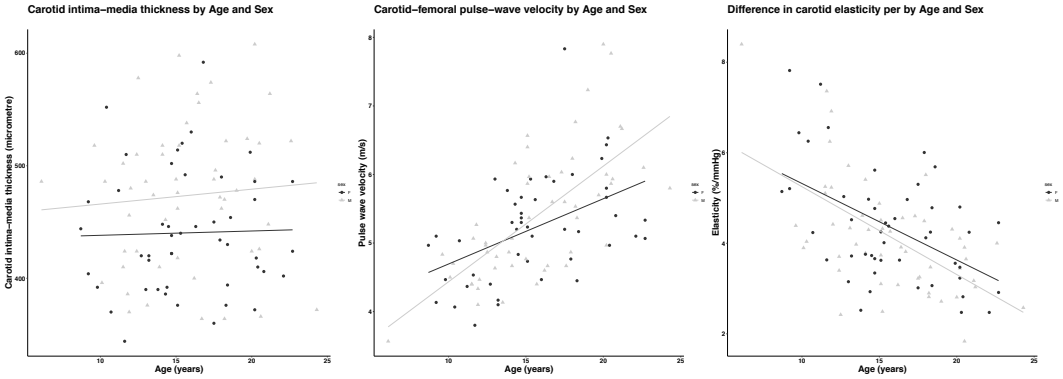
A graphical abstract, summarizing the main findings from study IV. With special thanks to Siroon Bekkering, co-author for her creativity.

5.5.1 Subclinical cardiovascular phenotypes by age and sex

In a linear regression model with each of the subclinical cardiovascular phenotypes as the independent variable and age, sex and SEIFA scores as dependent variables, cIMT measures were larger for males than females (mean difference for males 34 μm [95%CI 10-58 μm, p-value 0.006]), but did not change significantly for the observed age-interval [95%CI -2 to +2 μm, p-value 0.516] (see figure 19).

There were no sex differences for PWV (mean difference for males 0.163m/s [95%CI -0.122 to 0.448, p-value 0.259] and elasticity (mean difference for males -0.239 [95%CI -0.659 to 0.181%/10mmHg]). However, both measures changed with age: PWV increased by a mean of 0.136m/s [95%CI 0.098-0.174, p-value <0.001] per one year of ageing and elasticity decreased by a mean of -0.187%/10mmHg [95%CI -0.243 to – 0.130, p-value <0.001] per one year of ageing) (see figure 19).

Figure 19. Subclinical cardiovascular phenotypes stratified for sex and age



Scatterplots illustrating the change of each subclinical cardiovascular phenotypes per age, classified for sex. Units used for carotid intima-media thickness are micrometers, for pulse-wave velocity are meters per second and for elasticity are % per 10 millimetres of mercury. Results for males are shown in black, for females grey, lines illustrate the regression line.

5.5.2 Associations of changes in adiposity measures on subclinical cardiovascular phenotypes

The estimated associations of changes in adiposity measures over time with the subclinical cardiovascular phenotypes at follow-up are shown in table 23, adjusted for SEIFA scores, sex, age at the follow-up visit and the relevant adiposity measure, and age at the initial visit (mean interval 5.5 years).

Increasing levels for BMI, BMI z-score, the %>95th BMI-centile, WtH ratio and WC were associated with higher pulse wave velocity. All adiposity measures were inversely associated with carotid artery elasticity. There was no evidence for an association between adiposity measures and cIMT. Overall, results were similar for BMI and BMI z-score, WC and waist to height ratio and %BF and truncal fat percentage.

Table 23. Changes in adiposity measures and associations with subclinical cardiovascular phenotypes

Pulse Wave Velocity (m/s)				
Predictors	*Estimates*	*CI*	*P*	*N*
BMI	0.045	0.021 – 0.068	**<0.001**	98
BMI z-score	0.716	0.447 – 0.985	**<0.001**	96
%>95th Centile	0.011	0.004 – 0.017	**0.002**	98
Waist to height ratio	4.439	1.640 – 7.238	**0.002**	70
Waist circumference	0.028	0.014 – 0.042	**<0.001**	70
% Truncal fat	0.016	-0.010 – 0.041	0.231	70
% Body fat	0.004	-0.022 – 0.030	0.761	73
Elasticity (%/10mmHg)				
Predictors	*Estimates*	*CI*	*p*	*N*
BMI	-0.063	-0.100 – -0.026	**<0.001**	100
BMI z-score	-0.975	-1.351– -0.600	**<0.001**	98
%>95th Centile	-0.020	-0.032 – -0.007	**0.002**	100
Waist to height ratio	-4.399	-8.686– -0.112	**0.044**	70
Waist circumference	-0.035	-0.056 – -0.015	**0.001**	70
% Truncal fat	-0.052	-0.087– -0.018	**0.004**	71
% Body fat	-0.050	-0.085 – -0.016	**0.004**	74
Mean carotid intima-media thickness (μm)				
Predictors	*Estimates*	*CI*	*p*	*N*
BMI	0.985	-1.204 – 3.174	0.374	100
BMI z-score	12.093	-9.876 – 34.063	0.277	98
%>95th Centile	0.314	-0.311 – 0.940	0.321	100
Waist to height ratio	29.578	-230-968 – 290.123	0.821	70
Waist circumference	0.216	-1.092 – 1.523	0.734	70
% Truncal fat	-0.482	-2.772 – 1.807	0.675	71
% Body fat	0.090	-2.210 – 2.389	0.938	74

Regression coefficients per 1-unit change in predictor (1kg/m^2 for BMI, 1 z-score for BMI z-scores, 1% for %>95th BMI centile, 1cm for Waist circumference, 1 unit for waist to height ratio and 1% for truncal fat and body fat) over time. Linear regression models are adjusted for age and predictor at baseline, age at follow up, sex and SEIFA score. Models including BMI z-score and %>95th centile as predictor were only adjusted for %>95th centile at baseline and SEIFA scores. BMI: Body-mass index measured as weight in kg / height in m^2. %>95th centile: percentage above the 95th centile for BMI based on CDC growth charts. BMI z-score based on CDC growth charts

5.5.3 Associations between changes in cardiovascular risk factors on subclinical cardiovascular phenotypes

Table 24 reports the associations for sex, age and SEIFA-scores as the reference model. The coefficients for age and sex, each are given. The coefficient for SEIFA scores was not significant in any model and is therefore not shown. Adding to the reference model, subsequent models add changes in each cardiovascular risk factor over time on the subclinical cardiovascular phenotypes. Every year of ageing was associated with an increase in PWV by 0.136 m/s [95%CI 0.098-0.174], a decrease in carotid elasticity by 0.187%/10mmHg [95%CI -0.243 to – 0.130], but there was no evidence for an association with cIMT. In contrast, male sex was associated with a higher cIMT by 34 μm [95%CI 10-58], compared with females.

