Childhood Obesity and Metabolic Syndrome in Preschool Children

Early markers and identification of individuals at increased risk in a longitudinal perspective

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To my loving girls, you rock my world!

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Early markers and identification of individuals at risk in a longitudinal perspective

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ABSTRACT

BACKGROUND: Overweight and obesity have increased worldwide and affect children at ever younger ages, resulting in cardiovascular disease and type 2 diabetes even in adolescents. This illustrates the importance of identifying children at risk at an early stage.

AIM: The aim of this study was to explore metabolic health in preschool children. The specific aims were (i) to investigate whether 6-year-old children show signs of metabolic syndrome; (ii) to investigate whether the fat distribution in 7-year-old children is associated with their metabolic profile and whether there are any related sex differences; (iii) to study the profile of fatty acids in infancy and their influence on growth; (iv) to study the risk of developing adiposity and an impaired metabolic profile at 7 years of age as a result of early nutrition.

METHODS: This study is based on a longitudinal birth cohort (Halland Health and Growth Study) comprising 480 full-term infants, born at the regional hospital of Halmstad, Sweden, between 2008 and 2011. The children were monitored on regular visits for anthropometrics, biomarkers for growth, and nutrition and food diaries. From 6 years of age, examinations were extended to include fasting insulin, glucose, cholesterol, and blood pressure. At 7 years of age, magnetic resonance imaging (MRI) was performed on a subgroup of 81 children to quantify visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) volumes.

RESULTS: One key measure showed that about one fourth (26%) of the children had one or more risk factors for metabolic syndrome requiring action at 6 years of age. Children with obesity (3%) or overweight (14%) were more

likely to have insulin resistance (28% versus 5%, p <0.001) and higher triglycerides (8% versus 0%, p <0.001) than the normal-weight group. Children with high waist circumference had higher blood pressure than children with normal waist circumference (p <0.05). SAT showed a stronger correlation with metabolic risk factors than VAT, with the exception of triglycerides. Girls in general, showed a stronger correlation between adipose tissue and metabolic risk factors than boys.

Feeding modality, i.e. breastfeeding versus formula feeding, had an impact on n-6 and n-3 fatty acid profiles, with a higher linolenic acid and n-6/n-3 ratio in formula-fed infants at 4 months of age. We found n-6 fatty acids to be associated with insulin-like growth factor I (IGF-I), which was reflected in higher concentrations of IGF-I in formula-fed infants. IGF-I during infancy (0, 4, and 12 months) influenced BMI and waist circumference at 6 years of age. Associations were also seen between infancy IGF-I, in particular for 4-month IGF-I values, and insulin at 6 years of age. The adipokines leptin and adiponectin at 4 months of age were also associated with BMI and waist circumference at 6 years of age (positively for leptin and negatively for adiponectin). In addition, triglycerides in 6-year-olds were associated with concentrations of leptin at 4 months of age (p >0.001). High-density lipoprotein cholesterol in 6-year-olds was associated with the concentrations of adiponectin at 4 months (p = 0.01).

CONCLUSION: This thesis shows that a significant percentage of 6-year-old children, had abnormal metabolic profiles, including insulin resistance. Even at this young age, adipose tissue was positive associated with insulin resistance, stronger for SAT than VAT and stronger for girls than boys. Feeding modality at 4 months of age showed different fatty acid profiles and, in turn, fatty acids were associated with IGF-I and growth. Both IGF-I and adipokines at 4 months of age were associated with body composition and metabolic risk factors at 6 years of age, indicating that metabolic programming in early infancy is affected by nutrition.

KEYWORDS: Adiponectin, childhood obesity, insulin resistance, leptin, metabolic syndrome, n-6 fatty acids, SAT, VAT, waist circumference.

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SAMMANFATTNING PÅ SVENSKA

Meningen med denna avhandling var att undersöka metabol hälsa hos barn i förskoleåldern samt att identifiera tidiga markörer som kan hitta barn med ökad risk. Övervikt och fetma ökar i hela världen och påverkar barn i yngre och yngre åldrar. Fetma kan leda till påverkade metabola riskfaktorer så som nedsatt insulin känslighet, högt blodtryck och rubbningar av blodfetter. Dessa riskfaktorer tenderar att uppträda tillsammans med fetma och benämns samlat som metabola syndromet vilket medför en ökad risk att utveckla typ 2 diabetes samt hjärt- och kärlsjukdomar. Dessas följdsjukdomar kan förebyggas om symptomen påvisas och behandlas tidigt. Därmed är det viktigt att kunna identifiera barnen med en ökad risk att utveckla fetma och metabola rubbningar i en tidig ålder för att kunna förebygga sjuklighet och tidig död.
Denna avhandling hade som avsikt att utreda och besvara följande: 1) Undersöka om det finns tecken på metabola syndromet redan hos förskolebarn. 2) Utreda om fettfördelningen är associerad med metabola hälsan samt om det finns könsskillnader hos prepubertala barn. 3) Undersöka fettsyre sammansättningen under spädbarnsåldern samt att undersöka fettsyrornas påverkan på den tidiga tillväxten. 4) Undersöka nutritionens betydelse under första levnadsåret för att utveckla fetma och metabol ohälsa senare under barndomen.

Studien baserar sig på 480 fullgångna barn födda på Hallands Sjukhus Halmstad mellan 2008-2011 som en del av Tillväxt Projektet. Barnen är följda regelbundet från födelsen upp till 7 års ålder med bland annat tillväxt, blodprover med biomarkörer för tillväxt och nutrition samt matdagböcker. Från 6 års ålder utvidgades provtagningen med faste blodprover och blodtryck. Vid 7 års ålder utförde 81 av barnen en helkropps MRI för att mäta volymerna av underhudsfett (SAT) och visceralt fett (VAT) i buken.

Ett av huvudfynden var att 26% av barnen vid 6 års ålder hade en eller flera riskfaktorer kopplade till metabola syndromet. Barnen med fetma (3%) eller övervikt (14%) hade större benägenhet att ha utvecklat insulin resistens (28% versus 5%, $p < 0.001$) och förhöjda triglycerider (8% versus 0%, $p < 0.001$) än normalviktiga barn. Ytterligare så hade barnen med högt midjemått hade högre blodtryck än barnen med normalt midjemått ($p < 0.05$).

SAT korrelerade starkare med metabola riskfaktorerna än vad VAT gjorde med undantag för triglycerider där VAT hade starkaste korrelationen. Flickor

hade generellt starkare korrelationer mellan de olika fettdepoterna och metabola riskfaktorer än vad pojkarna hade.

Vid 4 månaders ålder hade nutritionen i form av bröstmjölk eller bröstmjölksersättning påverkan på omega-6 och omega-3 fettsyre profilen. Barn som fick mjölkersättning hade högre nivåer av den essentiella omega-6 fettsyran linolsyra (LA) samt högre kvot av omega-6/omega-3 vid 4 månaders ålder. Där blev funnet ett samband mellan omega-6 fettsyror och tillväxt hormonet insulin-like growth factor I (IGF-I) där barn som fick bröstmjölksersättning också hade högre nivåer av IGF-I. IGF-I nivåerna under spädbarnsåldern (0,4 och 12 månaders ålder) visade i sin tur påverkan på BMI och midjemått vid 6 års ålder. De tidiga IGF-I nivåerna, med starkaste associationerna vid 4 månader, associerade med insulin vid 6 års ålder. Också adpionektin och leptin som utsöndras från fettceller var vid 4 månaders ålder associerade med BMI och midjemått vid 6 års ålder (adiponektin negativt och leptin positivt associerat). Leptin vid 4 månader var även positivt associerat med triglycerid nivåerna vid 6 års ålder. Adiponektin vid 4 månader var negativt associerat med HDL kolesterol.

Sammanfattningsvis har denna avhandling kunnat visa att en betydande del av 6-åriga svenska barn hade påverkade metabola markörer som bland annat insulin resistens. Även innan puberteten var flickors fettvävnad starkare kopplad till insulin resistens än pojkars och SAT var stakare kopplat med insulin resistens och andra metabola markörer än vad VAT var. Avhandlingen har också visat på att amning kontra bröstmjölksersättning vid 4 månaders ålder påverkade barnens fettsyreprofil som i sin tur var kopplat till IGF-I och tillväxt. Både IGF-I, leptin och adiponektin var vid 4 månaders ålder associerat med kroppssammansättning och metabola riskfaktorer vid 6 års ålder vilket indikerar att metabol programmering under spädbarnsåldern påverkas av födan.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Emma Kjellberg**, Josefine Roswall, Stefan Bergman, Gerd Almquist-Tangen, Bernt Alm, Jovanna Dahlgren. **Longitudinal birth cohort study found that a significant proportion of children had abnormal metabolic profiles and insulin resistance at 6 years of age.** *Acta Paediatrica* 2018, Oct 17. DOI:10.1111/apa.14599.

II. **Emma Kjellberg**, Josefine Roswall, Jonathan Andersson, Stefan Bergman, Ann-Katrine Karlsson, Pär-Arne Svensson, Joel Kullberg, Jovanna Dahlgren. **Metabolic risk factors associate with visceral and subcutaneous adipose tissue in a sex-specific manner in seven-year-olds.** *Accepted with minor revision in Obesity. 2019.*

III. **Emma Kjellberg**, Josefine Roswall, Stefan Bergman, Birgitta Strandvik, Jovanna Dahlgren. **Serum n-6 and n-9 fatty acids correlate with serum IGF-1 and growth up to four months of age in healthy infants.** *J Pediatric Gastroenterology and Nutrition* 2018; 66: 141-146

IV. **Emma Kjellberg**, Josefine Roswall, Stefan Bergman, Gerd Almquist-Tangen, Bernt Alm, Jovanna Dahlgren. **Serum adipokines and insulin-like growth factor I during infancy are associated with future markers of the metabolic syndrome.** *Manuscript.*

CONTENTS

ABBREVIATIONS

AA	Arachidonic acid
AGA	Appropriate for gestational age
ALA	α-linolenic acid
BMI	Body mass index (kg/m^2)
BP	Blood pressure
CRP	C-reactive protein
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DXA	Dual-energy X-ray absorptiometry
EPA	Eicosapentaenoic acid
FFA	Free fatty acids
HDL	High-density lipoprotein
HOMA-IR	Homeostatic model assessment for IR
IGF-I	Insulin-like growth factor I
IR	Insulin resistance
IUGR	Intrauterine growth restriction
LA	Linoleic acid
LC-PUFA	Long chain polyunsaturated fatty acid
LDL	Low-density lipoprotein

LGA	Large for gestational age
LPL	Lipoprotein lipase
MRI	Magnetic resonance imaging
PUFA	Polyunsaturated fatty acid
SAT	Subcutaneous adipose tissue
SD	Standard deviation
SGA	Small for gestational age
VAT	Visceral adipose tissue
VLDL	Very-low-density lipoprotein
WC	Waist circumference
WHtR	Waist-to-height ratio

1 INTRODUCTION

1.1 CHILDHOOD OBESITY – A WORLDWIDE EPIDEMIC

Obesity is an ongoing epidemic, and according to the World Health Organization (WHO), the worldwide prevalence of obesity almost tripled during the latest four decades (1). During this period, childhood overweight and obesity have expanded from just 4% to as high as 18% worldwide (2-5). The increased number of children with overweight or obesity affects high-income countries as well as middle- and low-income countries. According to WHO, 41 million children under 5 years of age and 340 million children from 5 to 19 years of age were overweight or had obesity in 2014 (1). Sweden has a relatively low prevalence of childhood overweight and obesity compared to other European countries (6), but 19% of children aged 7 to 9 years were overweight and 3.3% had obesity in 2013 (7). Many children who become overweight do so during early childhood, and having obesity during childhood increases the likelihood of having obesity as an adult (8, 9).

1.2 BODY COMPOSITION

1.2.1 BMI

Overweight or obesity is commonly defined as high BMI and calculated as weight divided by height (kg/m^2). Obesity during childhood is a risk factor for physical morbidity and mortality throughout life (10). Moreover, childhood obesity is also associated with psychological morbidity in childhood (11). For more precise definitions of overweight and obesity during childhood, reference cut-offs for different ages are needed, as growing children continuously change their body composition. Several national growth curves and cut-offs have been produced over recent decades (12). In 1995, WHO recommended the use of Must et al.'s American child BMI references from the National Health and Nutrition Examination Survey I (NHANES I) (13). In 2006, WHO released new standards for children from birth up to 5 years of age (14). These references were presented as a standard of physiological growth rather than descriptive references and were derived from measurements of healthy breastfed children from six countries. This

standard was supplemented with growth curves for older children, aged 5 to 19 years, in 2007. These growth curves were based on US survey data (15) and on standard deviation (SD) scores and centiles specific for age and sex (16).

In 2006, the International Obesity Task Force (IOTF) proposed cut-offs for BMI based on previously published reference curves from six countries, including the USA (17). These charts are based on the adult cut-offs for thinness, overweight, and obesity, BMI ≤ 17, ≥ 25, and >30, respectively, at 18 years of age and extrapolated down to a corresponding BMI in children according to age and sex but not expressed as centiles. An extended reformulated version of the IOTF BMI charts from age 2 to 18 years came in 2012. The new charts have the benefit that they can be expressed as centiles or SD scores (18). The updated version of the IOTF charts made it possible to compare them with other BMI references, including the WHO standard.

In contrast to the IOTF BMI cut-offs based on centiles that define overweight as BMI 25 at 18 years of age, WHO has different cut-offs for different age groups. WHO defines overweight as +2 SDs in children below the age of 5 years and +1 SD for children aged 5 years or above. This results in a lower incidence of overweight in children below the age of 5 but a higher prevalence rate above this age when using the WHO definition instead of the IOTF definition (18).

1.2.2 WAIST CIRCUMFERENCE

Even though it is a widely used measure, BMI does not fully reflect body fat distribution and is thereby imprecise in describing health risks related to overweight. In adults, abdominal fat is known to be a risk factor for type 2 diabetes and metabolic syndrome, which can lead to cardiovascular disease (CVD) (19). Waist circumference is a simple anthropometric measure that gives more accurate predictions of metabolic risk and mortality than BMI (20) (21, 22). This seems to be the case in children as well (23, 24) (25). As with BMI, there is also a need for waist circumference references because body composition changes during childhood and adolescence. There are published national reference charts of waist circumference in children from several parts of the world but these mostly apply to school-aged children. In recent decades, several reference curves that also apply to younger children have been published (26-30), including Dutch (26), German (31), Norwegian (30), and Swedish (27) reference curves from northern Europe. In 2014, the

IDEFICS study presented curves based on children aged 2–10.9 years from eight European countries.