An 5 kg/m^2 increase in BMI and a 10 mmHg increase in systolic blood pressure was associated with a higher PWV (0.223m/s [95%CI 0.105-0.340] for BMI; 0.260m/s [95%CI 0.150-0.370] for SBP), adjusted for sex, SEIFA scores and age at both visits. For elasticity, a 5 kg/m^2 increase in BMI was associated with a lower carotid elasticity by 0.315 %/10mmHg [95%CI -0.130 to -0.500] and a 50mmol/l increase in GlycA was associated with a lower carotid elasticity by -0.162 %/10mmHg [95%CI -0.006 to -0.318]. There was no evidence to suggest LDL-C and fasting glucose associated with subclinical cardiovascular phenotypes (table 24).

Table 24. Association between changes in cardiovascular risk factors and subclinical cardiovascular phenotypes

Pulse Wave Velocity (m/s)				
Predictors	*β-coefficient*	*CI*	*p*	*N*
Age*, Sex, SEIFA scores	0.136	0.098 – 0.174	**<0.001**	98
Age, Sex*, SEIFA scores	0.163	-0.122 – 0.448	0.259	98
+ 5 kg/m^2 BMI	0.223	0.105 – 0.340	**<0.001**	98
+ 10mmHg systolic BP	0. 260	0.150 – 0.370	**<0.001**	92
+ 50 μmol/l GlycA	0.022	-0.093 – 0.137	0.709	62
+ 0.1 mmol LDL-C	0.008	-0.052 – 0.069	0.786	62
+ 0.1 mmol glucose	0.022	-0.013 – 0.057	0.209	79
Elasticity (%/10mmHg)				
Predictors	*β-coefficient*	*CI*	*p*	*N*
Age*, Sex, SEIFA scores	-0.187	-0.243 – -0.130	**<0.001**	100
Age, Sex*, SEIFA scores	-0.239	-0.659 – 0.181	0.261	100
+ 5 kg/m^2 BMI	-0.315	-0.500 – -0.130	**0.001**	100
+ 50 μmol/l GlycA	-0.162	-0.006 – -0. 318	**0.042**	64
+ 0.1 mmol LDL-C	0.004	-0.081 – 0.090	0.919	64
+ 0.1 mmol glucose	-0.007	-0.056 – 0.042	0.773	81
Mean carotid intima-media thickness (*μm*)				
Predictors	*β-coefficient*	*CI*	*p*	*N*
Age*, Sex, SEIFA scores	1.05	-2.15 – 4.26	0.516	100
Age, Sex*, SEIFA scores	34	10 – 58	**0.006**	100
+ 5 kg/m^2 BMI	5	-6 – 15	0.374	100
+ 10 mmHg systolic BP	0.7	-5 – 6	0.798	94
+ 50 μmol/l GlycA	3	-5 – 12	0.449	64
+ 0.1 mmol LDL-C	1	-3 – 6	0.573	64
+ 0.1 mmol glucose	1	-2 – 4	0.370	81

*Linear regression derived coefficients for total effects from cardiovascular risk factors on pulse wave velocity (in m/s), elasticity (in %/10mmHg) and cIMT (in μm). Initial model adjusted for age, sex and SEIFA scores. * indicates the coefficient illustrated. The coefficient for SEIFA scores was not significant in any model. Subsequent*

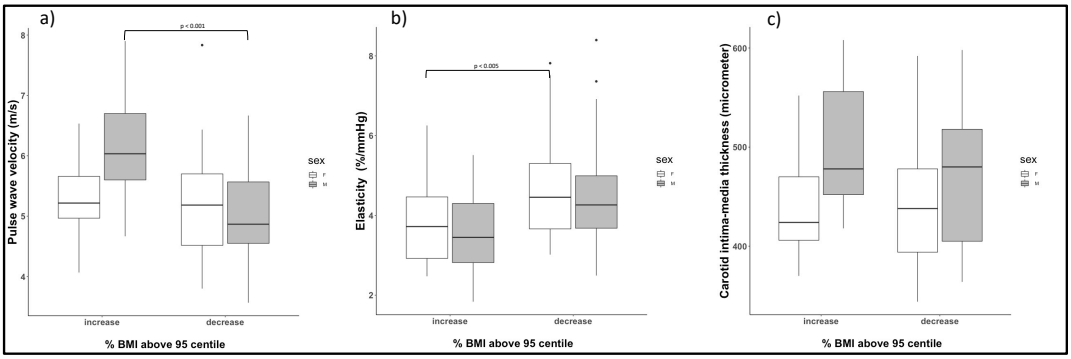
models include each specific cardiovascular risk factor separately. Coefficients are given for a per unit increase as indicated: For BMI: body-mass index in 5 kg/m²; for systolic BP: systolic blood pressure in 10 mmHg; for GlycA: glycoprotein acetyls in 50 µmol/l; for LDL-C: low-density lipoprotein cholesterol in 0.1 mmol/l; for Glucose: fasting glucose in 0.1 mmol/l.

5.5.4 Associations between changes in the severity of obesity over time with subclinical cardiovascular phenotypes

Figure 20 shows associations between changes in the percentage level above the 95th BMI-centile over time with subclinical cardiovascular phenotypes. The exposure variable was dichotomized into i) individuals who maintained or decreased their %>95th BMI centile and ii) individuals who increased their %>95th BMI centile over time. Using this approach, 62 participants maintained or decreased, whereas 37 participants increased their severity of adiposity over time.

Decreasing the %>95th BMI-centile from visit 1 (COBRA enrolment) to visit 2 (COBRA-CVR visit) was associated with a lower PWV in males (-0.750m/s [95%CI -1.137 to -0.363, p-value <0.001]), but there was no evidence for an association in females (0.014m/s [95%CI -0.408 to 0.435, p-value 0.95]). In corresponding regression models for carotid elasticity, decreasing the %>95th BMI-centile was associated with higher carotid elasticity in females (0.945%/10mmHg [95%CI 0.365 to 1.525, p-value < 0.005]), but there was no strong evidence for an association in males (0.429%/10mmHg [95%CI -0.214 to 1.073, p-value = 0.186]). There was no association between increasing or decreasing %>95th BMI centile with cIMT in either sex (see figure 20).

Figure 20. Association between changes in the %>95th BMI-centile from visit 1 to visit 2 and subclinical cardiovascular phenotypes, stratified by sex

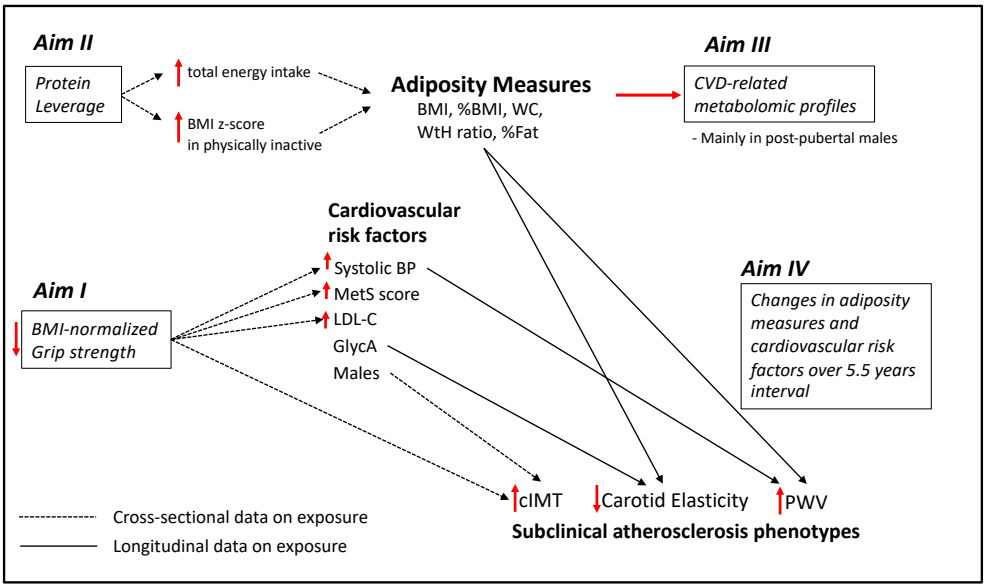


Boxplots for participants with increasing (left) versus decreasing (right) %>95th BMI-centile between visit 1 and 2, stratified for sex (females white, males grey). P-values are shown for the estimate of the association of the change in the %>95th BMI-centile between visit 1 and visit 2 (dichotomized as increasing or decreasing), adjusted for SEIFA scores at visit 2 and age at both visits.

6 Discussion

This study provides evidence that macronutrient composition is related to higher total energy intake in children and adolescents with obesity. Also, higher levels of obesity measures and lower levels of BMI-normalized grip strength were shown to associate with obesity-related adverse cardiometabolic outcomes and adverse subclinical cardiovascular phenotypes. An overview of the main findings is illustrated in figure 21. The main finding from aim I was that lower normalized grip strength identifies youth with adverse cardiometabolic risk factors and increased cIMT. The study related to aim II showed that protein leverage increases total energy intake in youth with obesity and contributes to increasing BMI levels in a physically inactive subgroup. The study for aim III showed that increasing adiposity measures, particularly in post-pubertal males, associate with adverse metabolomic profiles that are linked with CVD manifestation in adulthood. The main findings from aim IV were that changes in adiposity measures over a mean interval of 5.5 years between baseline (mean age 11.2 years) and follow-up (mean age 15.7 years) were associated with adverse arterial stiffness measures at follow-up. Additionally, changes in SBP was associated with increased PWV and changes in GlycA were inversely related to carotid elasticity. Male sex was the only cardiovascular risk factor associated with higher cIMT measures.

Figure 21. Overview of the main study results



Overview illustrating the main results of aims I to IV. Red arrows indicate the direction of associations from exposures towards outcomes. Abbreviations: BMI: body mass index; %BMI: percentage level above the 95th BMI centile; WC: waist circumference; WtH ratio: waist to height ratio; %Fat: % total body and truncal fat; BP: blood pressure; CVD: cardiovascular disease; CVR: cardiovascular disease; BP: blood pressure; MetS: Continuous metabolic syndrome score; LDL-C: Low-density lipoprotein cholesterol; GlycA; glycoprotein acetyls; cIMT; carotid intima-media thickness; PWV: pulse wave velocity.

6.1 Participants

The participants for the studies included in this thesis were from the Childhood Overweight BioRepository of Australia (COBRA), a prospective cohort study established in 2009 that included 438 children and adolescents with overweight and obesity (Sabin et al.,2010). Three studies (aims I, II and III) used a cross-sectionally study design, whereas aim IV included 101 participants from COBRA who were re-measured an average of 5.5 years later as part of the longitudinal study arm (COBRA-CVR).

For COBRA, all patients referred to the Weight Management Service, the largest tertiary-hospital based Weight Management Service across Australia (Spilchak et al.,2008) were approached to participate. COBRA was the first prospective cohort with children and adolescents with obesity to establish a biorepository including a comprehensive assessment of environmental and anthropometric measures, clinical data and blood samples that were "biobanked" for future batch analysis. Environmental measures were mainly assessed through self-completed, preferably validated and standardised questionnaires. Anthropometric measures (including weight, height, WC) were assessed by specialized nursing stuff using the same equipment to limit measurement error (Carsley et al.,2019).

The number of individuals included for the specific aims were mostly dependent on the availability of data and the eligibility criteria. The next section will briefly discuss factors that are to be considered to evaluate the representativeness of study populations and to evaluate the generalisability of study results, followed by sections to assess the comparability of the aim-specific subpopulations.

6.1.1 Representativeness and generalisability of the study participants

Depending on the research question, the representativeness of the cohort investigated is paramount to extrapolate the findings to individuals that have not been part of the study. In contrast, the generalisability of the study results is more a matter of statistical inference which in turn is a matter of available data to account for potential confounding (Richiardi et al.,2013).

Some factors related to the representativeness of the COBRA participants could not have been influenced by the study coordinators. First, the resources for weight management services for children and adolescents with obesity are limited despite the high prevalence of overweight and obesity in youth. Second, the rates of health-carers to refer a child with overweight or obesity to a weight management service vary considerably depending upon factors such as their sex, their specialty occupation, the socioeconomic status of the patients they care for and whether the referrer had an affiliation with the hospital or not (Imoisili et al.,2018). Third, once referred, the attendance rates of children and adolescents with obesity at weight management services are notoriously low due to factors such as the distance of the individual`s home from the treatment site, lower gross family income and youth self-report of depressive symptoms (Jensen et al.,2012).

Moreover, the eligibility criteria for the studies presented here were consistent, having a BMI at COBRA enrolment that classifies the individual as being obese and having an age of 6 years or over, with no other criteria that could introduce selection bias. Also, the COBRA biorepository collected comprehensive data that allowed adjustment for the main confounding factors. Having such a comprehensive dataset that included potential confounders is particularly important to evaluate the generalisability of the results. If the specific confounders to a relevant research question in a particular population are known, inference can be drawn and results may be generalisable for a sample size that is large enough to provide adequate power (Richiardi et al.,2013).

The next two sections discuss the participant`s comparability between the subpopulations investigated for the specific aims and the COBRA cohort from where the participants were selected.

6.1.2 Comparability between aim-specific characteristics with the COBRA cohort

Participant characteristics for aims II and III are listed in table 8. For aim II, investigating the protein leverage hypothesis, the main characteristics for age, sex and BMI did not show significant differences in their distribution between the participants for aim II and the COBRA cohort. Participants for aim III were slightly older (11.9 years versus 11.1 years, p-value 0.006) and had a slightly higher BMI value (34.5 kg/m^2 versus 32.6 kg/m^2, p-value 0.001) compared to the COBRA cohort without difference in the sex distribution. However, approximately 1 kg/m^2 can be explained by the difference in age, and the remaining difference (<1 kg/m^2) is considerably small to have major impact on the generalisability for the results amongst children and adolescents with obesity.

6.1.3 Comparability between participants for aim IV with the COBRA cohort

COBRA-CVR was designed as a longitudinal follow-up of the initial COBRA cohort that aimed to determine how changes in adiposity measures associated with subclinical cardiovascular phenotypes. A sample size estimation for aim IV found that a minimum sample size of 84 participants was needed to provide adequate statistical power to detect the expected differences in PWV.