There are some differences in the design of the curves, where some are presented as SD scores and others are presented as percentiles. There are also differences in cut-offs between the curves, which is probably related to differences in methods when constructing the curves, size of the studied cohort, representativeness, and secular trends over time. As an example, the mean waist circumference of the Swedish curves (27) was 2 cm larger than the mean of the Dutch curves (26) produced a decade earlier. The Norwegian curves also showed lower mean waist circumference than the Swedish ones (30), whereas the mean of the IDEFICS curves, from children in eight European countries including Sweden, was higher (28).

1.2.3 WAIST-TO-HEIGHT RATIO

To make waist circumference more independent of the effect of height, waist-to-height ratio (WHtR) has been proposed as a useful clinical parameter for measuring abdominal fat and identifying abdominal obesity, which has been found to be more closely associated than waist circumference with morbidity in adolescents (32).

A WHtR higher than 0.5 is considered to be a simple cut-off to indicate overweight and a risk of cardiometabolic health in both adults and schoolchildren of both genders (32, 33). But WHtR is decreasing with age through young childhood and is lower in girls than boys (32) which makes it not a relevant cut-off in children under age of 5 where instead age-specific reference charts are needed (27, 30).

In the Bogalusa heart study authors found WHtR to be a strong method for identifying children at risk of metabolic diseases from those with lower risk among the population with either overweight or normal weight (34). In another study from the same group they found no such advantages of WHtR but that age-adjusted BMI and WHtR showed similar associations with metabolic risk factors (35). There are other studies also showing that WHtR does not add on top of BMI or waist circumference to predict metabolic risk (36, 37).

1.2.4 VISCERAL AND SUBCUTANEOUS FAT

Anthropometric measures of waist circumference and WHtR are simple proxies for abdominal fat calculations. However, more accurate measures exist. Waist circumference is well associated with abdominal adiposity by direct measurements of subcutaneous adipose tissue (SAT) by dual-energy X-ray absorptiometry (DXA) (38) and MRI (39). Accumulation of body fat is most often accompanied with accumulation of SAT but the abdominal area is not the preferentially area for SAT accumulation in children.

Although both SAT and visceral adipose tissue (VAT) are strongly associated with each other, changes in one do not fully explain changes in the other (40). This is illustrated by the fact that change in body fat is associated with change in SAT, but only weakly with abdominal adipose tissue (40). The volume of VAT increases with age, mainly during puberty (41). In schoolchildren and adults, VAT is shown to be associated with metabolic risk factors such as low levels of high-density lipoprotein (HDL) cholesterol, high levels of fasting triglycerides, insulin, insulin resistance (IR), and high blood pressure (19, 42, 43). However, some claim that waist circumference correlates better with total body fat than with VAT in children (44).

In adolescents and adults, it is known that there are sex differences in the associations of VAT and SAT with metabolic risk factorswith stronger associations in females (45, 46). After puberty, VAT is larger in males than in females but, in contrast, SAT is larger in females during the whole life span (40, 41). Sex hormones are believed to be responsible for the sex differences in body fat distribution (40, 41). Whether these gender differences are found even in preschool children has not been established. Even at 5 years of age, boys born preterm were found to have higher levels of VAT than girls born preterm, despite adjusting for lean mass, which is known to be higher in boys (47). This gender difference may not apply to term-born children, as those born preterm or small for gestational age (SGA) have a different body fat distribution (48).

1.3 GROWTH

1.3.1 LONGITUDINAL GROWTH

The concept of growth includes longitudinal changes in anthropometry such as height, weight, and head circumference over time. Normal growth is not linear and is characterized by three growth phases: infancy, childhood, and puberty. This is well described by the *infancy, childhood, puberty* (ICP) model (49), which reflects the different hormonal phases and influences on the growth process.

Growth starts from conception and is driven by different mechanisms. In the early development of the fetus, growth is characterized by cell deletion, proliferation, and maturation. This is mainly driven by autocrine proteins such as insulin-like growth factor I (IGF-I), glucose, leptin, and thyroxine. This period, with a rapid growth phase mostly driven by IGF-I, is called *infancy growth*, starting with the fetus and ending before the age of 1 year. (50).

The next phase is the *childhood period*, characterized by linear growth influenced mainly by pulsatile secretion of growth hormone together with IGF-I. Growth hormone is the predominant regulator of growth during the childhood period, which is seen as the slowest of the three growth phases (51). In the third phase, *puberty*, the sex hormones have an impact on an accelerated growth which then declines until adult height (49, 52, 53). Environmental factors, especially nutrition, physiological health, and psychological health, are also important for normal growth, which makes a child's growth a good marker for health (54).

1.3.2 GROWTH PATTERNS

Longitudinal growth patterns, more than age-related cut-offs in the growth chart, may influence health outcomes (55). The dominant increase in weight over length during the first year of life gives a peculiar growth pattern. Children have an intense growth period during first year of life with a peak in BMI somewhere around 9–11 months of age. Both age and level of BMI at the "peak" is positively associated with BMI in childhood (56). After the peak there is a short period with decreasing BMI until a nadir is reached, normally around 6 years of age.

The point at which BMI starts to increase again is called adiposity rebound (56). The child's age at adiposity rebound is a good indicator for predicting later overweight and obesity, as an earlier adiposity rebound gives a higher risk of later overweight (57, 58). An early adiposity rebound mirrors the early and extreme weight gain that most children with obesity experience. It has also been associated with metabolic risk later in childhood, but is probably most strongly associated to the degree of adiposity (59).

1.4 METABOLIC SYNDROME

1.4.1 DEFINITION OF METABOLIC SYNDROME

Overweight and obesity are linked to risk factors for CVD and type 2 diabetes. They often appear alongside decreased glucose tolerance, IR, dyslipidemia, and hypertension (60). This combination of cardiovascular risk factors is called metabolic syndrome, as the risk factors appear to cluster in individuals with overweight or obesity. Obesity is a major part of the syndrome definition, and either BMI or waist circumference (61, 62) are mandatory risk factors.

The syndrome describes a risk of developing type 2 diabetes and CVD over time. Instead of describing all the risk factors in isolation (IR, high blood pressure, dyslipidemia, etc.), they are described as a syndrome and are driven by a shared pathophysiological mechanism. The components of metabolic syndrome are driven by visceral obesity (19) and the association with IR (63). Even though those who have overweight or obesity have a higher risk of developing metabolic syndrome, lean individuals can develop the syndrome as a result of genetic or epigenetic factors, such as being born preterm or SGA (64-66).

The body of knowledge about metabolic syndrome in younger children has so far been sparse. In adults, the syndrome is clearly defined and widely used (67), but there is no agreed definition in children, and the various existing definitions (61, 62, 68-70) are not consistent. Most of these definitions are modified models of adult definition. In some definitions adult cut-offs for risk factors are used and others apply age-specific cut-offs in at least some variables. They also differ in which centiles are used for cut-offs as well as which parameters to use for glucose tolerance and insulin sensitivity (table 1).

Table 1. *Different definitions of metabolic syndrome in children*

Definition by	Measure of adiposity	Blood pressure	Lipids	Insulin Sensitivity
Cook et al.	WC ≥90th percentile for age and gender	BP ≥90th percentile for age and gender	TG ≥1.24 mmol/L or HDL-C ≤1.03 mmol/L	Fasting glucose ≥6.11 mmol/L
Viner et al.	WC ≥95th percentile for age and gender	BP ≥95th percentile for age and gender	TG ≥1.69 mmol/L or HDL-C <0.91 mmol/L	Fasting insulin ≥15 mU/L or Fasting glucose ≥6.11 mmol/L
Weiss et al.	BMI z-score ≥ 2 SD	BP ≥95th percentile for age and gender	TG ≥90th percentile or HDL-C ≤5th percentile for age and gender	Fasting glucose >7.8 mmol/L
IDF	WC ≥90th percentile for age and gender	SBP ≥130 mmHg or DBP ≥85 mmHg	TG ≥1.69 mmol/L or HDL-C <1.03 mmol/L	Fasting glucose >5.55 mmol/L
IDEFICS	WC ≥90th or ≥95th percentile for age and gender	BP ≥90th or ≥95th percentile for age and gender	TG ≥90th or ≥95th percentile or HDL-C ≤ 10th or 5th percentile for age and gender	HOMA-IR or Fasting glucose ≥90th or ≥95th percentile for age and gender

Abbreviations: BMI, body mass index; BP, blood pressure; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment for insulin resistance; IDF, International Diabetes Federation; IDEFICS, Identification and prevention of Dietary- and lifestyle-induced health EFfects in Children and infantS; SBP, systolic blood pressure; TG, triglycerides; WC, waist circumference.

1.4.2 REFERENCE VALUES IN CHILDHOOD

In 2007, the International Diabetes Federation (IDF) made a proposal and reached a consensus definition for metabolic syndrome in children that should be clinically easier to use (70). From the age of 10 years, children fulfill the criteria of metabolic syndrome if they have three or more risk factors with modified adult reference values, such as high waist circumference, dyslipidemia, IR, and high blood pressure. They proposed that children under 10 years of age should not be diagnosed with metabolic syndrome and only needed monitoring of waist circumference. The reason for this recommendation was lack of good child-specific references. Since then,

several studies have found signs of metabolic syndrome in children much younger than 10 years of age (71).

In 2014, one study constructed reference values based on 18 169 European children aged 2–10 years for the different metabolic risk factors. They also made a proposal for a definition of metabolic syndrome based on these reference values for age and gender (28, 61, 72-74). In this study they had a higher prevalence of metabolic syndrome than previous studies, probably because the age- and gender-specific definitions. They presented both a monitor level at or above the 90th percentile for age and gender for the specific risk factors and an action level above the 95th percentile, where action for risk factors is recommended (61).

1.4.3 RISK FACTORS FOR THE METABOLIC SYNDROME

1. Waist circumference or body mass index

Abdominal obesity is known as a strong predictor of CVD in both adults (19, 22) and in children (75). It has been widely debated whether BMI or waist circumference is the better predictor for this increased risk. Even if waist circumference in children correlates better with whole body fat than abdominal visceral adipose tissue (44) it is a simple and accurat measure of abdominal obesity (38, 39). Waist circumference is used as one of the basic risk factors of the metabolic syndrome and is an essential part of most of the definitions (61, 68, 70). In some definitions, BMI is used instead, with the argument that a change in body proportions is normal during puberty and that there are racial differences in the proportions (62). Overweight and obesity in childhood often persevere into adulthood and thereby also increase the risk of metabolic syndrome and CVD (76).

2. Insulin resistance

Metabolic syndrome was first described 1988 by Reaven, who observed that hyperinsulinemia occurred simultaneously with several cardiovascular risk factors and this cluster of factors predicted a higher mortality (63). IR is initially associated with hyperinsulinemia and is the most important risk factor linked to the development of impaired glucose tolerance in childhood obesity (62). When hyperinsulinemia can no longer override IR due to beta-

cell failure, diabetes is established. IR is considered to be an essential component of the metabolic syndrome.

The gold standard for measuring IR is by euglycemic hyperinsulinemic clamp, where constant intravenous infusion of insulin is balanced by infusions of glucose and provides steady-state measures of insulin action (77). It is invasive and time-consuming, which makes it difficult to use in large studies, and this disadvantage is especially problematic in children. The homeostatic model assessment for IR (HOMA-IR) is a proxy for IR, in which both fasting insulin and glucose are included in the model (fasting insulin/fasting blood glucose)/22.5. Children retain normal fasting blood glucose for a long time, at the cost of hyperinsulinemia, which is an earlier sign of abnormal glucose homeostasis. HOMA-IR is therefore a sensitive predictor of IR in children with obesity (78).

3. Hypertension

High blood pressure is a cardiovascular risk factor, even in children, and overweight and obesity are strongly and directly associated with high blood pressure. Children with overweight or obesity have a threefold higher risk of high blood pressure and hypertension (79). In contrast, those with obesity during childhood who no longer have obesity as adults have risk profiles in adulthood similar to lean adults who have never had obesity (80).

Arterial stiffness increases with the degree of obesity and even more with the degree of the metabolic alterations in metabolic syndrome (81).

There are also associations between systolic blood pressure in children with metabolic syndrome and atherosclerotic vascular changes (82). Left ventricular hypertrophy and diastolic dysfunction are also seen in children with obesity prior to the development of sustained hypertension (83). Hypertension is one of the most important modifiable risk factors for CVD (84), and if not identified and treated, hypertension in childhood can lead to atherosclerosis in young adulthood (85, 86).

4. Dyslipidemia

Dyslipidemia may lead to hypertension and other CVDs (87). In particular, disturbed lipid accumulation in the vascular intima leads to atherosclerosis, which is found present already in childhood (88). Obesity leads to high triglyceride levels and low HDL cholesterol level, which are risk factors

included in definitions of metabolic syndrome (61, 70). High serum triglycerides are closely related to both IR and type 2 diabetes in adulthood (89).

Even though low-density lipoprotein (LDL) cholesterol is a risk factor for developing coronary heart disease during childhood (88), high LDL cholesterol is not considered to be a risk factor included in the definitions of metabolic syndrome in children.

See figure 1 for a simplified model of metabolic syndrome risk factors.

1.5 PATHOPHYSIOLOGY OF METABOLIC SYNDROME

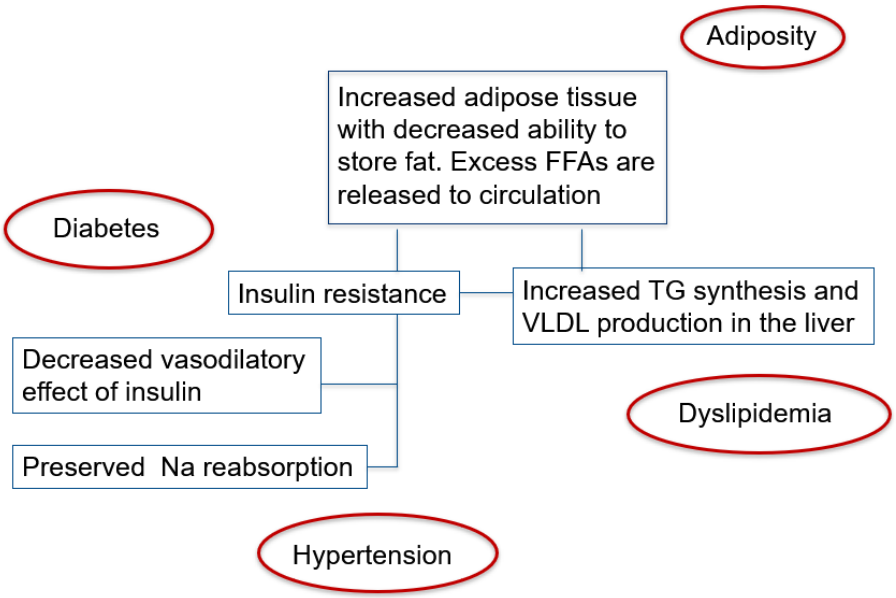

Figure 1. Pathophysiology of metabolic syndrome. Abbreviations: FFA, free fatty acid; TG, triglyceride; Na, sodium; VLDL, very-low-density lipoprotein.