As a principle, for conclusions to be valid for the original COBRA cohort, the loss to follow-up percentage shall be as low as possible to reduce selection bias (Howe et al.,2016). From computational modelling, a loss to follow-up for up to 60% did not result in bias (Kristman et al.,2004). In this study, from a total of 438 COBRA participants, 30 participants were excluded due to a BMI <95th BMI-centile or age below 6 years, resulting in 408 individuals suitable for recruitment. Of those, 96 (24 %) had outdated contact details (phone number disconnected or changed owner), 74 (18%) were excluded after three failed contact attempts, 115 (28%) declined to participate, 22 (5%) had relocated interstate and 101 (25%) agreed and consented to participate. Outdated contact details (24%), failed attempts (28%) and relocation (5%) are unlikely related to the exposures and outcomes of aim IV.

The main characteristics and cardiovascular risk factors in COBRA and the COBRA-CVR cohort are listed in tables 9 and 10. Based on the observational interval of 5.5 years from early to late adolescence, adiposity measures such as weight, BMI and WC increased over time. Also, 75% of the individuals participating in COBRA-CVR had completed puberty compared to 24% who had reached this level of maturity at baseline. However, the percentages of total body fat and truncal fat, and the severity of obesity (assessed by the percentage > 95th BMI-centile) remained comparable for individuals at 131.5% (SD 26.4) at baseline versus 135.3% (SD19.2) at follow-up. Similar to BMI and pubertal stage, SBP significantly increased from a mean SBP of 109 mmHg at baseline to a mean SBP of 126 mmHg at follow-up, a difference that is consistent with age-related changes (Flynn et al.,2017). Also, changes in LDL-C levels from a mean level of 2.54 mmol/l at baseline to a mean level of 1.76 at follow-up, most likely reflect puberty-related decreases (Elkins et al.,2019).

To conclude, neither the eligibility criteria nor the exposure characteristics between COBRA and COBRA-CVR seem to have introduced substantial bias beyond age-related effects. Therefore, the participants for each aim can be considered representative and – within the limits of the eligibility criteria – the results are generalisable for children and adolescents with obesity.

6.2 Methods

The assessment of exposure and outcome variables including demographic and anthropometric measures, nutritional data and grip strength, cardiovascular risk factors and subclinical cardiovascular phenotypes followed internationally standardised protocols, were derived from questionnaires that have undergone assessments for reproducibility and validity and - for data derived from the COBRA-CVR cohort - were performed by one examiner. A few aim-specific methods are discussed in the following sections.

6.2.1 Validity and reliability of grip strength measures

Grip strength data were assessed by a single examiner using a dynamometer with the highest criterion validity and reliability in adolescent populations (Espana-Romero et al.,2010). The assessment followed a highly standardized protocol that accounted for grip span (CDC,2011). Raw measures were adjusted for body weight due to reported weight-related influence (Aasa et al.,2003; Milliken et al.,2008) and subsequently categorized into low, moderate and high grip strength according to previously published normalized values (Peterson et al.,2016).

6.2.2 Validity and reliability of energy intake and energy expenditure data

Total energy intake was calculated using the Australian Child and Adolescent Eating Survey Food-frequency questionnaire (ACAES-FFQ) (Watson et al.,2009) which has undergone comprehensive evaluation for validity and reproducibility. Reproducibility and comparative validity for this FFQ have previously been established (Watson et al.,2009). In contrast to 24-hour recall or food records, FFQ are less time-consuming and do not require trained interviewers, meaning they are efficient and practical for use in epidemiologic studies (Watson et al.,2009). To address the problem of under-reporting of total energy intake those reporting a total energy intake below a Goldberg cut-off of 1.2 were excluded (see section 2.5.4.1). Although this reduced the number of participants for analysis, it increased the validity of the data.

Accelerometry data to assess physical activity was available from 57 individuals for aim II. The accelerometer was worn at the hip due to reported higher accuracy compared to devices worn at the ankle (Lynch et al.,2019). A period of one week was chosen and acceptability of data was evaluated based on published guidelines (Colley et al.,2010). Accelerometery-derived physical activity intensity was categorized according to published intensity cut-off points (Colley et al.,2011).

6.2.3 Validity and reliability of metabolome data

Reproducibility analysis for metabolomic results based on an NMR spectroscopy derived metabolomic platform used for this study revealed excellent results for both fasting and postprandial metabolite concentrations. The mean intra-class coefficients to assess variability were 0.99 (Li-Gao et al.,2019). In addition, blood samples were consistently collected in a fasting state, immediately processed and stored at the local biobanking facility within 2 hours of sample collection, to minimize pre-analytical errors.

Due to lower measure for NMR-derived LDL-C, HDL-C and total cholesterol levels compared to a standard lipid profile analyzed by the Royal Children`s Hospital pathology, 139 pairs of NMR and standard laboratory LDL-C levels were compared, available from analysis performed in COBRA participants using the Bland-Altmann method (Bland et al.,1986b). The mean difference and limits of agreement between the Nightingale LDL-C and standard laboratory LDL-C was -1.1mmol/l [95%CI -1.9 to -0.3mmol/l] with no changes to this difference throughout the range. Whereas this difference does not cause an error when included in statistical models, a regression model was used to calculated LDL-C levels for comparability to standard laboratory LDL-C levels.

6.2.4 Validity, reliability and accuracy of subclinical cardiovascular phenotypes

Risk factors for, and predictive abilities from, subclinical cardiovascular phenotypes are outlined in section 2.8. Available data on the validity, reliability and accuracy of the vascular phenotypes used in this study are summarized subsequently. The assessment of non-invasive subclinical cardiovascular phenotypes is safe and well-tolerated in children (Urbina et al.,2009a). The expertise at the study site to collect and analyze data on cIMT and carotid elasticity via ultrasound as well as PWVis considerable with more than 3500 infants, children and adults assessed. Standardized protocols have been developed and are in accordance with internationally published guidelines. This expertise is particularly important as it allowed reproducible measurements using standardized methods, dedicated software, and consideration in the statistical models for important covariates.

6.2.4.1 Carotid intima-media thickness

The assessment of cIMT is highly used and validated in adulthood and childhood (Burke et al.,1995; Zhao et al.,2019) with available reference data published (Doyon et al.,2013). If the same methodology is applied, excellent reproducibility has been reported in youth (Selamet Tierney et al.,2015). Reliability for mean cIMT measures was assessed for inter- and intra-rater reliability, with resulting intraclass correlations of 0.64 [95%CI 0.54-0.74] and 0.71 [95%CI 0.63-0.78]. Similar,inter-rater reliability, assessed by intraclass correlation obtained for a random sample of 30 de-identified cine loops for this study was 0.91 [95%CI 0.82-0.96]. Also, for this study, the Bland-Altman method was used (Bland et al.,1986b). The mean difference between two raters using was 14 μm and the 95% limits of agreement were -50 to 30 μm.

6.2.4.2 Carotid elasticity

Results for carotid elasticity are based on the same ultrasound assessment used for cIMT and are validated for children and adults (Gardner et al.,2010) with reference data reported (Doyon et al.,2013). Similar to cIMT, the use of software for edge-detection has increased the reproducibility of results (Selzer et al.,2001).

6.2.4.3 Pulse wave velocity

Pulse wave velocity (PWV) is a highly reproducible, non-invasive measure of arterial stiffness (Wilkinson et al.,1998). It is a well-tolerated measure in children and adolescents as the maximum brachial cuff only reaches systolic pressure to produce the central aortic pressure waveform.

6.3 Results

Results will be discussed in order of the main study aims:

Aim I) Associations between grip strength and cardiometabolic risk

This is the first study to relate hand grip strength in children and adolescents with obesity to cardiometabolic risk factors and subclinical cardiovascular phenotypes. Low normalized grip strength was shown to identify obese youth who have increased cardiometabolic risk. Youth with low normalized grip strength had higher systolic blood pressure (+13 mmHg), LDL-C (+0.26 mmol/l), MetS score, and cIMT (+50 μm), compared with those with moderate or high grip strength. These findings are important, because systolic blood pressure and LDL-C in youth are shown to independently predict coronary artery calcification in adulthood (Hartiala et al.,2012). In addition, youth with low normalized grip strength had higher MetS score, which has previously been associated with increased risk for future type 2 diabetes (Magnussen et al.,2010).

Previously, Melo et al. (Melo et al.,2015) demonstrated that grip strength was associated with cIMT in healthy weight children aged 11-12 years. This finding was extended by showing an inverse association of normalized grip strength and cIMT in a cohort of youth aged 8-19 years with severe obesity. The difference in cIMT between low and moderate/high normalized grip strength groups in the present study was +50 μm. This difference persisted after adjustment for BMI z-score, systolic blood pressure, and LDL-C, suggesting that differences in these factors are not on the pathway linking normalized grip strength to cIMT. Previously, in the Cardiovascular Risk in Young Finns Study, cIMT was shown to increase 5.7±0.4 μm/y in young adults (Juonala et al.,2008). Using the vascular age concept (Stein et al.,2004), the difference observed here means that participants with low normalized grip strength were almost 9 years older in terms of vascular age than those with moderate/high normalized grip strength.

In adults, poor grip strength and its decrease over time has been related to, and is predictive for, many health conditions, for an increased cause-specific mortality for CVD, respiratory diseases, various cancers, and all-cause mortality (Celis-Morales et al.,2018; Prasitsiriphon et al.,2018). Grip strength has also been shown to improve the prediction utility of a standard office-based risk score (Celis-Morales et al.,2018). Associations between hand grip strength with all-cause and CVD-specific mortality were even stronger than that of SBP or total physical activity, and the strength of associations with incidence of CVD was comparable to that for systolic blood pressure and stronger than that for physical activity (Roberts et al.,2011).

The physiologic mechanism behind these associations are not completely understood. Grip strength is a good marker for overall muscular strength (Bohannon et al.,2012), for nutritional status (Norman et al.,2011), for healthy patterns of eating including the intake of antioxidants, fish-oil and grams of protein (Robinson et al.,2008) and for physical activity and lower sedentary time (Hamer et al.,2013; Rantanen et al.,1997). Therefore, the advantage of grip strength may be based on unifying various information in one simple measure that is easy, cheap and reproducible in clinical practice compared to other measures of physical fitness (Roberts et al.,2011).

Aim II) To test the protein leverage hypothesis in children and adolescents with obesity

This cross-sectional study analysed macronutrient food composition data derived from FFQs in 137 children and adolescents with obesity, aged 6-18 years. The findings were that with decreasing percentages of total energy intake from protein sources, total daily energy intake increased, consistent with the mechanism of protein leverage.

In a physically inactive subgroup, lower percentage energy from protein sources remained the only macronutrient to increase total energy intake. Also, in the physically inactive subgroup, a diet high in protein and moderate in carbohydrate contents was associated with lower BMI z-score, whereas a diet moderate in protein and low in carbohydrates was associated with higher BMI z-scores. This is the first study to provide evidence for protein leverage in youth with obesity.

Evidence for the mechanism of protein leverage was found with an observed strength of leverage L = -0.48, indicating partial protein leverage that is comparable to available studies from adults (Raubenheimer et al.,2019). Also, results showed a positive association between TEI and BMI z-scores. However, macronutrient analysis was associated with BMI z-scores only in a physically inactive subgroup, but not in the whole cohort. Several factors may explain this. First, no information on dietary non-macronutrient composition was available, particularly on dietary fibre, which has shown to attenuate PL-effects on TEI and obesity in mice (Raubenheimer et al.,2019). Second, normative BMI centiles are steadily, in a near-linear manner, trending upwards from about 6 years of life throughout childhood and so too does the difference between the 50th and the 95th centile increase (the latter representing the threshold to determine obesity in childhood) (Kuczmarski et al.,2000). Hence, for protein leverage in this age group to be associated with increasing BMI z-scores, the effect size must exceed the one from adulthood, where overweight and obesity are determined by static thresholds (i.e. BMI 25kg/m^2 and 30kg/m^2). Third, protein targets vary through life and particularly throughout the childhood age-range, influenced by, amongst other factors, age, early nutritional experience and physical activity (Raubenheimer et al.,2019). Limited data collected for physical activity only allowed the mechanism of protein leverage and the protein leverage hypothesis to be tested in a physically inactive subgroup, where an effect of protein leverage on total energy intake and of dietary macronutrients (protein and carbohydrate) on BMI z-scores was found. Hence, the results suggest that the mechanism of protein leverage is necessary, but not sufficient for protein leverage to cause obesity (Raubenheimer et al.,2019).

Available data on total protein intake and the relation to later adiposity measures suggest that a higher protein intake in infancy is associated with higher adiposity at age 5 (Scaglioni et al.,2000) and 6 years (Gunnarsdottir et al.,2003). A European multicentre, double-blind, randomised clinical trial (The Childhood Obesity Project – CHOP-study) randomly assigned healthy infants to receive a high versus lower protein-content formula, differing in an average intake of 6-8 gr protein per day during the first year of life. At age 6 years, the high-protein formula fed children had higher BMI (0.51; [95%CI 0.13 - 0.90]) with an OR of 2.43 [95% CI 1.12-5.27] for obesity compared to the low-protein formula fed children (Weber et al.,2014b). Two mechanisms, one in agreement with the protein leverage hypothesis (Raubenheimer et al.,2015b) may explain this. If the protein intake target during infancy was set higher than normal due to habitually higher protein intakes, protein leverage may drive an increased total energy intake later in life to achieve this higher protein set point, therefore contributing to obesity (Raubenheimer et al.,2019). The other mechanism, termed 'the early protein hypothesis', assumes that higher protein intake in infancy triggers elevated levels of insulin-like growth factor 1 (IGF-1) and insulin (Socha et al.,2011) and therefore stimulates early weight gain. Studies in later childhood and early adolescence that investigate the effects of higher intake of protein on later body mass index and cardiometabolic risk factors (including hypertension, dyslipidaemia and insulin resistance) found limited and inconclusive results for adverse effects (Gow et al.,2014; Voortman et al.,2015). However, none of the aforementioned studies have used the method of compositional data analysis to investigate the effect of proportional intake of macronutrients on outcomes of interest in contrast to studies in adults.