1.5.1 FREE FATTY ACIDS AND INSULIN RESISTANCE

Weight gain leads to an accumulation of adipose tissue either by an increase in the number of adipocytes (hyperplasia) or an increase in the volume of each adipocyte (hypertrophy) (90). Adipose tissue was formerly known as the body's energy reserve that released free fatty acids (FFAs) to meet the energy demand in tissues and organs during fasting. Now adipose tissue is recognized as a complex and dynamic endocrine organ which interacts with energy homeostasis and with the whole body's homeostasis. The regulation of fat storage and mobilization is a highly coordinated minute-to-minute control which causes instant and dramatic shifts in metabolic flux (91).

Normally after a fat-containing meal, dietary fat that is not immediately oxidized is transported as chylomicron triglyceride fatty acids or very-low-density lipoprotein (VLDL) triglyceride to be stored in the adipocytes as triglycerides enhanced by insulin (figure 2) (60, 91).

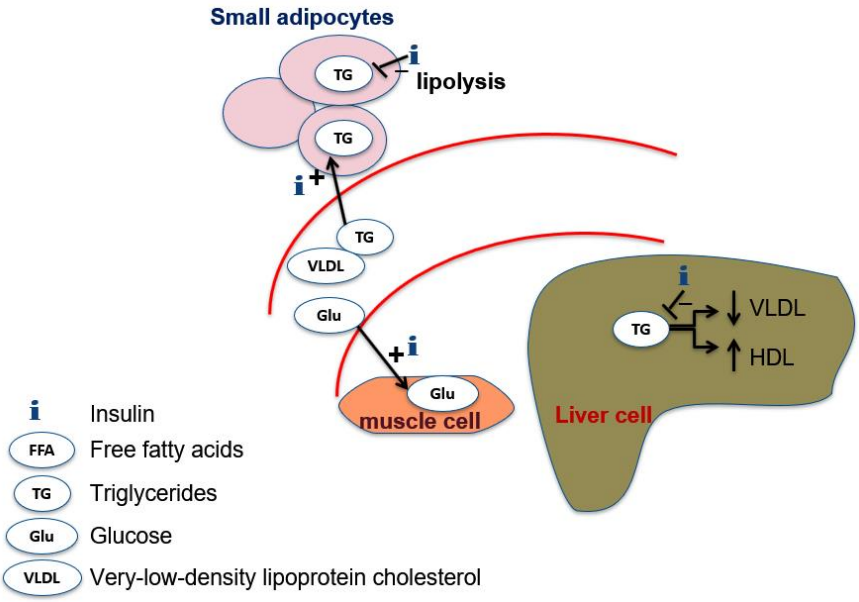

Figure 2. Individual with normal insulin sensitivity and healthy adipocytes storing triglycerides.

Excess adipose tissue with enlarged adipocytes causes IR with impaired cellular uptake of insulin as a result (92). Even deficiency of adipose tissue, as in lipodystrophy, is associated with IR. This suggests that healthy adipose tissue acts to maintain normal insulin sensitivity. In obesity, adipose tissue is

often associated with impaired function, with a lower capacity for storing fat and a lack of intracellular insulin due to impaired uptake. IR in adipose tissue is manifested by the inability to suppress lipolysis of stored triglycerides and FFAs are released (figure 3) (60). Excess lipids shunt to peripheral non-adipose tissue such as skeletal muscles, liver, heart, and pancreas, where they are stored as ectopic fat (93). Circulation of FFAs inhibits glucose uptake in skeletal muscles, which leads to peripheral IR and hyperglycemia (figure 3). This in turn will lead to increased insulin secretion of the pancreatic beta cells and systemic hyperinsulinemia to control the blood glucose levels in non-diabetic individuals. IR first contributes to hyperglycemia when pancreatic beta cells fail in insulin secretion (60, 93).

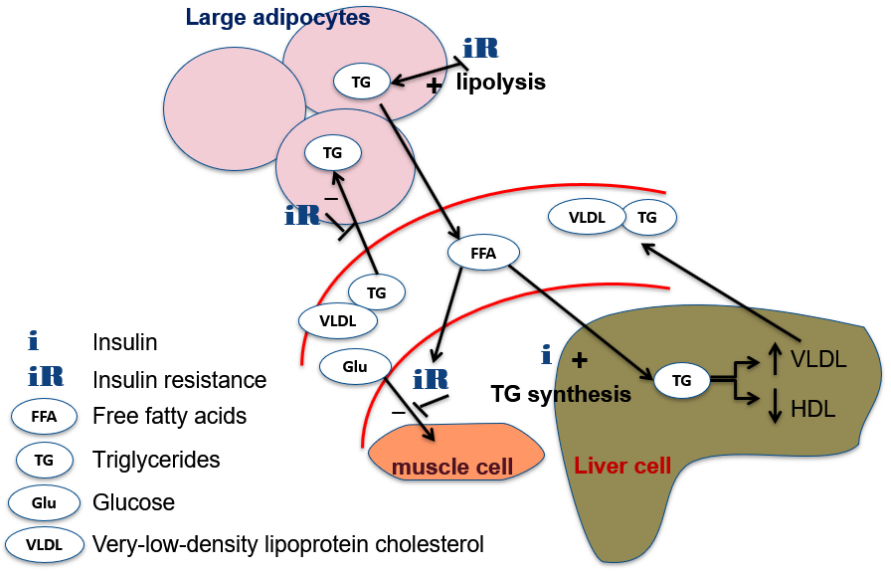

Figure 3. Individual with excess lipids, unhealthy large adipocytes and insulin resistance in peripheral tissue.

1.5.2 DYSLIPIDEMIA

FFAs reach the liver from the circulation through the portal vein and supply the liver with triglycerides. The liver can synthesize FFA and triglycerides to triglyceride-rich VLDL which are released to the circulation.

Triglyceride-rich chylomicrons are synthesized in the gut from dietary lipids. VLDL and chylomicrons will be lipolysed by the lipoprotein lipase in the endothelial wall, to deliver FFA to the circulation. FFA will then be used by muscle cells in heart and skeletal muscles for energy expenditure and to

adipocytes for storage (figure 4) (94, 95). The amount of FFA liberated from VLDL and chylomicrons depends on lipoprotein lipase activity which is stimulated by insulin. In healthy individuals, insulin suppresses the VLDL production in the liver (figure 2) (95). VLDL triglycerides are prone to penetrate blood vessels and accumulate in the extracellular matrix, where they trigger macrophages and inflammation (95).

Apo A-I is also synthesized in the liver and is the structural protein of HDL cholesterol. HDL cholesterol is an antiatherogenic lipoprotein which binds to vessel walls and removes fat molecules from cells and transport them back to the liver in a so called reversed cholesterol transport. Nascent HDL particles receive free cholesterol from peripheral tissues, and will be esterified within the HDL to cholesterol-esters. In this process, HDL cholesterol needs triglycerides from the triglyceride-rich VLDL in exchange of cholesterol esters. Triglycerides are then delivered back to the liver by HDL cholesterol (figure 4) (95).

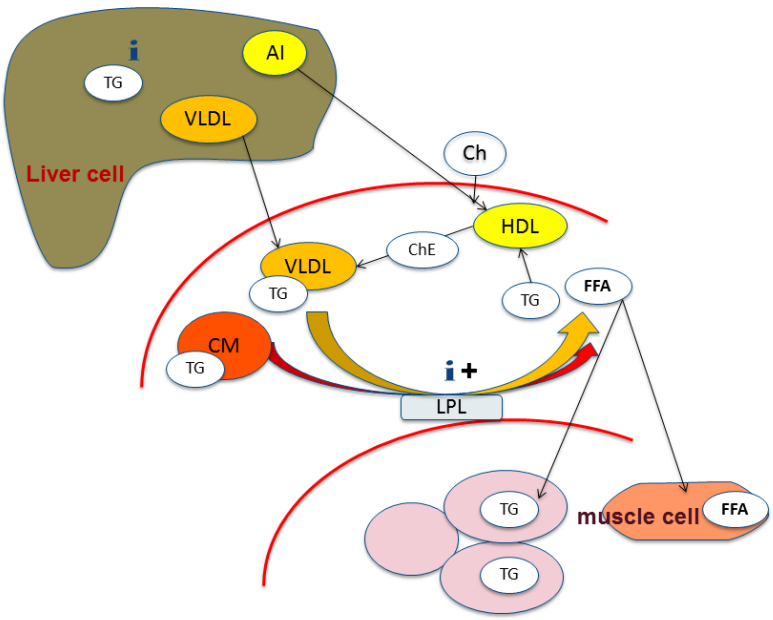

Figure 4. Healthy individual with insulin activated lipoprotein lipase to liberate FFAs for energy to muscle cells and storage in adipocytes.
Abbreviations: Apo-AI (AI), Cholesterol (Ch), Cholesterol ester (ChE), Chylomicron (CM), Free fatty acid (FFA),High density lipoprotein cholesterol (HDL), Lipoprotein lipase (LPL), Triglycerides (TG), Very low density lipoprotein cholesterol (VLDL)

In individuals with obesity and IR, there is a chronic hyperinsulinemic state which, together with high levels of triglycerides and abnormally high FFA levels reaching the liver, stimulates an overproduction instead, with secretion of triglyceride-rich VLDLs (figure 3). This increase in VLDL together with increased remnants from chylomicrons induces an increased exchange of triglycerides and cholesterol esters with HDL cholesterol. Lipolysis of these triglyceride-rich HDL cholesterols in the liver results in small HDL cholesterols with reduced affinity for Apo A-I which leads to a reduction of HDL cholesterol. This is manifested by increased plasma triglycerides and low HDL cholesterol concentrations (95-97). The IR in the liver and adipose tissue is the origin of dyslipidemia and the consequent development of atherosclerosis. IR further generates a low-grade inflammation with release of inflammatory markers, which will also affect blood pressure, endothelial cells, and macrophages (98).

1.5.3 INFLAMMATORY MARKERS

Adipose tissue produces and secretes both proinflammatory and anti-inflammatory adipokines. In obesity with hypertrophic adipocytes, there is an impaired adipokine secretion with increased proinflammatory factors including leptin, interleukin 6, and tumor necrosis factor alpha. At the same time there is reduced secretion of the insulin-sensitivity-related adipokines adiponectin and interleukin 10, which in combination promotes a low-grade inflammation (99).

Macrophages in the adipose tissue contribute to the adipose tissue stress response, with the risk that obesity will induce inflammation and metabolic alterations. Macrophages may also signal adipose tissue stress and inflammatory status to other organs as part of a systemic inflammation (100).

Interleukin 6 stimulates the production of C-reactive protein (CRP) in the liver. Obesity is strongly associated with CRP, and both elevated CRP and interleukin 6 are seen in young children with obesity (101). CRP is also found in atherosclerotic plaque and is associated with IR and other risk factors included in metabolic syndrome (102).

1.5.4 OXIDATIVE STRESS

When stressed, adipokines from adipose tissue induce the production of reactive oxygen species, which generates the process of systemic oxidative stress. Reactive oxygen species are proinflammatory and exacerbate cellular and vascular damage and endothelial dysfunction. In obesity there are different stressors, such as hyperglycemia, hyperleptinemia, inflammation, and FFAs, that may stimulate oxidative stress. Oxidative stress is involved in pathological processes such as IR, metabolic syndrome, CVD, and even hepatic and renal dysfunction and cancer (60, 103).

1.5.5 ENDOTHELIAL DYSFUNCTION

The endothelium, the inner layer of the vessels, actively regulates vascular tone and permeability, as well as many other functions such as control of coagulation and fibrinolysis. To manage these processes, the endothelium produces mediators such as nitric oxide, adhesion molecules, and cytokines (104). A disruption of the arterial endothelium and its functions, called endothelial dysfunction, is an early stage of atherosclerosis (104, 105).

Endothelial dysfunction causes impaired insulin action by altering the passage of insulin over the capillary vessels to reach target tissues. Overweight and obesity are associated with endothelial dysfunction through indirect mechanisms, including IR and hypertension, or directly by vascular damage caused by lipid deposition and oxidative stress, which triggers an inflammatory reaction. An inflammatory response with release of adipokines and cytokines worsens the IR (60). IR in endothelial dysfunction leads to an impaired insulin-mediated, nitric-oxide-dependent vasodilatation which results in an increased vasoconstriction (104, 105).

Endothelium-bound lipoprotein lipase is the rate-limiting enzyme for regulation of triglycerides and HDL cholesterol. A reduced functional endothelial surface area may result in reduced access of triacylglyceride-rich lipoprotein particles to lipoprotein lipase (104).

1.5.6 HYPERTENSION

The pathophysiology of hypertension in children with obesity is complex. Obesity in children is associated with an activation of the sympathetic nervous system (106). It has been hypothesized that selective leptin resistance maintains leptin-induced sympathetic activation in obesity, which permits leptin to play an important role in the pathogenesis of obesity-related hypertension and metabolic syndrome. Leptin secreted from the adipocytes has the direct central effect of increasing sympathetic outflow to the kidneys (106).

Children with obesity also have a disturbed sodium homeostasis, either in the form of sodium sensitivity or relative sodium retention (107). This, in combination with decreased relaxation of smooth muscles in blood vessels due to IR and the reduction of nitric-oxide-induced vasodilatation, is considered to be part of the cause of impaired blood pressure regulation in children with obesity (104, 106).

1.6 EARLY PROGRAMMING

Early programming is the concept that a stimulus or insult during a critical and sensitive developmental period can have long-lasting or even lifelong effects.

Metabolic syndrome and CVD are thought to have their origin *in utero*. This is a critical period of developmental plasticity during which the fetus can develop and adapt to the environment it will be born into. Both the prenatal and postnatal environment influence adult health, and this is described as early programming (108). Programming may occur as a normal part of the individual's biological development and adaptation. Early nutrition and hormones are known to be potent programming agents in many contexts.