In adulthood, population-based studies investigating macronutrient composition trends over the last four decades in the US (Hall,2019) have shown a decrease in the percentage intake of energy from protein sources accompanied by an increase in the overall intake of total energy, correlating with increasing trends in BMI. A recent NHANES analysis from data between 2009-2010 identified the most likely causative food product for this trend as being ultra-processed foods (UPF). UPF is a group of food products including soft drinks, industrialized desserts, reconstituted meat products, and "ready to consume" products, representing almost 60% of all energy intake in the US diet but containing just around 9.5% energy from protein sources (Martinez Steele et al.,2018). On a population level, the mechanism of the PLH may well contribute to an overall obesity epidemic as recently published according to a study investigating data from the US food supply since 1973 (Hall,2019).

Aim III) to investigate the relation between clinically assessed adiposity measures and a metabolomic platform with cardiometabolic interests in sera of children and adolescents with obesity, related to sex and pubertal development

This retrospective cohort study analysed the relation between adiposity measures and a NMR-spectroscopy derived metabolomic profile with cardiometabolic interest in 214 children and adolescents with obesity, aged 6-18 years. Findings were that an increase in a range of adiposity measures (BMI, BMI z-score, whole body and truncal fat percentage, WC and WtH) was associated with changes in the concentration of several metabolites and lipid subclass size. The strongest evidence for associations was observed in post-pubertal males.

A higher BMI z-score was positively associated with elevated concentrations for phenylalanine and negatively associated to log acetate after correction for FDR. Changes in lipid content in very large HDL lipoproteins, the estimated degree of unsaturation, the ratio of 22:6 docosahexaenoic acid and omega-3 fatty acids to total fatty acids were all negatively associated with BMI z-score in linear regression modelling, but these associations did not remain significant after adjustment for FDR.

In post-pubertal males, associations between increasing BMI z-score with elevated levels of triglyceride- and cholesterol-carrying VLDL and LDL particles, lower levels of larger HDL particles, increased levels of fatty acids, higher levels of BCAA (i.e. leucine, isoleucine and valine) and aromatic amino acids (AAA, e.g. phenylalanine, tyrosine, histidine) and elevated levels for a marker of chronic inflammation - glycoprotein acetyls - were found.

Several of those metabolites are critically involved in the process of atherosclerosis: i) elevated levels of VLDL lipoproteins, their cholesterol- and triglyceride-content and the ratio of apolipoprotein B/AI are key initial steps of atherosclerosis, from endothelial penetration and subendothelial retention of modified lipoprotein particles to the initiation of an inflammatory cascade (Back et al.,2019); ii) decreased ratios of docosahexaenoic acid/total fatty acids and omega-6 fatty acids/total fatty acids have recently been related to a decreased inhibition of the NLRP3 inflammasome, a core inflammatory pathway involved in atherosclerosis (Lopategi et al.,2019); iii) decreasing levels of larger and increasing levels of small HDL particles. Recent studies have shown that NMR spectroscopy based differentiation of HDL particles revealed superior cardiovascular risk assessment as compared to the classical HDL-C concentration (Santos-Gallego,2015). And iv), GlycA is a measure for chronic inflammation. Chronic inflammation is a major mechanism underlying obesity-related comorbidities including increased risk for cardiovascular (MI and intracranial haemorrhage) and all-cause mortality (Akinkuolie et al.,2014; Lawler et al.,2016; Wurtz et al.,2015), and for incident CVD (MI, ischaemic stroke, coronary revascularization, and CVD death) in initially healthy women followed up for 17.2 years (Akinkuolie et al.,2014). Also, weight loss over 12 months after bariatric surgery (Roux-en-Y gastric bypass or sleeve gastrectomy) was associated with a significant reduction in GlycA (Manmadhan et al.,2019). In this study, GlycA showed a positive trend with increasing BMI, total and truncal body fat percentages, although not after FDR correction. Association between higher BMI, BMI z-score or waist to height ratio with GlycA were primarily found amongst post-pubertal males.

Metabolomic patterns and CVD risk

Changes in metabolomic profiles are suggested to lay on the causal pathway, located between a risk factor for CVD and the manifestation of CVD, thereby offering an opportunity for early risk stratification. Evidence for this is based on two studies. The first, a longitudinal study over 6 years in adults, used Mendelian randomisation to show casual effects from increasing adiposity on multiple metabolites including elevated levels of triglyceride- and cholesterol-carrying VLDL and LDL particles, lower levels of larger HDL particles, increased levels of fatty acids, higher levels of BCAA and AAA and elevated levels for a marker of chronic inflammation - glycoprotein acetyls (Wurtz et al.,2014). The second study used data from the China Kadoorie Biobank, a cohort of more than half a million Chinese adults aged 30-79 years (Holmes et al.,2018). The authors related a similar metabolomic platform to CVD outcomes including MI and IS. Positive associations with MI and IS were found for a 1-SD increase in VLDL and LDL lipoprotein particles, mean VLDL diameter, cholesterol in VLDL particles, the ratio of apolipoprotein B / apolipoprotein A1, total fatty acids, omega-3 and omega-6 fatty acids, mono and polyunsaturated fatty acids, saturated fatty acids and GlycA. Similar, triglycerides levels in all lipoprotein subclasses containing apolipoprotein B were positively associated with MI and triglycerides in VLDL lipoproteins, isoleucine and leucine were positively associated with IS. Negative OR with MI and IS were found for a 1-SD increase in HDL particle size, and solely for MI from cholesterol in HDL2 (larger HDL particles) (Holmes et al.,2018). Many associations found in the China Kadoorie Biobank study were observed with increasing BMI z-score in post-pubertal males in this study.

Metabolomic patterns and T2DM risk

A study using an untargeted metabolomic approach in a paediatric cohort (Viva La Familia Study, n=803, 56% with obesity, mean age 11y), reported positive associations between increasing weight and circulating branched chain amino acids and insulin resistance (Butte et al.,2015). In adults, higher BCAA and AAA (including phenylalanine and tyrosine) have been positively associated with insulin resistance and metabolic syndrome (Wiklund et al.,2014; Wurtz et al.,2012). In the whole cohort, associations between BMI and BMI z-score with phenylalanine, BMI and waist circumference with tyrosine and a negative association between total body fat percentages and histidine were found. In females, negative associations for histidine and positive associations for tyrosine and phenylalanine that attenuated towards the null following FDR correction were found. In post-pubertal males, associations between increasing BMI z-score and leucine and isoleucine were significant, after adjustment for FDR.

Aim IV) to investigate the effect of adiposity measures and cardiovascular risk factors over time on a comprehensive, non-invasive subclinical cardiovascular phenotype assessment in children and adolescents with obesity

This prospective study of 101 children and adolescents with severe obesity followed over 5.5 years, found that age and changes in most adiposity measures were associated with arterial stiffness. Of the cardiovascular risk factors, changes in GlycA were inversely associated with carotid elasticity; second, increased systolic blood pressure was positively associated with faster PWV; and third, male sex was the only factor associated with higher cIMT. A maintenance or decrease in the severity of obesity over 5.5 years during adolescence was associated with improved measures of carotid elasticity in females, and reduced PWV results in males.

Arterial stiffness measures for early detection of functional and structural abnormalities
These prospective results support previous associations between age, male sex, cardiovascular risk factors and increased PWV (Urbina et al.,2010). In contrast, the largest study investigating over 6500 children aged 10-11 years, showed a lower brachial PWV in children with overweight or obesity compared to leaner individuals (Charakida et al.,2012). However, that study investigated younger children and the BMI range was limited to 15.7-18.8 kg/m^2, whereas in the current study participants were older (aged 6-25 years) with a higher BMI range 18.2 to 60.9 kg/m^2. Also, brachial PWV may not be comparable to carotid femoral PWV as the structural component (elastin) varies with the arterial segment. Moreover, when a subgroup of adolescents (n=28) was followed up at ages 14 and 19 years, brachial PWV increased in individuals with overweight and obesity compared to lean controls, despite similar baseline values for PWV (Dangardt et al.,2013), suggesting that a minimum required age and duration of exposure to an elevated BMI may be needed to detect increased arterial stiffness measures in children with obesity.

The finding for an inverse effect of increasing weight and reduced carotid elasticity are consistent with a study in children with obesity with lower carotid elasticity compared to those with healthy weight (Tounian et al.,2001). In addition, obesity in childhood is predictive for decreased elasticity in adulthood 21-years later (Juonala et al.,2005). The findings of lower carotid elasticity with increasing adiposity measures amongst youth with severe obesity are consistent with, and extend, these results. The present study is the first to measure GlycA – a composite measure indicative for chronic inflammation (Connelly et al.,2017) – in relation to subclinical cardiovascular phenotypes in youth with severe obesity. In adulthood, GlycA has been associated with future CVD and mortality (Akinkuolie et al.,2014; Kettunen et al.,2018) and is suggested to be a better predictor for the identification and stratification of individuals at risk for future CVD than acute phase proteins, such as high sensitivity C-reactive protein (Connelly et al.,2017). However, there are few studies comparing GlycA and established inflammatory markers in childhood.

Arterial structure - carotid intima-media thickness in adolescence
Studies in the International Childhood Cardiovascular Cohort Consortium have shown improvements in cardiovascular risk factor profiles and cIMT for children and adolescents who were overweight or obese (mean age 11.4 years) who were non-obese adults (mean age 31years) (Juonala et al.,2011). In the current study of children and adolescents with severe obesity, male sex was the only factor associated with increased cIMT. No association with cIMT was found for those who decreased or increased their %>95th BMI-centile over a mean follow-up time of 5.5 years, when assessed at a mean age of 15.7 years. This may be because the appearance of fatty streaks in the carotid artery (in pathology studies) occurs after the second decade of life (Kawauchi,1965). Also, the exposure and the severity of concomitant cardiovascular risk factors may not have been of sufficient duration to manifest as differences in cIMT in this young cohort (Ayer et al.,2015; Park et al.,2015). Moreover, the time-period investigated in this cohort includes pubertal transition, when the average increase in sitting height (an approximate for the increase in the thoracic arteries length) is ~10 cm (Fredriks et al.,2005). This

increase in the longitudinal axis of an elastic artery may limit the detection of changes in cIMT, however this remains hypothetical. In line with the latter argument, including pubertal stage in the analysis had no effect in this study.

Associations for increasing versus decreasing and maintaining the severity of obesity over time
In this population of severely obese children and adolescents, an improvement in the %>95th BMI-centile over a relatively short period was associated with better PWV in males and carotid elasticity in females, even if normal weight was not achieved. As data from a systematic review in adults suggest that an increase in PWV of 1 m/s results in a 14-15% increased risk of cardiovascular events, cardiovascular mortality and all-cause mortality (Vlachopoulos et al.,2010), the results observed in the present study suggest that the 0.75 m/s lower PWV at follow-up for those who reduced or maintained their %>95th BMI centile is likely to have a meaningful impact on subsequent CVD risk.

6.4 Strengths and limitations

Strengths of this study are the clear focus on children and adolescents with obesity. This is a vulnerable population with still growing trends in prevalence in preschool children and countries with lower socioeconomic status. A particular strength was the collection of extensive exposure data including several measures of anthropometry, key cardiovascular risk factors, quantitative data on physical activity and detailed information on nutrition for the COBRA cohort and follow-up anthropometry, demography, and cardiovascular risk factor measures as well as hand grip strength and subclinical cardiovascular phenotypes added for the COBRA-CVR cohort. This enabled the investigation of associations from various exposures, and allowed for the adjustment of various confounders, which is an important determinant for the generalizability of study findings. Also, the longitudinal study design for aim IV provides a higher level of evidence as exposure data were assessed over time and hence, allow to assess effects from changes of exposures over time on the subclinical cardiovascular phenotypes.

Limitations of this study include the cross-sectional assessments of exposures and outcomes, which does only provide associational level of evidence. However, the tools and measures used for aims I, II and III were novel in their application in youth with obesity and adjusted for available confounders. Hence, the resulting findings provide the background to generate new hypotheses that warrant testing in larger cohorts over a longer period. Also, this study had no data available on a control group with a weight category in the normal range, which limits the extrapolation of our findings to individuals with normal-weight BMI values. However, the aims were focussed on a population of youth with obesity, which is at higher risk for adverse cardiometabolic outcomes. Another limitation, albeit inevitable in clinical cohort studies, was the low number of participants with a complete dataset for all variables of interest. Similar, the availability of blood samples in research with children was a limiting factor. Aim-specific eligibility criteria have sanctioned the number of participants further. However, they also increased the validity of the findings. This study was also not designed to provide the physiologic background to explain the findings, e.g. what is the physiology behind the association between hand grip strength and adverse cardiometabolic outcomes, or why are males more susceptible for early, adverse vascular changes. Finally, this study used subclinical cardiovascular phenotypes that represent intermediate outcomes on the pathway of arteriosclerosis towards manifest clinical CVD. Nevertheless, they are validated, reproducible, well-tolerated and valuable for CVD risk prediction. The manifestation of CVD is a process lasting decades, warranting the use of well-validated intermediate outcomes when seeking methods for early risk-stratification.

6.5 Implications for clinicians and policy makers

The main outcome of this study is that children and adolescents with obesity represent a vulnerable group, with manifestation of adverse cardiometabolic risk factors and adverse vascular changes already during adolescence. Effective treatment options in youth are limited, and the persistence of obesity and obesity-related cardiometabolic risk factors into adulthood highlights the importance of primary and secondary prevention.