Children born with intrauterine growth restriction (IUGR) either due to placental insufficiency or maternal nutrient deficiency can be born SGA. If the milieu does not match the programmed phenotype, it can result in later non-communicable diseases.

Children born SGA have an increased risk of metabolic syndrome as adults (109). Despite their usually small stature, children born SGA have less lean

mass but proportionally more fat mass than other children (65). Even though children born SGA have the same BMI in adulthood as those born appropriate for gestational age (AGA), they have an increased risk of IR and thus all other parameters of metabolic syndrome as well in adulthood. The catch-up of adiposity and not catch-up growth is believed to cause this increased risk (64).

Children born preterm are at risk of metabolic syndrome due to extrauterine growth retardation as well as incomplete hormonal supply in the absence of the placental endocrine source. These children have, despite their typically small size, higher waist circumference and visceral fat (47), known to be linked to IR. Some studies suggest that the catch-up growth during childhood seen in children born preterm or SGA is the driving factor (64, 110).

1.6.1 EARLY PROGRAMMING BY NUTRITION

During neonatal life and infancy, nutrition is important for later health. Fetal life and early infancy are periods of rapid growth and development in which nutrition plays a crucial role. Early nutrition is believed to be one important factor in metabolic programming, and nutrients *in utero* affect the growth of the infant after birth. Both maternal nutrition, which affects fetal growth, and postnatal nutrition (breastfeeding, formula feeding and the weaning period), are believed to be of importance.

Both undernutrition during prenatal life and placental dysfunction are associated with IR and metabolic syndrome in adulthood (111-113). When the maternal nutrient supply to the fetus is impaired, either by placental deficiency or nutrient insufficiency, it causes IUGR, shown as lower birth weight and birth length.

Children born SGA may have suffered undernutrition based on several different etiologies (109, 111), but low birth weight *per se* increases the risk of developing IR and metabolic syndrome later in life (114, 115). This is hypothesized to be an *in utero* adaptation of the fetus to maximize the chances of survival if born into a postnatal nutritional deficiency, known as the "thrifty phenotype hypothesis" (114). In a nutritionally excessive postnatal environment this will instead lead to an increased risk of obesity and metabolic alterations such as IR and metabolic syndrome in adult life. Both the well-studied Dutch famine (116) and Chinese famine (117) studies show that famine during prenatal life increases the risk of hyperglycemia in

adulthood, which is aggravated by an unhealthy lifestyle and obesity as adults.

An imbalanced maternal diet during pregnancy also seems to have an impact on the fetus. A balanced energy and protein supplementation improves fetal growth, although high protein intake might be harmful, and low protein concentrations in diets are seen as a risk factor for later metabolic health (111, 118). The worsening in future insulin sensitivity after low protein intake has been shown in rat studies to be a result of reduced beta-cell proliferation in neonates whose mothers were fed a low-protein diet during pregnancy (118).

Furthermore, maternal intake of polyunsaturated fatty acids (PUFAs) during pregnancy and lactation has developmental and possibly programming effects on the infant (119).

1.6.2 EARLY PROGRAMMING BY PROTEINS

Proteins are of major importance, and a sufficient intake in fetal life and early infancy is necessary for normal growth and neurodevelopment. Conversely, high protein intake is associated with accelerated growth and increased body fat and an earlier adiposity rebound (120, 121). This accelerated growth can partly be explained by higher IGF-I concentrations that are driven by nutrition and proteins. Dietary protein supply in fetal life and infancy has an effect on metabolic and endocrine response in infants (122). Randomized controlled trials have shown that infant formula with a high protein content compared to formula with a lower protein content increased the plasma concentration of essential amino acids, whereas non-essential amino acids were lower (122). Breast milk has a lower protein content compared to modern formula composition (122). The higher protein intake in formula-fed infants is believed to be one explanation for the higher risk of obesity (120), hypertension (123), and type 2 diabetes (124) in individuals who were formula-fed compared to those who were breastfed during infancy.

1.6.3 EARLY PROGRAMMING BY POLYUNSATURATED FATTY ACIDS

Nutrient fat is important for growth and development. Dietary lipids, including their PUFAs, are much more than a source of energy. Fatty acids

are included in every cell membrane and regulate protein function and signaling functions to regulate appetite, energy balance, and inflammation. Fatty acids modulate gene expression and thereby respond to the metabolic environment (125). During prenatal life and infancy, the essential fatty acids play an important role in rapid growth (126). The PUFAs α-linolenic acid (ALA) and LA are essential fatty acids and must be supplied through the diet. They can then be elongated to long-chain PUFAs (LC-PUFA). The essential n-3 PUFA ALA can be synthesized to docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and the n-6 LA can further be synthesized to arachidonic acid (AA) (figure 4) (127).

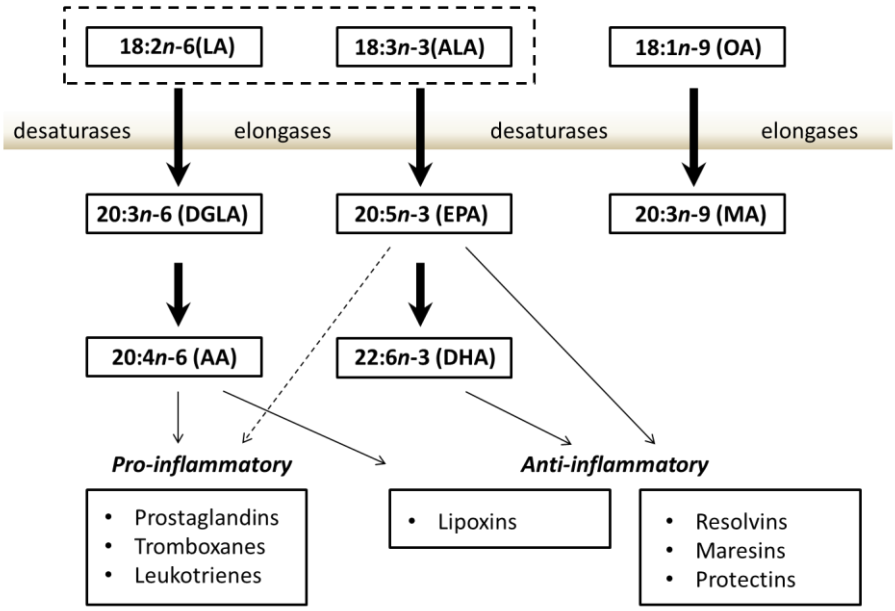

Figure 5. Overview of the major transformation steps for essential fatty acids, linoleic acid (LA) and α-linolenic acid (ALA), to the main long-chain polyunsaturated fatty acids arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) and their most important metabolic products. The body can synthesize oleic acid (OA) and its transformation product mead acid (MA) to balance a deficiency of the essential fatty acids in the membranes

ALA, LA, and AA are supplied mainly from vegetable oils, and DHA and EPA have mainly marine origins. N-3 and n-6 PUFAs compete for the same enzyme systems for elongation to LC-PUFAs, which includes EPA, DHA, and AA, making the ratio between n-3 and n-6 important (127).

Fetuses and infants have difficulties converting LA to AA, and they get their supply of LC-PUFA through the placenta, mainly during the last trimester (128), and then through breast milk. The placenta is, by selective uptake, able to concentrate the important LC-PUFAs from serum and transfer a higher concentration to the child than the mother's own serum contains (128). The mother's serum and breast milk concentration of LC-PUFA depends on her choice of food and varies in quality worldwide. There is also wide regional variation in the mean ratio of DHA to AA in breast milk. The highest DHA concentration in breast milk is primarily found in coastal populations and is associated with marine food consumption (129). In a Swedish cohort study, the breast milk n-6/n-3 LC-PUFA ratio ranged from 3.4 to 9 (130), and a study of rural Chinese women found a breast milk n-6/n-3 ratio as high as 17.6 (131).

In animal studies, LC-PUFAs have been shown to influence body composition (132). In human cohort studies, however, there have been conflicting and inconclusive results that have raised the possibility that the nutritional influence might differ through different phases of perinatal development of fat storages (133-135). It is shown that low plasma LC-PUFA status at birth is associated with increased early infancy weight gain (136).

1.6.4 EARLY PROGRAMMING BY MATERNAL FACTORS

Mothers with obesity have a fourfold higher risk of having children with obesity. If the father also has obesity, the risk increases to over tenfold (137). Regardless of whether it is genetically determined or a consequence of lifestyle, most of the obesity risk is due to increased weight gain during childhood. The worst combination for increased obesity risk is the offspring born SGA to mothers with obesity, in connection with postnatal catch-up growth. These children have a significantly increased risk of obesity and CVD in later life (138). Not only the mother's weight but also her weight gain during pregnancy is a risk factor for later obesity in the child (139).

Gestational diabetes mellitus is another maternal risk factor for increased risk of obesity and metabolic syndrome in the child. The hyperglycemic diabetic intrauterine environment is an important risk factor for programming of later obesity and IR in the child. Gestational diabetes increases the risk of childhood overweight and abdominal adiposity by approximately 60%–80% (140). Children born large for gestational age (LGA) to mothers with

gestational diabetes have an increased risk of developing metabolic risk factors and metabolic syndrome during childhood and adolescence, independently of maternal BMI (141-143).

Smoking during pregnancy is associated with IUGR and an increased risk of the child being born SGA (144), and later during childhood and adolescence there is an increased risk of overweight and obesity (145).

1.6.5 EARLY PROGRAMMING BY RAPID GROWTH

1. Catch-up growth by height

Children born LGA have an increased risk of childhood overweight that progresses into adulthood. In particular, LGA children have more lean mass and not fat mass compared to other children, but their risk of metabolic alterations in prepubertal years is still higher. Children born SGA are often smaller during childhood than children born AGA or LGA, but the smaller size is composed of less lean mass and equal fat mass, which gives a higher fat ratio in children born SGA (65, 146).The difference in lean mass and fat mass persists throughout the lifespan and is typically already present during childhood in the form of increased abdominal adipose tissue (47).

Children born SGA often have a catch-up weight gain during the first months of life, and the majority has accomplished this before the age of 2 years. This rapid weight gain is strongly associated with later adiposity and overweight (147, 148). Children with catch-up growth within the first years are shown to be larger and fatter with higher waist circumference than other children by the age of 5 years (65). Catch-up growth during first years of life is also a significant risk factor for developing metabolic syndrome and later CVD (64, 148). The paradox is that the mortality of SGA children is decreased by the nutritional recovery and catch-up growth early in life (147).

2. Rapid weight gain in infancy

Rapid weight gain as early as during the first six months of life is not necessarily catch-up growth due to intrauterine undernutrition; it may just be postnatal rapid weight gain. This accelerated gain in weight is commonly defined as a gain greater than 0.67 z-scores (149). Rapid weight gain itself is associated with overweight and metabolic risk in later childhood. Together

with an early adiposity rebound, rapid weight gain crossing several growth percentiles is hypothesized to be a factor in early programming of later obesity and ill health (65, 150).

Rapid weight gain during the first months of life and a major crossing of several growth percentiles is rare and correlates with some monogenic entities such as leptin deficiency or MC4-receptor deficiency (151).

1.6.6 EARLY PROGRAMMING BY HORMONES

1. Insulin-like growth factor-I

Most organs in the body produce IGF-I, but the main production of circulating IGF-I is in the liver. Its structure is reminiscent of pro-insulin and, like insulin, it promotes glucose uptake in the cell. IGF-I is predominantly a mediator of growth and differentiation, and it initiates intracellular signaling through multiple pathways binding to the IGF-I receptor in the tissue (152).

Secretion of IGF-I in fetal life and infancy is dependent on nutrition and, in turn, IGF-I stimulates fetal and infant growth. The level of IGF-I increases with gestational age, but may be affected if the fetal nutritional supply is disturbed (153). The secretion of IGF-I is besides the energy intake from nutrients also shown to be triggered by protein content (122, 154, 155).

There are reports that both low and high levels of IGF-I during infancy might influence health. In preterm infants, low cord levels of IGF-I are associated with increased risk of vascular abnormalities (156) and retinopathy of prematurity (157). These abnormalities are known to predispose for hypertension in adulthood (158). On the other hand, in childhood, high IGF-I levels were found to be associated with higher weight gain and BMI (159). One study suggested that IGF-I during infancy protects against later obesity (160), while another concluded that it might just reflect an early adiposity rebound (159).

Children born IUGR are known to have lower cord IGF-I concentrations compared to children born AGA but also compared to SGA children, which may reflect altered growth hormone or IGF-I sensitivity when exposed to nutrient deficiency (161). Additionally, children born IUGR are known to have higher long-term disease risk (109), which is hypothesized to be a consequence of a mismatch between the predictive future environment and

the actual nutritional environment after birth (161, 162). It is not fully known when exactly the critical window for programming occurs, but evidence points to prenatal and possibly early postnatal life, when it is beneficial to have a high IGF-I (156).

2. Adipokines

The adipocyte produces anti-inflammatory cytokines such as adiponectin and leptin (163).

Adiponectin is an insulin-sensitizing hormone and has a negative effect on atherosclerosis and inflammation (164, 165). At birth, adiponectin correlates positively with birth size and negatively with gestational age. At about one year of age, adiponectin reaches a plateau and thereafter the levels decrease with increasing weight gain (164, 166). A low adiponectin level in children is associated with a negative metabolic risk profile. The concentration of adiponectin at baseline is inversely associated with metabolic risk factors several years later, both in children (167) and in adults (168). Furthermore, hypoadiponectinemia is believed to play a causal role in obesity-induced IR, type 2 diabetes, and ultimately atherosclerosis (167, 169).

Leptin regulates energy balance through the hypothalamic-pituitary axis and thereby regulates the appetite and satiety. Leptin correlates positively with birth weight. On the other hand, low leptin levels at birth are associated with increased weight gain and growth during the first months of life (170). Maintenance of leptin levels during early development is important for normal maturation and signaling pathways for the metabolic homeostasis. Hypo- or hyperleptinemia during this sensitive period can induce some of the metabolic adaptations involved in developmental programming (171, 172). Alterations in leptin concentrations during fetal and neonatal life have been associated with obesity and metabolic syndrome in adulthood (171).

2 AIMS

General aims

The aim of this study was to explore metabolic health in preschool children. A further aim was to investigate whether there are biomarkers in infancy that can identify children at risk of developing obesity and metabolic syndrome later in childhood.