Obesity in childhood is a multifactorial health condition that warrants a multidisciplinary approach to prevent primarily the development of obesity and secondarily prohibit obesity-related comorbidities. Policy makers, state and local organizations, school, childcare, healthcare professionals, families and individuals must work together to create an environment that supports primary and secondary prevention of this pandemic. The majority of overweight and obesity in childhood is caused by an obesogenic environment that is modifiable. Research on identifying determinants for an obesogenic environment and markers or methods that allow risk stratification for the child with obesity provide the tools for primary and secondary prevention. And often, the nature of such determinants will inform the relevant responsible discipline or individuals to adopt measures to make a change.

Findings from this study provide evidence that increasing physical fitness and consuming a diet balanced in macronutrients may provide an opportunity to reduce the severity of adiposity and to identify youth with obesity who are at higher risk for severe cardiometabolic consequences. Whereas the individual`s family and the treating physician is usually thought the first-line responsible to encourage physical activity and a healthy, well-balanced nutrition, such individual approaches are unlikely to be effective for the whole population. Therefore, higher levels of responsibility are clearly needed to address these problems. These include: i) governmental structures that prioritize childhood obesity as a major public health problem and provide adequate funding to combat and prevent obesity in childhood; and ii) governmental laws, regulations and taxes that influence the food environment, e.g. with restrictions to the marketing of ultra-processed foods, and children-targeted advertising of unhealthy, calorie-dense foods. Similar, governmental policies and regulations that facilitate and promote physical activity from early childhood on, e.g. in childcare, schools and workplaces later in adulthood.

Several findings from this study have implications for paediatric primary health carers, specialty physicians, dietitians and physiotherapist providing care for weight management services. Simple tools such as a dynamometer to assess and monitor grip strength as well as counselling on methods to improve physical fitness are likely to have an impact on the cardiometabolic risk profile in children and adolescents with obesity. Similar, rather than focussing on single food items, the study investigating the protein leverage hypothesis provided evidence for prioritizing a balanced diet with a relevant proportion of proteins to reduce total energy intake which will likely reduce the severity of obesity. Both these methods are cheap, do not necessitate higher levels of expertise and could be easily implemented in practise.

Findings from the metabolomic analysis in this study for risk-stratification are promising. For adults, metabolomics showed valuable results to identify those at higher risk for many diseases and mortality at an early timepoint, where preventative measures are likely to have highest benefits. However, well-designed longitudinal studies are needed to assess the true impact of metabolomics. Despite the promising results, metabolomic analysis is intense in their requirement for funds to provide the necessary expertise to collect, analyse and interpret the results.

Implications for the clinician from findings related to aim IV are that irrespective of the adiposity measure assessed, an increase in any of them over a 5.5 years period during adolescence is associated with adverse arterial stiffness outcomes. Also, the findings highlighted the potential importance of assessing BP and markers for chronic inflammation, as changes over time in both of these cardiovascular risk factors were associated with adverse changes in arterial stiffness measures.

Last, our results showed that reducing or even maintaining the severity of obesity (assessed as the percentage level above the 95th centile, i.e. the definition for obesity) over time was associated with beneficial subclinical cardiovascular phenotypes. Translating this into clinical practice, it is important to consider that the 95th centile for an 11-year corresponds to a BMI of ~ 24 kg/m^2, whereas for a 16-year old, this corresponds to a BMI of ~ 29 kg/m^2. As per our study results, the 11-year old with severe obesity who can maintain or increase his BMI by no more than ~5 kg/m^2 between 11 to 16 years is likely to have better arterial stiffness measures.

6.6 Future research perspectives

The findings from the grip strength study provide evidence on a level of association. Available evidence from studies in adults showed that grip strength is strongly associated and highly predictive for a variety of diseases and mortality. Longitudinal studies starting from early childhood are needed to evaluate the predictive ability and the capacity for risk stratification from hand grip strength in youth. Also, studies investigating a series of potentially contributing factors to overall physical fitness are warranted to help explain the mechanistic background of this measure of global fitness. Grip strength is easy to collect with low risk for inter and intra-observer errors and is inexpensive.

Findings from the protein leverage hypothesis warrant confirmation in an extended BMI range including children and adolescents with normal BMI categories. Also, studies investigating the protein leverage mechanism with cardiometabolic risk factors and CVD would be interesting to extend the existing literature to disease risk factors and disease outcomes rather than just to an anthropometric measure. Findings from such studies may provide the background for policy makers to guide the food industry in their production and marketing of foods with unfavourable macronutrient composition. However, such studies warrant an assessment of large quantities of data to allow adjustment for confounders.

Findings from the metabolomics study warrant confirmation in a wider age and BMI spectrum in children and adolescents with long-term follow up data on specific disease outcomes. Such studies are needed to confirm the existing value of metabolomics in risk stratification and risk prediction. Also, once a connection between early adverse metabolomic profiles with specific diseases is established, this may guide health carers towards early, preventive treatments for such diseases.

Findings from the subclinical cardiovascular phenotypes study warrant replication in youth with a BMI range also extending into the normal weight category. This would allow to identify proportional contributing effects from ageing versus BMI. Further, such studies would need to be designed longitudinally in large populations to detect small effect sizes. Further research is also warranted regarding the subclinical cardiovascular risk factors. Whereas it seems plausible from a wide range of observations in nature that form fits function, further large-scale research on vascular phenotypes is necessary to detect if functional changes are preceding the structural changes in large arteries. This would inform on the age-appropriateness of measures for risk stratification and risk prediction. Such studies could also provide evidence as to whether there are sex differences from early adolescence, as observed in this study.

7 Summary and Conclusions

Aim I. The findings related to grip strength suggest that grip strength may be a useful, cheap and efficient method to identify those children and adolescents with obesity with a high risk for a wide range of diseases, particularly for future CVD.

Aim II. The findings related to the macronutrient analysis provide evidence for the mechanism of protein leverage in youth with obesity. They extend the existing literature on effects from proportions of macronutrients on health outcomes in adults to a vulnerable population of youth with obesity. Assessing the proportion of energy from proteins may be a useful tool to help decrease total energy intake, and as a consequence, to support weight management in youth with obesity.

Aim III. The findings related to the investigation of a metabolomic platform with focus on cardiovascular disease provide evidence for an association between various measures for obesity in youth with adverse metabolomic profiles that themselves are associated with increased risk for adverse cardiometabolic outcomes. Particularly, post-pubertal males with increasing adiposity were identified as being at risk for adverse profiles. These findings support the importance of preventing excessive weight gain prior to puberty to avoid metabolomic changes that are likely an intermediate on the pathway to adult health consequences, including CVD.

Aim IV. The findings related to the association of changes in adiposity measures and cardiovascular risk factors over time on a comprehensive set of subclinical cardiovascular phenotypes in children and adolescents with severe obesity were that those individuals who improve or maintain their severity-level of obesity from early to late adolescence had reduced arterial stiffness, even though a normal weight status was not achieved. Changes in adiposity were identified as the main modifiable contributor to arterial stiffness, together with changes in an inflammatory marker and blood pressure. Male youth with severe obesity had poorer functional and structural markers and should be targeted for early CVD prevention.

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