Specific aims

- To investigate whether 6-year-old children show signs of metabolic syndrome (Paper I).
- To investigate whether the fat distribution in 7-year-old children is associated with their metabolic profile, and whether there are sex differences in associations between fat distribution and metabolic risk factors at this age (Paper II).
- To study the fatty acid profile and its influence on growth between birth and 4 months of age (Paper III).
- To investigate whether biomarkers in infancy can identify children at risk of obesity and metabolic syndrome in 6-year-old children (Paper IV).

Hypotheses

We hypothesized that metabolic syndrome can be found in pre-school children with overweight or obesity. We also hypothesized that children at risk can be identified with early biomarkers during their first year of life. Early nutrition with healthy composition of fatty acids is important for growth and later health.

3 PATIENTS AND METHODS

3.1 STUDY DESIGN

This study was part of a larger ongoing population-based Swedish longitudinal birth cohort, the Halland Health and Growth study (13). Children born in one of the included birth clinics, Halland Hospital Halmstad, were followed more thoroughly with, among other measures, anthropometry and blood sampling. All pregnant women received written information about the study when they visited maternal health care units during the last trimester of their pregnancy.

The children were first enrolled into a five-year study recording anthropometry, collecting breast milk samples (from the first week and at 4 months), and blood samples at birth, day 2, day 4, and at 12, 36, and 60 months (figure 5). Parents answered questionnaires about health before and during pregnancy and brought food diaries. Those who still were in the study at 5 years of age were invited to a follow-up study of five more years with the first visit at 6.5 years of age. At the 6.5-year visit, the examinations were extended to include resting blood pressure and fasting blood sampling. At 7 years of age, a subpopulation were invited to MRI in order to quantify SAT and VAT.

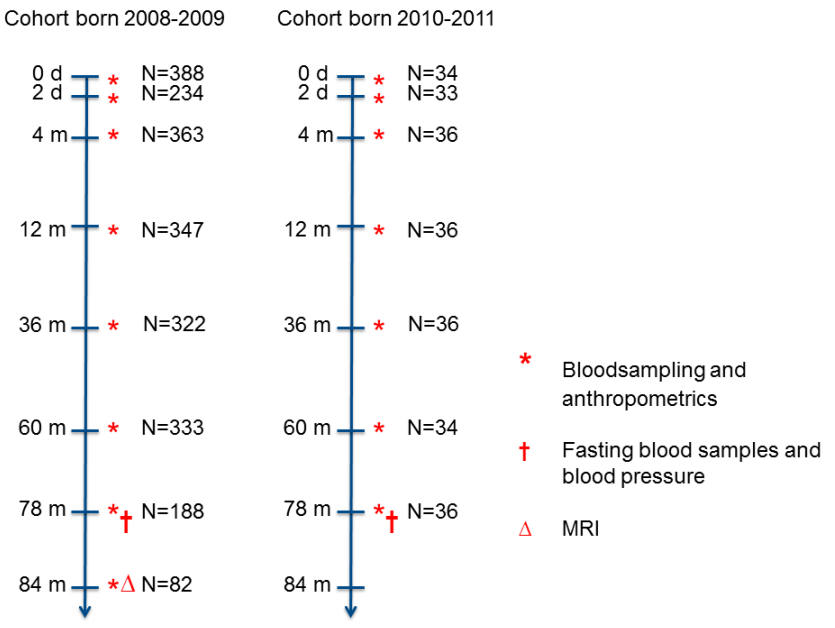

Figure 6. Study participants at the different examinations.
Abbreviations: d, days; m, months; N, number of participants

3.2 STUDY PARTICIPANTS

From April 2008 to June 2009 and from June 2010 to August 2011, 506 infants were recruited at Halland Hospital Halmstad, Sweden. During these periods, 2450 full-term infants were born in Halmstad. The exclusion criteria used were severe maternal illness, home birth, prematurity, Down syndrome, and difficulties with communication in Swedish.

The first period included children born to mothers registered at the maternity ward. Of these, 11% were born by cesarean section but none by elective cesarean section.

The second period included only children born by cesarean sections. In total, 24% of the children were born by cesarean section. The sectio cohort was included in Paper I.

Twenty-six preterm children were excluded from the study. The 480 healthy infants (50.0% boys) included in the study were born full term with mean

gestational age 39+5 ±1.1 weeks (range 37–42 weeks). Of these children, 27 (5.3%) were born SGA and 13 (2.7%) were born LGA.

Among the mothers, 25% were overweight (n = 45) and 8% had obesity (n = 14).

Table 2. *Early demographic characteristics of the follow-up study population at 6.5 years visit compared to dropouts and to children not included in the study*

	Follow-up cohort (n=212)	**Dropouts (n=268)**	**Non-cohort (n = 1970)**
Male gender (N (%))	110 (52)	130 (49)	
Gestational age (days)	280 ± 8	280 ± 9	279 ± 9*
Birth weight (g)	3605 ± 534	3598 ± 553	3529 ± 542**
Birth length (cm)	50.9 ± 2.2	50.8 ± 2.1	50.5 ± 2.2***
Breastfeeding at 4 months (N (%)):			
exclusive	132 (62)	148 (63)	
mixed	41 (19)	42 (18)	
formula	39 (18)	44 (19)	

Data are presented as mean ± SD unless otherwise stated. P < 0.05 was considered significant.
* p <0.05, ** p <0.01, *** p <0.001 for cohort versus non-cohort

3.3 METHODS

3.3.1 MEASUREMENTS

Anthropometrics

All children were measured by the same two research nurses at the same site during all visits. Measurements of weight, length, and head circumferences were recorded using a standard procedure. Length/height was measured to the nearest 0.1 cm with a stadiometer. Up to 12 months of age, length was measured in the supine position, and from 3 years of age, height was measured with a wall-mounted stadiometer. Weight was measured to the nearest 0.1 kg. At birth, at 4 and at 12 months of age, infants were weighed naked on baby scales in the supine position. From 3 years of age, the children were weighed in underclothes and measured on electronic step scales. Waist circumference was measured midway between the iliac crest and lowest rib

after a gentle expiration. Weight, length, and head circumference SD scores were calculated. BMI was calculated as kg/m².

Blood samples

A cord blood sample was taken at birth. On about day 2, a blood sample was taken together with the screening test all newborn babies undergo between day 2 and day 5. Infants were pacified by either breastfeeding or oral glucose. From 4 months of age the blood samples were taken after local anesthetic using EMLA (Aspen Pharma trading limited, Citywest Business Campus, Dublin 24, Ireland) (figure 6). At the 6.5-year visit, children came after a night's fasting to give fasting blood samples. Afterwards they were served a light breakfast.

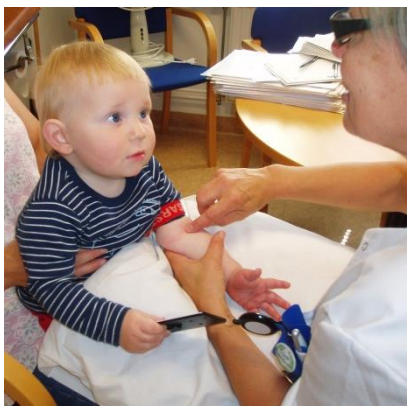

Figure 7. Blood sampling was taken with local anesthetic.

Breast milk samples

Breast milk samples were collected during the first week (median 3 days, range 1–14 days) and at 4 months of age (median 119 days, range 83–163 days). The milk sample was collected using a breast cup during breastfeeding.

Blood pressure

At the 6.5-year visit, blood pressure was measured by a Welch Allyn Spot Vital Signs digital monitor (Welch Allyn, New York, USA) on the upper right arm after a minimum of 10 minutes seated. Cuffs (child size 9 or small

adult size 10) were chosen according to arm size. Thereafter, fasting serum samples were collected.

Pubertal stage

At 6.5 years of age, pubertal stage was determined according to the definition by Tanner and Whitehouse (173). All children were prepubertal (in girls, breast stage <2 and in boys, testicular volume <4 mL) (19).

3.3.2 MRI

For quantifying VAT and SAT in the studies in this thesis, magnetic resonance imaging (MRI) was used. Dual-energy X-ray absorptiometry (DXA) (13) and MRI (14) are the most common methods for measuring abdominal fat distribution. Less commonly, ultrasound and computed tomography (CT) are used. CT is as accurate as MRI for measuring fat distribution, but is not a preferred method in children because it exposes the subjects to ionizing radiation. DXA is easy to use but less accurate in identifying abdominal fat than MRI, which makes MRI the gold standard method for identifying VAT (15).

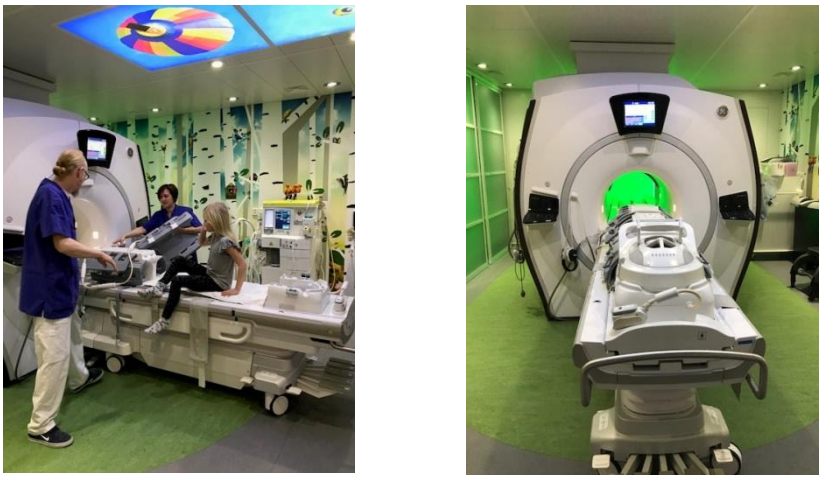

Figure 8. MRI at Queen Silvia Children's Hospital

On the day of the MRI, the children had eaten a fat-free breakfast. Seventy children underwent whole-body MRI scans and the remaining 12 underwent

only abdominal MRI scans. Imaging was performed with the child in the supine position, without sedation or anesthesia (figure 7). The same radiographer conducted all scans and sent the results to radiographers at the University of Uppsala for processing and analysis.

MRI protocol

All imaging was performed on a clinical 3.0 T MR system (Discovery MR 750w, GE Medical Systems, Waukesha, WI, USA). Most children were examined with a whole-body 3D fast SPGR 6-echo sequence, producing water and fat images. MRI was performed at 10 stations of 28 transversal slices each. At four stations, images were acquired during breath-hold. Those 12 children who did not undergo the whole-body imaging procedure were examined using a T1 weighted two-point Dixon 3D gradient-echo technique (LAVA-Flex) to acquire fat and water images. Sixteen transversal slices over the abdomen, centered over L4, were acquired. Imaging was performed during one breath held for 5 seconds.

Image analysis

For the subjects who had undergone whole-body scanning, measurements of VAT and SAT were centered on the umbilicus and spanned 162 mm in the feet–head direction. For the subjects who only underwent abdominal scanning, the entire scanned volume was used for the measurements.

In the image analysis, the body was first segmented from the background using threshold levels determined slice-wise on the sum of the water and fat images (figure 8). Arms were automatically removed by considering only the largest connected component. VAT and SAT were automatically segmented using the inside lean-tissue filter (174), with voxels of fat fraction at or above 50% considered as adipose tissue. Bones and adipose tissue near the spine belonging to neither depot were manually removed. Errors of the automated method were manually corrected and the volumes were then measured.

Measuring adipose tissue in children with MRI is challenging because it is hard to gain their compliance with the procedure, which requires a minimum of movement and sometimes even breath-holding. Moreover, children have less adipose tissue than adolescents and adults, which reduces the accuracy of the method. The method uses fast water and fat imaging in combination with automated assessment of VAT and SAT volumes. The water and fat imaging

takes advantage of the chemical shift between MR signals from these components that in combination with multi-echo acquisition and model-based reconstruction generates water and fat images. These images give a good water/fat contrast and allow analysis of fat content (175).

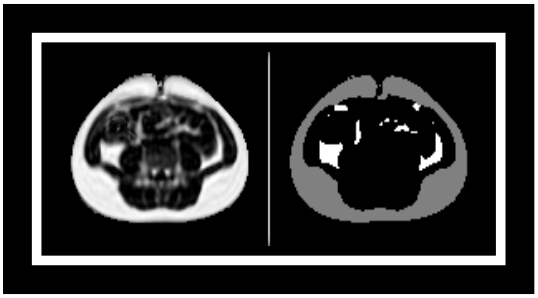

Figure 9. Transverse images centered on the umbilicus from one of the children who underwent a whole body scan. Left: Fat fraction image, arms and background removed. Right: The segmentation mask. White represents VAT and grey represents SAT.

3.3.3 LABORATORY

All blood samples were immediately centrifuged and sera were frozen at –80 ºC within the following hour until analyses could be performed. Plasma glucose and insulin were analyzed immediately. Glucose, insulin, total cholesterol, triglycerides, HDL cholesterol, and LDL cholesterol were analyzed at Halland Hospital Halmstad. IGF-I, total adiponectin, leptin, and fatty acids were analyzed at the laboratory at Gothenburg Pediatric Growth Research Center.

IGF-I was analyzed in duplicate using the IDS-iSYS technique (176). All samples were analyzed in the same batch, and the intra-assay coefficient of variation (CV) for IGF-I was 2.8% (5.8% for levels <28 ng/mL).

Leptin concentrations were measured by RIA (Linco Research, St. Charles, USA). Adiponectin concentrations were measured by enzyme-linked immunosorbent assay (R&D System Inc., Minneapolis, MI, USA) and expressed as µg/mL.

Glucose was measured by an enzymatic method with hexokinase, and insulin was measured by an electrochemiluminescence immunoassay, both on the

Cobas 6000 analyzer (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-69305 Mannheim, Germany). IR was estimated using the homeostatic model assessment for IR (HOMA-IR) calculated as (fasting insulin x fasting glucose)/22.5.

Total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides were measured by an enzymatic colorimetric assay on a Cobas 6000 analyzer (Roche, see above). For determination of fatty acids, 100 µL of milk was lyophilized overnight and lipids were extracted three times using sonication and centrifugation at 2000 g. Butylated hydroxytoluene plus 0.1 mg/mL chloroform was added and the supernatant was evaporated under nitrogen and redissolved in 3 mL chloroform before methylation, as previously described (177). Phospholipids from serum were fractionated on a single SEP-PAK aminopropyl cartridge (Waters Corp., Beverly, MA, USA), transmethylated, and separated by capillary gas chromatography in a Hewlett-Packard 6890 (177). C 21:0 was used as an internal standard and the fatty acid methyl esters were identified according to the retention times of pure reference substances.

3.3.4 QUESTIONNAIRES

All parents filled a questionnaire about background variables such as health during pregnancy, medical history, heredity for diabetes, overweight and cardiovascular heart disease, maternal diet, and parental education.

At the 4-month visit they had brought a three-day food diary and answered questions about breastfeeding and formula feeding with answers as seen below in figure 9.

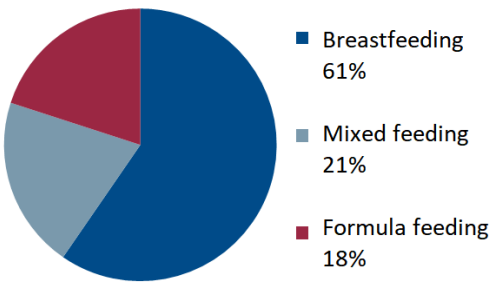

Figure 10. Feeding pattern at 4 months of age.

3.4 STATISTICS

Data analyses were performed using SPSS Statistics, version 22 (IBM Corp, Armonk, NY, USA). Normally distributed measurements are presented as mean ± SD, whereas non-normal distributions are presented as median and interquartile range (25–75). P-values <0.05 were considered statistically significant.

Paper I

Student's t-test, ANOVA, chi-square test, or Fisher's exact test were used for comparisons between groups as appropriate. Pearson's correlation coefficient was used for correlation analyses.

Paper II

Sex differences were first studied by interaction analyses, then variables were stratified by sex. Student's t-test or the Mann Whitney U-test were used for comparisons of continuous variables. Linear and multiple linear regression models were conducted to explore associations between the anthropometric variables (BMI, waist circumference, and WHtR), with VAT and SAT as dependent variables. Models were adjusted for sex, birth weight, maternal BMI, and education. SAT and VAT were then analyzed as independent variables, stratified by sex, in linear regression analyses with the metabolic risk factors (insulin, HOMA-IR, triglycerides, HDL cholesterol, and blood pressure) as dependent variables.

Paper III

The Friedman test for repeated samples was used for comparisons of fatty acids over time and the Wilcoxon signed-rank test was used for comparisons between two time points. The Kruskal–Wallis one-way analysis of variance and the Mann–Whitney U test were used for comparisons between groups with different feeding patterns. Spearman's rank correlation coefficient was used for correlations. Due to multiple testing, the Holm–Bonferroni method was used on bivariate correlations for significance levels. Multiple linear regression analyses were used for possible associations adjusted for confounding factors.

Paper IV

Student's t-test and the Mann–Whitney U-test were used for group comparisons. Linear regression models were used for associations. In multiple linear regression analyses, results were adjusted for confounding factors such as gender, weight at birth, BMI at 4 or 12 months and BMI at 6 years, feeding modality at 4 months, and maternal BMI.

Due to multiple testing, Holm–Bonferroni adjustments were made and used for significance levels.

3.5 ETHICS

The longitudinal study was conducted according to the Helsinki Declaration and was approved by the Regional Ethical Review Board in Lund (ethics approval number: 44/2008), and the MRI study was approved by the Regional Ethical Review Board in Gothenburg (number 375/15). All pregnant women received written information when they visited the maternity health care unit during the last trimester of their pregnancy. If they wanted to take part, written informed consent was obtained when they registered with the maternity ward. Written information was sent out to families prior to all visits. Written informed consent was obtained from all participating parents and oral assent from the children at 6 and 7 years of age.

Before blood sampling, children were anesthetized with local anesthetic. All measurements and blood sampling were conducted in a child-friendly environment by the same two pediatric nurses at all times. After each visit the children received an age-specific present.

During visits, parents were given the opportunity to ask the two experienced pediatric nurses questions about the study or about child health.

If any disease or other health-related symptoms were detected during an examination, the child was remitted for appropriate further examinations.

4 RESULTS

4.1 METABOLIC SYNDROME IN 6-YEAR-OLD CHILDREN (PAPER I)

In this relatively small cohort of 212 6-year-olds, four children (2%) fulfilled all criteria for the diagnosis of metabolic syndrome according to the IDEFICS action level of the definition of metabolic syndrome. Those four children had three or more risk factors over the 95th percentile for age and gender. Only one of those children had obesity; the other three were overweight. Using the IDEFICS monitor level instead of the action level of the definition of metabolic syndrome, there were instead nine children (4%) with three or more risk factors.

A significant percentage of children in this study showed an abnormal metabolic profile, increasing their risk of CVD. All risk factors of the syndrome were represented in the cohort, with a higher incidence in children with overweight or obesity.

Most of the children with overweight or obesity (62%) also had high waist circumference. Likewise, out of 183 children with normal waist circumference only 8% had high BMI (table 3). This gives a sensitivity of 97% and a specificity of 79%.

Table 3. *Waist circumference in children with normal BMI and with high BMI (overweight or obesity).*

	WC normal	WC high	Total
BMI normal	169	6	175
BMI high	14	23	37
Total	183	29	212

Abbreviations: BMI, body mass index; WC, waist circumference.

Metabolic risk factor clustering was strongly correlated with both BMI ($r = 0.53$, $p < 0.001$) and waist circumference ($r = 0.62$, $p < 0.001$). In total, 55 (26%) of the 212 children had one or more risk factors. Clustering of two or more risk factors was almost exclusively present in children with overweight or obesity as can be seen below in figure 10.

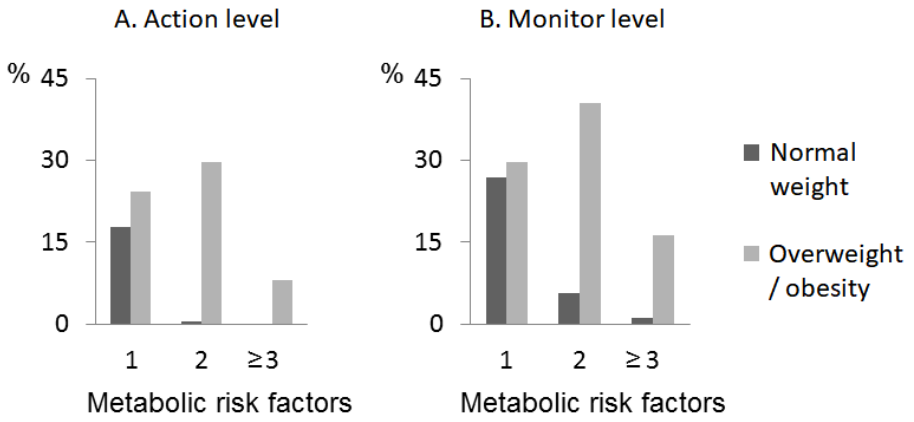

Figure 11. Distribution of metabolic risk factors in 6-year-old children according to weight groups (normal weight versus overweight or obesity) based on the 95th percentile for the IDEFICS action level (A) and the 90th percentile for the IDEFICS monitor level (B). The X axis represents the number of metabolic risk factors and the Y axis represents the proportion within each weight group. Kjellberg et al., Acta Paediatrica 2018.

Figure 11 shows the individual insulin levels versus BMI at 6 years of age, subdivided by sex. Insulin increased with BMI, and children with overweight or obesity had higher insulin levels than children with normal weight did ($p < 0.001$). Almost all children at this age had a normal glucose level and there was no significant difference between weight groups. Triglycerides increased ($p = 0.02$) and HDL cholesterol decreased ($p = 0.04$) with BMI but not until obesity was reached, and there were no significant differences between children with normal weight and overweight.

In stepwise multiple regression models, both high waist circumference ($\beta = 0.06$, $R^2 = 0.25$, $p < 0.001$) and high BMI ($\beta = 0.15$, $R^2 = 0.23$, $p < 0.001$) had an impact on IR.

One boy and four girls out of 212 children (2%) had triglycerides at or above the 95th percentile and all but one were overweight or had obesity. Although there were lower HDL cholesterol levels in children with obesity ($p = 0.04$), none had HDL cholesterol below the 5th percentile.

There was no significant difference in blood pressure across weight groups but both systolic and diastolic blood pressure were higher in children with high waist circumference ($p = 0.004$ and $p = 0.04$, respectively).

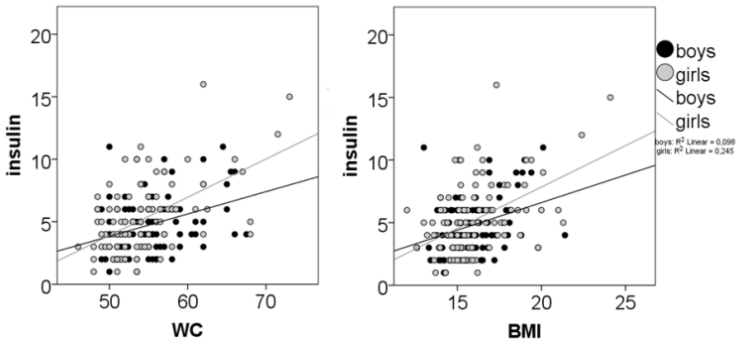

Figure 12. Left panel: the association between insulin and waist circumference (WC) stratified by sex (p <0.001 for girls, p = 0.003 for boys). Right panel: the association between insulin and BMI stratified by sex (p <0.001 for girls, p = 0.003 for boys)

4.1.1 HEREDITY

Among the children in this study, only four mothers had gestational diabetes during pregnancy. Two of these offspring had one metabolic risk factor and the other two had no metabolic risk factors. There were no associations between gestational diabetes and any metabolic risk factors.

Stated heredity for overweight, diabetes, and/or CVD showed a weak significant correlation between heredity and measured HDL cholesterol (r = 0.2, p = 0.005). However, in a multiple regression model (including birth weight, heredity, paternal BMI, maternal BMI, maternal smoking, and maternal education) with R^2 0.10, heredity could explain only 1.8% of metabolic clustering in children (β = 0.23, p = 0.045) and maternal BMI explained 3.2% (β = 0.38, p = 0.02). Paternal BMI showed no impact

4.2 ANTHROPOMETRY AND METABOLIC RISK FACTORS BY SEX (PAPER I AND II)

Of the 81 children who participated in the MRI study at 7 years of age (Paper II), six had obesity, five of whom were girls (table 4). Even though there were no significant differences in BMI or waist circumference between sexes, there was a trend in the cohort for higher BMI and waist circumference in girls. Fat distribution was significantly different, with girls having more SAT than boys (1.25 (0.75–1.79) L versus 0.83 (0.58–1.37) L, p = 0.02). There were no significant differences in VAT. Thus, even at the age of 7 years there were sex differences in the unadjusted associations between the metabolic risk factors and SAT and VAT.

Table 4. *Anthropometry for the cohort from Paper II (expressed as mean ± SD or median (interquartile range) for skewed distributions.*

	Boys (N = 39)	Girls (N = 42)
Normal weight (N)	26	27
Overweight (N)	12	10
Obesity (N)	1	5
BMI (kg/m2)	16.7 ± 2.0	17.0 ± 2.5
WC (cm)	55.8 ± 8.4	56.4 ± 5.6
VAT (L)	0.13 (0.08, 0.21)	0.17 (0.11, 0.26)
SAT (L)	0.83 (0.58, 1.37)	1.25 (0.75, 1.79) *

Abbreviations: BMI, body mass index; WC, waist circumference; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue.
*p-value < 0.05.

Generally, SAT showed a stronger association with most of the metabolic risk factors than VAT did. Interaction analyses showed significant interactions between VAT and insulin, HOMA-IR and systolic blood pressure ($p < 0.05$). There were close to significant interactions between SAT and insulin and HOMA-IR ($p \leq 0.1$).

Table 5 shows that the associations between anthropometry and metabolic risk factors were generally stronger in girls than boys. For example, in adjusted models, insulin was associated with both SAT and VAT in girls but no significant associations were seen in boys. In contrast, blood pressure only showed associations with SAT in boys. Moreover, triglycerides were associated with both SAT and VAT in girls. When the model was further adjusted for insulin there were no longer significant associations.

Table 5. *Adjusted regression models explaining insulin and triglycerides. Each variable was adjusted for birth weight, maternal BMI, and education.* (From Paper II)

	Triglycerides			Insulin			Diastolic BP		
	R^2	beta	p-value	R^2	beta	p-value	R^2	beta	p-value
Boys									
BMI (kg/m^2)	0.16	0.01	0.69	0.24	0.72	**0.03**†	0.26	0.55	0.36
WC (cm)	0.15	0.00	0.93	0.36	0.35	**0.004**†	0.26	0.20	0.39
SAT (liters)	0.23	0.08	0.15	0.19	2.08	0.06	0.34	4.22	**<0.05**
VAT (liters)	0.21	0.20	0.20	0.04	-0.77	0.81	0.31	9.97	0.11
Girls									
BMI (kg/m^2)	0.32	0.04	**0.03***	0.56	0.85	**<0.001**†	0.27	-0.11	0.78
WC (cm)	0.37	0.02	**0.01***	0.66	0.39	**<0.001**†	0.28	0.07	0.66
SAT (liters)	0.31	0.12	**0.04***	0.62	2.96	**<0.001**†	0.28	0.65	0.61
VAT (liters)	0.37	0.71	**0.01***	0.51	12.74	**0.001**†	0.28	4.28	0.50

*No significance when also adjusted for insulin
†Still significant when also adjusted for triglycerides
Abbreviations: BMI, body mass index; WC, waist circumference; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; BP, blood pressure.

4.3 BIOMARKERS DURING INFANCY (PAPER III AND IV)

4.3.1 POLYUNSATURATED FATTY ACIDS

The major PUFAs LA, AA, DHA, and EPA all changed during the first year of life (figure 12). The ratio of n-6/n-3 fatty acids in serum phospholipids increased from birth to 2 days (19%) and increased more to 4 months (56%) ($p < 0.001$), which reflected the increased number of formula-fed infants with time. Between the ages of 2 days and 4 months, serum phospholipids DHA and MA decreased 15% and 76%, respectively, whereas LA increased by 164% ($p < 0.001$ for all changes over time). Concentrations of LC-PUFA and the n-6/n-3 ratio varied widely in breast milk between mothers in the first postpartum week (6.5 ± 1.7, range 3.3–13.0) and at 4 months (6.6 ± 2.0, range 3.5–14.1).

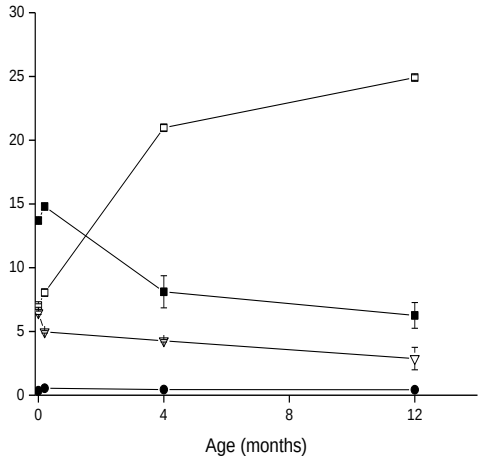

Figure 13. Changes in polyunsaturated fatty acids(mol%) from birth to 12 months. White squares represent linoleic acid (LA), black squares arachidonic acid (AA), triangles docosahexaenoic acid (DHA), and circles eicosapentaenoic acid (EPA).

The n-6/n-3 ratio as well as the decrease in DHA and increase in LA concentration were significantly less pronounced in breastfed than formula-fed infants ($p = 0.01$ and $p < 0.001$, respectively) (figure 13).

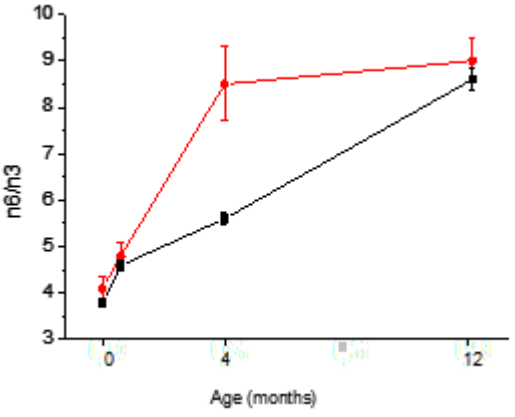

Figure 14. The ratio of n-6 to n-3 fatty acids from birth to 12 months in breastfed infants (black squares) compared to formula-fed infants (red circles).

4.3.2 IGF-I, FATTY ACIDS, ADIPOKINES, AND WEIGHT GAIN

At birth, SGA children had lower IGF-I than children born LGA but there were no differences in IGF-I concentrations at 4 months of age (SGA 44.5 µg/L and LGA 43.4 µg/L, p = 0.91). Delta IGF-I between birth and 4 months was negatively correlated with birth weight (r = -0.52, p <0.001) but positively correlated with weight gain from birth to 4 months (r = 0.49, p <0.001). IGF-I at 4 months of age was 65.0 (47.0, 87.0) µg/L for formula-fed infants and 38.5 (28.5, 50.8) µg/L for breastfed infants (p <0.001) (figure 14). Formula-fed infants had a higher weight gain from birth to 4 months and a higher BMI at 4 months (p = 0.04). A smaller group of infants (23%), independently of feeding modality, had higher concentrations of IGF-I at 4 months than at birth, compared with the normal pattern of lower IGF-I at birth than at 4 months. These infants had a larger weight gain during this period (207% vs 185%, p <0.001), higher LA (mean 22.7 vs 20.5, p <0.001) and lower AA (mean 7.3 vs 8.4, p <0.001) concentrations at 4 months of age.

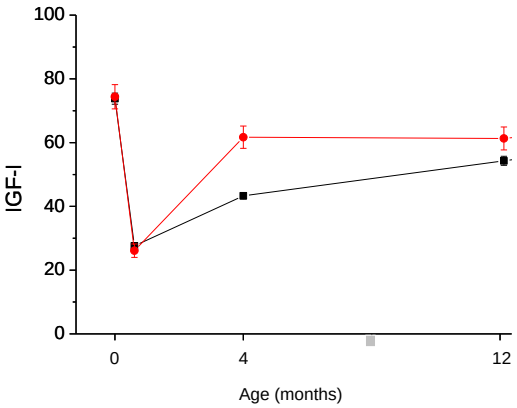

Figure 15. Insulin-like growth factor I (IGF-I) concentrations(µg/L) from birth to 12 months in breastfed infants (black squares) compared to formula-fed infants (red circles).

There were associations between IGF-I and fatty acids. At the age of 2 days, there were positive correlations between IGF-I and concentrations of LA and DGLA, and a negative correlation between IGF-I and AA. At 4 months, LA still had a positive correlation and AA a negative correlation with IGF-I. Additionally, the increase of IGF-I from day 2 to 4 months correlated with the increase in LA (r = 0.52, p <0.001) and with the decrease in AA (r = 0.44, p <0.001) during the same time period. The n-9 fatty acid MA was positively

correlated with IGF-I in cord blood and weakly negatively correlated at 4 months. Multiple regression analyses confirmed these associations.

Maternal BMI was associated with cord blood leptin (r = 036, p <0.001) but there were no associations with either cord blood IGF-I or adiponectin. Both IGF-I and leptin from cord blood were strongly correlated with birth weight (p <0.001 for both). At 4 months they were both associated with weight (p <0.01 for both) and waist circumference (p <0.001 for both) but associated negatively with gain in BMI from 4 to 12 months (p<0.001 for both). Adiponectin only showed an association with waist circumference at 4 months of age (p <0.05) but not at 12 months.

4.3.3 BIOMARKERS IN INFANCY AND OUTCOMES AT 6 YEARS OF AGE

We found no associations between fatty acids in infancy and metabolic risk factors at 6 years of age (data not shown). BMI and waist circumference at 6 years of age were associated with IGF-I (figure 15) and adipokines during infancy, especially the 4-month values (leptin positively and adiponectin negatively). After adjustment for sex, birth weight, and BMI, 4-month adiponectin accounted for 3% of BMI and 2% of waist circumference at 6 years of age. Likewise, IGF-I but not leptin at 4 months of age accounted for 2% of BMI and 4% of waist circumference.

IGF-I at 4 months of age was positively associated with fasting insulin (p = 0.005) at 6 years, even after adjustments for background variables. Delta IGF-$I_{0\text{-}4m}$ was also associated with insulin at 6 years of age (figure 15).

Serum triglycerides in children at 6 years of age were associated with leptin independently of weight at 4 months of age. Adjusted, leptin accounted for 14% of triglycerides in 6-year-olds. Adiponectin at 4 months of age had a weak positive association with 6-year-olds' HDL cholesterol levels (p = 0.01). It remained significant after adjustments in the regression model but not after Holm–Bonferroni adjustments for multiple tests.

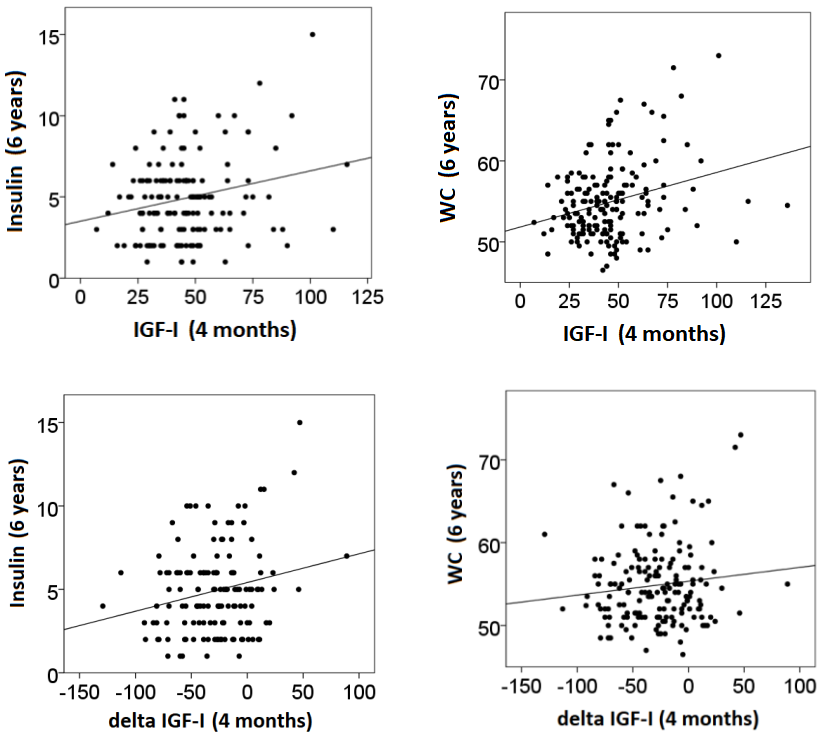

Figure 16. Insulin-like growth factor I (IGF-I) at 4 months of age was associated with insulin concentrations (top left panel, p = 0.005) and waist circumference (WC) (top right panel, p <0.001) at 6 years of age. Delta IGF-I $_{0\text{-}4m}$ was associated with insulin at 6 years (bottom left panel, p = 0.02) and a trend was seen an association between delta IGF-I $_{0\text{-}4m}$ and waist circumference at 6 years (bottom right panel, p=0.13).

5 DISCUSSION

Main findings

One of the main findings of this thesis is that metabolic alterations and metabolic syndrome can be found in 6-year-old children. Although only in a small percent of an otherwise healthy population, the classic signs of the syndrome were fulfilled and, in addition, a larger number of children had several signs of the syndrome. It is also noteworthy that the anthropometric measurements commonly used in clinical practice, such as BMI and waist circumference, are good predicting markers for developing the metabolic syndrome before adolescence.

Early nutrition, here with focus on breastfeeding, was important regarding the association of PUFAs with IGF-I and growth during infancy, which have implications for later metabolic health. There were significant differences between breastfed and formula-fed infants at 4 months of age. The differences were seen in both fatty acid profile and hormonal status. Illustrating the importance of infant levels of adipokines and IGF-I, there were associations with body composition and metabolic risk factors at the age of 6 years. These results add to the existing knowledge about early programming of later health and diseases.

Identifying metabolic syndrome in young children

The presence of metabolic syndrome and the need to examine young children with overweight or obesity for the presence of risk factors has been questioned. Even as recently as 2007, the IDF consensus (70) recommended that children under the age of 10 years should not be diagnosed with metabolic syndrome and should only have their waist circumference measured. This absence of interest in metabolic syndrome in children is partly explained by the previous lack of reliable reference values for the included components of the syndrome. This was also the reason behind inconsistent definitions of the syndrome. Another major cause of the consistency of the definition is the large variety in timing of biological changes related to growth and maturation of the individual child.

Since the IDF consensus, there have been few studies in children younger than 10 years of age, which highlights the need for a useful definition and references even in younger children (71). The IDEFICS study produced a clear proposal for a definition of metabolic syndrome in 2014 (61). Due to the large number of children in each age group, the study was able to provide reliable data with cut-offs divided according to both age and sex (28, 72-74). Thanks to these newly available data on European children, we based the studies in this thesis on the IDEFICS definitions and reference values. Using another definition would have given a different result, as shown in Paper I, where the results were tested against the IDF definition of metabolic syndrome in children over 10 years of age. Others have also tested the same cohort with different definitions of metabolic syndrome. Reinehr et al. compared eight different definitions and found a prevalence of metabolic syndrome ranging between 6% and 39% among children and adolescents with overweight or obesity (178). As in all fields of pediatrics, definitions and references based on age, gender, and pubertal stage are crucial when investigating and measuring metabolic health.

IR is a fundamental risk factor for metabolic syndrome. In this study, 28% of the 6-year-olds with overweight or obesity had IR and were thereby considered to have a high risk of later developing metabolic syndrome and CVD. More than every fourth 6-year-old child with overweight or obesity has this increased risk, which should not be neglected because of their young age. Looking at the clinical implications of the definition and diagnosis of obesity and metabolic syndrome in children, the whole picture has to be considered; overweight or obesity alone cannot determine the child's risk of morbidity. The ideal intervention for a specific child can be found by considering together the risk factors, their shared pathophysiology, family heredity, and lifestyle. Thus, establishing an age- and gender-related definition of metabolic syndrome, with references that are applicable to young children, is the necessary first step to identifying individuals at risk.

We used fasting insulin as a proxy for IR. Fasting glucose is still very stable at this age, as healthy beta mass in the pancreas still compensates for IR with more insulin secretion. That makes fasting glucose an inferior method for measuring IR in this age group. The gold standard for measuring IR is hyperinsulinemic euglycemic clamping, which is not suitable in young children due to the risk of hypoglycemia (179). Oral glucose tolerance tests with measurement of glucose and insulin would probably have given more precise data on IR in the children in our studies but this is still not the recommended method for diagnosing metabolic syndrome.

A surprisingly high incidence of high blood pressure was observed in this cohort and was significantly associated with high waist circumference. The association between high blood pressure and overweight in prepubertal children is known (107). It is a weakness of this thesis that resting blood pressure was only measured once in the children. For many of the children it was the first time their blood pressure had been measured. This possible stress could explain the high incidence of hypertension. If three measurements had been collected and the mean value used, lower blood pressure levels might have been shown. However, even this would be no guarantee against a child's high blood pressure being an artifact. Indeed, "white coat hypertension" (when a patient's anxiety in a medical environment results in an abnormally high blood pressure reading) is a known risk factor for CVD (180).

Dyslipidemia was seen in some children (Paper I), and four out of five of these were overweight or had obesity and had high waist circumference. The negative association between triglycerides and HDL cholesterol was expected because the hepatic pathway with high triglycerides conducts lower HDL cholesterol (96, 97). Although lower HDL cholesterol was observed in children with high triglycerides in this study, none had HDL cholesterol under the 5^{th} percentile.

Sex differences

The study in Paper II showed sex differences in fat distribution, with girls having more SAT than boys, but no differences in VAT. There were also stronger associations in girls between both SAT and VAT and metabolic risk factors. This could be due to the non-significant tendency to higher BMI and higher volume of VAT in girls. Moreover, five of six children with obesity at 6 years of age were girls. Whether this is a coincidence in this cohort or a new trend in the community needs further confirmation. Historically, boys have been heavier and taller, but in modern overfed societies this pattern might disappear.

Anthropometrics are enough to identify high-risk children

We found BMI and waist circumference to be anthropometric measures that are easy to use in the clinic and as accurate as MRI quantifications of VAT and SAT for identifying adiposity and cardiometabolic risk. Moreover, waist circumference was shown to be the best anthropometric measure for IR. Similar findings have previously been found in adolescents (181).

Early programming

Barker showed that the early nutritional state affects the development of CVD in later life (111). Even though only six children born SGA participated in the 6-year follow-up, we found significantly higher fasting insulin in these children.

Early rapid weight gain, possibly as a result of intrauterine undernutrition, is one risk factor for the development of obesity and metabolic syndrome. Rapid weight gain during the first half of infancy seems to be much more predictive of later obesity than weight gain in later infancy (182). In the present thesis, IGF-I at 4 months of age was positively associated with BMI and waist circumference at 6 years of age but not with the weight gain from 4 to 12 months of age. On the other hand, IGF-I at 4 months was associated with weight gain from birth to 4 months. This indicates that the earlier weight gain has a higher impact on the development of overweight.

LC-PUFA

Besides growth, the nutrient content influences infant weight gain. The protein content in breast milk and formula is a known factor that affects the levels of IGF-I and weight gain in infants (122). Fatty acids are also shown to have an important role in early growth and development (126) but their association with IGF-I in infants has not earlier been described. Over several millennia, the balance of fatty acid intake has changed toward a higher n-6/n-3 ratio. During the last hundred years, fatty acid intake and balance have changed tremendously and simultaneously as cardiovascular and metabolic diseases have increased (125).

We showed not only how fatty acids are associated with IGF-I levels at 4 months of age, but also the importance of changes in IGF-I levels from birth to 4 months of age. During this period, n-6 fatty acids have a larger impact on IGF-I than n-3 fatty acids have. The major findings reported in Paper III were an early positive association between IGF-I and the n-6 fatty acids LA and DGLA but a negative association between IGF-I and AA. These results are consistent with other studies, where AA concentrations were negatively associated with BMI in 2- to 6-year-old children (183) and the perinatal n-6/n-3 ratio was positively associated with BMI at 3 years of age (184). A study on maternal PUFA intake indicated lower BMI and height in children of mothers with a high intake of EPA, DHA, and AA. The study showed no association between maternal PUFA intake and the child's metabolic profile (185). Animal studies have shown that rats given a diet with LA content

similar to a western diet developed symptoms related to metabolic syndrome. However, we found no associations between fatty acids and metabolic risk factors at 6 years of age.

Earlier publications focused more on n-3 fatty acids than on n-6 fatty acids or the ratio between them, but there has lately been a new focus on n-6 fatty acids. For example, a recent study found that, high postnatal AA levels in preterm infants decreased the risk of developing retinopathy of prematurity, a serious condition known to arise when rapid changes in IGF-I levels suddenly appear (186).

Associations between IGF-I and n-3 fatty acids in the current thesis were weak but still there was a negative association between AA/DHA and IGF-I. Poor LC-PUFA status with, in particular, low concentrations of n-3 DHA and EPA levels in cord blood has previously been found to be associated with gain in BMI during early infancy (136). This strengthens our results, given that low n-3 LC-PUFA levels can be a consequence of high levels of LA which was associated with increased IGF-I and growth. An intervention study of fish oil compared to sunflower oil between 9 and 18 months investigated the relation of n-3 to IGF-I. They found that n-3 was positively associated with IGF-I in boys only. IGF-I changes during the intervention were positively associated with BMI, with the most pronounced association in girls. Another study found that DHA content in breast milk postponed the timing of adiposity rebound (187), which might indicate a later effect of n-3 fatty acids on overweight and obesity.

The choice of a measuring point at 4 months of age in Paper III and IV was partly to be able to separate exclusively breastfed infants from exclusively formula-fed infants. Formula-fed infants had at that age higher IGF-I concentrations than breastfed infants, which is well established from other studies (122). Formula-fed infants also had a different fatty acid profile than breastfed infants, with a higher n-6/n-3 ratio; in addition, the increase in LA and decrease in DHA from birth to 4 months were more pronounced in formula-fed infants.

Modern formula aims to mimic breast milk, with a lower protein content and added fatty acids. However, we saw distinct differences in IGF-I and fatty acid profile in formula-fed infants compared to breastfed infants. In view of the increase of LA in breast milk during recent decades (188) and the high concentration of LA in formula compared to breast milk (177), our results support earlier concerns about the high LA content in formula (189, 190).

Leptin

We found leptin to be positively associated with weight status at birth and at 4 months of age. Furthermore, infancy leptin levels were associated with BMI and waist circumference at 6 years of age, independently of birth weight or weight status at 4 months of age.

Maternal nutritional status is known to influence leptin levels of the newborn (172), and an association between maternal BMI and neonatal leptin was also seen in the present study.

In this thesis a positive association was found, independently of weight and maternal BMI, between leptin during infancy and triglycerides at 6 years of age. This is not surprising, as leptin in infancy has been shown to correlate with leptin later in childhood, and previously seen associations between leptin and triglycerides are seen in both children and adolescents (59).

Unadjusted, leptin at 12 months was associated with diastolic blood pressure. Obesity leads to high leptin levels and induces a leptin resistance in the satiety pathways but not in the renal sodium retention pathway, with high blood pressure as a consequence (191). However, the positive association in our study (Paper IV) did not remain significant when adjusted for weight of the infant and mother. This can possibly be explained by the known association between maternal BMI and blood pressure in prepubertal children (172).

Adiponectin

In contrast to leptin, we saw no associations between adiponectin and weight, length, or BMI during the first year. Interestingly, we did find that adiponectin at 4 and 12 months of age was negatively associated with both BMI and waist circumference at 6 years of age. These associations were independent of the infant's weight. This was in line with the known decrease of adiponectin in overweight and obesity after the neonatal phase (164, 166). Additionally, those children with overweight or obesity at 6 years of age had significantly lower levels of adiponectin during infancy. Although it is not known at what level adiponectin contributes to overweight, these results indicate an early programming role of adiponectin on the development of overweight. A previous study that investigated the programming role of adiponectin on overweight found no associations between cord blood adiponectin and BMI at 3 years of age (170). However, they found cord blood adiponectin to be negatively associated with weight gain during the

first six months of life. Another study investigating the role of adiponectin on programing growth, did find positive associations between cord blood adiponectin and body fat at 3 and 4 years of age but not at 5 years. There was no association between adiponectin at 3 years of age and adiposity at 3 or 5 years of age in that study (192). A possible explanation for this contrast with our results concerning adiponectin in infancy and adiposity in childhood is the timing: the critical window of programming might be narrow and closed after infancy.

Paper VI showed positive associations between adiponectin during infancy and HDL cholesterol in 6-year-olds. The association was weak and did not remain significant after Holm–Bonferroni testing due to multiple testing; nonetheless, did remain significant in multiple regression models after adjusting for factors such as weight, gender, and maternal BMI. Adiponectin upregulates HDL cholesterol either by increasing HDL assembly in the liver or by downregulating hepatic lipase activity and stimulating the activity of lipoprotein lipase in muscles (193). Adiponectin is known to be negatively associated with metabolic syndrome (194). The role of adiponectin and HDL cholesterol in metabolism and their anti-atherogenic characteristics overlap, or it may be that the action of one is mediated by the other. This thesis adds to this knowledge, by showing that adiponectin might have a programming role for HDL cholesterol, already evident during infancy.

Adiponectin has also been negatively associated with LDL cholesterol and triglycerides (193), but this was not found nor was any associations to fatty acids or IR found.

IGF-I

In Paper III, children with higher IGF-I concentrations at 4 months of age had an increased weight gain, measured by delta BMI, from birth to 4 months of age compared to children with lower levels of IGF-I at 4 months. In contrast, Paper IV showed that low levels of IGF-I at 4 months were associated with an increased weight gain between 4 and 12 months, but the weight gain during this period was not associated with higher BMI in 6-year-olds. Moreover, IGF-I levels at 4 months of age were positively associated with BMI and waist circumference at 6 years of age. This indicates that the rapid weight gain during the first four months of life had a higher impact on adiposity later in childhood than the weight gain during later infancy had. This is in line with the “rapid weight gain” hypothesis that points to an early rapid weight gain as one of the risk factors for later development of obesity (65, 150).

The positive associations between IGF-I levels at 4 months of age and insulin at 6 years of age that were shown in Paper IV could be a first sign of metabolic alterations due to early programming by IGF-I. Even though there were few SGA children in the study, they had significantly lower levels of IGF-I at birth and higher insulin concentrations at 6 years of age. These children were found to have a rapid normalization of IGF-I concentrations from birth to 4 months of age. This rapid change in IGF-I was accompanied by rapid weight gain during the same time period.

Maternal factors

Maternal factors known to have an impact on children's growth and development include gestational weight gain, maternal adiposity, smoking, low vitamin D levels, maternal dietary patterns, and short duration of breastfeeding (195). In this thesis we found that maternal pre-pregnancy BMI, measured at the first visit to the maternity health care unit, was a significant predictor of birth weight and of the child's BMI and waist circumference at 6 years of age. It was a stronger marker than both gestational diabetes and heredity for clustering of metabolic risk factors (Paper I). In fact, gestational diabetes did not show any associations with the various outcomes.

In this study, only five of the mothers of children still in the study at 6 years of age smoked. Maternal smoking was not associated with either weight or metabolic outcome, but this finding is likely to be an artefact because there were too few smokers in the study to achieve power.

Even though breastfeeding was associated with lower levels of IGF-I at 4 months of age, it was not found to be directly associated with a lower risk of adiposity in children in this cohort. This could be due to the small cohort or it may indicate that breastfeeding at 4 months of age is not a good parameter for measuring the possible association of breast milk with lower adiposity. But nonetheless, four out of five children with three or more risk factors for metabolic syndrome were formula fed.

Other possible impacts of early nutrition than breastfeeding and fatty acid concentration were not examined in this study.

Strength and weaknesses

The main strength of this thesis is the longitudinal design of the cohort study. It made interpretations of causations in a longitudinal perspective possible. It is a rather small cohort but nevertheless representative for background variables and birth data for the larger population, with the exception of maternal age.

Another strength is that measurements of weight, length / height, and waist circumference were conducted by the same two experienced pediatric nurses at the same site during all the examinations. The families had met the nurses before they left the hospital after the birth of the child. Being followed up by the same two nurses over several years established trust and provided a reason for the families to continue in the study. The other advantage of the same two nurses conducting all measurements was that it increased the reliability of the data. The only exception was at the MRI examinations at the university hospital in Gothenburg (Paper II), where no anthropometric measurements were taken. This delay of six months in gathering height and weight data is a weakness. However, all individual growth curves were studied with measurements before and after 7 years of age, and the vast majority of the children followed their own growth curve. Thus, the anthropometry for Paper II was considered reliable. Even so, the power of VAT and SAT in associations with metabolic parameters might be weakened due to the delayed examination since blood sampling. This was not the case for BMI and waist circumference, which might falsely have given these measures stronger associations with risk factors than VAT and SAT had.

Finally, the fact that after the first five years there was a larger dropout rate, making the cohort at 6 years of age smaller is a weakness. There were only six children with obesity at the follow up at 6 years of age.

6 CONCLUSION

This thesis indicates that a substantial percentage of 6-year-old children show an abnormal metabolic profile, increasing the risk of CVD. Even in our small cohort of children born full-term, we found children who fulfilled all criteria for metabolic syndrome. Risk factors for metabolic syndrome were mostly detected in children with high BMI or high waist circumference. BMI was shown to be a good marker for childhood obesity but waist circumference showed a tendency toward being a stronger marker for IR and metabolic risk factor clustering in this age group.

Even at 7 years of age there were sex differences in abdominal fat volumes measured with MRI, namely that girls had more SAT than boys. In girls, fasting insulin associated more strongly with both SAT and VAT than in boys. In children at this age, SAT seems to account even more than VAT for increased metabolic risk. SAT showed stronger associations with metabolic risk factors than VAT did.

This thesis investigated factors of early metabolic programming. The n-6 fatty acids LA and AA, which mainly come from the diet, interact with IGF-I from birth and up to 4 months of age. The results suggest the importance of a fatty acid balance, both within the n-6 series for LA and AA and between the n-6 and n-3 series. Formula, which is richer in LA and less rich in AA compared with breast milk, was associated with higher serum IGF-I levels and increased weight gain from birth to 4 months of age.

IGF-I at 4 months of age was negatively associated with weight gain between 4 and 12 months of age but positively associated with BMI and waist circumference at the age of 6 years. Furthermore, IGF-I was associated with IR at 6 years of age. The adipokines leptin and adiponectin during infancy were associated with adiposity and risk factors for metabolic syndrome at 6 years of age.

The fact that it was possible to show that nutrition as well as fatty acids during infancy were associated with IGF-I, which in turn was positively associated with adiposity and IR in 6-year-olds, opens up for early interventions. To identify children with increased risk of metabolic impairment, traditional measures such as BMI and waist circumference are still valuable.

7 FUTURE PERSPECTIVES

The importance of early programming on metabolic health should be highlighted in initiatives that aim to prevent later negative metabolic alterations. This would hopefully lead to improved guidance and focused individualized interventions. More studies are needed to clarify the relationships that nutrition and hormonal status in fetal life and early infancy have on long-term health, including the development of metabolic syndrome. This could be used for improved information, guidance and even very early interventions in connection with maternity health care.

The overall finding of this thesis highlights the importance of focusing on the early development of overweight and what the consequences can be for later health. BMI and waist circumference are excellent and easy to use measures in the screening of children at risk, with the advantage that they can be monitored longitudinally using a BMI or waist chart. If these measures were used on regular basis in child health care centers as well as in school health care, the metabolic impairments of many children could be detected at an early state. Metabolic alterations in children of pre-school and early school age are still reversible.

As I now resume my clinical work in the pediatric department, I aim to take all my knowledge from the work in this thesis with me. That involves translating the scientific results to clinical practice, as well as bringing the evidence-based approach to searching for information and new knowledge.

But as I have been allowed to immerse myself in this research, I cannot leave it all behind. This cohort can still provide answers to many unsolved questions. Some of the research questions waiting for answers are the fascinating connection between the PUFAs and our gut microbiota and a possibly mutual association with metabolic syndrome. In addition, the role of nutrition and PUFAs in bone metabolism opens up topics for future research.

"*Once you have traveled, the voyage never ends, but is played out over and over again in the quietest chambers. The mind can never break off from the journey*." **—** Pat Conroy (The Prince of Tides, 2002)

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A journey has in one sense come to an end. This was a path I never thought I would walk, and once in a while I thought I would never reach the end. But life has a funny way of sneaking up on you, and here I am, at the finish line. But once here I realize it is not the final goal, but rather just a milestone on the path I chose. Where the path is taking me I do not know, but I will bring my backpack with me and I will be prepared whenever the path of science will take me on another journey.

"*It is good to have an end to journey toward; but it is the journey that matters, in the end*." (Ursula K. Le Guin, The Left Hand of Darkness, 1969)

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